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Neonatal levels of acute phase proteins and risk of autism spectrum disorders

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Abstract

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

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

Results: C reactive protein was consistently associated with odds of ASD in case-control comparisons, with higher odds associated with the highest quintile compared to the middle quintile (OR 1.50, 95% CI 1.10 – 2.04) in adjusted analyses. In contrast, the lowest quintiles of alpha-2-macroglobulin (3.71, 1.21 – 11.33), ferritin (4.20, 1.40 – 12.65), and Serum Amyloid P (3.05, 1.16 – 8.01) were associated with odds of ASD in the matched sibling comparison. Neonatal acute phase proteins varied with perinatal environmental factors and maternal/fetal phenotypes. Significant interactions in terms of risk for ASD were observed between neonatal acute phase proteins and maternal infection in late pregnancy, maternal anemia, and maternal psychiatric history.

Conclusions: Indicators of the neonatal innate immune response are associated with risk for ASD, though 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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Introduction

Perturbations in immune signaling pathways are hypothesized to influence neurodevelopment, as 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 ASD compared to controls (3, 4), though 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).

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 already *in utero* (15-19). Acute phase proteins (APP) are key components of the innate immune response. We investigated the associations between neonatal APP and later diagnosis of ASD, and how environmental exposures (e.g., maternal infections) may affect such associations. We also compared individuals with ASD to their unaffected siblings to understand the role of shared familial factors (e.g., genetic background).

Material and Methods

Study population

The Stockholm Youth Cohort (SYC) is a register-based cohort of all children aged 0-17 years living in Stockholm County (20, 21). Of the individuals born 1996-2000, we selected all individuals diagnosed with ASD before 12-31-2011, 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 12-31-2016. Ethical approval was obtained the Stockholm regional review board (DNR 2010/1185-31/5). Individual consent was not required for this anonymized register-based study.

ASD ascertainment

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We implemented a case-finding procedure covering all pathways to child and adolescent psychiatric care and habilitation services in Stockholm County (20, 21). Ascertainment of ASD, ID, and ADHD in the SYC is described in Table S1. The outcome of ASD was stratified by presence of co-occurring ADHD and ID: ASD with ID, ASD with ADHD, and ASD without ID or ADHD (ASD only). Individuals diagnosed with both co-occurring ID and ADHD (n=111) were included in the ASD with ID group.

Laboratory analysis

Neonatal dried blood spots (NDBS) were collected from the national biobank at Karolinska University Hospital, Solna. Blood spots were originally collected at approximately 3-5 days after birth (mean=4.1 days, SD=1.3). We selected a smaller sample (born 1998-2000) of the NDBS collected for analysis of immune markers. A sample (12.5 %) of those born 1996-1997 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).

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 two hours. Samples for the case-cohort comparison were eluted in three batches over three subsequent days and stored at -80°C until analysis. For the sibling comparison, a new punch was taken for each affected sibling and their 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 diluted 1:4 for analysis of α-2 macroglobulin (A2M), C reactive protein (CRP), haptoglobin (HAP), and serum amyloid P (SAP) using a premixed, multiplex panel and the Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). Coefficients of variation for manufacturer-provided controls varied by analyte from 9.0% to 20.8% (Table S2). The percentage of samples below the lower limit of quantification (LLOQ) varied by analyte from 0.00% to 0.45% (Table S2), and were assigned the value of LLOQ/$\sqrt{2}$.

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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 (22), country of origin (23), BMI (24), psychiatric history (25), hospitalization for infection during late pregnancy (3rd trimester) (15), anemia (26), and supplement use during pregnancy (27); parental income at the time of birth and education level (highest of mother or father) (28); children's birth order, sex, gestational age at birth (29), size for gestational age (30), mode of delivery (31), and Apgar score (32).

Statistical analysis

We $\log_2$-transformed the APP values as 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 cut-offs.

We examined the association of each covariate with the 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 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; 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 to 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.

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We conducted sensitivity analyses to determine whether variation in APP measurements may have influenced results. First, we additionally 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 multi-level modeling to account for both plate and batch.

Results

Association of APP with covariates

All APPs were correlated with each other and with total protein yield ($p<0.05$) (Figure S3). Other than parental education, maternal BMI, and maternal psychiatric history, each covariate was also associated with at least one APP ($p<0.05$; Figure 1; Table S4), though the patterns of association varied for each APP. For example, CRP was the only APP associated with sex, with a higher z-score for males (0.03) than females (-0.16; $p=0.001$). The patterns of association between APP and covariates differed among cases versus controls (Figure S4, Table S5). For example, levels of most APP, particularly FER, FIB, PCT, and tPA, were lower among cases born to mothers who had been hospitalized for infection during the third trimester compared to levels among cases whose mothers had not been hospitalized for infections and levels of FER were lower among cases born to mothers diagnosed with anemia (Figure S4), in contrast to the patterns observed among controls (Figure 1).

Association of APP with odds of ASD

Median levels of all APP except FER were higher in ASD cases compared to controls (Table 1), though the overall distributions of APP 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 evidence for non-linear associations for CRP, FIB, and PCT (Table S6). The relationships between FIB, PCT, and SAA and ASD were attenuated in the fully adjusted models. Similar patterns of association were observed when we considered APP as a categorical variable, with increased odds of ASD associated with the highest quintiles of CRP (OR 1.50, 95% CI 1.10 – 2.04) and PCT (1.35, 0.99 – 1.85) compared to the middle quintile in adjusted models (Figure S6A).

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Overall the patterns of association with APP were similar among the stratified outcomes. The odds associated with the highest quintile of CRP were greatest for ASD only (1.80, 1.15–2.82) compared to ASD with co-occurring ID (1.29, 0.83 – 2.01) or with co-occurring ADHD (1.34, 0.85–2.11), with similar results in continuous analyses (Figures S7–10).

APP and Odds of ASD Among Matched Sibling Pairs

In contrast to the case-control comparison, we found that median levels of all APP except CRP, HAP, and PCT were lower in ASD cases compared to their unaffected siblings (Table S3), with distinct distributions of particularly A2M, SAP, and FER among ASD cases compared to their unaffected siblings (Figure S5). In matched regression analyses, we observed decreasing odds with increasing levels of A2M, FER, SAA, SAP, and tPA (Figure 3, Figure S6B). Associations with SAA, SAP, and tPA were attenuated in adjusted models, though the pattern associated with A2M and FER persisted (Figure 3). Compared to the middle quintile, the lowest quintile of A2M (3.71, 1.21 – 11.33), FER (4.20, 1.40 – 12.65), and SAP 3.05 (1.16 – 8.01) were associated with increased odds of ASD in the matched sibling comparison (Figure S6B).

Interaction Between APP and Other Risk Factors for ASD

Since many of the covariates we studied (e.g., maternal infection, mode of delivery) represent environmental influences that may influence 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 APP and a number of other risk factors for ASD in our study (Figure 4, Figure S11, Table S7). We observed interactions between maternal hospitalization for infections during the third trimester and neonatal levels of APP (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 late in pregnancy (Figure 4A-E), in contrast to those whose mothers were not hospitalized for infection. 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). The relationship between elevated CRP and ASD was stronger among children whose

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mother had no history of psychiatric illness compared to those whose mother did have a psychiatric history (Figure 4H).

Sensitivity analyses

Sensitivity analyses were designed to evaluate whether variation in APP measurements may have influenced results. Estimates from models in sensitivity analyses were similar to the results of the main analysis (Figure S12).

Discussion

Our results indicate that of the 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 for potential confounders. We observed significant interactions between environmental exposures that could plausibly influence the neonatal innate immune system and levels of APP. With the exception of PCT, higher levels of these markers also tended to be associated with lower risk of ASD when comparing ASD cases to 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 cases of ASD are identified, using validated ascertainment methods (21). We included two comparison groups in the study. Unaffected siblings share aspects of the early life environment with ASD-affected siblings 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 APP. While the use of multiple markers of the innate immune response and information on many putative risk factors for ASD is a strength of this study, there is still a possibility for residual confounding and the analysis of such markers results in multiple statistical comparisons.

The use of APP 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,

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33). Cytokines and chemokines have short half-lives relative to acute phase proteins (34, 35). 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, 33).

Interpretation

APP 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 (36). Moreover, many of the APP 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 (37-40). Expression of APP 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 (41-44). Fetal liver produces APP, with expression detectable from early in the second trimester (45). APP are not believed to cross the placenta (46). We consider APP in NDBS as specific indicators of the neonatal innate immune status.

CRP is a pattern recognition molecule, opsonin, and activator of the complement pathway (39). Of the APP studied, CRP had the strongest association with risk of ASD in the case-control comparison, displaying a U-shaped association with odds of ASD. 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 confidence intervals 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 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 stimuli that increase CRP production may be more important to the etiology of ASD among individuals with lower genetic liability for the disorder. Moreover, both positive and negative genetic correlations between psychiatric disorders (e.g., bipolar disorder, schizophrenia) and CRP levels have been reported (47, 48). Thus, levels of CRP in children born to mothers with a high genetic risk for psychiatric illness may exhibit larger variation than children to unaffected mothers.

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Levels of multiple APP were elevated among neonates whose mothers were hospitalized for infections in 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 in late pregnancy. The actions of the APP 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 (49). We previously reported that low levels of APP in combination with maternal exposure to cytomegalovirus and *Toxoplasma gondii* were associated with greater risk for non-affective psychosis compared to those with higher APP levels (50). Taken together with the results of the current study, this suggests that the degree of protection a neonate's innate immune system offers against an environmental insult may be important in modulating the influence of such insults on neurodevelopmental processes. Of the APP that interacted with infection in terms of risk for ASD, higher levels of all but PCT were also associated with lower odds of ASD in the sibling comparison study. In other words, we observe that, within matched pairs, the sibling who produced higher levels of these APP 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 (36), in the absence of this response, FER reflects the body's store of iron in both adults and neonates (51). We recently reported that maternal diagnosis with anemia was associated with ASD, ID, and ADHD in offspring (26). 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 evidence that among individuals whose mothers were affected by anemia during pregnancy, higher levels of ferritin 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 ferritin when comparing ASD cases to their unaffected siblings, in line with sibling comparisons of exposure to maternal anemia (26).

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Numerous studies have posited adverse effects of intrauterine inflammatory signals to explain the associations consistently observed between elevated maternal BMI and risk for ASD (52-55). 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 in later pregnancy.

Conclusion

Together, our findings suggest that it is not the levels of inflammatory or regulatory markers *per se* that may influence ASD risk. Rather, the strength of those signals in the genetic and environmental context of the developing nervous system must be considered. Correspondingly, understanding on a molecular level the individual's ability to resolve environmental insults may help to identify those that are particularly susceptible to such insults.

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Disclosures

The authors declare no conflicts of interest.

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References

1. Boulanger LM (2009): Immune proteins in brain development and synaptic plasticity. *Neuron*. 64:93-109.

2. Deverman BE, Patterson PH (2009): Cytokines and CNS development. *Neuron*. 64:61-78.

3. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ (2015): Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Mol Psychiatry*. 20:440-446.

4. Saghazadeh A, Ataeinia B, Keynejad K, Abdolalizadeh A, Hirbod-Mobarakeh A, Rezaei N (2019): A meta-analysis of pro-inflammatory cytokines in autism spectrum disorders: Effects of age, gender, and latitude. *J Psychiatr Res*. 115:90-102.

5. Ashwood P, Wills S, Van de Water J (2006): The immune response in autism: a new frontier for autism research. *J Leukoc Biol*. 80:1-15.

6. Davignon MN, Qian Y, Massolo M, Croen LA (2018): Psychiatric and Medical Conditions in Transition-Aged Individuals With ASD. *Pediatrics*. 141:S335-S345.

7. Alexeeff SE, Yau V, Qian Y, Davignon M, Lynch F, Crawford P, et al. (2017): Medical Conditions in the First Years of Life Associated with Future Diagnosis of ASD in Children. *J Autism Dev Disord*. 47:2067-2079.

8. Nazeen S, Palmer NP, Berger B, Kohane IS (2016): Integrative analysis of genetic data sets reveals a shared innate immune component in autism spectrum disorder and its co-morbidities. *Genome Biol*. 17:228.

9. Abdallah MW, Larsen N, Grove J, Bonefeld-Jorgensen EC, Norgaard-Pedersen B, Hougaard DM, et al. (2013): Neonatal chemokine levels and risk of autism spectrum disorders: findings from a Danish historic birth cohort follow-up study. *Cytokine*. 61:370-376.

10. Abdallah MW, Larsen N, Mortensen EL, Atladottir HO, Norgaard-Pedersen B, Bonefeld-Jorgensen EC, et al. (2012): Neonatal levels of cytokines and risk of autism spectrum disorders: an exploratory register-based historic birth cohort study utilizing the Danish Newborn Screening Biobank. *Journal of neuroimmunology*. 252:75-82.

11. Krakowiak P, Goines PE, Tancredi DJ, Ashwood P, Hansen RL, Hertz-Picciotto I, et al. (2015): Neonatal Cytokine Profiles Associated with Autism Spectrum Disorder. *Biol Psychiatry*.

12. Zerbo O, Yoshida C, Grether JK, Van de Water J, Ashwood P, Delorenze GN, et al. (2014): Neonatal cytokines and chemokines and risk of Autism Spectrum Disorder: the Early Markers for Autism (EMA) study: a case-control study. *J Neuroinflammation*. 11:113.

13. Abdallah MW, Larsen N, Grove J, Norgaard-Pedersen B, Thorsen P, Mortensen EL, et al. (2011): Amniotic fluid chemokines and autism spectrum disorders: an exploratory study utilizing a Danish Historic Birth Cohort. *Brain Behav Immun*. 26:170-176.

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14. Abdallah MW, Larsen N, Grove J, Norgaard-Pedersen B, Thorsen P, Mortensen EL, et al. (2011): Amniotic fluid inflammatory cytokines: Potential markers of immunologic dysfunction in autism spectrum disorders. *World J Biol Psychiatry*.

15. Lee BK, Magnusson C, Gardner RM, Blomstrom A, Newschaffer CJ, Burstyn I, et al. (2015): Maternal hospitalization with infection during pregnancy and risk of autism spectrum disorders. *Brain Behav Immun*. 44:100-105.

16. Brown AS, Sourander A, Hinkka-Yli-Salomaki S, McKeague IW, Sundvall J, Surcel HM (2014): Elevated maternal C-reactive protein and autism in a national birth cohort. *Mol Psychiatry*. 19:259-264.

17. Jones KL, Croen LA, Yoshida CK, Heuer L, Hansen R, Zerbo O, et al. (2016): Autism with intellectual disability is associated with increased levels of maternal cytokines and chemokines during gestation. *Mol Psychiatry*.

18. Zerbo O, Traglia M, Yoshida C, Heuer LS, Ashwood P, Delorenze GN, et al. (2016): Maternal mid-pregnancy C-reactive protein and risk of autism spectrum disorders: the early markers for autism study. *Translational psychiatry*. 6:e783.

