Neonatal Levels of Acute Phase Proteins and Risk of Autism Spectrum Disorder

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ABSTRACT

BACKGROUND: Immune signaling pathways influence neurodevelopment and are hypothesized to contribute to the etiology of autism spectrum disorder (ASD). We aimed to assess risk of ASD in relation to levels of neonatal acute phase proteins (APPs), key components of innate immune function, measured in neonatal dried blood spots.

METHODS: We included 924 ASD cases, 1092 unaffected population-based controls, and 203 unaffected siblings of ASD cases in this case-control study nested within the register-based Stockholm Youth Cohort. Concentrations of 9 different APPs were measured in eluates from neonatal dried blood spots from cases, controls, and siblings using a bead-based multiplex assay.

RESULTS: Neonatal C-reactive protein was consistently associated with odds of ASD in case-control comparisons, with higher odds associated with the highest quintile compared with the middle quintile (odds ratio [OR] = 1.50, 95% confidence interval [CI] = 1.10–2.04) in adjusted analyses. In contrast, the lowest quintiles of α2-macroglobulin (OR = 3.71, CI = 1.21–11.33), ferritin (OR = 4.20, CI = 1.40–12.65), and serum amyloid P (OR = 3.05, CI = 1.16–8.01) were associated with odds of ASD in the matched sibling comparison. Neonatal APPs varied with perinatal environmental factors and maternal/fetal phenotypes. Significant interactions in terms of risk for ASD were observed between neonatal APPs and maternal infection during late pregnancy, maternal anemia, and maternal psychiatric history.

CONCLUSIONS: Indicators of the neonatal innate immune response are associated with risk of ASD, although the nature of these associations varies considerably with factors in the perinatal environment and the genetic background of the comparison group.

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Perturbations in immune signaling pathways are hypothesized to influence neurodevelopment because many immune effector molecules have pleiotropic effects in the developing nervous system (1,2). Peripheral concentrations of cytokines and chemokines involved in the innate and adaptive immune responses vary in individuals already diagnosed with autism spectrum disorder (ASD) compared with control subjects (3,4), although the prevalence of multiple disorders of the immune system (e.g., asthma, infections, allergy, autoimmune disorders) is also higher in individuals with ASD (5–7). Integrative analyses of genetic and transcriptomic data suggest that ASD and several of these commonly co-occurring conditions may be related via shared mechanisms involving innate immunity (8). The innate immune system is our first-line defense against invading microbes and internal danger signals and is subject to variation by both heritable and nonheritable factors. The shared disruption of innate immune signaling pathways between ASD and immune disorders may implicate shared genetic liabilities between these disorders but also may represent pathways relevant for gene–environment interactions.

In studies employing biological samples collected from etiologically relevant periods, deviations in neonatal and fetal markers of inflammation and innate immunity exist in some individuals later diagnosed with ASD (9–14). Such differences may be attributable to genetic background or differential exposure to environmental insults in utero (15–19). Altered levels of cytokines, chemokines, and acute phase proteins (APPs) have been reported in the maternal peripheral circulation during pregnancy in mothers of children later diagnosed with ASD, with some evidence indicating that associations vary with the presence of co-occurring intellectual disability (ID) (16–18). It has been posited that alterations in such signaling molecules in the maternal circulation could potentially influence the fetal immune and nervous system signaling and therefore development. Experimental models indicate that maternal immune activation alters fetal brain development and that maternal interleukin-6 (IL-6) plays a critical role in mediating these effects (20,21).

APPs are key components of the innate immune response and are produced primarily in response to IL-6 and other inflammatory cytokines (22). IL-6, among other cytokines, was elevated in mid-pregnancy serum levels in mothers of children with ASD and co-occurring ID compared with general population control subjects (17), although studies of the association between maternal mid-pregnancy levels of one APP, C-reactive protein (CRP), and risk of ASD have been inconsistent (16,19). Together, the APPs are a diverse group of proteins that recognize and opsonize invading
pathogens and cellular debris, regulate blood viscosity and clotting, and sequester nutrients (e.g., iron) from pathogens (23). We investigated the associations between neonatal APPs and later diagnosis of ASD and how environmental exposures (e.g., maternal infections) may affect such associations. We also compared individuals with ASD with their unaffected siblings to understand the role of shared familial factors (e.g., genetic background).

METHODS AND MATERIALS

Study Population

The Stockholm Youth Cohort (SYC) is a register-based cohort of all children up to 17 years of age living in Stockholm County, Sweden (24,25). Of the individuals born from 1996 to 2000 and living in Stockholm County for a minimum of 4 years (to ensure adequate follow-up time for ASD ascertainment), we selected all individuals diagnosed with ASD before December 31, 2011, a random sample of individuals in the SYC, and unaffected siblings to ASD cases (Figure S1). Diagnostic information in the SYC was updated after sample collection to include follow-up until December 31, 2016. Ethical approval was obtained from the Stockholm regional review board. Individual consent was not required for this anonymized register-based study.

ASD Ascertainment

We implemented a case-finding procedure covering all pathways to child and adolescent psychiatric care and habilitation services in Stockholm County (24,25). Ascertainment of ASD, ID, and attention-deficit/hyperactivity disorder (ADHD) in the SYC is described in Table S1. The outcome of ASD was stratified by the presence of co-occurring ADHD and ID: ASD with ID, ADHD with ID, and ADHD without ID or ADHD (ASD only). Individuals diagnosed with both co-occurring ID and ADHD (n = 111) were included in the ASD with the ID group.

