ARTICLE

Maternal mid-gestational and child cord blood immune signatures are strongly associated with offspring risk of ASD

Xiaoyu Che[1,2,10], Mady Hornig[3,10], Michaeline Bresnahan[3,4], Camilla Stoltenberg[5,6], Per Magnus[5], Pål Surén[5], Siri Mjaaland[5], Ted Reichborn-Kjennerud[5,7], Ezra Susser[3,4]✉ and W. Ian Lipkin[1,3,8,9]✉

Epidemiological studies and work in animal models indicate that immune activation may be a risk factor for autism spectrum disorders (ASDs). We measured levels of 60 cytokines and growth factors in 869 maternal mid-gestational (MMG) and 807 child cord blood (CB) plasma samples from 457 ASD (385 boys, 72 girls) and 497 control children (418 boys, 79 girls) from the Norwegian Autism Birth Cohort. We analyzed associations first using sex-stratified unadjusted and adjusted logistic regression models, and then employed machine learning strategies (LASSO + interactions, Random Forests, XGBoost classifiers) with cross-validation and randomly sampled test set evaluation to assess the utility of immune signatures as ASD biomarkers. We found prominent case–control differences in both boys and girls with alterations in a wide range of analytes in MMG and CB plasma including but not limited to IL1RA, TNFα, Serpin E1, VCAM1, VEGFD, EGF, CSF1, and CSF2. MMG findings were most striking, with particularly strong effect sizes in girls. Models did not change appreciably upon adjustment for maternal conditions, medication use, or emotional distress ratings. Findings were corroborated using machine learning approaches, with area under the receiver operating characteristic curve values in the test sets ranging from 0.771 to 0.965. Our results are consistent with gestational immunopathology in ASD, may provide insights into sex-specific differences, and have the potential to lead to biomarkers for early diagnosis.

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INTRODUCTION

Autism spectrum disorders (ASDs) comprise a set of pervasive neurodevelopmental conditions characterized by restricted and repetitive behavior patterns and impairments in social interaction and communication [1]. ASD diagnosis is based on clinical criteria and requires specialized expertise [2]. Although caregivers may detect behavioral abnormalities at earlier time points, the mean age for diagnosis is age 4–5 years [3]. ASDs have a profound impact on public health. The Autism and Developmental Disabilities Monitoring Network, comprising 11 sites in the United States, reported a prevalence of 1 in 54 in 2016 [4]. World Health Organization estimates are lower at 1 in 270. This may represent a true difference in prevalence or in the efficiency of case ascertainment. ASDs are four times more common in boys than in girls. One proffered explanation for sex bias is a multiple-threshold multifactorial liability model wherein the minimum genetic liability sufficient to cause ASD is greater in females than in males [5].

Twin studies as early as the 1970s indicate that ASD are heritable. Nonetheless, extragenetic factors, including prenatal medications, toxins, nutrients, fever, and immune activation, may have an important role. Valproic acid exposure during the first trimester, for example, is associated with an increased risk of ASD [6, 7]. Anatomical and behavioral outcomes of maternal immune activation in animal models vary with exposure timing [8] and as a function of sex-specific differences in microglial responses [5, 9–13]. Gestational exposure of rodents and non-human primates to the proinflammatory cytokines IL6 and IL17 results in structural and behavioral disturbances reminiscent of ASD [14–17]. Cytokines regulate intrauterine immune responses, neurogenesis, neuronal migration, and synaptogenesis, and have the capacity to signal through cognate receptors on microglial cells and other neural components distributed throughout brain circuitry [18–22]. Immune molecules, including VEGF, serpin E1, VCAM1, and TNFa, may be produced by neural progenitors during fetal brain development, encourage angiogenesis, and contribute to the sculpting of the brain during development; disruptions in these immune molecules may contribute to the abnormalities in angiogenesis in ASD [23].

There is only sparse literature on prenatal cytokine surveys in ASD. Most studies assayed dried neonatal blood spots instead of umbilical cord blood (CB) [24–28]. Few have described large population-based samples or prospective designs with extended longitudinal follow-

[1]Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, NY, USA. [2]Department of Biostatistics, Mailman School of Public Health, Columbia University, New York, NY, USA. [3]Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA. [4]New York State Psychiatric Institute, New York, NY, USA. [5]Norwegian Institute of Public Health, Oslo, Norway. [6]Department of Global Public Health, University of Bergen, Bergen, Norway. [7]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. [8]Department of Neurology, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, USA. [9]Department of Pathology and Cell Biology, Vagelos College of Physicians and Surgeons, Columbia University, New York, NY, USA. [10]These authors contributed equally: Xiaoyu Che, Mady Hornig. ✉email: ess8@cumc.columbia.edu; wil2001@cumc.columbia.edu

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up to ensure capture of ASD cases or subsets that elude earlier diagnosis. Furthermore, none have had access to the serial samples needed to examine exposures at different time points during brain development. To investigate potential associations between immune activation during gestation and ASD risk, we characterized cytokine profiles early in gestation (maternal mid-gestation or MMG) and at birth CB in cases and controls from the Autism Birth Cohort study (ABC Study), a case–control study nested within a population-based pregnancy cohort [29].

METHODS

Study design, participants, and specimen collection

The Norwegian Mother, Father and Child Cohort Study (MoBa). The MoBa is a population-based pregnancy cohort administered by the Norwegian Institute of Public Health [30], comprising 114,473 children born in 1999–2009 and more than 95,000 mothers and 75,000 fathers. Pregnant women attending routine ultrasound examinations at approximately 18 weeks of gestation were recruited between 1999 and 2008 throughout Norway, with an overall participation rate of 41% [31]. Biological samples collected included maternal samples at MMG and birth, paternal samples, and child umbilical CB.

The ABC Study. The ABC Study is a sub-study nested within the MoBa cohort and is comprised of cases of ASD and a random sample of the cohort selected as controls [32].

ASD cases. The ABC Study protocol defined cases according to DSM-IV-TR criteria for any ASD (autistic disorder, Asperger's disorder, pervasive developmental disorder-not otherwise specified). ASD cases in the ABC cohort were ascertained by multiple methods, including MoBa questionnaire screening (child ages 3, 5, and 7 years), referrals (parental or professional), and annual linkages to the Norwegian Patient Register (NPR). The ABC methods for diagnosis and confirmation are outlined in Stoltenberg et al. [32]. Of the cases included in cytokine analyses reported here, 146 children were diagnosed with ASD in the ABC Study Clinic by trained clinical psychologists and child psychiatrists. The assessments included the ADI-R and the ADOS, medical history, neurological examination, and tests of intellectual and adaptive functioning and language capacity. Via the NPR, 309 children were diagnosed with ASD by a physician or a psychologist using ICD-10 F84 criteria corresponding to DSM-IV-TR criteria. Patient records included developmental, medical, and neurological exams, the ADI-R, the ADOS, language assessments, adaptive functioning, and direct observation in a nursery, school, or clinic. Cases were additionally classified according to comorbidity with intellectual disability or attention deficit hyperactivity disorder, combining data from ABC clinic assessments, record review, and the NPR [12]. A record review study was conducted to determine the validity of NPR-registered ASD diagnoses. Suren et al. [33] found that 95% of NPR-sourced ASD diagnoses were consistent with the ABC Study case definitions, both for the subset of children examined at the ABC Study Clinic and for those undergoing record review.