19. Al-Haddad BJS, Jacobsson B, Chabra S, Modzelewska D, Olson EM, Bernier R, et al. (2019): Long-term Risk of Neuropsychiatric Disease After Exposure to Infection In Utero. *JAMA Psychiatry*. 76:594-602.

20. Idring S, Lundberg M, Sturm H, Dalman C, Gumpert C, Rai D, et al. (2015): Changes in prevalence of autism spectrum disorders in 2001-2011: findings from the Stockholm youth cohort. *J Autism Dev Disord*. 45:1766-1773.

21. Idring S, Rai D, Dal H, Dalman C, Sturm H, Zander E, et al. (2012): Autism spectrum disorders in the Stockholm Youth Cohort: design, prevalence and validity. *PloS one*. 7:e41280.

22. Idring S, Magnusson C, Lundberg M, Ek M, Rai D, Svensson AC, et al. (2014): Parental age and the risk of autism spectrum disorders: findings from a Swedish population-based cohort. *Int J Epidemiol*. 43:107-115.

23. Magnusson C, Rai D, Goodman A, Lundberg M, Idring S, Svensson A, et al. (2012): Migration and autism spectrum disorder: population-based study. *Br J Psychiatry*. 201:109-115.

24. Gardner RM, Lee BK, Magnusson C, Rai D, Frisell T, Karlsson H, et al. (2015): Maternal body mass index during early pregnancy, gestational weight gain, and risk of autism spectrum disorders: Results from a Swedish total population and discordant sibling study. *Int J Epidemiol*. 44:870-883.

25. Xie S, Karlsson H, Dalman C, Widman L, Rai D, Gardner RM, et al. (2019): Family History of Mental and Neurological Disorders and Risk of Autism. *JAMA Netw Open*. 2:e190154.

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26. Wiegersma AM, Dalman C, Lee BK, Karlsson H, Gardner RM (2019): Association of Prenatal Maternal Anemia With Neurodevelopmental Disorders. *JAMA Psychiatry*.1-12.

27. DeVilbiss EA, Magnusson C, Gardner RM, Rai D, Newschaffer CJ, Lyall K, et al. (2017): Antenatal nutritional supplementation and autism spectrum disorders in the Stockholm youth cohort: population based cohort study. *BMJ (Clinical research ed)*. 359:j4273.

28. Rai D, Lewis G, Lundberg M, Araya R, Svensson A, Dalman C, et al. (2012): Parental socioeconomic status and risk of offspring autism spectrum disorders in a Swedish population-based study. *J Am Acad Child Adolesc Psychiatry*. 51:467-476 e466.

29. Xie S, Heuvelman H, Magnusson C, Rai D, Lyall K, Newschaffer CJ, et al. (2017): Prevalence of Autism Spectrum Disorders with and without Intellectual Disability by Gestational Age at Birth in the Stockholm Youth Cohort: a Register Linkage Study. *Paediatr Perinat Epidemiol*. 31:586-594.

30. Abel KM, Dalman C, Svensson AC, Susser E, Dal H, Idring S, et al. (2013): Deviance in fetal growth and risk of autism spectrum disorder. *The American journal of psychiatry*. 170:391-398.

31. Curran EA, Dalman C, Kearney PM, Kenny LC, Cryan JF, Dinan TG, et al. (2015): Association Between Obstetric Mode of Delivery and Autism Spectrum Disorder: A Population-Based Sibling Design Study. *JAMA Psychiatry*. 72:935-942.

32. Modabbernia A, Sandin S, Gross R, Leonard H, Gissler M, Parner ET, et al. (2019): Apgar score and risk of autism. *European journal of epidemiology*. 34:105-114.

33. Heuer LS, Croen LA, Jones KL, Yoshida CK, Hansen RL, Yolken R, et al. (2019): An Exploratory Examination of Neonatal Cytokines and Chemokines as Predictors of Autism Risk: The Early Markers for Autism Study. *Biol Psychiatry*. 86:255-264.

34. Kuribayashi T (2018): Elimination half-lives of interleukin-6 and cytokine-induced neutrophil chemoattractant-1 synthesized in response to inflammatory stimulation in rats. *Lab Anim Res*. 34:80-83.

35. Dandona P, Nix D, Wilson MF, Aljada A, Love J, Assicot M, et al. (1994): Procalcitonin increase after endotoxin injection in normal subjects. *J Clin Endocrinol Metab*. 79:1605-1608.

36. Gabay C, Kushner I (1999): Acute-phase proteins and other systemic responses to inflammation. *The New England journal of medicine*. 340:448-454.

37. Cox N, Pilling D, Gomer RH (2014): Serum amyloid P: a systemic regulator of the innate immune response. *J Leukoc Biol*. 96:739-743.

38. Sack GH, Jr. (2018): Serum amyloid A - a review. *Mol Med*. 24:46.

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39. Volanakis JE (2001): Human C-reactive protein: expression, structure, and function. *Mol Immunol*. 38:189-197.

40. Sander LE, Sackett SD, Dierssen U, Beraza N, Linke RP, Muller M, et al. (2010): Hepatic acute-phase proteins control innate immune responses during infection by promoting myeloid-derived suppressor cell function. *The Journal of experimental medicine*. 207:1453-1464.

41. Heinrich PC, Castell JV, Andus T (1990): Interleukin-6 and the acute phase response. *The Biochemical journal*. 265:621-636.

42. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, et al. (2009): Genetic Loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA*. 302:37-48.

43. Huang J, Huffman JE, Yamakuchi M, Trompet S, Asselbergs FW, Sabater-Lleal M, et al. (2014): Genome-wide association study for circulating tissue plasminogen activator levels and functional follow-up implicates endothelial STXBP5 and STX2. *Arteriosclerosis, thrombosis, and vascular biology*. 34:1093-1101.

44. Langlois MR, Delanghe JR (1996): Biological and clinical significance of haptoglobin polymorphism in humans. *Clin Chem*. 42:1589-1600.

45. Bauer J, Lengyel G, Thung SN, Jonas U, Gerok W, Acs G (1991): Human fetal hepatocytes respond to inflammatory mediators and excrete bile. *Hepatology*. 13:1131-1141.

46. de Villiers WJ, Louw JP, Strachan AF, Etsebeth SM, Shephard EG, de Beer FC (1990): C-reactive protein and serum amyloid A protein in pregnancy and labour. *British journal of obstetrics and gynaecology*. 97:725-730.

47. Ligthart S, Vaez A, Vosa U, Stathopoulou MG, de Vries PS, Prins BP, et al. (2018): Genome Analyses of >200,000 Individuals Identify 58 Loci for Chronic Inflammation and Highlight Pathways that Link Inflammation and Complex Disorders. *Am J Hum Genet*. 103:691-706.

48. Prins BP, Abbasi A, Wong A, Vaez A, Nolte I, Franceschini N, et al. (2016): Investigating the Causal Relationship of C-Reactive Protein with 32 Complex Somatic and Psychiatric Outcomes: A Large-Scale Cross-Consortium Mendelian Randomization Study. *PLoS Med*. 13:e1001976.

49. van Rossum AM, Wulkan RW, Oudesluys-Murphy AM (2004): Procalcitonin as an early marker of infection in neonates and children. *Lancet Infect Dis*. 4:620-630.

50. Blomstrom A, Gardner RM, Dalman C, Yolken RH, Karlsson H (2015): Influence of maternal infections on neonatal acute phase proteins and their interaction in the development of non-affective psychosis. *Translational psychiatry*. 5:e502.

51. Siddappa AM, Rao R, Long JD, Widness JA, Georgieff MK (2007): The assessment of newborn iron stores at birth: a review of the literature and standards for ferritin concentrations. *Neonatology*. 92:73-82.

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52. Krakowiak P, Walker CK, Bremer AA, Baker AS, Ozonoff S, Hansen RL, et al. (2012): Maternal metabolic conditions and risk for autism and other neurodevelopmental disorders. *Pediatrics*. 129:e1121-1128.

53. Li M, Fallin MD, Riley A, Landa R, Walker SO, Silverstein M, et al. (2016): The Association of Maternal Obesity and Diabetes With Autism and Other Developmental Disabilities. *Pediatrics*. 137:e20152206.

54. Getz KD, Anderka MT, Werler MM, Jick SS (2016): Maternal Pre-pregnancy Body Mass Index and Autism Spectrum Disorder among Offspring: A Population-Based Case-Control Study. *Paediatr Perinat Epidemiol*. 30:479-487.

55. Windham GC, Anderson M, Lyall K, Daniels JL, Kral TVE, Croen LA, et al. (2019): Maternal Pre-pregnancy Body Mass Index and Gestational Weight Gain in Relation to Autism Spectrum Disorder and other Developmental Disorders in Offspring. *Autism research : official journal of the International Society for Autism Research*. 12:316-327.

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Figures:

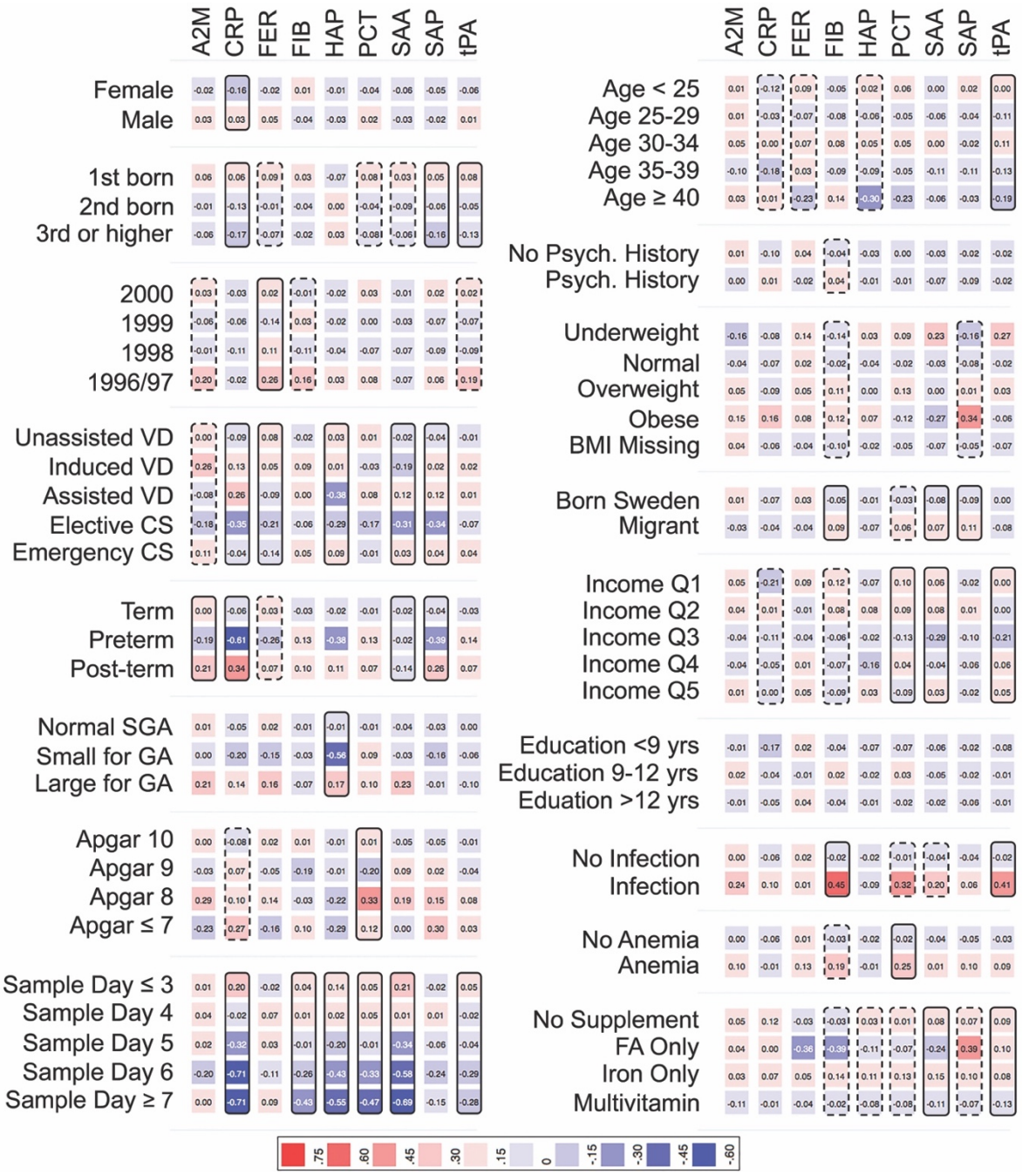

Fig. 1. Heat map showing the mean APP z-score according to each category of the covariates. among 1092 unaffected individuals in the cohort. Solid boxes indicate that the APP is associated with the covariate at $p<0.05$. Dashed boxes indicate that the APP is associated with the covariate at $p<0.20$. Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator; **VD**: vaginal delivery; **CS**: Caesarean section delivery; **SGA**: size for gestational age; **GA**: gestational age; **Psych:** Psychiatric; **BMI**: body mass index; **Income Q**: income quintile; **FA**: folic acid.