Laboratory Analysis

Neonatal dried blood spots (NDBSs) were collected from the national biobank at Karolinska University Hospital in Solna, Sweden. Blood spots were originally collected approximately 3 to 5 days after birth (mean = 4.1 days, SD = 1.3). For economic reasons, we selected a smaller sample (born 1998–2000) of the NDBSs collected for analysis of immune markers. A sample (12.5%) of those born in 1996 or 1997 was also included. Our final sample consisted of 924 ASD cases and 1092 controls as well as 203 unaffected siblings to ASD cases (Figure S1). The characteristics of those included in the final sample were similar to the overall characteristics of the source cohort SYC (Table S2).

A 3.2-mm-diameter disc was punched from each NDBS, immersed in 200 µL of phosphate-buffered saline, and incubated at room temperature on a rotary shaker (600 rpm) for 2 hours. Samples for the case-cohort comparison were eluted in 3 batches over 3 subsequent days and stored at −80 °C until analysis. For the sibling comparison, a new punch was taken for each affected sibling and the unaffected sibling, placed in adjacent wells on the same plate for elution, and analyzed immediately. Total protein concentration in eluates was measured using Direct Detect infrared spectroscopy (Merck, Darmstadt, Germany).

Eluates were analyzed for procalcitonin (PCT), ferritin (FER), tissue plasminogen activator (TPA), fibrinogen (FIB), and serum amyloid A (SAA) or were diluted 1:4 for analysis of α2-macroglobulin (A2M), CRP, haptoglobin, and serum amyloid P (SAP) using a premixed multiplex panel and the Bio-Plex 200 System (Bio-Rad, Hercules, CA). Coefficients of variation for manufacturer-provided controls varied by analyte from 9.0% to 20.8% (Table S3). The percentages of samples below the lower limit of quantification varied by analyte from 0.00% to 0.45% (Table S3) and were assigned the value of lower limit of quantification/√2.

Covariates

Covariates were chosen based on previous associations with ASD. Covariate data were extracted from the Medical Birth Register, the Integrated Database for Labor Market Research, and the National Patient Register. We considered maternal age (26), country of origin (27), body mass index (BMI) (28), psychiatric history (29), hospitalization for infection during late pregnancy (third trimester) (15), anemia (30), and supplement use during pregnancy (31); parental income at the time of birth and education level (highest of mother or father) (32); and children’s birth order, sex, gestational age at birth (33), size for gestational age (34), mode of delivery (35), and Apagar score (36).

Statistical Analysis

We log2-transformed the APP values because the distributions were right-skewed. Because of differences in total protein and APP distributions according to elution batch (n = 3) and analytical plate (n = 24) (Figure S2), we created plate-specific standardized z scores for each APP by subtracting the plate-specific mean from each observation and dividing by the plate-specific standard deviation. We created quintiles of each APP, using the distribution of z scores among unaffected individuals to set cutoffs.

We examined the association of each covariate with the neonatal APP z scores, separately for ASD cases and controls, estimating mean APP z score over categories of the covariates with linear regression, followed by a Wald test as a general test of the association between the APP and the covariate.

We used conditional logistic regression models stratified by plate to calculate the odds of ASD associated with each neonatal APP. For categorical analysis, the middle quintile was the referent group. For continuous analysis, we used restricted cubic spline models with three knots. Adjusted models included terms for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; parental income; and children’s birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. We tested for interactions between APP levels and the covariates, comparing models including the cross-product terms with those without the cross-product terms using a likelihood ratio test. Matched sibling analysis used conditional logistic regression models clustered by family and adjusted for sex, birth order, and total protein concentration.

We conducted sensitivity analyses to determine whether variation in APP measurements may have influenced results. First, we also adjusted for factors that influence APP levels but were not associated with ASD (total protein, birth year, and age at neonatal blood sample). Second, we excluded samples with low total protein (i.e., those within the lowest quartile of total protein). Finally, we used multilevel modeling to account for both plate and batch.
RESULTS

Association of APPs With Covariates

All APPs were correlated with each other and with total protein yield \((p < .05)\) (Figure S3). Other than parental education, maternal BMI, and maternal psychiatric history, each covariate was also associated with at least one neonatal APP \((p < .05)\) (Figure 1 and Table S4), although the patterns of association varied for each APP. For example, CRP was the only APP associated with sex, with a higher z score for boys \((0.03)\) than for girls \((-0.16)\) \((p = .001)\). The patterns of association between neonatal APPs and covariates differed among cases versus controls (Figure S4 and Table S5). For example, levels of most neonatal APPs, particularly FER, FIB, PCT, and tPA, were lower among cases born to mothers who had been hospitalized for infections, and levels of FER were lower among cases born to mothers diagnosed with anemia (Figure S4) compared with the patterns observed among controls (Figure 1).