ABC controls. Approximately 1.63% of those reaching 37.5 months of age in a given week were randomly selected from the MoBa cohort to serve as a pool of eligible controls, principally for laboratory studies (original $n = 1811$) [32]. Controls assigned an ASD diagnosis at the ABC clinic or ascertained with an ASD by the NPR were reclassified as cases ($n = 8$). MMG plasma was collected at 17–21 weeks gestation. CB plasma was collected on the day of birth.

Study sample selection for cytokine analyses. The sample derives from ASD cases ascertained through 2015 and ABC controls meeting inclusion criteria (singleton birth, continued participation in the cohort, and survival to at least age 3 years, availability of 120 µl or more of MMG and/or umbilical CB plasma), and subjects with data available from MoBa questionnaires containing covariates relevant to both MMG and CB analyses (completed by mothers at gestational weeks 17 and 30, and 6 months post partum). MMG and CB study samples were selected based on ASD case availability for each sample type, stratified on sex. Subjects with both MMG and CB samples were prioritized for the current analyses. Male and female ABC control samples were then randomly selected from among the pool of eligible male and female ABC controls in numbers equal to that of each sex- and sample type-stratified ASD case group. Demographic details of the subjects are shown in Table 1. The derivation of the final analytic study sample appears in Fig. 1.

Covariates. Maternal illness and medications consumed during pregnancy have the potential to influence both the risk of having altered immune marker concentrations in MMG or CB plasma, and the risk of ASD. Accordingly, we extracted data from MoBa questionnaires covering the time period prior to the maternal mid-gestational blood draw (MMG analyses), or for all of pregnancy up until birth (CB analyses) including maternal report of fever, respiratory infection, other infection, autoimmune/allergic disorders, emotional distress ratings (Hopkins Symptom Check List) (SCL-5) [34], and use of acetaminophen and related non-NSAID-type antipyretic drugs (ATC codes N02BE01, N02BA01, N02BA51, N02BB51). We also included maternal age (dichotomized as <30 years or ≥30 years). MMG analyses additionally included gestational age (in days) at maternal mid-gestational blood draw. For MMG sensitivity analyses, to examine the possibility that some questionnaire data were provided beyond ±28 days from the timing of the acquisition of the maternal blood sample, we also extracted the dates of return of the MoBa questionnaires containing data regarding the period preceding the MMG blood draw, calculated the difference in days between the MMG blood draw and the return of the relevant MoBa questionnaire, and categorized subjects as within or outside the 28-day window between questionnaire completion and blood sample collection. For CB sensitivity analyses, we extracted data regarding preeclampsia/eclampsia, mode of delivery (Caesarean section or not), and gestational age at birth (categorized as <37 weeks; ≥37 weeks and <42 weeks; ≥42 weeks).

Human subjects. Studies were approved by the Regional Committee for Medical and Health Research Ethics for Southeastern Norway and the Columbia University Medical Center Institutional Review Board (protocol number AAA2258). All samples were obtained using informed consent from mothers for both MMG plasma and CB.

MMG and CB samples. MMG and umbilical CB samples were collected via syringe into K2 EDTA tubes, processed and stored at −80 °C [31], with quality assurance procedures as previously described [35]. MMG and CB plasma samples were shipped from the MoBa Biobank to Columbia on dry ice, stored at −80 °C until aliquoting and returned to −80 °C storage until use.

Immune profiling assays. We assayed a wide range of cytokines, chemokines, cellular, and growth factors reflecting key processes relating to systemic activation of inflammatory/immune signaling pathways involved in autoimmunity and anti-inflammatory responses as well as others implicated in CNS inflammation, neurovascular disruption, and neurogenesis. Immune molecules within this panel are also found to be dysregulated during infection with certain pathogens, including those that trigger autoimmunity [36], as well as in some studies in ASD [27, 28, 37] (Supplementary Table 1). The fluorescent intensity levels of the following immune molecules were determined using a bead-based, 60-plex immunoassay: *interleukin (IL)1 superfamily*, IL1α, IL1β, IL18, IL1RA; *IL2 family*, IL2, IL4, IL7, IL9, IL15, IL21; *IL6 (gp130) family*, IL6, IL31, LIF; *IL12 family*, IL12p40, IL12p70, IL23, IL27; *IL17 family*, IL17A, IL17F, IL22; *Th2 type*, IL5, IL10, IL13; *tumor necrosis factor (TNF) superfamily*, TNFα (TNFSF2), TNFβ (TNFSF1), sFasL (TNFSF6), TRAIL (TNFSF10); *type I interferons (IFN)*, IFNα2, IFNβ; *type II IFN*, IFNγ; *CC chemokines*, CCL2 (MCP1), CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES), CCL7 (MCP3), CCL11 (eotaxin); *CXC chemokines*, CXCL1 (GROα), CXCL8 (IL8), CXCL9 (MIG), CXCL10 (IP10), CXCL12a (SDF1); *Other growth factors*, βNGF, EGF, HGF, TGFα, TGFβ, FGFb; *PDGF family/VEGF subfamily*, PDGFBB, VEGFA, VEGFD; *Serine protease inhibitor*, PAI1 (serpin E1); *Cell adhesion molecules*, sICAM1 (CD54), VCAM1 (CD106); *Neurotrophic/stimulating factors*, BDNF, CSF1 (MCSF), CSF2 (GMCSF), CSF3 (GCSF), SCF; *Adipose-derived hormones*, leptin, resistin (customized Procarta immunoassay, Affymetrix/eBioscience, Santa Clara, CA, USA, Thermo Fisher Scientific).

Plasma samples from male and female ASD cases and ABC controls were coded, randomized, and assayed in duplicate. Median fluorescence intensities (MFI) of each analyte-specific immunoassay bead set were detected by the flow- and fluorescence-based Luminex 200™ detection platform (Luminex Corporation, Austin, TX, USA) [38]. Data were processed using a quality control (QC) algorithm that calibrates performance of an expanded set of serial standard curves and in-house plasma controls included on every plate, and monitors intra- and inter-plate coefficient of variation (CV) and bead counts. Only plates with mean intra-assay %CV <

Table 1. Subject characteristics.