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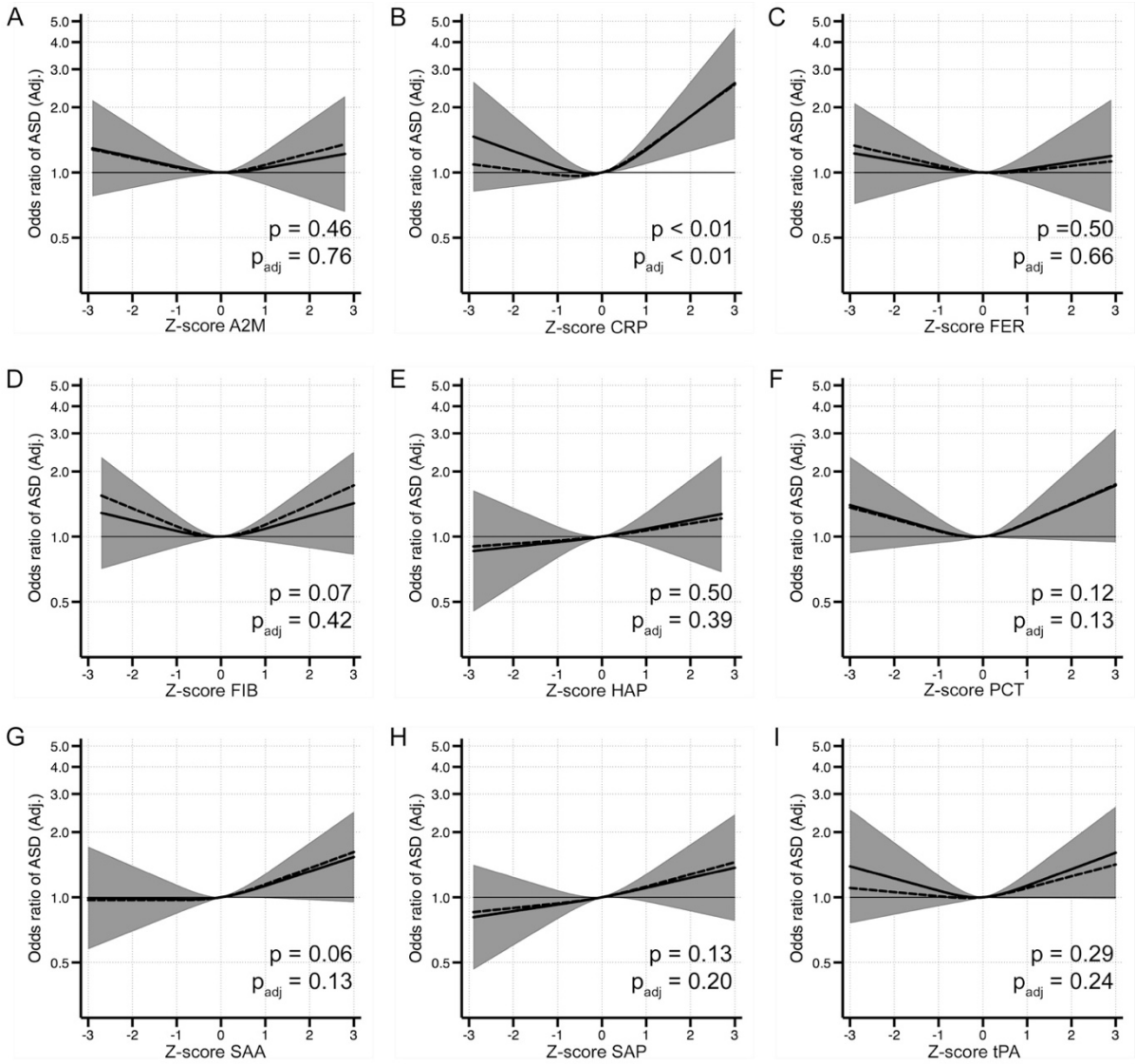

Fig. 2. The relationship between APP and odds of ASD when comparing 924 ASD cases to 1092 unaffected individuals selected from the cohort. Each panel displays the odds of ASD according to APP z-score, flexibly fit using restricted cubic spline models with three knots and a z-score=0 as the referent. The dashed line represents the unadjusted estimate of the relationship between each APP and odds of ASD. The solid line represents the fully adjusted model, adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. The gray bands represent the 95% confidence interval for the fully adjusted model. 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. P values are shown for spline terms in both the unadjusted models (p) and the adjusted models (p_{adj}). Abbreviations: **ASD**: autism spectrum disorders; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

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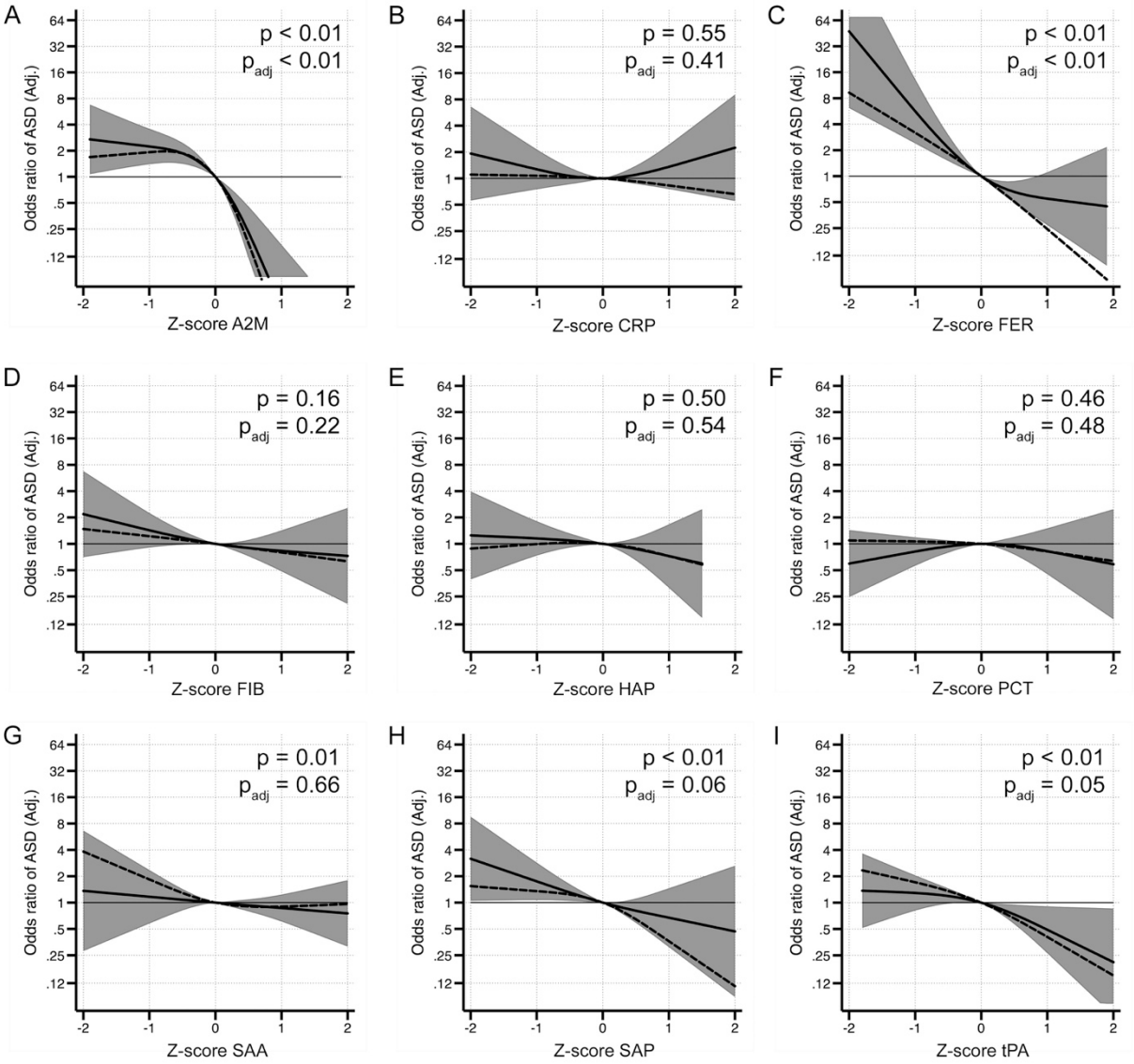

Fig. 3. The relationship between APP and odds of ASD when comparing 203 ASD cases to their matched, unaffected sibling. Each panel displays the odds of ASD according to APP z-score, flexibly fit using restricted cubic spline models with three knots and a z-score=0 as the referent. The dashed line represents the unadjusted estimate of the relationship between each APP and odds of ASD. The solid line represents the adjusted model, adjusted for children's birth order, and the total protein concentration of the sample. The gray bands represent the 95% confidence interval for the fully adjusted model. 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. P values are shown for spline terms in both the unadjusted models (p) and the adjusted models (p_{adj}). Abbreviations: **ASD**: autism spectrum disorders; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

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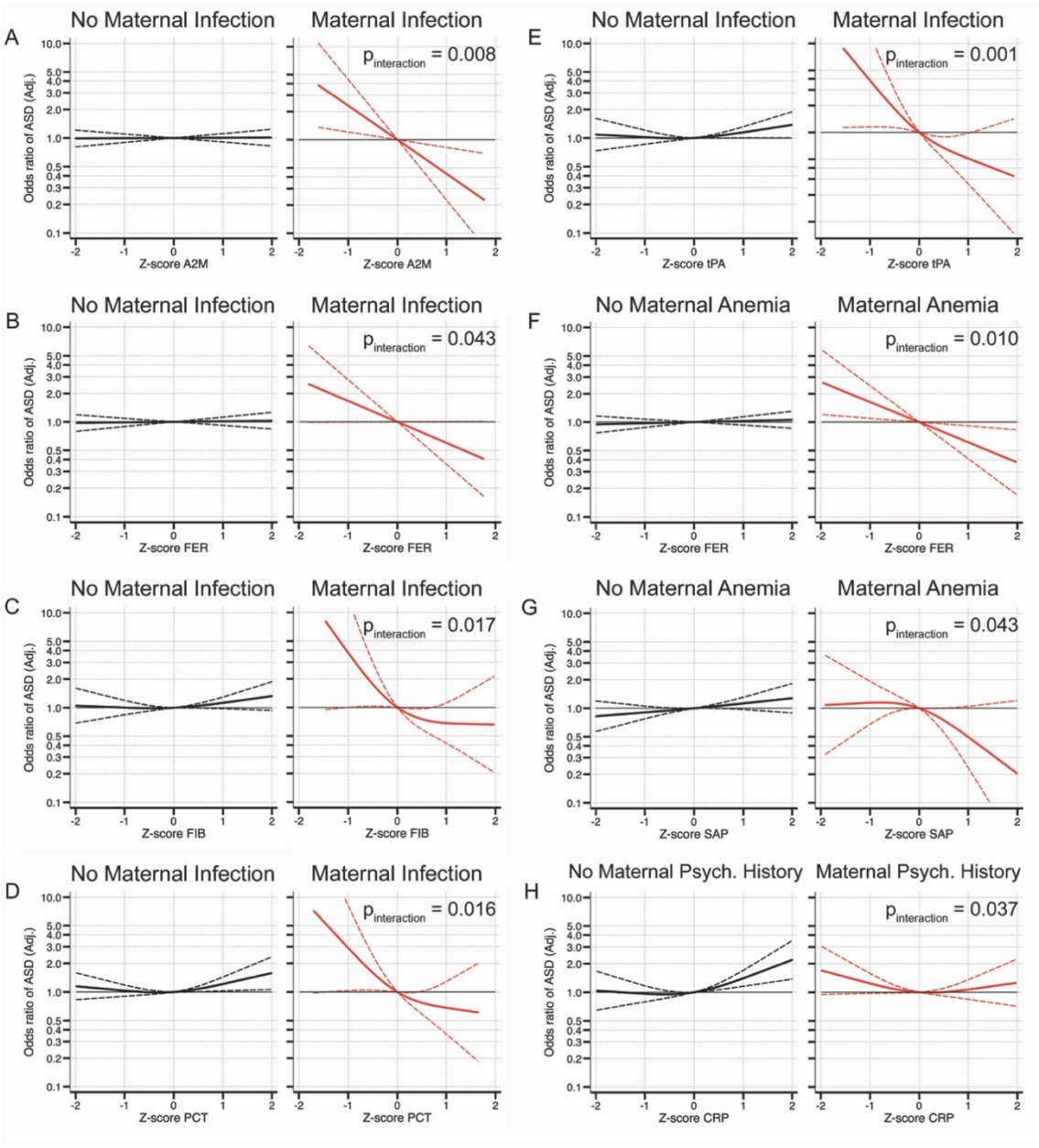

Fig. 4. Interaction between APP and other risk factors. Odds ratios for ASD over the range of APP are shown separately for those who were not exposed to maternal hospitalization for infection in late pregnancy (3rd trimester) compared to those who were (A-E), those who were not exposed to maternal anemia compared to those who were (F-G), and those whose mother had no history of psychiatric diagnoses compared to those whose mother did have a psychiatric history (H). Solid lines represent the OR estimate for each group and dashed lines represent the 95% confidence interval for the fully adjusted model. P-values for interaction (a likelihood ratio test comparing a model with interaction terms to a model without interaction terms) are shown.

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Table 1. Characteristics of individuals diagnosed with ASD and unaffected individuals in the study sample.

	Unaffected (n = 1092)	ASD (n = 924)	p-value[a]	ASD with ID (n = 321)	ASD with ADHD (n = 322)	ASD only (n = 281)	p-value[b]
Sex							
Female	538 (49.3%)	214 (23.2%)	<0.001	84 (26.2%)	70 (21.7%)	60 (21.4%)	<0.001
Male	554 (50.7%)	710 (76.8%)		237 (73.8%)	252 (78.3%)	221 (78.6%)	
Birth Order							
1st born	443 (40.6%)	419 (45.3%)	0.030	124 (38.6%)	158 (49.1%)	137 (48.8%)	0.006
2nd born	385 (35.3%)	289 (31.3%)		114 (35.5%)	87 (27.0%)	88 (31.3%)	
3rd or higher	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							
Unassisted VD	712 (65.2%)	532 (57.6%)	0.014	175 (54.5%)	180 (55.9%)	177 (63.0%)	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%)	
Gestational Age							
≤36 weeks	53 (4.9%)	63 (6.8%)	0.12	23 (7.2%)	21 (6.5%)	19 (6.8%)	0.59
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 Gestational Age							
Normal size for GA	960 (87.9%)	772 (83.5%)	0.17	274 (85.4%)	258 (80.1%)	240 (85.4%)	0.032
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							
10	861 (78.8%)	685 (74.1%)	0.064	219 (68.2%)	243 (75.5%)	223 (79.4%)	<0.001
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)							
≤3	423 (38.7%)	346 (37.4%)	0.19	109 (34.0%)	127 (39.4%)	110 (39.1%)	0.13
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)							
>25	145 (13.3%)	116 (12.6%)	0.011	51 (15.9%)	40 (12.4%)	25 (8.9%)	<0.001
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							
No	723 (66.2%)	483 (52.3%)	<0.001	190 (59.2%)	145 (45.0%)	148 (52.7%)	<0.001
Yes	369 (33.8%)	441 (47.7%)		131 (40.8%)	177 (55.0%)	133 (47.3%)	
Maternal BMI							
Normal	538 (49.3%)	364 (39.4%)	<0.001	130 (40.5%)	110 (34.2%)	124 (44.1%)	<0.001
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							
No	807 (73.9%)	647 (70.0%)	0.053	168 (52.3%)	262 (81.4%)	217 (77.2%)	<0.001