Association of Neonatal APP With Odds of ASD

Median levels of all neonatal APPs except FER were higher in ASD cases compared with controls (Table 1), although the overall distributions of neonatal APPs were similar between cases and controls (Figure S5). In regression analyses, we observed modest U-shaped associations between CRP, FIB, PCT, and SAA and odds of ASD, with the strongest associations between CRP and odds of ASD (Figure 2 and Table S6) and evidence for nonlinear associations for CRP, FIB, and PCT (Table S7). The relationships between FIB, PCT, and SAA and ASD were attenuated in the fully adjusted models. CRP levels 1 SD above the mean were associated with higher odds of ASD compared with the mean (odds ratio \([OR] = 1.23\), 95% confidence interval \([CI] = 1.06–1.42\)) in fully adjusted models (Table S6). Similar patterns of association were observed when we considered each APP as a categorical variable, with increased odds of ASD associated with the highest quintiles of CRP \([OR = 1.50, CI = 1.10–2.04]\) and PCT \([OR = 1.35, CI = 0.99–1.85]\) compared with the middle quintile in adjusted models (Figure S6A).

Overall, the patterns of association with neonatal APPs were similar among the stratified outcomes. The odds associated with the highest quintile of CRP were greatest for ASD only \([OR = 1.80, CI = 1.15–2.82]\) compared with ASD with co-occurring ID \([OR = 1.29, CI = 0.83–2.01]\) or with co-occurring ADHD \([OR = 1.34, CI = 0.85–2.11]\), with similar results in continuous analyses (Figures S7–S10).

Neonatal APPs and Odds of ASD Among Matched Sibling Pairs

In contrast to the case-control comparison, we found that median levels of all neonatal APPs except CRP, haptoglobin, and PCT were lower in ASD cases compared with their unaffected siblings (Table S8), with distinct distributions of particularly A2M, SAP, and FER among ASD cases compared with their unaffected siblings (Figure S5). In matched regression analyses, we observed decreasing odds with increasing levels of A2M, FER, SAP, and tPA (Figure 3, Figure S6B, and Table S6). Associations with SAA, SAP, and tPA were attenuated in adjusted models, although the pattern associated with A2M and FER persisted (Figure 3). A2M levels 1 SD below the mean were associated with higher odds of ASD compared with the mean \((OR = 2.24, CI = 1.40–3.58)\) in adjusted analyses, as were FER levels 1 SD below the mean \((OR = 5.66, CI = 2.40–13.35)\) (see Table S6). Compared with the middle quintile, the lowest quintile of A2M \((OR = 3.71, CI = 1.21–11.33)\), FER \((OR = 4.20, CI = 1.40–12.65)\), and SAP \((OR = 3.05, CI = 1.16–8.01)\) was associated with increased odds of ASD in the matched sibling comparison (Figure S6B).

Interaction Between Neonatal APPs and Other Risk Factors for ASD

Because many of the covariates we studied (e.g., maternal infection, mode of delivery) represent environmental influences that may elicit responses from the innate immune system and are also risk factors for ASD, we hypothesized that variation in the innate immune response to these environmental influences may be associated with risk for ASD. We observed interactions between neonatal APPs and a number of other risk factors for ASD in our study (Figure 4, Figure S11, and Tables S9 and S10). We observed interactions between maternal hospitalization for infections during the third trimester and neonatal levels of APPs (Figure 4). Lower odds of ASD were associated with increasing levels of A2M, FER, FIB, PCT, and tPA among children whose mothers were hospitalized for infection during late pregnancy, in contrast to those whose mothers were not hospitalized for infection (Figure 4A–E). For example, neonates with A2M values 1 SD above the mean who were exposed to maternal infection had lower odds of ASD \((OR = 0.43, CI = 0.23–0.83)\) compared with those with mean A2M levels. Neonates with A2M values 1 SD above the mean who were not exposed to maternal infection, on the other hand, had similar odds compared with mean A2M levels (OR = 1.01, CI = 0.91–1.11) (see Table S10). Similarly, we observed lower odds of ASD associated with increasing levels of FER and SAP among children whose mothers were diagnosed with anemia during pregnancy, in contrast to children whose mothers were not diagnosed with anemia (Figure 4F, G). Neonates with FER values 1 SD above the mean who were exposed to maternal anemia had lower odds of ASD \((OR = 0.62, CI = 0.42–0.91)\) compared with those with mean FER levels, while neonates with FER values 1 SD above the mean who were not exposed to maternal anemia had similar odds compared with mean FER levels \((OR = 1.03, CI = 0.93–1.14)\) (see Table S10). The relationship between elevated CRP and ASD was stronger among children whose mothers had no history of psychiatric illness (1 SD above the mean: OR = 1.40, CI = 1.16–1.70) compared with those whose mothers did have a history of psychiatric illness (1 SD above the mean: OR = 1.07, CI = 0.84–1.35) (Figure 4H and Table S10).