Subject characteristics		Sex	MMG plasma ($n = 854$)						
			ASD cases $n = 414$ 64 F/350 M		Controls $n = 440$ 69 F/371 M		Total MMG 133 F/721 M		p value[a]
			n	%	*n*	%	*n*	%	
Maternal characteristics									
Maternal age	<30 years	F	27	42.2	23	33.3	50	37.6	0.292
		M	177	50.6	144	38.8	321	44.5	0.001
Parental education[b]	<12 years	F	9	14.1	2	2.9	11	8.3	0.070
		M	17	4.9	11	3.0	28	3.9	0.098
	12 years	F	13	20.3	12	17.6	25	18.9	
		M	102	29.3	89	24.1	191	26.6	
	13–16 years	F	21	32.8	21	30.9	42	31.8	
		M	126	36.2	134	36.2	260	36.2	
	≥17 years	F	21	32.8	33	48.5	54	40.9	
		M	103	29.6	136	36.8	239	33.3	
Maternal exposures any time in pregnancy	Non-NSAID medications	F	22	34.4	29	42.0	51	38.3	0.364
		M	138	39.4	128	34.5	266	36.9	0.171
	Fever	F	5	7.8	3	4.3	8	6.0	0.401
		M	22	6.3	17	4.6	39	5.4	0.312
	Infection	F	40	62.5	42	60.9	82	61.7	0.847
		M	231	66.0	218	58.8	449	62.3	0.045
	Autoimmune/allergic disorders	F	16	25.0	18	26.1	34	25.6	0.886
		M	97	27.7	100	27.0	197	27.3	0.819
Obstetrical/perinatal factors									
Mode of delivery	Caesarean section	F	5	7.8	5	7.2	10	7.5	0.902
		M	27	7.7	25	6.7	52	7.2	0.613
Child characteristics									
Birth year	2000	F	4	6.3	2	2.9	6	4.5	0.021
		M	7	2.0	4	1.1	11	1.5	<0.001
	2001	F	3	4.7	5	7.2	8	6.0	
		M	26	7.4	15	4.0	41	5.7	
	2002	F	11	17.2	3	4.3	14	10.5	
		M	52	14.9	30	8.1	82	11.4	
	2003	F	17	26.6	10	14.5	27	20.3	
		M	62	17.7	44	11.9	106	14.7	
	2004	F	7	10.9	9	13.0	16	12.0	
		M	56	16.0	52	14.0	108	15.0	
	2005	F	9	14.1	9	13.0	18	13.5	
		M	50	14.3	51	13.7	101	14.0	
	2006	F	5	7.8	11	15.9	16	12.0	
		M	44	12.6	69	18.6	113	15.7	
	2007	F	7	10.9	9	13.0	16	12.0	
		M	30	8.6	53	14.3	83	11.5	
	2008	F	0	0.0	9	13.0	9	6.8	
		M	22	6.3	45	12.1	67	9.3	
	2009	F	1	1.6	2	2.9	3	2.3	
		M	1	0.3	8	2.2	9	1.2	
Birth season	Winter (Dec–Feb)	F	13	20.3	18	26.1	31	23.3	0.347
		M	87	24.9	76	20.5	163	22.6	0.093
	Spring (Mar–May)	F	27	42.2	19	27.5	46	34.6	
		M	93	26.6	113	30.5	206	28.6	
	Summer (Jun–Aug)	F	14	21.9	17	24.6	31	23.3	
		M	74	21.1	96	25.9	170	23.6	
	Fall (Sept–Nov)	F	10	15.6	15	21.7	25	18.8	
		M	96	27.4	86	23.2	182	25.2	
GA[c]	<37 weeks	F	4	6.3	5	7.2	9	6.8	0.812
		M	27	7.7	16	4.3	43	6.0	0.041
	37–<42 weeks	F	56	88.9	59	85.5	115	87.1	

Table 1. continued

Subject characteristics		Sex	MMG plasma ($n = 854$)						
			ASD cases $n = 414$ 64 F/350 M		Controls $n = 440$ 69 F/371 M		Total MMG 133 F/721 M		p value[a]
			n	%	*n*	%	*n*	%	
		M	276	79.1	318	85.9	594	82.6	
	≥42 weeks	F	3	4.8	5	7.2	8	6.1	
		M	46	13.2	36	9.7	82	11.4	
Birth weight[d]	<1500 g	F	0	0.0	0	0.0	0	0.0	0.597
		M	4	1.1	2	0.5	6	0.8	0.363
	1500–<2500 g	F	0	0.0	1	1.5	1	0.8	
		M	13	3.7	8	2.2	21	2.9	
	2500–<4000 g	F	51	79.7	52	76.5	103	78.0	
		M	233	66.8	242	65.2	475	66.0	
	≥4000 g	F	13	20.3	15	22.1	28	21.2	
		M	99	28.4	119	32.1	218	30.3	
Subject characteristics		**Sex**	**CB plasma ($n = 793$)**						
			ASD cases $n = 398$ 63 F/335 M		Controls $n = 395$ 62 F/333 M		Total MMG 125 F/668 M		p value[a]
			n	%	*n*	%	*n*	%	
Maternal characteristics									
Maternal age	<30 years	F	31	49.2	21	33.9	52	41.6	0.082
		M	171	51.0	128	38.4	299	44.8	0.001
Parental education[b]	<12 years	F	8	12.7	2	3.2	10	8.0	0.140
		M	19	5.7	12	3.6	31	4.7	0.059
	12 years	F	11	17.5	9	14.5	20	16.0	
		M	99	29.6	73	22.1	172	25.9	
	13–16 years	F	23	36.5	21	33.9	44	35.2	
		M	114	34.1	129	29.0	243	36.5	
	≥17 years	F	21	33.3	30	48.4	51	40.8	
		M	102	30.5	117	35.3	291	32.9	
Maternal exposures any time in pregnancy	Non-NSAID medications	F	29	46.0	28	45.2	57	45.6	0.922
		M	167	49.9	162	48.6	329	49.3	0.756
	Fever	F	14	22.2	6	9.7	20	16.0	0.056
		M	76	22.7	66	19.8	142	21.3	0.365
	Infection	F	53	84.1	49	79.0	102	81.6	0.462
		M	273	81.5	278	83.5	551	82.5	0.498
	Autoimmune/allergic disorders	F	17	27.0	14	22.6	31	24.8	0.569
		M	117	34.9	115	34.5	232	34.7	0.916
Obstetrical/perinatal factors									
Mode of delivery	Caesarean section	F	6	9.5	2	3.2	8	6.4	0.150
		M	19	5.7	15	4.5	34	5.1	0.493
Child characteristics									
Birth year	2000	F	2	3.2	2	3.2	4	3.2	0.058
		M	8	2.4	3	0.9	11	1.6	<0.001
	2001	F	3	4.8	5	8.1	8	6.4	
		M	27	8.1	15	4.5	42	6.3	
	2002	F	11	17.5	3	4.8	14	11.2	
		M	53	15.8	27	8.1	80	12.0	
	2003	F	18	28.6	11	17.7	29	23.2	
		M	63	18.8	45	13.5	108	16.2	
	2004	F	10	15.9	8	12.9	18	14.4	
		M	51	15.2	44	13.2	95	14.2	
	2005	F	9	14.3	8	12.9	17	13.6	
		M	46	13.7	47	14.1	93	13.9	
	2006	F	3	4.8	10	16.1	13	10.4	
		M	38	11.3	60	18.0	98	14.7	
	2007	F	6	9.5	7	11.3	13	10.4	