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Yes	285 (26.1%)	277 (30.0%)		153 (47.7%)	60 (18.6%)	64 (22.8%)	
Family Income Quintile							
1st (Lowest)	154 (14.1%)	122 (13.2%)	0.003	64 (19.9%)	29 (9.0%)	29 (10.3%)	<0.001
2nd	234 (21.4%)	240 (26.0%)		92 (28.7%)	82 (25.5%)	66 (23.5%)	
3rd	240 (22.0%)	214 (23.2%)		68 (21.2%)	84 (26.1%)	62 (22.1%)	
4th	229 (21.0%)	205 (22.2%)		64 (19.9%)	82 (25.5%)	59 (21.0%)	
5th	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%)	
Parental Education							
<9 years	165 (15.1%)	167 (18.1%)	0.078	74 (23.1%)	59 (18.3%)	34 (12.1%)	<0.001
9-12 years	474 (43.4%)	411 (44.5%)		140 (43.6%)	155 (48.1%)	116 (41.3%)	
>12 years	453 (41.5%)	344 (37.2%)		106 (33.0%)	108 (33.5%)	130 (46.3%)	
Missing	0 (0.0%)	2 (0.2%)		1 (0.3%)	0 (0.0%)	1 (0.4%)	
Maternal Hospitalization for Infection (3rd trimester)							
No	1000 (91.6%)	811 (87.8%)	0.024	279 (86.9%)	283 (87.9%)	249 (88.6%)	0.050
Yes	31 (2.8%)	43 (4.7%)		19 (5.9%)	11 (3.4%)	13 (4.6%)	
Missing	61 (5.6%)	70 (7.6%)		23 (7.2%)	28 (8.7%)	19 (6.8%)	
Maternal Anemia During Pregnancy							
No	1030 (94.3%)	866 (93.7%)	0.57	298 (92.8%)	302 (93.8%)	266 (94.7%)	0.75
Yes	62 (5.7%)	58 (6.3%)		23 (7.2%)	20 (6.2%)	15 (5.3%)	
Maternal Dietary Supplementation							
None	433 (39.7%)	383 (41.5%)	0.23	119 (37.1%)	137 (42.5%)	127 (45.2%)	0.39
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 NDBS (median [IQR])							
A2M (ng/ml)	533.0 (252.3, 911.3)	563.3 (266.0, 1052.0)	0.017	574.4 (249.7, 1051.9)	596.0 (307.1, 1123.1)	513.6 (251.4, 1004.0)	0.037
CRP (ng/ml)	1.3 (0.6, 3.1)	1.6 (0.7, 4.1)	<0.001	1.5 (0.7, 4.1)	1.7 (0.6, 4.1)	1.7 (0.7, 4.2)	0.006
FER (ng/ml)	1.8 (0.7, 3.3)	1.7 (0.6, 3.9)	0.27	1.7 (5.9, 3.6)	2.2 (0.8, 4.2)	1.5 (0.5, 4.0)	0.067
FIB (ng/ml)	27.1 (16.5, 53.1)	30.6 (16.5, 60.6)	0.074	30.7 (16.9, 63.1)	32.1 (17.5, 61.1)	27.1 (15.4, 55.9)	0.098
HAP (ng/ml)	27.5 (9.3, 76.8)	29.9 (11.0, 85.4)	0.036	27.6 (10.6, 78.2)	33.7 (12.1, 87.9)	29.1 (9.8, 88.5)	0.10
PCT (pg/ml)	10.9 (7.4, 15.6)	11.7 (7.5, 17.1)	0.013	11.0 (7.7, 16.9)	12.0 (7.7, 17.9)	11.1 (7.0, 17.6)	0.038
SAA (ng/ml)	1.3 (0.8, 2.2)	1.4 (0.9, 2.5)	0.019	1.4 (0.8, 2.7)	1.5 (0.9, 2.7)	1.3 (0.8, 2.2)	0.030
SAP (ng/ml)	10.5 (6.8, 15.5)	11.5 (7.5, 17.7)	<0.001	11.1 (8.1, 18.4)	12.0 (7.5, 17.5)	11.2 (7.0, 16.9)	0.007
tPA (pg/ml)	9.6 6.2, 14.7)	10.6 (6.6, 15.9)	0.010	9.9 (6.6, 15.0)	11.5 (7.2, 17.4)	10.1 (6.3, 16.1)	0.004
Total protein (mg/ml)	1.3 (1.0, 1.7)	1.3 (1.0, 1.7)	0.76	1.2 (0.9, 1.6)	1.3 (1.0, 1.7)	1.3 (1.0, 1.7)	0.19

a. Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distributions among all ASD-affected individuals. Kruskal-Wallis tests were used for continuous variables, as the distributions of the APP concentrations were strongly skewed.

b. Pearson's chi-squared test was used for categorical variables, comparing the frequency distributions among unaffected individuals to the distribution among the stratified ASD outcome groups. Kruskal-Wallis tests were used for continuous variables, as the distributions of the APP concentrations were strongly skewed.

Abbreviations: **ASD**: autism spectrum disorders; **ADHD**: attention-deficit/hyperactivity disorder; **ID**: intellectual disability; **VD**: vaginal delivery; **CS**: Caesarean section delivery; **GA**: gestational age; **BMI**: body mass index; **IQR**: interquartile range; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

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Supplementary Materials for:

Neonatal levels of acute phase proteins and risk of autism spectrum disorders

Supplemental Figures:

Figure S1. Selection of individuals and NDBS samples.

Figure S2. Levels of total protein and one APP according to analytic plate and elution batch.

Figure S3. The Spearman rho correlations of nine acute phase proteins with each other and with total protein measurements, as measured in neonatal dried blood spots of 1092 unaffected controls selected from the cohort.

Figure S4. Heat map showing the mean APP z-score according to each category of the covariates, among 924 ASD-affected individuals in the cohort.

Figure S5. The distribution of each APP measured in neonatal dried blood spot samples, comparing ASD-unaffected individuals to ASD-affected individuals.

Figure S6. The relationship between APP and odds of ASD when comparing 924 ASD cases to 1092 unaffected individuals selected from the cohort and when comparing 203 ASD cases to their matched, unaffected siblings.

Figure S7. The relationship between APP and odds of ASD when the outcome is stratified by co-occurring ID and ADHD.

Figure S8. The relationship between APP and odds of ASD with co-occurring ID when comparing 321 individuals affected by ASD with co-occurring ID to 1092 unaffected individuals selected from the cohort.

Figure S9. The relationship between APP and odds of ASD with co-occurring ADHD when comparing 322 individuals affected by ASD with co-occurring ADHD to 1092 unaffected individuals selected from the cohort.

Figure S10. The relationship between APP and odds of ASD without co-occurring ID or ADHD ("ASD only") when comparing 281 individuals affected by ASD and without a diagnosis of co-occurring ID or ADHD to 1092 unaffected individuals selected from the cohort.

Figure S11. Interaction models for categorical variables. Odds ratios for ASD over the range of APP are shown separately according to strata of parental education level, Apgar score at 5 minutes, and parental income at birth.

Figure S12. The relationship between APP and odds of ASD in sensitivity analyses.

Supplemental Tables:

Table S1. Diagnostic codes used in the ascertainment of ASD, ADHD, and ID in the Stockholm Youth Cohort.

Table S2. Quality control statistics for multiplex assays to analyze acute phase protein concentrations in neonatal dried blood spots.

Table S3. Characteristics of unaffected and ASD-affected siblings included in the matched sibling comparison.

Table S4. P-values for the association of APP with other covariates among 1092 unaffected individuals.

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Table S5. P-values for the association of APP with other covariates among 924 ASD affected individuals.

Table S6. P-values for non-linearity in restricted cubic spline analyses.

Table S7. P-values for interaction using a likelihood ratio test comparing a model without interaction terms nested in model with interaction terms.

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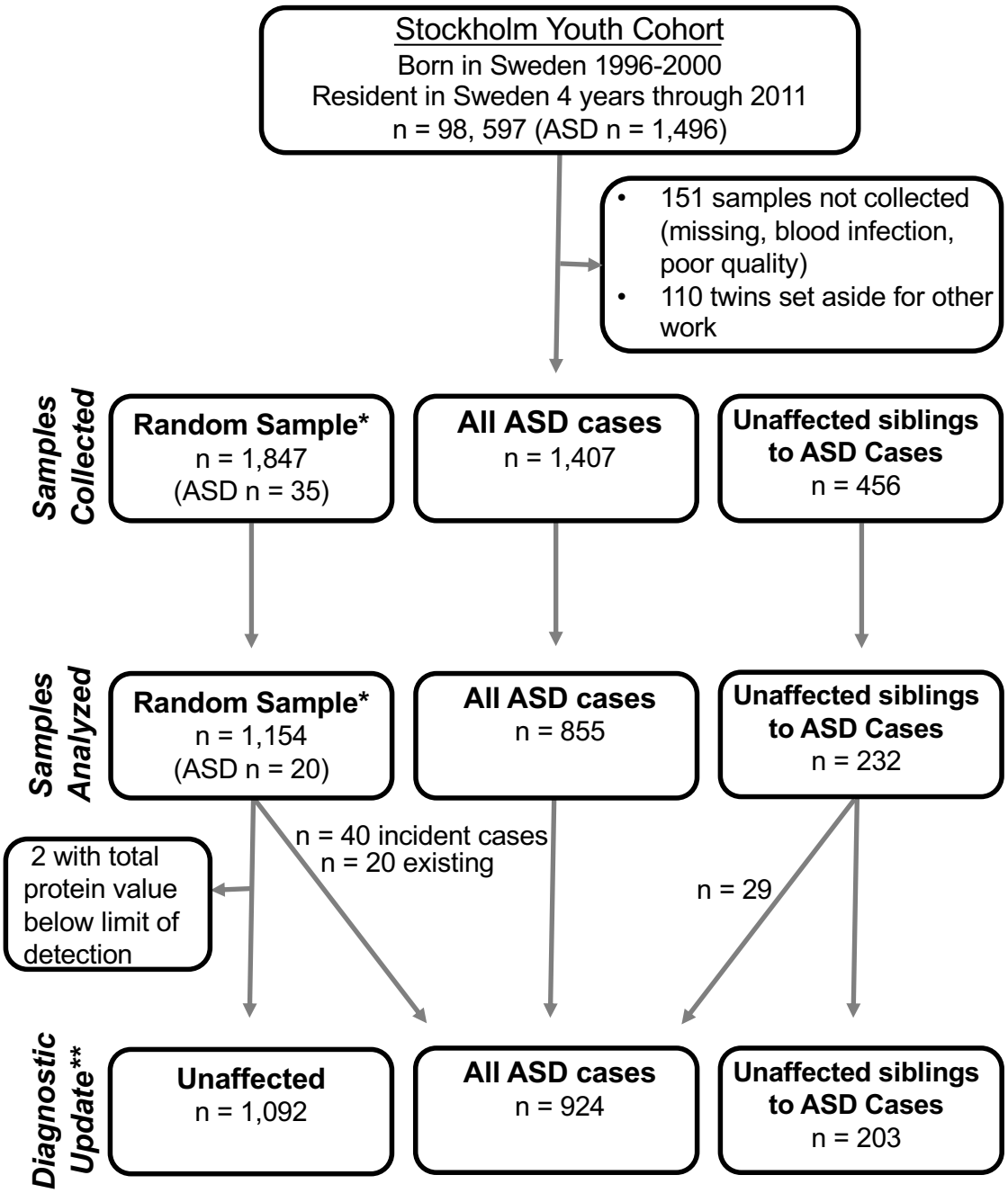
Figure S1. Selection of individuals and NDBS samples.



*A random sample of the SYC and, as such, includes ASD also included in the total sample of ASD cases.

**ASD diagnostic data updated as of December 31, 2016 for the SYC, resulting in 69 incidence cases of ASD: 29 individuals sampled as siblings to an ASD case and 40 individuals sampled as part of the random sample of the SYC.

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Figure S2. Levels of total protein (**A**) and one APP (**B** Ferritin) according to analytic plate and elution batch. Protein elution batches are demarcated by red dotted lines in **A**. Plate-specific z-scores were calculated to allow comparisons across batches and plates (**C**).

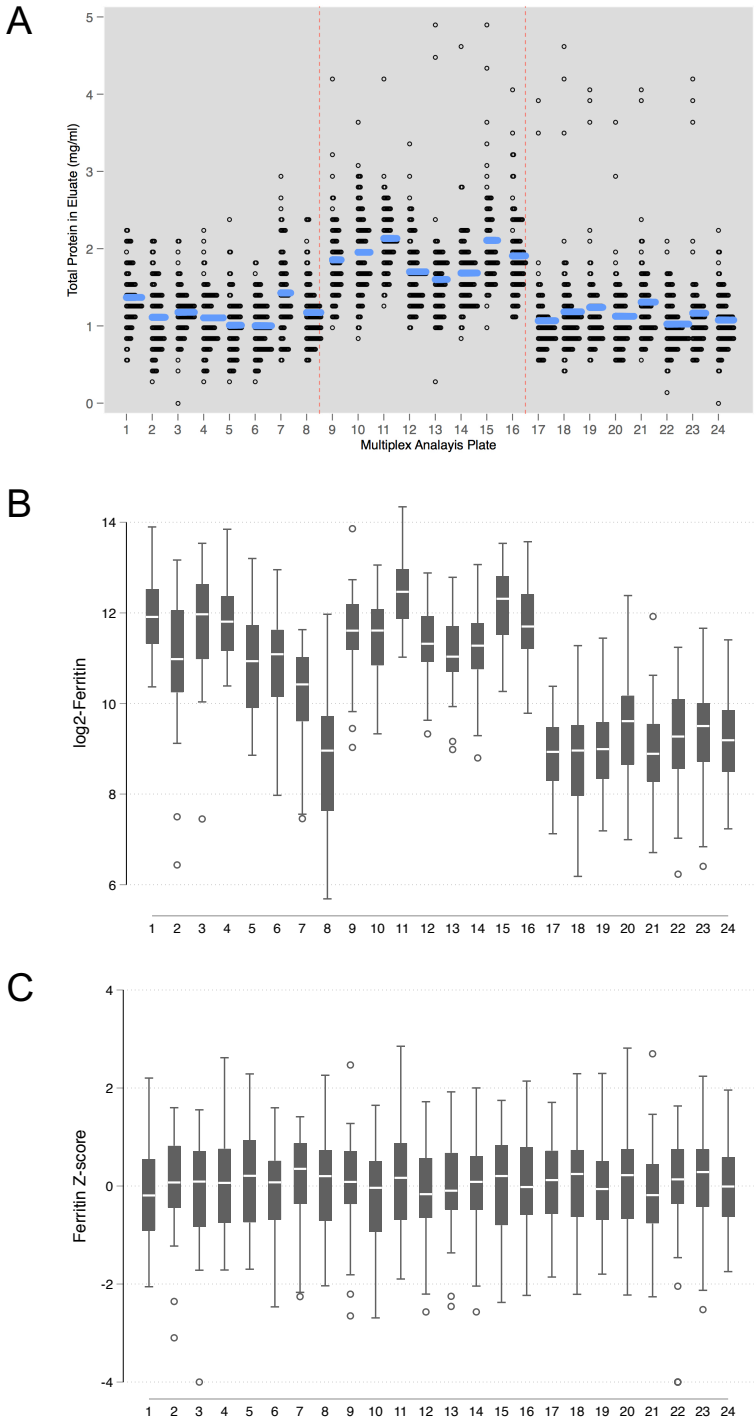

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Figure S3. The Spearman rho correlations of nine acute phase proteins with each other and with total protein measurements, as measured in neonatal dried blood spots of 1092 unaffected controls selected from the cohort. Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; **tPA**: tissue plasminogen activator; and **TP**: total protein.