Sensitivity Analyses

Sensitivity analyses were designed to evaluate whether variation in neonatal APP measurements may have influenced results. Estimates from models in sensitivity analyses were similar to the results of the main analysis (Figure S12).
Figure 1. Heat map showing the mean acute phase protein (APP) z score according to each category of the covariates among 1092 unaffected individuals in the cohort. Solid boxes indicate that the APP is associated with the covariate at $p < .05$. Dashed boxes indicate that the APP is associated with the covariate at $p < .20$. A2M, α-2-macroglobulin; BMI, body mass index; CRP, C-reactive protein; CS, Caesarean section delivery; FA, folic acid; FER, ferritin; FIB, fibrinogen; GA, gestational age; HAP, haptoglobin; Income Q, income quintile; PCT, procalcitonin; Psych., Psychiatric; SAA, serum amyloid A; SAP, serum amyloid P; SGA, size for gestational age; tPA, tissue plasminogen activator; VD, vaginal delivery; yrs, years.
|                          | Unaffected | ASD (n = 924) | p Value<sup>a</sup> | ASD With ID (n = 321) | ASD With ADHD (n = 322) | ASD Only (n = 281) | p Value<sup>b</sup> |
|--------------------------|------------|---------------|---------------------|-----------------------|------------------------|-------------------|-------------------|
| Sex, n (%): Male         | 554 (50.7%)| 710 (76.8%)   | .001                | 237 (73.8%)           | 252 (78.3%)            | 221 (78.6%)       |                   |
|                          | Female     | 538 (49.3%)   | 214 (23.2%)         | 84 (26.2%)            | 70 (21.7%)             | 60 (21.4%)        | <.001             |
| Birth Order, n (%):      |            |               |                     |                       |                        |                   |                   |
| First born               | 443 (40.6%)| 419 (45.3%)   | .030                | 124 (38.6%)           | 158 (49.1%)            | 137 (48.8%)       | .006              |
| Second born              | 385 (35.3%)| 289 (31.3%)   |                     | 114 (35.5%)           | 87 (27.0%)             | 88 (31.3%)        |                   |
| Third or later born      | 205 (18.8%)| 148 (16.0%)   |                     | 60 (18.7%)            | 49 (15.2%)             | 39 (13.9%)        |                   |
| Missing                  | 59 (5.4%)  | 68 (7.4%)     |                     | 23 (7.2%)             | 28 (8.7%)              | 17 (6.0%)         |                   |
| Mode of Delivery, n (%): |            |               |                     |                       |                        |                   |                   |
| Unassisted VD            | 712 (65.2%)| 532 (57.6%)   | .014                | 175 (54.5%)           | 180 (55.9%)            | 177 (63.0%)       | .042              |
| Induced VD               | 63 (5.8%)  | 71 (7.7%)     |                     | 23 (7.2%)             | 30 (9.3%)              | 18 (6.4%)         |                   |
| Assisted VD              | 102 (9.3%) | 90 (9.7%)     |                     | 37 (11.5%)            | 29 (9.0%)              | 24 (8.5%)         |                   |
| Elective CS              | 65 (6.0%)  | 71 (7.7%)     |                     | 26 (8.1%)             | 21 (6.5%)              | 24 (8.5%)         |                   |
| Emergency CS             | 91 (8.3%)  | 92 (10.0%)    |                     | 37 (11.5%)            | 34 (10.6%)             | 21 (7.5%)         |                   |
| Missing                  | 59 (5.4%)  | 68 (7.4%)     |                     | 23 (7.2%)             | 28 (8.7%)              | 17 (6.0%)         |                   |
| GA, n (%):               |            |               |                     |                       |                        |                   |                   |
| <=36 weeks               | 53 (4.9%)  | 63 (6.8%)     | .12                 | 23 (7.2%)             | 21 (6.5%)              | 19 (6.8%)         | .59               |
| 37–41 weeks              | 886 (81.1%)| 723 (78.2%)   |                     | 249 (77.6%)           | 250 (77.6%)            | 224 (79.7%)       |                   |
| 42 weeks                 | 92 (8.4%)  | 69 (7.5%)     |                     | 26 (8.1%)             | 23 (7.1%)              | 20 (7.1%)         |                   |
| Missing                  | 61 (5.6%)  | 69 (7.5%)     |                     | 23 (7.2%)             | 28 (8.7%)              | 18 (6.4%)         |                   |