Table 1. continued

Subject characteristics		Sex	CB plasma ($n = 793$)						
			ASD cases $n = 398$ 63 F/335 M		Controls $n = 395$ 62 F/333 M		Total MMG 125 F/668 M		p value[a]
			n	%	*n*	%	*n*	%	
		M	30	9.0	43	12.9	73	10.9	
	2008	F	1	1.6	6	9.7	7	5.6	
		M	19	5.7	39	11.7	58	8.7	
	2009	F	0	0.0	2	3.2	2	1.6	
		M	0	0.0	10	3.0	10	1.5	
Birth season	Winter (Dec–Feb)	F	12	19.0	16	25.8	28	22.4	0.490
		M	79	23.6	69	20.7	148	22.2	0.042
	Spring (Mar–May)	F	24	38.1	18	29.0	42	33.6	
		M	89	26.6	103	30.9	192	28.7	
	Summer (Jun–Aug)	F	18	28.6	15	24.2	33	26.4	
		M	69	20.6	89	26.7	158	23.7	
	Fall (Sept–Nov)	F	9	14.3	13	21.0	22	17.6	
		M	98	29.3	72	21.6	170	25.4	
GA[c]	<37 weeks	F	4	6.5	3	4.8	7	5.6	0.927
		M	22	6.6	9	2.7	31	4.7	0.005
	37–<42 weeks	F	54	87.1	55	88.7	109	87.9	
		M	265	79.3	293	88.3	558	83.8	
	≥42 weeks	F	4	6.5	4	6.5	8	6.5	
		M	47	14.1	30	9.0	77	11.6	
Birth weight[d]	<1500 g	F	0	0.0	0	0.0	0	0.0	0.474
		M	1	0.3	0	0.0	1	0.1	0.229
	1500–<2500 g	F	1	1.6	0	0.0	1	0.8	
		M	9	2.7	3	0.9	12	1.8	
	2500–<4000 g	F	51	81.0	47	77.0	98	79.0	
		M	224	67.1	222	66.7	446	66.9	
	≥4000 g	F	11	17.5	14	23.0	25	20.2	
		M	100	29.9	108	32.4	208	31.2	

ASD autism spectrum disorder, *CB* cord blood, *F* female, *GA* gestational age at birth, *M* male, *MMG* maternal mid-gestation.
[a]χ^2, two-sided p value.
[b]Parental education missing: MMG, $n = 3$ M, 1 F; CB, $n = 3$ M.
[c]Gestational age missing: MMG, $n = 2$ M; CB, $n = 2$ M, 1 F.
[d]Birth weight missing: MMG, $n = 1$ M, 1 F; CB, $n = 1$ M, 1 F.

15% were accepted. Samples failing to meet QC criteria were designated for re-run when feasible (CVs >25%, bead counts <30). MFI values exceeding machine limits of reliable detection (>25,000) were excluded. Because interpolated concentrations can introduce bias [39–42] for samples with very low or high values in relation to the serial standard curves, we based our analyses on MFI rather than concentration estimates. Analyte concentrations in Luminex assays are derivatives of MFI values. As noted by Breen et al., low abundance analytes are frequently not in the linear range of the standard curve [39]. Accordingly, accurate measurements of analyte concentration would require individualized, calibrated standard curves for each of the 60 analytes on every plate. Moreover, MFI has a lower inter-assay CV [40]. Thus, analyses based on MFI are more reliable than analyses based on estimates of analyte concentrations. Averaged MFI values meeting QC criteria for all 60 cytokines were used in the final statistical analyses. The final dataset (test samples) had mean intra-assay %CV of 5.5% (SD 5.2%) and 0.73% of all possible intra-assay % CV values were >25%, across all cytokines. All per-cytokine intra-assay %CV values were similarly <10%, with the exception of CCL2, for which the mean intra-assay %CV was 10.5%. The medians and ranges of the MFI values of all 60 cytokines in MMG and CB are reported in Supplementary Table 2.

Statistical analysis

Immune data processing: missing data, imputation, outliers, and transformations. Missing data and imputation: Five data points were lacking in MMG immunoassay data due to insufficient bead counts (<0.01% of 98,820 potential values derived from the MMG and CB immunoassays). These included one MMG boy lacking both CCL2 and IL22, one MMG boy lacking CCL2, and one girl lacking both CCL2 and IL22. No CB immunoassay values were missing. For logistic regression analyses, procedures were pursued using all 60 analytes, excluding subjects with these missing data; for the predictive modeling through machine learning, the missing data points were imputed using the mean value of the corresponding analyte.

Outliers and data transformations: Outliers were identified through principal component analysis (PCA) [43]. After eliminating samples identified by PCA as outliers (visual inspection, MMG female $n = 5$, MMG male $n = 10$, CB female $n = 4$, CB male $n = 10$; for these samples, data from all 60 analytes were excluded from analyses), levels of each analyte were natural log-transformed and divided by the standard deviation of that analyte within the control group.

Data analyses: We used logistic regression models separately for boys and girls, and within each sample type to test for an association between each analyte and ASD risk. Two models were explored for MMG samples: one unadjusted, and another adjusting for the questionnaire- and MBRN-derived covariate data reflecting maternal age, illnesses (fever, infection, inflammatory, autoimmune, allergic disorders), emotional distress scores (SCL-5), and use of non-NSAID antipyretic medications (e.g., acetaminophen) in pregnancy up until sample acquisition, as well as gestational age at MMG blood sample collection. Two models were applied for CB samples: one unadjusted, and another adjusting for the questionnaire-derived