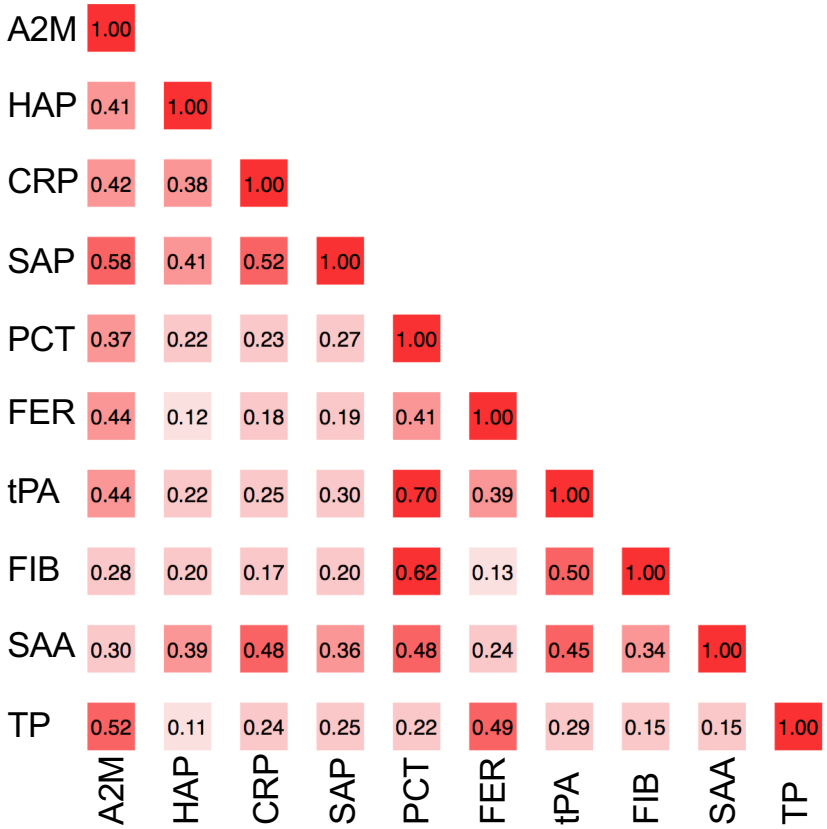

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Figure S4. Heat map showing the mean APP z-score according to each category of the covariates, among 924 ASD-affected individuals in the cohort. Solid boxes indicate that the APP is associated with the covariate at p<0.05. Dashed boxes indicate that the APP is associated with the covariate at p<0.20. Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator; **VD**: vaginal delivery; **CS**: Caesarean section delivery; **SGA**: size for gestational age; **GA**: gestational age; **Psych:** Psychiatric; **BMI**: body mass index; **Income Q**: income quintile; **FA**: folic acid.

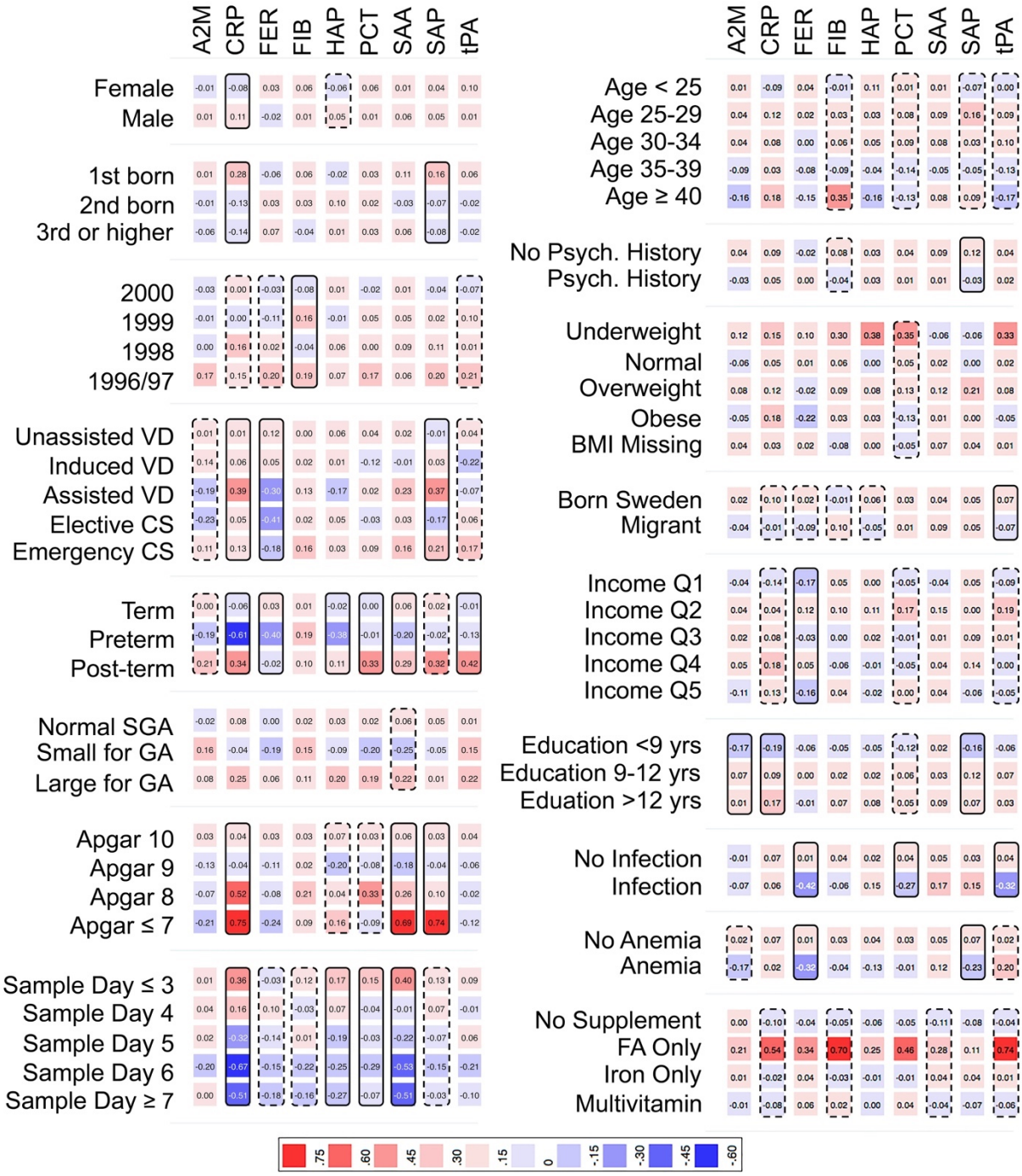

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Figure S5. The distribution of each APP measured in neonatal dried blood spot samples, comparing ASD-unaffected individuals (0) to ASD-affected individuals (1). In (**A**), 924 ASD cases are compared to 1092 unaffected individuals selected from the cohort. In (**B**), 203 ASD cases are compared to their unaffected siblings.

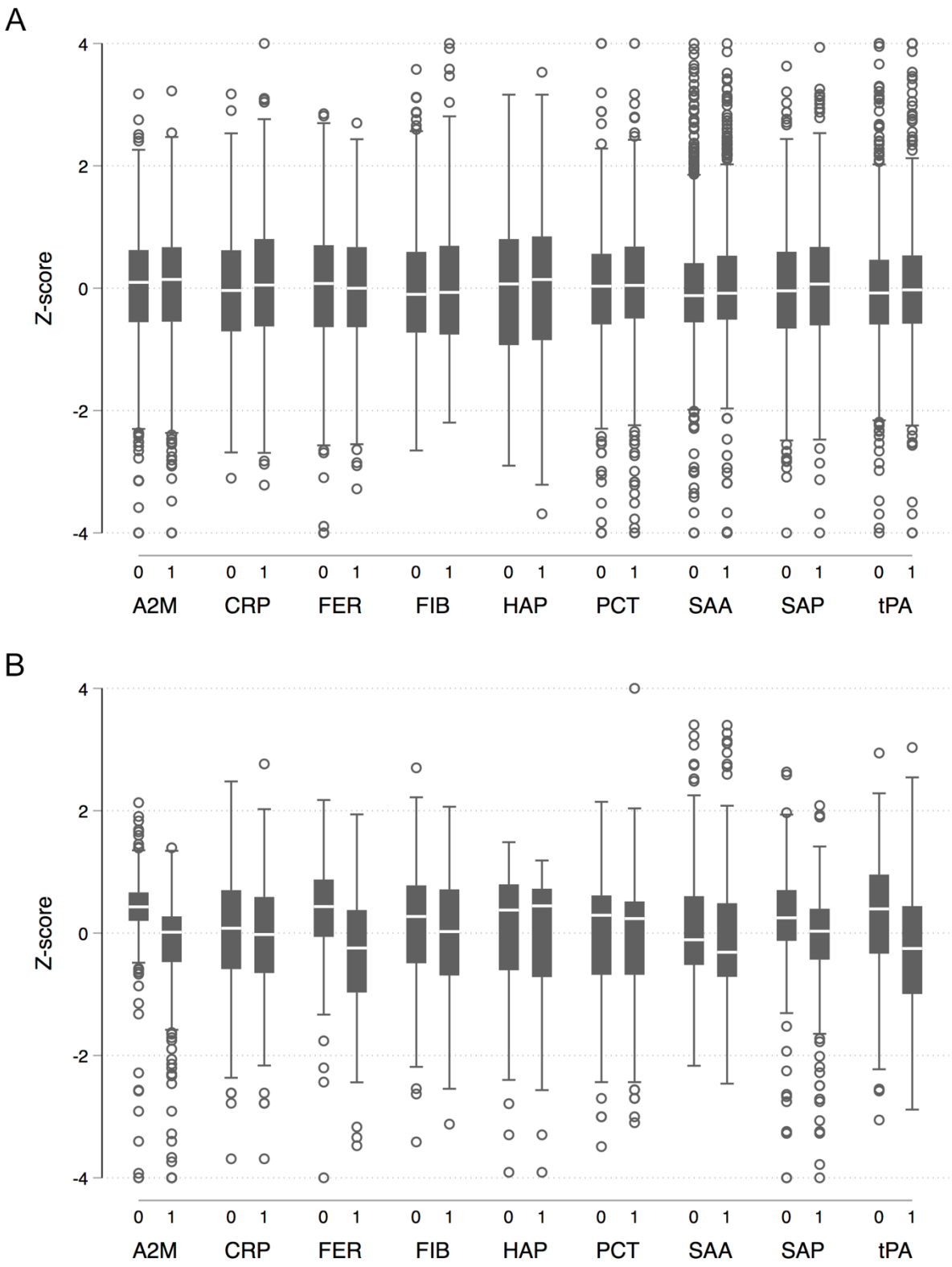

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Figure S6. The relationship between APP and odds of ASD when comparing 924 ASD cases to 1092 unaffected individuals selected from the cohort (**A**) and when comparing 203 ASD cases to their matched, unaffected siblings (**B**). Quintiles of each APP were created using the distribution of z-scores among unaffected individuals to set the cut-offs and the middle quintile was used as the referent category. Results are shown for unadjusted estimates (open circles) and fully adjusted estimates (solid circles). Models were adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. P-values are shown for a Wald test with a null hypothesis that all APP categorical terms were jointly equal to zero, as a test of whether each APP was generally associated with the outcome. Truncated confidence intervals in the matched sibling comparison are indicated with an arrow. Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

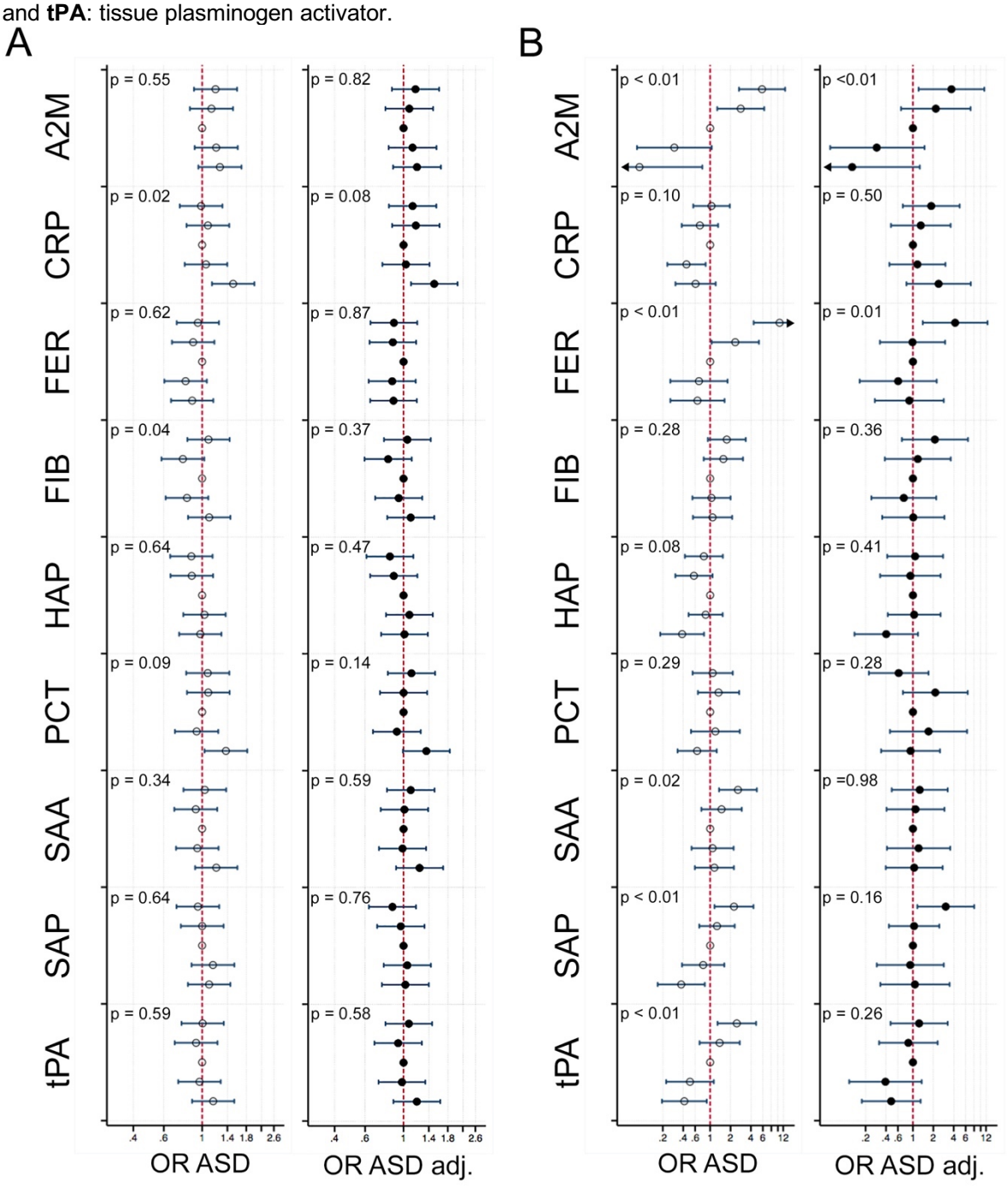

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Figure S7. The relationship between APP and odds of ASD when the outcome is stratified by co-occurring ID and ADHD. Results are shown for ASD with co-morbid ID, ASD with co-morbid ADHD, and ASD without co-occurring ID or ADHD ("ASD only"), compared to 1092 unaffected individuals selected from the cohort. Quintiles of each APP were created using the distribution of z-scores among unaffected individuals to set the cut-offs and the middle quintile was used as the referent category. Models were adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. P-values are shown for a Wald test with a null hypothesis that all APP categorical terms were jointly equal to zero, as a test of whether each APP was generally associated with the outcome. Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