| Size for GA, n (%):      |            |               | .17                 |                        |                        |                   |                   |
| Normal for GA            | 960 (87.9%)| 772 (83.5%)   |                     | 274 (85.4%)           | 258 (80.1%)            | 240 (85.4%)       | .032              |
| Small for GA             | 29 (2.7%)  | 29 (3.1%)     |                     | 14 (4.4%)             | 10 (3.1%)              | 5 (1.8%)          |                   |
| Large for GA             | 36 (3.3%)  | 43 (4.7%)     |                     | 8 (2.5%)              | 20 (6.2%)              | 15 (5.3%)         |                   |
| Missing                  | 67 (6.1%)  | 80 (8.7%)     |                     | 25 (7.8%)             | 34 (10.6%)             | 21 (7.5%)         |                   |
| Apgar Score at 5 Minutes, n (%): |             |               | .064                |                        |                        |                   |                   |
| 10                       | 861 (78.8%)| 685 (74.1%)   |                     | 219 (68.2%)           | 243 (75.5%)            | 223 (79.4%)       | <.001             |
| 9                        | 110 (10.1%)| 101 (10.9%)   |                     | 35 (10.9%)            | 36 (11.2%)             | 30 (10.7%)        |                   |
| 8                        | 33 (3.0%)  | 36 (3.9%)     |                     | 20 (6.2%)             | 10 (3.1%)              | 6 (2.1%)          |                   |
| <=7                      | 15 (1.4%)  | 25 (2.7%)     |                     | 19 (5.9%)             | 3 (0.9%)               | 3 (1.1%)          |                   |
| Missing                  | 73 (6.7%)  | 77 (8.3%)     |                     | 28 (8.7%)             | 30 (9.3%)              | 19 (6.8%)         |                   |
| Age at Neonatal Blood Sample, Days, n (%): |             |               | .19                 |                        |                        |                   |                   |
| 3                        | 423 (38.7%)| 346 (37.4%)   |                     | 109 (34.0%)           | 127 (39.4%)            | 110 (39.1%)       | .13               |
| 4                        | 357 (32.7%)| 312 (33.8%)   |                     | 106 (33.0%)           | 114 (35.4%)            | 92 (32.7%)        |                   |
| 5                        | 195 (17.9%)| 167 (18.1%)   |                     | 68 (21.2%)            | 51 (15.8%)             | 48 (17.1%)        |                   |
| 6                        | 84 (7.7%)  | 55 (6.0%)     |                     | 17 (5.3%)             | 19 (5.9%)              | 19 (6.8%)         |                   |
| >=7                      | 31 (2.8%)  | 41 (4.4%)     |                     | 21 (6.5%)             | 10 (3.1%)              | 10 (3.6%)         |                   |
| Missing                  | 2 (0.2%)   | 3 (0.3%)      |                     | 0 (0.0%)              | 1 (0.3%)               | 2 (0.7%)          |                   |
| Maternal Age, Years, n (%): |            |               | .011                |                        |                        |                   |                   |
| <25                      | 145 (13.3%)| 116 (12.6%)   |                     | 51 (15.9%)            | 40 (12.4%)             | 25 (8.9%)         | <.001             |
| 25–29                    | 293 (26.8%)| 307 (33.2%)   |                     | 108 (33.6%)           | 118 (36.6%)            | 81 (28.8%)        |                   |
| 30–34                    | 393 (36.0%)| 291 (31.5%)   |                     | 86 (26.8%)            | 102 (31.7%)            | 103 (36.7%)       |                   |
| 35–39                    | 229 (21.0%)| 173 (18.7%)   |                     | 56 (17.4%)            | 56 (17.4%)             | 61 (21.7%)        |                   |
| >=40                     | 32 (2.9%)  | 37 (4.0%)     |                     | 20 (6.2%)             | 6 (1.9%)               | 11 (3.9%)         |                   |
| Maternal Psychiatric History, n (%): |             |               | .001                |                        |                        |                   |                   |
| No                       | 723 (66.2%)| 483 (52.3%)   |                     | 190 (59.2%)           | 145 (45.0%)            | 148 (52.7%)       | <.001             |
| Yes                      | 369 (33.8%)| 441 (47.7%)   | .001                | 131 (40.8%)           | 177 (55.0%)            | 133 (47.3%)       |                   |
## Table 1. Continued