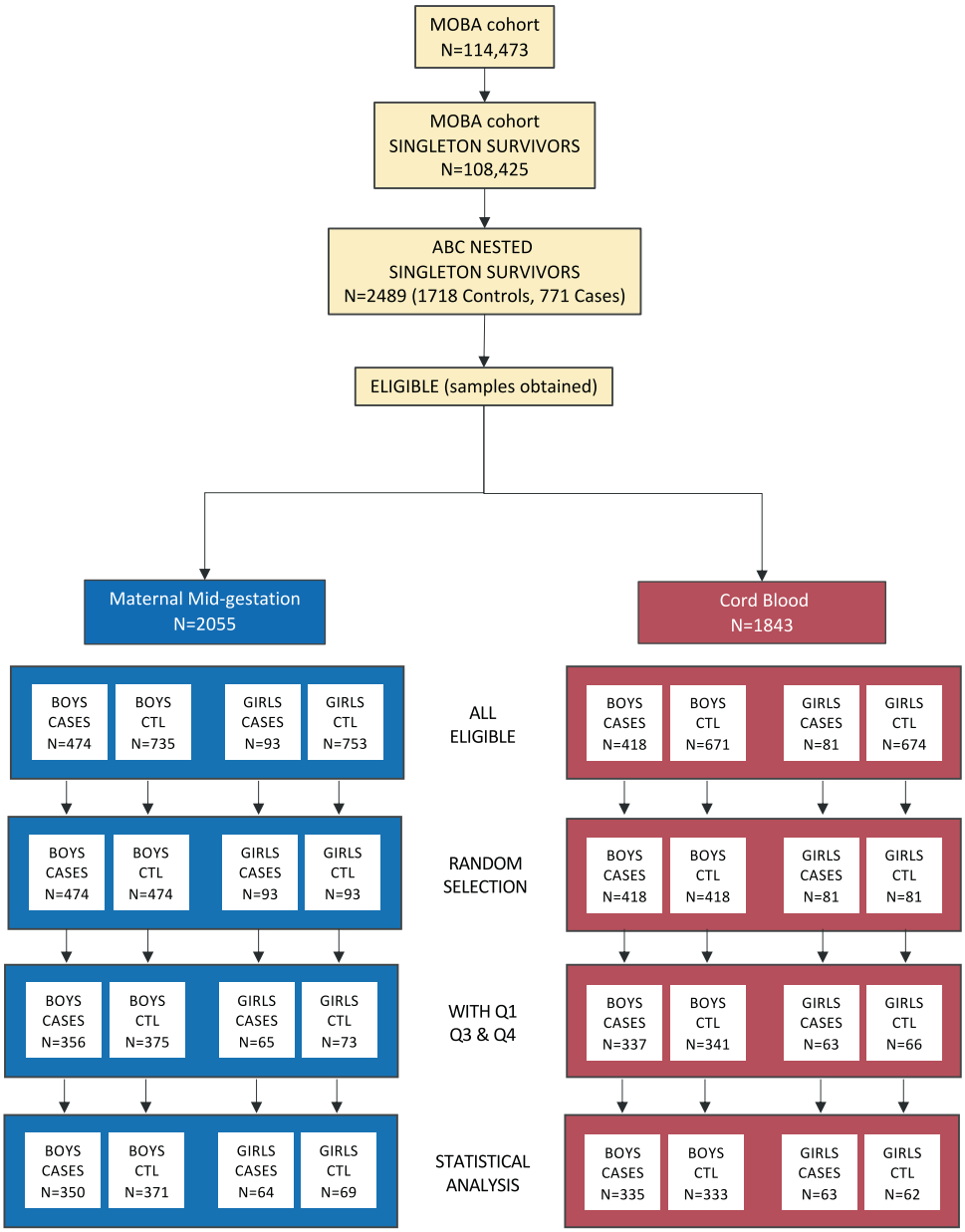

Fig. 1 Pipeline for sample selection.

covariate data reflecting maternal age, maternal illnesses during pregnancy (fever, infection, inflammatory/autoimmune/allergic disorders), maternal emotional distress scores, and use of non-NSAID antipyretic medications in pregnancy. We imputed missing values within the maternal variables including gestational age at MMG blood draw (female $n = 1$, male $n = 2$), and gestational age at birth (female $n = 1$, male $n = 2$). Missing items for maternal emotional distress scores (SCL-5) were addressed by mean imputation ($n = 10$ missing all SCL-5 items on the gestational week 17 questionnaire; $n = 6$ subjects missing all SCL-5 items on the gestational week 30 questionnaire). The standard errors reduced by mean/mode imputations are negligible given the low prevalence of missing information. For MMG samples, we repeated the adjusted models in one sensitivity analysis restricting the study population to those whose mid-gestational sample collection times were within ±28-day window from the dates of return of the MoBa questionnaires. Three sensitivity analyses were explored for CB samples: one restricting the study population to those not born by Caesarean section, one restricting to those whose mothers did not experience preeclampsia or eclampsia during the pregnancy, and one restricting to those with gestational ages between weeks 37 and 41 of gestation.

Multiple comparisons over the 60-plex immunoassay panel were corrected using the Benjamini–Hochberg procedure [44], controlling the overall false discovery rate (FDR) at the level of 0.05. Odds ratios (ORs) and their associated 95% confidence intervals (CIs) were calculated. To explore the utility of the multiplex panel as a biomarker tool for ASD, we employed three machine learning algorithms: LASSO (least absolute shrinkage and selection operator) [45], Random Forests (RF) [46], and XGBoost [47]. For LASSO, we fitted the 60 immune signature analytes, with and without their two-way interaction terms, as predictors in two separate models. Models were built and evaluated within each sample type and sex, separately. The models were first trained in the 80% randomly selected training set using ten-fold cross-validation, and the remaining 20% of the study population was used as the independent test set to validate model performance. We also applied the Bayesian Model Averaging method that combines the predictions of multiple models using weighted averages in which the weights are Bayesian posterior probabilities that the given model is the true model, conditional on the training data [48]. The predictive performance of the five models (LASSO without interaction terms, Lasso with interaction terms, RF, XGBoost, and Model Average) in the test set was evaluated using area under the receiver operating characteristic curve

Table 2. Estimates from logistics regression models testing for ASD association with each analyte.