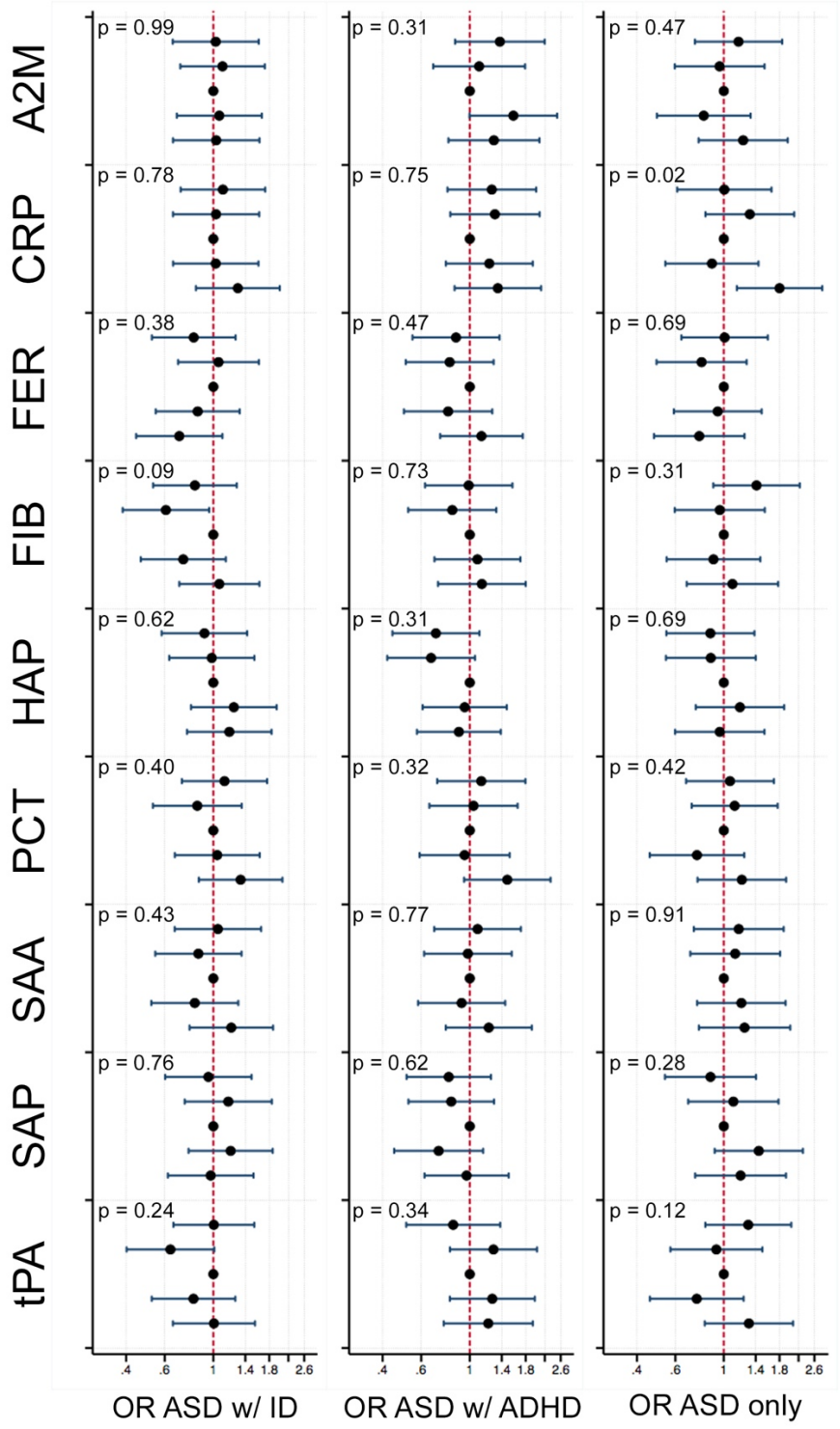

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Figure S8. The relationship between APP and odds of ASD with co-occurring ID when comparing 321 individuals affected by ASD with co-occurring ID to 1092 unaffected individuals selected from the cohort. Each panel displays the odds of ASD according to APP z-score, flexibly fit using restricted cubic spline models with three 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 fully adjusted model, adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. The gray bands represent the 95% confidence interval for the fully adjusted model. 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. P values are shown for spline terms in both the unadjusted models (p) and the adjusted models (p_{adj}). Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

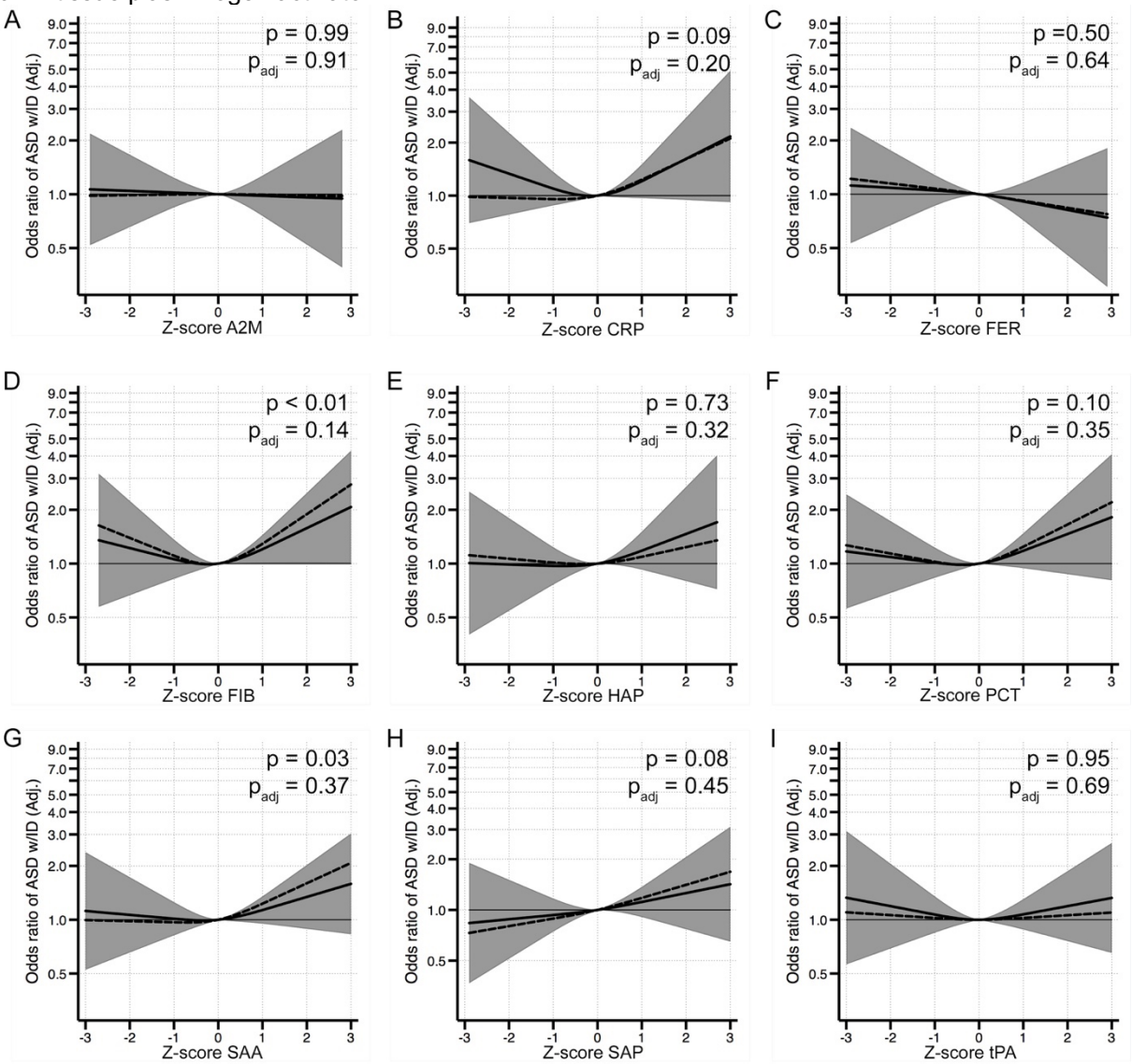

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Figure S9. The relationship between APP and odds of ASD with co-occurring ADHD when comparing 322 individuals affected by ASD with co-occurring ADHD to 1092 unaffected individuals selected from the cohort. Each panel displays the odds of ASD according to APP z-score, flexibly fit using restricted cubic spline models with three knots and 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 fully adjusted model, adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. The gray bands represent the 95% confidence interval for the fully adjusted model. 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. P values are shown for spline terms in both the unadjusted models (p) and the adjusted models (p_{adj}). Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

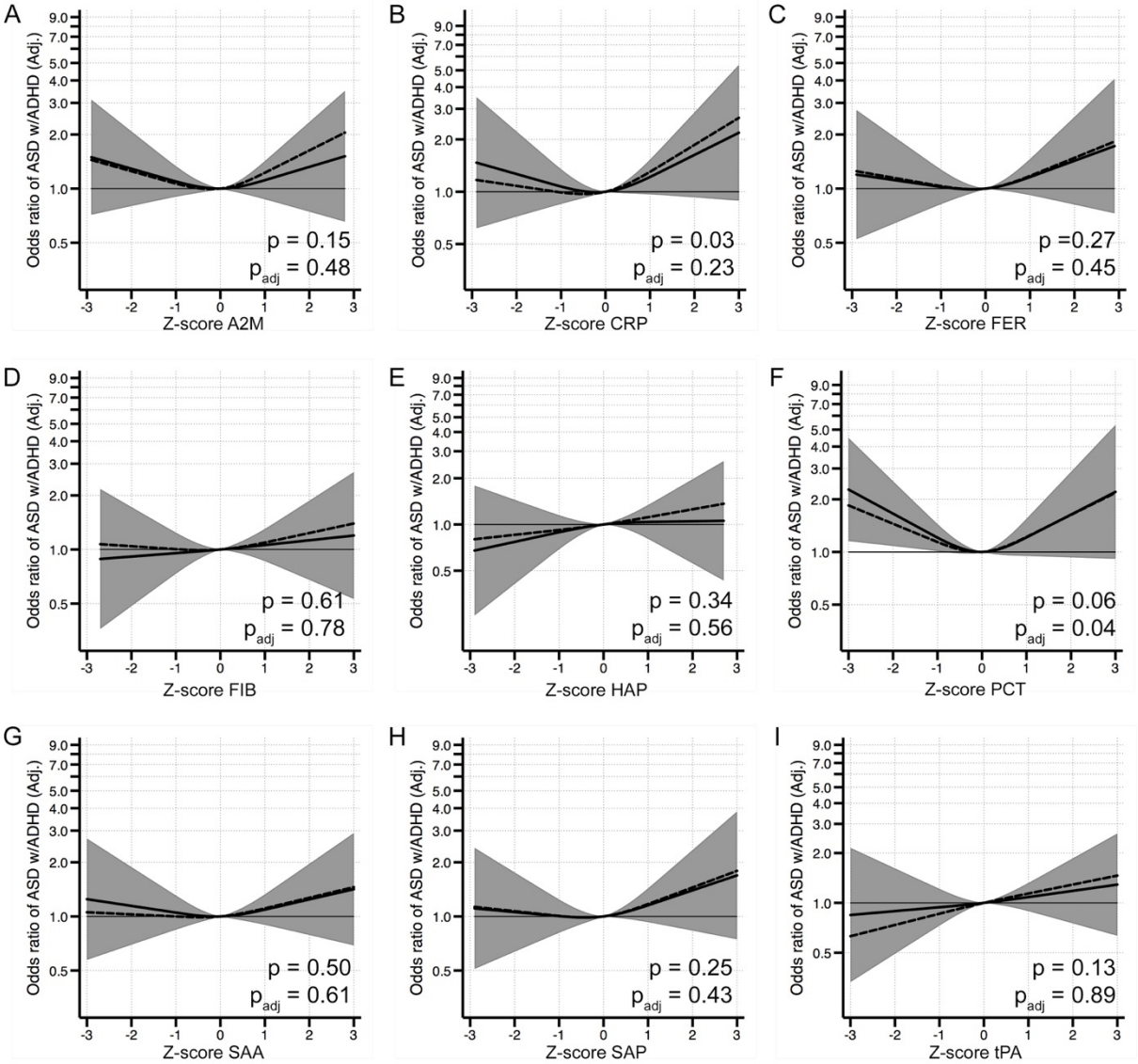

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Figure S10. The relationship between APP and odds of ASD without co-occurring ID or ADHD ("ASD only") when comparing 281 individuals affected by ASD and without a diagnosis of co-occurring ID or ADHD to 1092 unaffected individuals selected from the cohort. Each panel displays the odds of ASD according to APP z-score, flexibly fit using restricted cubic spline models with three 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 fully adjusted model, adjusted for maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery. The gray bands represent the 95% confidence interval for the fully adjusted model. 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. P values are shown for spline terms in both the unadjusted models (p) and the adjusted models (p_{adj}). Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