|                                | Unaffected  | ASD  | p Valuea | ASD With ID | ASD With ADHD | ASD Only | p Valueb |
|--------------------------------|-------------|------|----------|-------------|---------------|----------|----------|
|                                | (n = 1092)  | (n = 924) |          | (n = 321)   | (n = 322)      | (n = 281) |          |
| Maternal BMI, n (%)            |             |      |          |             |               |          |          |
| Normal                         | 538 (49.3%) | 364 (39.4%) | <.001    | 130 (40.5%) | 110 (34.2%)   | 124 (44.1%) | <.001    |
| Underweight                    | 22 (2.0%)   | 22 (2.4%)    |          | 8 (2.5%)   | 10 (3.1%)     | 4 (1.4%)   |
| Overweight                     | 184 (16.8%) | 165 (17.9%)  |          | 61 (19.0%) | 62 (19.3%)    | 42 (14.9%) |
| Obese                          | 49 (4.5%)   | 77 (8.3%)    |          | 25 (7.8%)  | 35 (10.9%)    | 17 (6.0%)  |
| Missing                        | 299 (27.4%) | 296 (32.0%)  |          | 97 (30.2%) | 105 (32.6%)   | 94 (33.5%) |
| Mother Born Outside Sweden, n (%) |             |      |          |             |               |          |          |
| No                             | 807 (73.9%) | 647 (70.0%)  | .053     | 168 (52.3%) | 262 (81.4%)   | 217 (77.2%) | <.001    |
| Yes                            | 285 (26.1%) | 277 (30.0%)  |          | 153 (47.7%) | 60 (18.6%)    | 64 (22.8%) |
| Family Income Quintile, n (%)  |             |      |          |             |               |          |          |
| First, lowest                  | 154 (14.1%) | 122 (13.2%)  | .003     | 64 (19.9%)  | 29 (9.0%)     | 29 (10.3%) |
| Second                         | 234 (21.4%) | 240 (26.0%)  |          | 92 (28.7%)  | 82 (25.5%)    | 66 (22.1%) |
| Third                          | 240 (22.0%) | 214 (23.2%)  |          | 68 (21.2%)  | 84 (26.1%)    | 62 (22.1%) |
| Fourth                         | 229 (21.0%) | 205 (22.2%)  |          | 64 (19.9%)  | 82 (25.5%)    | 59 (21.0%) |
| Fifth                          | 235 (21.5%) | 141 (15.3%)  |          | 32 (10.0%)  | 45 (14.0%)    | 64 (22.8%) |
| Missing                        | 0 (0.0%)    | 2 (0.2%)     |          | 1 (0.3%)   | 0 (0.0%)      | 1 (0.4%)   |
| Maternal Hospitalization for Infection During Third Trimester, n (%) |             |      |          |             |               |          |          |
| No                             | 1000 (91.6%)| 811 (87.8%)  | .024     | 279 (86.9%) | 283 (87.9%)   | 249 (88.6%) | .050     |
| Yes                            | 31 (2.8%)   | 43 (4.7%)    |          | 19 (5.9%)  | 11 (3.4%)     | 13 (4.6%)  |
| Maternal Anemia During Pregnancy, n (%) |             |      |          |             |               |          |          |
| No                             | 1030 (94.3%)| 866 (93.7%)  | .57      | 298 (92.8%) | 302 (93.8%)   | 266 (94.7%) | .75      |
| Yes                            | 62 (5.7%)   | 58 (6.3%)    |          | 23 (7.2%)  | 20 (6.2%)     | 15 (5.3%)  |
| Maternal Dietary Supplementation, n (%) |             |      |          |             |               |          |          |
| None                           | 433 (39.7%) | 383 (41.5%)  | .23      | 119 (37.1%) | 137 (42.5%)   | 127 (45.2%) | .39      |
| Folic acid only                | 10 (0.9%)   | 14 (1.5%)    |          | 4 (1.2%)   | 6 (1.9%)      | 4 (1.4%)   |
| Iron only                      | 336 (30.8%) | 294 (31.8%)  |          | 109 (34.0%) | 102 (31.7%)   | 83 (29.5%)  |
| Multivitamin                   | 313 (28.7%) | 233 (25.2%)  |          | 89 (27.7%) | 77 (23.9%)    | 67 (23.8%)  |
| Proteins Measured in NDBSs, Median (IQR)c |             |      |          |             |               |          |          |
| A2M, ng/mL                     | 533.0 (252.3, 911.3) | 563.3 (266.0, 1052.0) | <.001 | 574.4 (249.7, 1051.9) | 596.0 (307.1, 1123.1) | 513.6 (251.4, 1004.0) | .037 |
| CRP, ng/mL                     | 1.3 (0.6, 3.1) | 1.6 (0.7, 4.1) | <.001 | 1.5 (0.7, 4.1) | 1.7 (0.6, 4.1) | 1.7 (0.7, 4.2) | .006 |
| FER, ng/mL                     | 1.8 (0.7, 3.3) | 1.7 (0.6, 3.9) | .27  | 1.7 (5.9.3.6) | 2.2 (0.8, 4.2) | 1.5 (0.5, 4.0) | .067 |
| FIB, ng/mL                     | 27.1 (16.5, 53.1) | 30.6 (16.5, 60.6) | .074 | 30.7 (16.9, 63.1) | 32.1 (17.5, 61.1) | 27.1 (15.4, 55.9) | .098 |
| HAP, ng/mL                     | 27.5 (9.3, 76.8) | 29.9 (11.0, 85.4) | .036 | 27.6 (10.6, 78.2) | 33.7 (12.1, 87.9) | 29.1 (9.8, 88.5) | .10   |
| PCT, pg/mL                     | 10.9 (7.4, 15.6) | 11.7 (7.5, 17.1) | .013 | 11.0 (7.7, 16.9) | 12.0 (7.7, 17.9) | 11.1 (7.0, 17.6) | .038 |
| SAA, ng/mL                     | 1.3 (0.8, 2.2) | 1.4 (0.9, 2.5) | .019 | 1.4 (0.8, 2.7) | 1.5 (0.9, 2.7) | 1.3 (0.8, 2.2) | .030 |
| SAP, ng/mL                     | 10.5 (6.8, 15.5) | 11.5 (7.5, 17.7) | <.001 | 11.1 (8.1, 18.4) | 12.0 (7.5, 17.5) | 11.2 (7.0, 16.9) | .007 |
| tPA, pg/mL                     | 9.6 (6.2, 14.7) | 10.6 (6.5, 15.9) | .010 | 9.9 (6.6, 15.0) | 11.5 (7.2, 17.4) | 10.1 (6.3, 16.1) | .004 |
| Total protein, mg/mL           | 1.3 (1.0, 1.7) | 1.3 (1.0, 1.7) | .76  | 1.2 (0.9, 1.6) | 1.3 (1.0, 1.7) | 1.3 (1.0, 1.7) | .19   |