		MMG: Boys (n=350 ASD, 371 controls)									
Immune family	Immune molecule	Unadjusted model					Adjusted model[a]				
		OR	95% CI		*p*-value	Adj *p* [c]	aOR	95% CI		*p*-value	Adj *p* [c]
IL1 superfamily	IL1α	1.31	1.13	1.54	<0.001	0.001	1.34	1.14	1.56	<0.001	<0.001
	IL1β	1.58	1.35	1.85	<0.0001	<0.0001	1.58	1.35	1.85	<0.0001	<0.0001
	IL18	1.14	0.98	1.33	0.086	0.105	1.17	1.00	1.36	0.051	0.068
	IL1RA	2.35	1.93	2.86	<0.0001	<0.0001	2.33	1.91	2.84	<0.0001	<0.0001
IL2 family	IL2	1.84	1.56	2.16	<0.0001	<0.0001	1.83	1.55	2.15	<0.0001	<0.0001
	IL7	1.48	1.27	1.72	<0.0001	<0.0001	1.47	1.26	1.71	<0.0001	<0.0001
	IL9	1.47	1.24	1.74	<0.0001	<0.0001	1.47	1.24	1.75	<0.0001	<0.0001
IL12 family	IL27	1.35	1.15	1.59	<0.001	<0.001	1.37	1.17	1.62	<0.001	<0.001
TNF superfamily	TNFα	2.58	2.14	3.10	<0.0001	<0.0001	2.63	2.18	3.18	<0.0001	<0.0001
	sFasL	1.20	1.02	1.41	0.026	0.039	1.19	1.01	1.40	0.039	0.056
IL6 (gp130) cytokine family	IL6	0.96	0.82	1.12	0.585	0.627	0.96	0.83	1.13	0.644	0.678
	IL31	1.18	1.01	1.39	0.035	0.049	1.17	1.00	1.38	0.051	0.068
Th2 type	IL5	1.69	1.43	1.99	0.0000	<0.0001	1.68	1.42	1.99	<0.0001	<0.0001
	IL13	1.76	1.47	2.09	0.0000	<0.0001	1.74	1.46	2.08	<0.0001	<0.0001
	IL10	1.27	1.09	1.48	0.002	0.004	1.27	1.09	1.49	0.003	0.005
CC chemokines	CCL5 (RANTES)	1.76	1.49	2.06	<0.0001	<0.0001	1.77	1.50	2.09	<0.0001	<0.0001
	CCL3 (MIP1a)	1.35	1.15	1.59	<0.001	<0.001	1.35	1.15	1.59	<0.001	<0.001
	CCL4 (MIP1b)	1.38	1.18	1.61	<0.0001	<0.001	1.39	1.19	1.63	<0.0001	0.0001
	CCL11 (eotaxin)	1.24	1.06	1.44	0.008	0.012	1.30	1.11	1.53	0.001	0.002
CXC chemokines	CXCL8 (IL8)	1.61	1.35	1.93	<0.0001	<0.0001	1.60	1.33	1.92	<0.0001	<0.0001
	CXCL9 (MIG)	1.11	0.96	1.29	0.152	0.172	1.15	0.99	1.34	0.075	0.091
	CXCL10 (IP10)	1.63	1.38	1.93	<0.0001	<0.0001	1.68	1.42	1.99	<0.0001	<0.0001
	CXCL12a (SDF1)	1.30	1.11	1.51	<0.001	0.002	1.33	1.14	1.55	<0.001	<0.001
Neurotrophic/stimulating factors	CSF3 (G-CSF)	1.90	1.61	2.25	<0.0001	<0.0001	1.90	1.61	2.25	<0.0001	<0.0001
	SCF (KITLG)	1.27	1.08	1.48	0.003	0.005	1.31	1.12	1.54	<0.001	0.001
Serine protease inhibitors	Serpin E1 (PAI-1)	2.41	2.01	2.90	<0.0001	<0.0001	2.44	2.03	2.93	<0.0001	<0.0001
Cell adhesion molecules	VCAM1 (CD106)	2.56	2.13	3.06	<0.0001	<0.0001	2.58	2.15	3.10	<0.0001	<0.0001
	sICAM1 (CD54)	1.15	0.99	1.34	0.074	0.092	1.19	1.02	1.40	0.027	0.040
PDGF family/VEGF subfamily	VEGFD	1.53	1.32	1.78	<0.0001	<0.0001	1.59	1.36	1.86	<0.0001	<0.0001
	VEGFA	1.40	1.17	1.66	<0.001	<0.001	1.41	1.18	1.68	<0.001	<0.001
	PDGFBB	1.20	1.03	1.39	0.016	0.025	1.19	1.02	1.38	0.025	0.039
Other growth factors	EGF	1.54	1.33	1.79	<0.0001	<0.0001	1.51	1.30	1.75	<0.0001	<0.0001
	FGFb	1.37	1.16	1.61	<0.001	<0.001	1.36	1.15	1.60	<0.001	<0.001
	βNGF	1.22	1.02	1.46	0.034	0.048	1.21	1.01	1.45	0.042	0.059
	TGFα	1.12	0.96	1.31	0.149	0.172	1.15	0.98	1.35	0.083	0.099
	TGFβ	1.12	0.97	1.31	0.132	0.156	1.13	0.97	1.32	0.116	0.134
Neurotrophic/stimulating factors	BDNF	1.11	0.96	1.29	0.174	0.193	1.09	0.93	1.26	0.292	0.325
Adipose-derived hormones	Resistin	1.16	1.00	1.35	0.050	0.066	1.19	1.02	1.38	0.028	0.041
Type I IFN	IFNβ	1.13	0.97	1.32	0.118	0.141	1.17	1.00	1.37	0.053	0.069
IL2 family	IL4	0.58	0.49	0.68	<0.0001	<0.0001	0.59	0.50	0.70	<0.0001	<0.0001
	IL15	0.85	0.73	1.00	0.044	0.060	0.86	0.74	1.01	0.068	0.085
	IL21	0.97	0.83	1.13	0.668	0.691	0.97	0.83	1.14	0.750	0.776
IL6 (gp130) cytokine family	LIF	0.86	0.74	1.00	0.056	0.072	0.87	0.75	1.02	0.087	0.103
IL12 family	IL12p40	0.54	0.46	0.63	<0.0001	<0.0001	0.54	0.46	0.64	<0.0001	<0.0001
	IL12p70	0.83	0.71	0.98	0.027	0.039	0.85	0.72	1.00	0.055	0.070
	IL23	0.79	0.67	0.93	0.005	0.008	0.80	0.68	0.95	0.009	0.015
IL17 family	IL17A	0.77	0.66	0.90	0.001	0.002	0.78	0.67	0.92	0.002	0.004
	IL17F	1.00	0.85	1.16	0.964	0.964	1.02	0.87	1.19	0.805	0.805
	IL22	0.43	0.35	0.52	<0.0001	<0.0001	0.43	0.35	0.53	<0.0001	<0.0001
Type I IFN	IFNα2	0.86	0.74	1.00	0.047	0.062	0.89	0.76	1.04	0.139	0.157
Type II IFN	IFNγ	0.47	0.39	0.56	<0.0001	<0.0001	0.48	0.40	0.57	<0.0001	<0.0001
CC chemokines	CCL2 (MCP1)	0.44	0.37	0.53	<0.0001	<0.0001	0.45	0.37	0.53	<0.0001	<0.0001
	CCL7 (MCP3)	1.04	0.90	1.21	0.566	0.618	1.06	0.91	1.23	0.463	0.506
CXC chemokines	CXCL1 (GROa)	0.76	0.64	0.90	0.001	0.003	0.75	0.63	0.89	<0.001	0.002
Neurotrophic/stimulating factors	CSF1 (M-CSF)	0.42	0.35	0.50	<0.0001	<0.0001	0.42	0.36	0.51	<0.0001	<0.0001
	CSF2 (GM-CSF)	0.51	0.43	0.60	<0.0001	<0.0001	0.51	0.43	0.60	<0.0001	<0.0001
Other growth factors	HGF	0.63	0.53	0.74	<0.0001	<0.0001	0.64	0.54	0.76	<0.0001	<0.0001
TNF superfamily	TNFβ (LTA)	0.78	0.67	0.92	0.003	0.005	0.79	0.67	0.92	0.004	0.006
	TRAIL	1.03	0.89	1.20	0.658	0.691	1.05	0.91	1.22	0.498	0.533
Adipose-derived hormones	Leptin	1.02	0.87	1.19	0.805	0.819	1.02	0.87	1.20	0.792	0.805