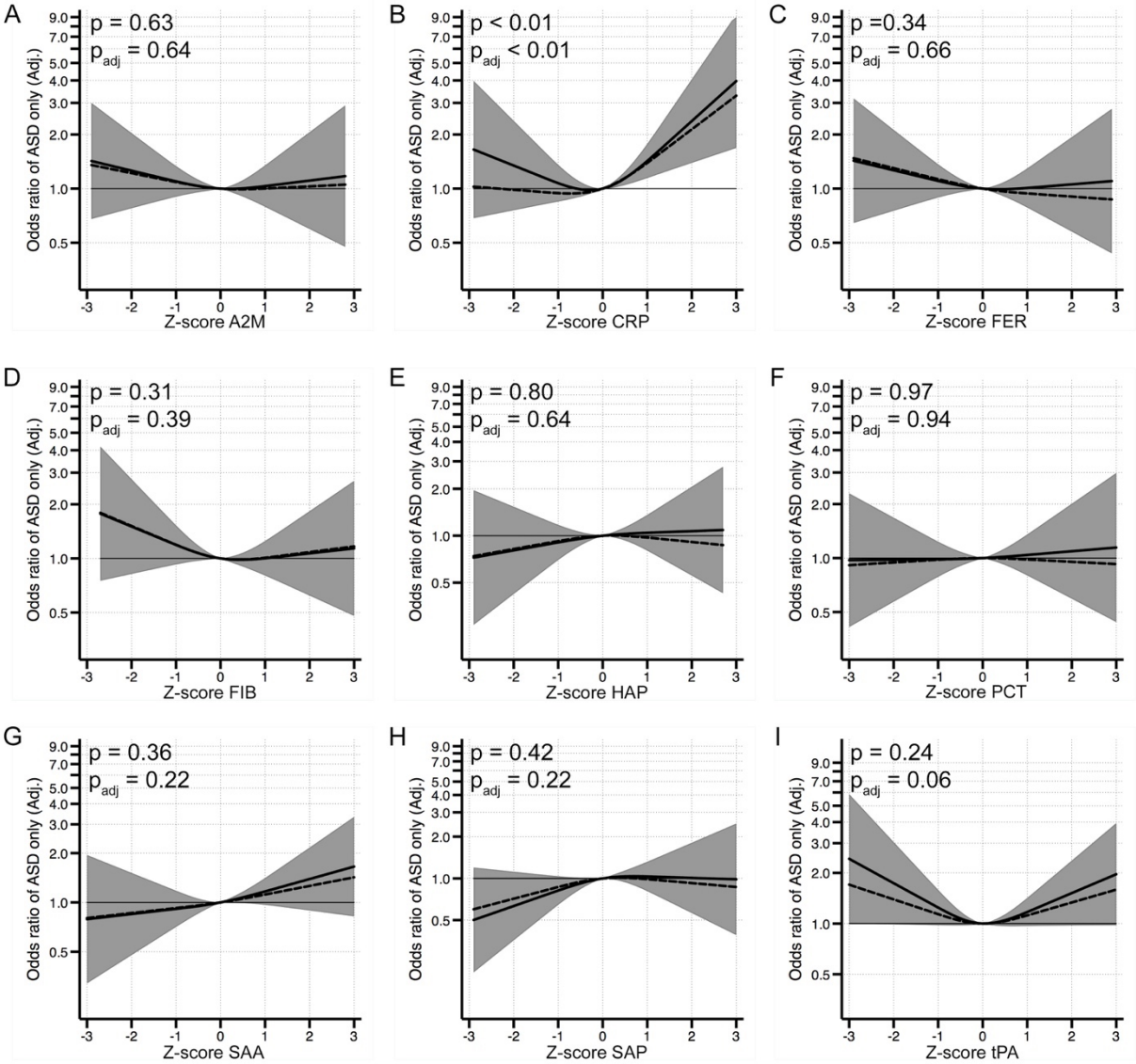

Figure S11. Interaction models for categorical variables. Odds ratios for ASD over the range of APP are shown separately according to strata of parental education level **(A)**, Apgar score at 5 minutes **(B)**, and parental income at birth **(C)**. Solid lines represent the OR estimate for each group and dashed lines represent the 95% confidence interval for the fully adjusted model. P-values for interaction (a likelihood ratio test comparing a model with interaction terms to a model without interaction terms) are shown. Abbreviations: **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

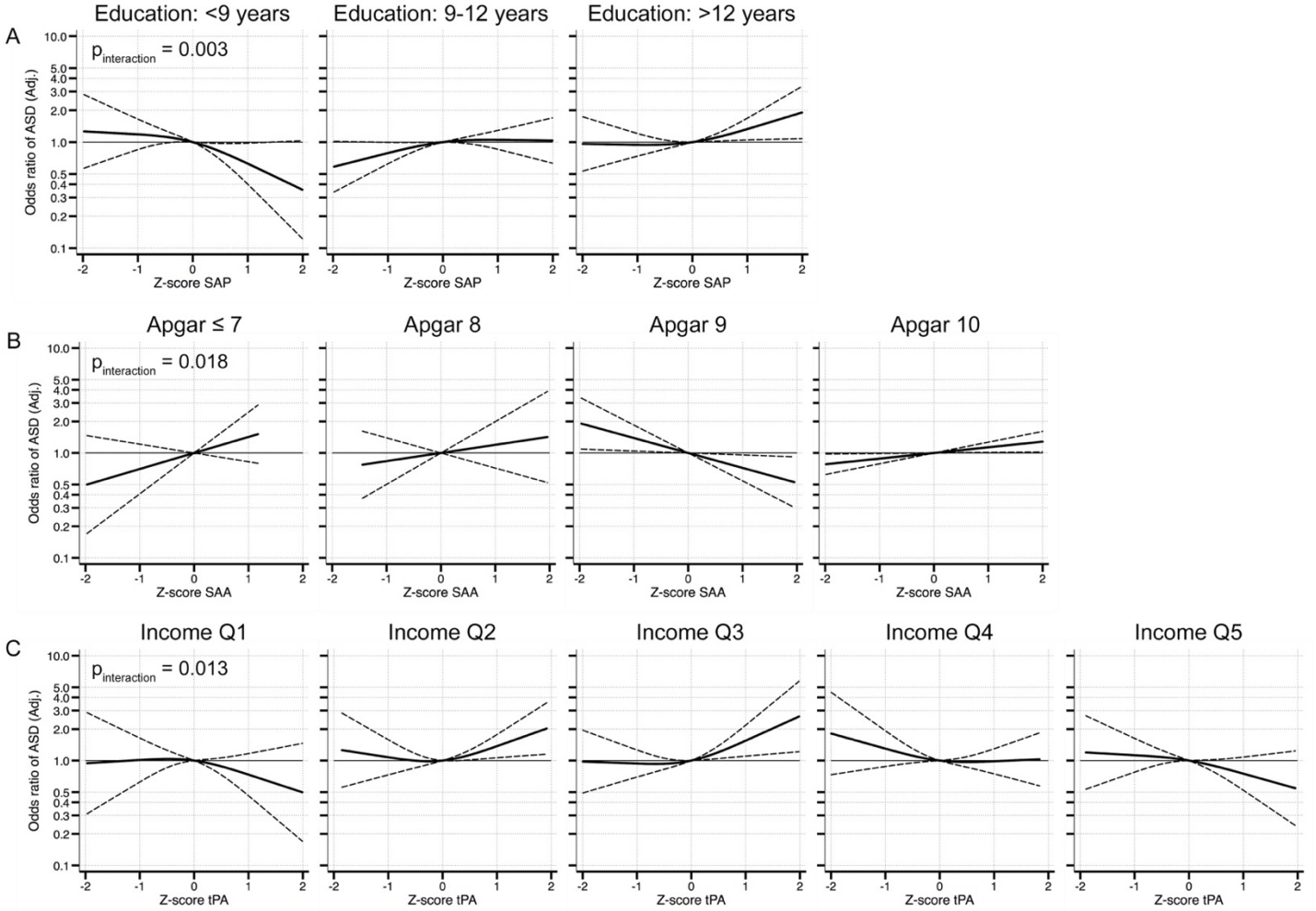

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Figure S12. The relationship between APP and odds of ASD in sensitivity analyses. In (**A**), 924 ASD cases are compared to 1092 unaffected individuals selected from the cohort, adjusting for birth year, total protein content of the eluate from the neonatal dried blood spot sample, and age (in days) at neonatal blood sample, in addition to the covariates of the fully adjusted model (maternal age, psychiatric history, country of origin, and hospitalization for infection during pregnancy; children's birth order, sex, gestational age at birth, size for gestational age, and mode of delivery). In (**B**), individuals with a total protein concentration in the lowest quartile were excluded, and results are shown for the fully adjusted model comparing 688 ASD cases to 819 unaffected individuals selected from the cohort. In (**C**), results are shown for the fully adjusted model using a multi-level modeling approach that accounts for the clustering of APP observations in elution batches and multiplex analysis plate, comparing 924 ASD cases to 1092 unaffected individuals. We used generalized linear and mixed models to fit a 3-level random intercept model with logit link, allowing for the intercepts of both plates and batch to vary, but assuming that the relationship between the log-odds of ASD and each APP does not vary across plates or batches.

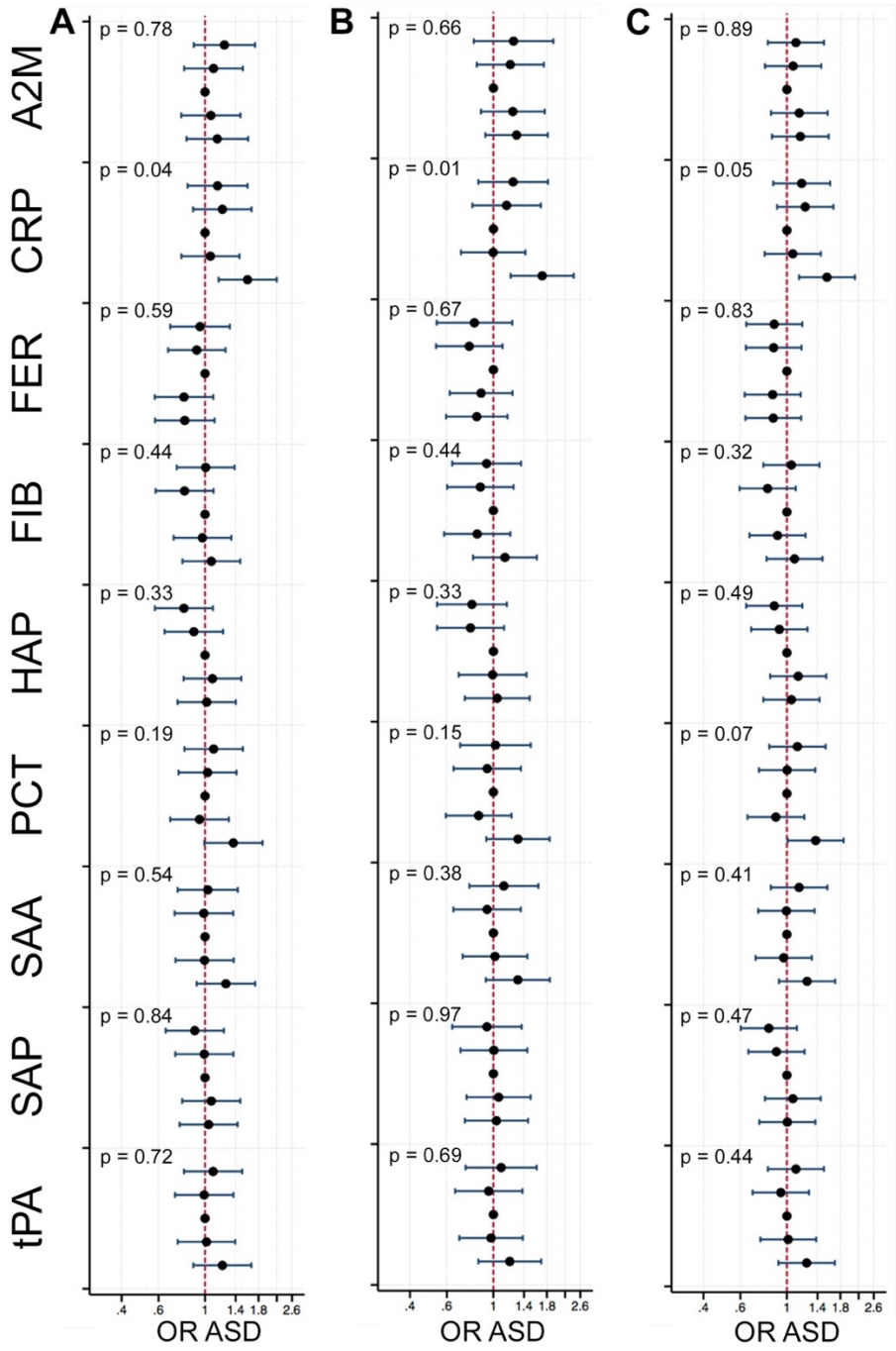

Table S1. Diagnostic codes used in the ascertainment of ASD, ADHD, and ID in the Stockholm Youth Cohort.

Coding system	Autism Spectrum Disorders (ASD)	Attention Deficit/Hyperactivity Disorder (ADHD)	Intellectual Disability (ID)
ICD-10[a,b,c]	F84	F90	F70-F79
DSM-IV[b]	299	314	317-319
Other	Habilitation Register[d]	Prescription Drug Register[e]: methylphenidate [N06BA04] or atomoxetin [N06BA09]	Habilitation Register[d]

[a] The National Patient Register (NPR): including inpatient care from 1973, outpatient physician visits in specialist care from 1997, and children and adolescent psychiatry from 2006.
[b] Stockholm Clinical Database for Child and Adolescent Psychiatry: DSM-IV was used until 2008; ICD-10 was used thereafter.
[c] VAL register: Stockholm county health care databases including in- and outpatient care, regardless of specialty/primary health care.
[d] The Habilitation Register (HAB): Provides data on utilization of Stockholm County Habilitation services according to type of disability, such as pervasive developmental disorders and intellectual disability (recorded as present or absent). HAB register was used until 2008; since 2008 it has been included in the VAL databases as ICD-coded diagnoses (see C above).
[e] The Prescription Drug Register (PDR) contains data on medications dispensed to the entire population in Sweden since 1 July 2005. Receipt of a prescription for ADHD medications is a useful proxy for an ADHD diagnosis, as Swedish medical guidelines mandate that ADHD medications should only be prescribed by a psychiatric specialist and after other (non-pharmacological) interventions have failed.

Table S2. Quality control statistics for multiplex assays to analyze acute phase protein concentrations in neonatal dried blood spots.

Analyte	%CV (controls)[a]	Below LLOQ	LLOQ[b]	Above ULOQ	ULOQ[c]	Imputed[d]
α-2-macroglobulin	11.5	0 (0.00%)	0.40 ng/ml	0 (0.00%)	1658.00 ng/ml	2 (0.10%)
Haptoglobin	23.3	3 (0.15%)	0.09 ng/ml	2 (0.10%)	99.05 ng/ml	11 (0.55%)
C Reactive Protein	15.4	5 (0.25%)	0.01 ng/ml	0 (0.00%)	22.95 ng/ml	19 (0.94%)
Serum Amyloid P	9.1	1 (0.05%)	0.01 ng/ml	0 (0.00%)	185.20 ng/ml	1 (0.05%)
Procalcitonin	22.3	9 (0.45%)	4.71 pg/ml	0 (0.00%)	5430 pg/ml	95 (4.71%)
Ferritin	11.7	0 (0.00%)	2.39 pg/ml	0 (0.00%)	39698.50 pg/ml	0 (0.00%)
Tissue Plasminogen Activator	23.3	4 (0.20%)	1.07 pg/ml	0 (0.00%)	4884 pg/ml	41 (2.03%)
Fibrinogen	17.0	0 (0.00%)	3.04 ng/ml	0 (0.00%)	2911.50 ng/ml	1 (0.05%)
Serum Amyloid A	22.0	0 (0.00%)	0.50 ng/ml	0 (0.00%)	374 ng/ml	82 (4.07%)

a. The percent CV is based on standardized controls that were run in duplicate on each of the 24 assay plates.
b. Lower Limit of Quantitation, average across 24 assay plates.
c. Upper Limit of Quantitation, average across 24 assay plates.
d. The absolute and relative frequency of observations that were imputed, including those values that were below the LLOQ, above the ULOQ, or that the Bio-plex Manager software indicated were near the LLOQ and thus less certain than values higher in the range of quantitation.