ADHD, attention-deficit/hyperactivity disorder; APP, acute phase protein; ASD, autism spectrum disorder; A2M, α-2-macroglobulin; BMI, body mass index; CRP, C-reactive protein; CS, Cesarean section delivery; FER, ferritin; FIB, fibrinogen; GA, gestational age; HAP, haptoglobin; ID, intellectual disability; IQR, interquartile range; NDBS, neonatal dried blood spot; PCT, procalcitonin; SAA, serum amyloid A; SAP, serum amyloid P; tPA, tissue plasminogen activator; VD, vaginal delivery.

*Pearson’s χ² test was used for categorical variables, comparing the frequency distributions among unaffected individuals with the distributions among all individuals affected by ASD. Kruskal–Wallis tests were used for continuous variables because the distributions of the APP concentrations were strongly skewed.*

*Pearson’s χ² test was used for categorical variables, comparing the frequency distributions among unaffected individuals with the distributions among the stratified ASD outcome groups. Kruskal–Wallis tests were used for continuous variables because the distributions of the APP concentrations were strongly skewed.*

*The IQR data are the 25th and 75th percentiles.*
Our results indicate that of the neonatal innate immune markers that we studied, only CRP was associated with risk for ASD, with levels of CRP above the population mean associated with increased odds of ASD even after accounting for a range of potential confounders. We observed significant interactions between environmental exposures that could plausibly influence the neonatal innate immune system and levels of APPs. With the exception of PCT, higher levels of these markers also tended to be associated with lower risk of ASD when comparing ASD cases with their unaffected siblings.

**DISCUSSION**

Our results indicate that of the neonatal innate immune markers that we studied, only CRP was associated with risk for ASD, with levels of CRP above the population mean associated with increased odds of ASD even after accounting for a range of potential confounders. We observed significant interactions between environmental exposures that could plausibly influence the neonatal innate immune system and levels of APPs. With the exception of PCT, higher levels of these markers also tended to be associated with lower risk of ASD when comparing ASD cases with their unaffected siblings.
Strengths and Weaknesses

The setting of the study within a universal healthcare system with developmental screening provided to families free of charge increases the likelihood that ASD cases are identified using validated ascertainment methods (25). We included two comparison groups in the study. Unaffected siblings share aspects of the early-life environment with ASD cases as well as on average 50% of their common genetic variants. The large population-based comparison group of unrelated individuals randomly sampled from the larger cohort included in the study is also a strength, allowing us to study the relationship between a large number of potential confounders and neonatal levels of APPs. While the use of multiple markers of the neonatal innate immune response and information on many

Figure 3. (A–I) The relationship between acute phase protein (APP) and odds of autism spectrum disorders (ASD) when comparing 203 ASD cases with their matched unaffected siblings. Each panel displays the odds of ASD according to APP z score, flexibly fit using restricted cubic spline models with 3 knots and a z score = 0 as the referent. The dashed line represents the unadjusted estimate of the relationship between each APP and odds of ASD. The solid line represents the model fully adjusted for children’s birth order, sex, and the total protein concentration of the sample. The gray bands represent the 95% confidence interval for the fully adjusted model. The p values are shown for a Wald test with a null hypothesis that all APP spline terms were jointly equal to zero as a test of whether each APP was generally associated with the outcome. The p values are shown for spline terms in both the unadjusted models (p) and adjusted models (p_adj). Adj., adjusted; A2M, α-2-macroglobulin; CRP, C-reactive protein; FER, ferritin; FIB, fibrinogen; HAP, haptoglobin; PCT, procalcitonin; SAA, serum amyloid A; SAP, serum amyloid P; tPA, tissue plasminogen activator.
Figure 4. Interaction between acute phase protein (APP) and other risk factors. Odds ratios for autism spectrum disorder (ASD) over the range of APP are shown separately for those who were not exposed to maternal hospitalization for infection during late pregnancy (third trimester) compared with those who were (A–E), those who were not exposed to maternal anemia compared with those who were (F, G), and those whose mothers did not have a history of psychiatric (Psych.) diagnoses compared with those whose mothers did (H). Solid lines represent the odds ratio estimate for each group, and dashed lines represent the 95% confidence interval for the fully adjusted model. The $p$ values for interaction (a likelihood ratio test comparing a model with interaction terms with a model without interaction terms) are shown. Adj., adjusted; A2M, α2-macroglobulin; CRP, C-reactive protein; FER, ferritin; FIB, fibrinogen; PCT, procalcitonin; SAP, serum amyloid P; tPA, tissue plasminogen activator.
Neonatal Acute Phase Proteins and ASD

Appropriate risk factors for ASD is a strength of this study, there is still a possibility of residual confounding, and the analysis of such markers results in multiple statistical comparisons for which we have not corrected.

The use of APPs as markers of the neonatal innate immune response is also a strength of this study. Previous studies have measured cytokines and chemokines, which is technically challenging (9–12,37). Cytokines and chemokines have short half-lives relative to APPs (38,39). Therefore, it is difficult to interpret the cytokine profile with little information about the environmental factors that may influence such levels, with some studies indicating opposing effects of the same cytokines (10,11,37). While we have considered interaction between neonatal APP levels and putative environmental risk factors, it is also possible to consider neonatal APPs as mediators of the relationship between such environmental exposures and risk of ASD. Such an analysis was beyond the scope of this study, although future studies will evaluate evidence of mediation.

Interpretation

APPs are a diverse group of proteins that collectively recognize and opsonize invading pathogens and cellular debris, regulate blood viscosity and clotting, and sequester nutrients (e.g., iron) from pathogens (23). Moreover, many of the APPs play key roles in the regulation of the innate and adaptive immune responses and are required for resolution of an acute inflammatory response in experimental models (40–43). Synthesis of APPs occurs primarily in the liver, is influenced by genetic background, and increases by orders of magnitude in response to IL-6 and other inflammatory cytokines (22,44–46). Fetal liver produces APPs, with expression detectable from early in the second trimester (47). APPs are not believed to cross the placenta (48–51). However, transplacental immune regulation and iron transport may lead to correlation between maternal APPs and neonatal APPs, and strong correlations have been noted in certain circumstances. For example, strong correlations were noted for CRP (but not PCT) levels between neonates affected by intrauterine growth restriction and their mothers (52) and in FER levels between neonates and their mothers in populations affected by iron deficiency anemia (53).