Table 2. continued

Immune family	Immune molecule	MMG: Girls (n=64 ASD, 69 controls)									
		Unadjusted model					Adjusted model[a]				
		OR	95% CI		*p*-value	Adj *p*[c]	aOR	95% CI		*p*-value	Adj *p*[c]
IL1 superfamily	IL1α	2.21	1.48	3.31	<0.001	<0.001	2.21	1.46	3.33	<0.001	<0.001
	IL1β	4.33	2.62	7.17	<0.0001	<0.0001	5.00	2.84	8.80	<0.0001	<0.0001
	IL18	1.28	0.95	1.73	0.103	0.126	1.26	0.93	1.70	0.134	0.164
	IL1RA	5.48	3.14	9.58	<0.0001	<0.0001	6.14	3.37	11.18	<0.0001	<0.0001
IL2 family	IL2	4.37	2.68	7.14	<0.0001	<0.0001	4.70	2.78	7.93	<0.0001	<0.0001
	IL7	3.04	1.95	4.74	<0.0001	<0.0001	3.56	2.18	5.79	<0.0001	<0.0001
	IL9	2.91	1.77	4.80	<0.0001	<0.0001	3.07	1.84	5.14	<0.0001	<0.0001
IL12 family	IL27	2.51	1.57	4.03	<0.001	<0.001	2.54	1.56	4.13	<0.001	<0.001
TNF superfamily	TNFα	7.45	3.78	14.68	<0.0001	<0.0001	8.42	4.03	17.59	<0.0001	<0.0001
	sFasL	2.09	1.30	3.34	0.002	0.004	2.11	1.30	3.42	0.002	0.004
IL6 (gp130) cytokine family	IL6	1.15	0.82	1.61	0.417	0.455	1.11	0.79	1.57	0.547	0.597
	IL31	2.38	1.54	3.67	<0.0001	<0.001	2.43	1.56	3.80	<0.0001	<0.001
Th2 type	IL5	4.41	2.63	7.39	<0.0001	<0.0001	4.67	2.72	8.04	<0.0001	<0.0001
	IL13	4.34	2.61	7.22	<0.0001	<0.0001	4.79	2.77	8.30	<0.0001	<0.0001
	IL10	2.19	1.42	3.40	<0.001	<0.001	2.22	1.41	3.48	<0.001	<0.001
CC chemokines	CCL5 (RANTES)	2.47	1.67	3.63	<0.0001	<0.0001	2.64	1.76	3.95	<0.0001	<0.0001
	CCL3 (MIP1a)	2.95	1.88	4.62	<0.0001	<0.0001	2.97	1.88	4.70	<0.0001	<0.0001
	CCL4 (MIP1b)	2.64	1.75	3.99	<0.0001	<0.0001	2.69	1.77	4.09	<0.0001	<0.0001
	CCL11 (eotaxin)	1.95	1.33	2.85	<0.001	0.001	1.97	1.34	2.90	<0.001	<0.001
CXC chemokines	CXCL8 (IL8)	3.37	2.03	5.57	<0.0001	<0.0001	3.65	2.15	6.21	<0.0001	<0.0001
	CXCL9 (MIG)	1.54	1.05	2.27	0.026	0.036	1.56	1.05	2.32	0.029	0.039
	CXCL10 (IP10)	2.86	1.82	4.48	<0.0001	<0.0001	3.19	1.97	5.18	<0.0001	<0.0001
	CXCL12a (SDF1)	2.61	1.70	3.99	<0.0001	<0.0001	2.63	1.70	4.08	<0.0001	<0.0001
Neurotrophic/stimulating factors	CSF3 (G-CSF)	6.51	3.40	12.45	<0.0001	<0.0001	7.82	3.76	16.25	<0.0001	<0.0001
	SCF (KITLG)	3.33	2.00	5.52	<0.0001	<0.0001	3.46	2.03	5.89	<0.0001	<0.0001
Serine protease inhibitors	Serpin E1 (PAI-1)	5.73	3.30	9.96	<0.0001	<0.0001	7.64	3.90	14.96	<0.0001	<0.0001
Cell adhesion molecules	VCAM1 (CD106)	5.37	3.15	9.16	<0.0001	<0.0001	5.84	3.30	10.35	<0.0001	<0.0001
	sICAM1 (CD54)	1.50	0.97	2.32	0.067	0.084	1.65	1.03	2.64	0.038	0.049
PDGF family/VEGF subfamily	VEGFD	2.35	1.64	3.37	<0.0001	<0.0001	2.53	1.72	3.72	<0.0001	<0.0001
	VEGFA	3.62	2.21	5.93	<0.0001	<0.0001	3.87	2.30	6.50	<0.0001	<0.0001
	PDGFBB	1.87	1.27	2.76	0.002	0.003	2.10	1.38	3.22	<0.001	0.001
Other growth factors	EGF	2.67	1.87	3.82	<0.0001	<0.0001	3.10	2.07	4.65	<0.0001	<0.0001
	FGFb	2.93	1.83	4.69	<0.0001	<0.0001	3.30	2.00	5.44	<0.0001	<0.0001
	βNGF	3.59	2.19	5.88	<0.0001	<0.0001	4.00	2.35	6.81	<0.0001	<0.0001
	TGFα	1.64	1.10	2.44	0.015	0.023	1.64	1.09	2.47	0.019	0.028
	TGFβ	1.90	1.25	2.90	0.003	0.005	1.95	1.26	3.01	0.003	0.004
Neurotrophic/stimulating factors	BDNF	1.68	1.18	2.39	0.004	0.006	1.77	1.22	2.56	0.003	0.004
Adipose-derived hormones	Resistin	1.06	0.74	1.52	0.736	0.789	1.06	0.73	1.54	0.770	0.825
Type I IFN	IFNβ	1.58	1.05	2.36	0.028	0.037	1.57	1.03	2.39	0.034	0.045
IL2 family	IL4	0.54	0.37	0.80	0.002	0.004	0.53	0.35	0.79	0.002	0.003
	IL15	1.25	0.86	1.82	0.234	0.265	1.23	0.84	1.80	0.294	0.340
	IL21	1.48	1.06	2.06	0.022	0.031	1.47	1.04	2.06	0.027	0.038
IL6 (gp130) cytokine family	LIF	1.30	0.88	1.92	0.187	0.220	1.27	0.85	1.89	0.245	0.288
IL12 family	IL12p40	0.37	0.25	0.53	<0.0001	<0.0001	0.35	0.24	0.52	<0.0001	<0.0001
	IL12p70	1.21	0.81	1.81	0.343	0.382	1.18	0.78	1.77	0.441	0.490
	IL23	0.97	0.65	1.43	0.860	0.905	0.98	0.65	1.46	0.909	0.940
IL17 family	IL17A	1.01	0.70	1.47	0.946	0.979	0.98	0.67	1.44	0.936	0.952
	IL17F	1.27	0.89	1.81	0.186	0.220	1.26	0.87	1.82	0.219	0.262
	IL22	0.43	0.29	0.65	<0.0001	<0.0001	0.41	0.27	0.62	<0.0001	<0.0001
Type I IFN	IFNα2	0.68	0.48	0.96	0.029	0.038	0.66	0.46	0.94	0.023	0.033
Type II IFN	IFNγ	0.36	0.24	0.53	<0.0001	<0.0001	0.32	0.21	0.49	<0.0001	<0.0001
CC chemokines	CCL2 (MCP1)	0.22	0.13	0.38	<0.0001	<0.0001	0.22	0.13	0.37	<0.0001	<0.0001
	CCL7 (MCP3)	1.61	1.07	2.43	0.022	0.031	1.59	1.04	2.41	0.031	0.041
CXC chemokines	CXCL1 (GROa)	1.23	0.88	1.74	0.226	0.261	1.21	0.84	1.74	0.304	0.345
Neurotrophic/stimulating factors	CSF1 (M-CSF)	0.21	0.13	0.35	<0.0001	<0.0001	0.20	0.12	0.34	<0.0001	<0.0001
	CSF2 (GM-CSF)	0.32	0.21	0.49	<0.0001	<0.0001	0.27	0.16	0.43	<0.0001	<0.0001
Other growth factors	HGF	0.71	0.49	1.02	0.063	0.080	0.69	0.48	1.01	0.057	0.071
TNF superfamily	TNFβ (LTA)	1.00	0.68	1.47	0.997	0.997	1.00	0.67	1.49	0.992	0.992
	TRAIL	1.50	1.08	2.10	0.016	0.024	1.49	1.06	2.09	0.020	0.030
Adipose-derived hormones	Leptin	0.99	0.73	1.35	0.969	0.985	0.97	0.70	1.34	0.844	0.888