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Table S3. Characteristics of unaffected and ASD-affected siblings included in the matched sibling comparison.

	Unaffected siblings (n = 203)	ASD affected siblings (n = 203)
Sex		
Female	116 (57.1%)	43 (21.2%)
Male	87 (42.9%)	160 (78.8%)
Birth Order		
1st born	100 (49.3%)	39 (19.2%)
2nd born	68 (33.5%)	105 (51.7%)
3rd or higher	29 (14.3%)	41 (20.2%)
Missing	6 (3.0%)	18 (8.9%)
Mode of Delivery		
Unassisted VD	129 (63.5%)	127 (62.6%)
Induced VD	10 (4.9%)	13 (6.4%)
Assisted VD	19 (9.4%)	12 (5.9%)
Elective CS	15 (7.4%)	13 (6.4%)
Emergency CS	24 (11.8%)	20 (9.9%)
Missing	6 (3.0%)	18 (8.9%)
Gestational Age		
≤36 weeks	7 (3.4%)	8 (3.9%)
37-41 weeks	173 (85.2%)	165 (81.3%)
≥42 weeks	17 (8.4%)	12 (5.9%)
Missing	6 (3.0%)	18 (8.9%)
Size for Gestational Age		
Normal size for GA	182 (89.7%)	172 (84.7%)
Small for GA	9 (4.4%)	4 (2.0%)
Large for GA	6 (3.0%)	7 (3.4%)
Missing	6 (3.0%)	20 (9.9%)
Apgar Score at 5 Minutes		
10	158 (77.8%)	152 (74.9%)
9	24 (11.8%)	24 (11.8%)
8	9 (4.4%)	4 (2.0%)
≤7	5 (2.5%)	4 (2.0%)
Missing	7 (3.4%)	19 (9.4%)
Age at Neonatal Blood Sample (Days)		
≤3	79 (38.9%)	66 (32.5%)
4	73 (36.0%)	73 (36.0%)
5	16 (7.9%)	43 (21.2%)
6	20 (9.9%)	11 (5.4%)
≥7	13 (6.4%)	10 (4.9%)
Missing	2 (1.0%)	0 (0.0%)
Maternal Age (Years)		
>25	35 (17.2%)	25 (12.3%)
25-29	67 (33.0%)	71 (35.0%)
30-34	75 (36.9%)	64 (31.5%)
35-39	20 (9.9%)	38 (18.7%)
≥40	6 (3.0%)	5 (2.5%)
Maternal Psychiatric History		
No	115 (56.7%)	114 (56.2%)
Yes	88 (43.3%)	89 (43.8%)
Maternal BMI		
Normal	94 (46.3%)	80 (39.4%)
Underweight	3 (1.5%)	2 (1.0%)
Overweight	41 (20.2%)	35 (17.2%)
Obese	15 (7.4%)	13 (6.4%)
Missing	50 (24.6%)	73 (36.0%)
Mother Born Outside Sweden		
No	130 (64.0%)	130 (64.0%)
Yes	73 (36.0%)	73 (36.0%)
Family Income Quintile		
1st (Lowest)	28 (13.8%)	28 (13.8%)
2nd	47 (23.2%)	50 (24.6%)

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3rd	45 (22.2%)	56 (27.6%)
4th	44 (21.7%)	45 (22.2%)
5th	38 (18.7%)	24 (11.8%)
Parental Education		
<9 years	41 (20.2%)	41 (20.2%)
9-12 years	92 (45.3%)	92 (45.3%)
>12 years	70 (34.5%)	70 (34.5%)
Maternal Hospitalization for Infection (3rd trimester)		
No	190 (93.6%)	176 (86.7%)
Yes	7 (3.4%)	9 (4.4%)
Missing	6 (3.0%)	18 (8.9%)
Maternal Anemia During Pregnancy		
No	191 (94.1%)	192 (94.6%)
Yes	12 (5.9%)	11 (5.4%)
Maternal Dietary Supplementation		
None	95 (46.8%)	94 (46.3%)
Folic Acid only	4 (2.0%)	1 (0.5%)
Iron only	62 (30.5%)	56 (27.6%)
Multivitamin	42 (20.7%)	52 (25.6%)
Proteins measured in NDBS (median [IQR])		
A2M (ng/ml)	212.4 (34.1, 290.0)	135.0 (25.0, 223.0)
CRP (ng/ml)	1.2 (0.4, 3.8)	1.2 (0.3, 3.7)
FER (ng/ml)	4.8 (3.3, 6.5)	2.9 (1.9, 4.2)
FIB (ng/ml)	282.1 (142.5, 461.9)	220.4 (117.0, 420.2)
HAP (ng/ml)	13.5 (5.1, 78.9)	18.1 (5.5, 68.7)
PCT (pg/ml)	11.2 (8.6, 16.0)	11.2 (8.6, 15.8)
SAA (ng/ml)	2.0 (1.2, 3.9)	1.7 (1.1, 3.4)
SAP (ng/ml)	17.5 (3.2, 37.8)	13.5 (3.0, 26.9)
tPA (pg/ml)	10.6 (8.1, 15.9)	9.2 (6.5, 13.7)
Total protein (mg/ml)	3.4 (3.0, 4.0)	2.9 (2.4, 3.3)

Abbreviations: **ASD**: autism spectrum disorders; **ADHD**: attention-deficit/hyperactivity disorder; **ID**: intellectual disability; **VD**: vaginal delivery; **CS**: Caesarean section delivery; **GA**: gestational age; **BMI**: body mass index; **IQR**: interquartile range; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

Table S4. P-values for the association of APP with other covariates among 1092 unaffected individuals. We examined the association of each covariate with the APP z-scores by regressing each APP over the categories of the covariate, followed by a joint Wald test of the hypothesis that all coefficients for the categorical indicators are equal to zero, a test of whether each APP was generally associated with the covariate.

	A2M	*CRP*	*FER*	*FIB*	*HAP*	*PCT*	*SAA*	*SAP*	*tPA*
Sex	0.413	**0.001**	0.226	0.397	0.745	0.301	0.597	0.629	0.244
Birth Order	0.290	**0.002**	0.109	0.571	0.359	0.051	0.184	**0.030**	**0.012**
Birth Year	0.179	0.754	**0.001**	0.077	0.956	0.420	0.835	0.338	0.055
Mode	0.071	**<0.001**	**0.040**	0.844	**<0.001**	0.521	**0.039**	**0.039**	0.959
Gestational Age	**0.043**	**<0.001**	0.088	0.265	**0.013**	0.414	0.527	**0.000**	0.298
Size for GA	0.458	0.350	0.425	0.926	**0.006**	0.688	0.243	0.756	0.796
Apgar	0.266	0.170	0.677	0.202	0.466	**0.023**	0.258	0.303	0.933
Sample Age	0.359	**<0.001**	0.515	**0.011**	**<0.001**	**<0.001**	**<0.001**	0.294	**0.017**
Maternal Age	0.447	0.174	0.166	0.118	0.175	0.275	0.672	0.769	**0.006**
Maternal Psych. Hist.	0.919	0.067	0.371	0.189	0.798	0.887	0.509	0.265	0.951
Maternal BMI	0.499	0.572	0.777	0.164	0.937	0.201	0.251	0.059	0.448
Maternal Migration	0.552	0.620	0.300	**0.037**	0.353	0.173	**0.030**	**0.003**	0.266
Income	0.802	0.156	0.739	0.082	0.083	**0.020**	**<0.001**	0.785	**0.012**
Education	0.842	0.298	0.777	0.584	0.796	0.388	0.883	0.777	0.681
Maternal Infection	0.172	0.349	0.955	**0.006**	0.683	0.052	0.174	0.569	**0.010**
Anemia	0.403	0.658	0.358	0.078	0.926	**0.025**	0.672	0.256	0.322
Supplement	0.207	0.476	0.360	0.054	0.199	0.092	**0.022**	0.117	**0.045**

Abbreviations: **GA**: gestational age; **Psych:** Psychiatric; **BMI**: body mass index; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

Table S5. P-values for the association of APP with other covariates among 924 ASD affected individuals. We examined the association of each covariate with the APP z-scores by regressing each APP over the categories of the covariate, followed by a joint Wald test of the hypothesis that all coefficients for the categorical indicators are equal to zero, a test of whether each APP was generally associated with the covariate.

	A2M	*CRP*	*FER*	*FIB*	*HAP*	*PCT*	*SAA*	*SAP*	*tPA*
Sex	0.820	**0.019**	0.480	0.526	0.142	0.562	0.482	0.926	0.259
Birth Order	0.710	**<0.001**	0.311	0.591	0.268	0.994	0.214	**0.004**	0.488
Birth Year	0.533	0.174	0.134	**0.017**	0.887	0.566	0.652	0.268	0.123
Mode	0.055	**0.029**	**<0.001**	0.608	0.402	0.707	0.363	**0.002**	0.137
Gestational Age	0.068	**<0.001**	**0.006**	0.354	**<0.001**	**0.035**	**0.024**	0.053	**0.002**
Size for GA	0.540	0.465	0.554	0.697	0.428	0.287	0.172	0.835	0.316
Apgar	0.285	**<0.001**	0.350	0.753	0.091	0.198	**0.001**	**0.005**	0.716
Sample Age	0.504	**<0.001**	0.066	0.104	**<0.001**	**0.013**	**<0.001**	0.143	0.238
Maternal Age	0.501	0.346	0.738	0.178	0.560	0.100	0.632	0.129	0.066
Maternal Psych. Hist.	0.264	0.542	0.718	0.083	0.964	0.597	0.218	**0.022**	0.686
Maternal BMI	0.469	0.732	0.382	0.218	0.443	0.104	0.848	0.230	0.555
Maternal Migration	0.367	0.113	0.113	0.165	0.123	0.827	0.491	0.991	**0.049**
Income	0.562	0.098	**0.029**	0.546	0.704	0.126	0.486	0.394	0.055
Education	**0.025**	**0.001**	0.770	0.404	0.406	0.116	0.627	**0.010**	0.406
Maternal Infection	0.687	0.957	**0.006**	0.558	0.416	**0.050**	0.470	0.468	**0.023**
Anemia	0.169	0.728	**0.015**	0.633	0.205	0.816	0.579	**0.030**	0.197
Supplement	0.896	0.144	0.358	0.082	0.701	0.236	0.148	0.323	0.055

Abbreviations: **GA**: gestational age; **Psych:** Psychiatric; **BMI**: body mass index; **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

Table S6. P-values for non-linearity in restricted cubic spline analyses. Not all relationships we considered are necessarily non-linear. To assess evidence for non-linear relationships, we tested the null-hypothesis that all spline terms that would indicate a change in the slope of the relationship (i.e., all but the first spline term) are equal to zero using a Wald test.

	Any ASD		***ASD w/ ID***		***ASD w/ ADHD***		***ASD only***		***Siblings***	
	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted	unadjusted	adjusted
A2M	0,212	0,355	0,946	0,996	0,067	0,232	0,594	0,478	**0,000**	**0,001**
CRP	**0,028**	**0,010**	0,274	0,094	0,085	0,136	0,079	**0,014**	0,668	0,179
FER	0,361	0,449	0,927	0,792	0,187	0,329	0,707	0,549	0,507	0,051
FIB	**0,030**	0,233	**0,015**	0,151	0,551	0,956	0,270	0,341	0,915	0,649
HAP	0,849	0,861	0,572	0,491	0,883	0,705	0,591	0,799	0,334	0,731
PCT	**0,045**	0,063	0,082	0,248	**0,016**	**0,015**	0,812	0,889	0,560	0,284
SAA	0,240	0,321	0,167	0,320	0,431	0,355	0,823	0,686	0,056	0,980
SAP	0,634	0,846	0,757	0,816	0,245	0,366	0,342	0,358	**0,034**	0,725
tPA	0,280	0,081	0,751	0,394	0,893	0,901	0,095	**0,019**	0,210	0,221

Abbreviations: **ASD**: autism spectrum disorders; **ADHD**: attention-deficit/hyperactivity disorder; **ID**: intellectual disability**; A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator.

Table S7. P-values for interaction using a likelihood ratio test comparing a model without interaction terms nested in model with interaction terms. A low p-value on this test indicates evidence to reject the null hypothesis of the absence of interaction between the APP and the covariates tested.

	A2M	***CRP***	***FER***	***FIB***	***HAP***	***PCT***	***SAA***	***SAP***	***tPA***
Sex	0.473	0.726	0.097	0.746	0.360	0.340	0.975	0.385	0.239
Birth order	0.869	0.296	0.103	0.281	0.764	0.345	0.856	0.179	0.371
Mode	0.887	0.474	0.240	0.991	0.202	0.801	0.226	0.520	0.640
Gestational age	0.929	0.556	0.860	0.583	0.872	0.455	0.153	0.165	0.125
Size for gestational age	0.188	0.607	0.810	0.621	0.341	0.640	0.600	0.365	0.223
Apgar score	0.312	0.241	0.784	0.543	0.233	0.867	**0.018**	0.674	0.415
Maternal Age	0.872	0.593	0.779	0.473	0.449	0.718	0.999	**0.026**	0.352
Maternal Psychiatric History	0.331	**0.037**	0.619	0.093	0.710	0.911	0.369	0.293	0.575
Maternal BMI	0.810	0.112	0.408	0.298	0.828	0.710	0.801	0.485	0.989
Maternal Migration	0.538	0.235	0.612	0.440	0.352	0.321	0.131	0.143	0.778
Parental Income	0.515	0.410	0.098	0.640	0.956	0.242	0.157	0.387	**0.013**
Parental Education	0.070	0.215	0.321	0.737	0.399	0.849	0.707	**0.003**	0.174
Maternal Infection	**0.008**	0.511	**0.043**	**0.017**	0.799	**0.016**	0.218	0.741	**0.001**
Maternal Anemia	0.100	0.864	**0.010**	0.185	0.526	0.204	0.816	**0.043**	0.311
Maternal Supplement Use	0.907	0.283	0.631	0.500	0.546	0.116	0.078	0.565	0.917

Abbreviations: **A2M**: α-2 macroglobulin; **CRP**: C reactive protein; **FER**: ferritin; **FIB**: fibrinogen; **HAP**: haptoglobin; **PCT**: procalcitonin; **SAA**: serum amyloid A; **SAP**: serum amyloid P; and **tPA**: tissue plasminogen activator