However, in studies where maternal–child pairs have not been selected on the basis of such pregnancy complications, levels of these APPs do not appear to correlate between maternal serum and cord blood samples (48–51,54,55). In studies of paired mothers and neonates, cord blood levels of APPs were associated with DNA methylation changes in genes relevant to inflammation and angiogenesis in the neonate, while maternal levels measured at multiple points over the course of pregnancy were not associated with DNA methylation in the neonate, implying that fetal levels of CRP were of particular relevance to epigenetic programming in comparison with maternal levels (55). We consider APPs in NDBSSs as specific indicators of the neonatal innate immune status.

CRP is a pattern recognition molecule, opsonin, and activator of the complement pathway (42). Of the neonatal APPs studied, CRP had the strongest association with risk of ASD in the case-control comparison, displaying a U-shaped association with odds of ASD, with the strongest association between increasing neonatal CRP levels above the mean and higher odds of ASD. While the relationship of CRP below the mean and odds of ASD was weaker in comparison with that above the mean, the U-shaped relationship of this APP (and the similar tendency observed among other neonatal APPs in this study) implies that high levels of APPs may indicate ongoing inflammation or immune responses potentially detrimental to neurodevelopment but that levels that are too low may indicate an inability to respond appropriately to the environment that may also be detrimental to neurodevelopment. While the shape of the association between CRP and odds of ASD in the sibling comparison was similar, the association was less apparent with considerably wider CIs in the sibling analysis, possibly reflecting shared common genetic variation in innate immune signaling and risk of ASD (8). We also observed a weaker relationship between neonatal CRP and risk of ASD among those whose mothers had a previous history of psychiatric illness in the case-control comparison, with a significant interaction detected between maternal psychiatric history and levels of CRP. Environmental exposures that increase CRP production may be more important to the etiology of ASD among individuals with lower genetic liability for the disorders. Moreover, both positive and negative genetic correlations between psychiatric disorders (e.g., bipolar disorder, schizophrenia) and CRP levels have been reported (56,57). Thus, levels of CRP in children born to mothers with a high genetic risk for psychiatric illness may exhibit larger variation than children born to unaffected mothers.

Levels of multiple APPs were elevated among neonates whose mothers were hospitalized for infections during late pregnancy, although these markers were not elevated to the same extent among children later diagnosed with ASD. In interaction analyses, higher levels of A2M, FER, FIB, PCT, and tPA were associated with lower risk of ASD among those exposed to maternal infections during late pregnancy. The actions of the APPs indicated here are diverse. A2M, FIB, and tPA regulate blood coagulation, while FER binds to iron to limit the growth of pathogens. The physiological function of PCT is poorly defined, but its rapid rise in response to bacterial infections makes PCT useful when diagnosing sepsis, particularly in neonates (58). While the direct antimicrobial functions of the APPs may help to protect the developing nervous system from harm, the APPs have a wider role in the regulation of the innate immune system. In particular, a robust acute phase response is necessary in experimental studies for the appropriate resolution of the inflammatory response (43). We previously reported that low levels of APPs in combination with maternal exposure to cytomegalovirus and Toxoplasma gondii were associated with greater risk for nonaffective psychosis compared with those with higher APP levels (59). Taken together with the results of the current study, this suggests that the degree of protection a neonate’s innate immune system offers against environmental insults may be important in modulating the influence of such insults on neurodevelopmental processes. Of the neonatal APPs that interacted with infection in terms of risk of ASD, higher levels of all but PCT were also associated with lower odds of ASD in the sibling comparison study. In other words, we observed that within matched pairs, the sibling who produced higher levels of these APPs in response to a presumably similar early-life environment was less likely to develop ASD.

While FER concentrations rise to withhold iron from pathogens during the acute phase response (23), in the absence of
this response. FER reflects the body’s store of iron in both adults and neonates (60). We recently reported that maternal diagnosis with anemia was associated with ASD, ID, and ADHD in offspring (30). The results of that study indicated that the increased risk associated with maternal anemia may be limited to cases where anemia was severe and long-lasting. Here we report that among individuals whose mothers were affected by anemia during pregnancy, higher levels of FER were associated with lower odds of ASD. These results suggest that among mothers affected by anemia, an adequate iron store in the fetus may be protective with regard to risk of ASD. We also observed a similar protective association with increasing levels of FER when comparing ASD cases with their unaffected siblings, in line with sibling comparisons of exposure to maternal anemia (30).

Numerous studies have posited adverse effects of intra-uterine inflammatory signals to explain the associations consistently observed between elevated maternal BMI and risk of ASD (61–64). Here we report that maternal BMI was not associated with APPs in cases or controls measured only a few days after birth, raising the question of whether this particular mechanism to link maternal BMI to risk of ASD is relevant, at least during later pregnancy.

Conclusions

Indicators of the neonatal innate immune response are associated with risk of ASD, although the nature of these associations varies considerably with factors in the perinatal environment and the genetic background of the comparison group. Our findings suggest that it is not the levels of early-life inflammatory or regulatory markers per se that may influence ASD risk. Rather, the strength of those signals in the genetic and environmental contexts of the developing nervous system must be considered. Correspondingly, understanding, on a molecular level, individuals’ ability to resolve environmental insults may help to identify those who are particularly susceptible to such effects.

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