Table 2. continued

Immune family	Immune molecule	CB: Boys (n=355 ASD, 333 controls)									
		Unadjusted model					Adjusted model[b]				
		OR	95% CI		*p*-value	Adj *p*[c]	aOR	95% CI		*p*-value	Adj *p*[c]
IL1 superfamily	IL1α	1.06	0.92	1.22	0.440	0.528	1.05	0.90	1.21	0.543	0.651
	IL1β	1.17	1.02	1.34	0.023	0.038	1.15	1.00	1.32	0.043	0.069
	IL18	1.02	0.88	1.18	0.769	0.824	1.00	0.86	1.16	0.962	0.962
	IL1RA	1.37	1.17	1.60	<0.0001	<0.001	1.35	1.15	1.59	<0.001	<0.001
IL2 family	IL2	1.25	1.09	1.45	0.002	0.004	1.24	1.08	1.44	0.003	0.006
	IL7	1.70	1.45	1.99	<0.0001	<0.0001	1.68	1.43	1.97	<0.0001	<0.0001
	IL9	1.12	0.96	1.31	0.165	0.230	1.09	0.93	1.28	0.276	0.376
IL12 family	IL27	0.95	0.82	1.11	0.525	0.617	0.96	0.82	1.12	0.593	0.679
TNF superfamily	TNFα	1.75	1.48	2.07	<0.0001	<0.0001	1.71	1.44	2.03	<0.0001	<0.0001
	sFasL	0.96	0.83	1.11	0.579	0.668	0.96	0.83	1.11	0.600	0.679
IL6 (gp130) cytokine family	IL6	1.12	1.01	1.23	0.031	0.049	1.11	1.00	1.23	0.042	0.068
	IL31	0.90	0.77	1.05	0.174	0.237	0.88	0.76	1.03	0.123	0.184
Th2 type	IL5	1.38	1.18	1.62	<0.0001	<0.001	1.37	1.17	1.60	<0.001	<0.001
	IL13	1.24	1.07	1.43	0.004	0.006	1.23	1.07	1.43	0.005	0.009
	IL10	0.86	0.75	1.00	0.046	0.070	0.86	0.75	1.00	0.048	0.074
CC chemokines	CCL5 (RANTES)	1.41	1.21	1.64	<0.0001	<0.0001	1.41	1.21	1.64	<0.0001	<0.0001
	CCL3 (MIP1a)	1.06	0.94	1.21	0.342	0.446	1.05	0.92	1.19	0.471	0.587
	CCL4 (MIP1b)	1.13	0.98	1.30	0.095	0.142	1.12	0.97	1.29	0.126	0.184
	CCL11 (eotaxin)	0.97	0.83	1.13	0.679	0.767	0.98	0.84	1.14	0.783	0.839
CXC chemokines	CXCL8 (IL8)	1.09	0.94	1.26	0.274	0.365	1.06	0.91	1.23	0.458	0.585
	CXCL9 (MIG)	0.75	0.64	0.88	<0.001	<0.001	0.75	0.64	0.88	<0.001	0.001
	CXCL10 (IP10)	1.28	1.10	1.49	0.002	0.003	1.28	1.10	1.50	0.002	0.004
	CXCL12a (SDF1)	0.90	0.78	1.04	0.155	0.221	0.90	0.78	1.04	0.148	0.207
Neurotrophic/stimulating factors	CSF3 (G-CSF)	1.19	1.04	1.37	0.012	0.019	1.17	1.02	1.34	0.028	0.046
	SCF (KITLG)	0.75	0.65	0.88	<0.001	<0.001	0.77	0.66	0.90	<0.001	0.002
Serine protease inhibitors	Serpin E1 (PAI-1)	1.95	1.63	2.34	<0.0001	<0.0001	1.92	1.60	2.31	<0.0001	<0.0001
Cell adhesion molecules	VCAM1 (CD106)	1.80	1.53	2.12	<0.0001	<0.0001	1.78	1.51	2.10	<0.0001	<0.0001
	sICAM1 (CD54)	0.97	0.85	1.12	0.691	0.767	0.98	0.85	1.12	0.735	0.817
PDGF family/VEGF subfamily	VEGFD	1.37	1.18	1.58	<0.0001	<0.0001	1.38	1.19	1.59	<0.0001	<0.0001
	VEGFA	1.07	0.92	1.24	0.3712	0.4640	1.06	0.91	1.23	0.480	0.587
	PDGFBB	1.13	0.98	1.31	0.0975	0.1427	1.12	0.96	1.30	0.144	0.205
Other growth factors	EGF	1.75	1.49	2.04	<0.0001	<0.0001	1.71	1.46	2.00	<0.0001	<0.0001
	FGFb	1.27	1.08	1.48	0.003	0.006	1.25	1.06	1.47	0.007	0.011
	βNGF	0.93	0.81	1.08	0.351	0.448	0.92	0.80	1.07	0.289	0.385
	TGFα	1.00	0.86	1.15	0.975	0.985	0.98	0.84	1.13	0.765	0.834
	TGFβ	0.78	0.67	0.91	0.002	0.004	0.78	0.67	0.92	0.002	0.005
Neurotrophic/stimulating factors	BDNF	1.00	0.86	1.16	0.985	0.985	0.99	0.85	1.15	0.919	0.951
Adipose-derived hormones	Resistin	1.00	0.87	1.14	0.945	0.978	0.99	0.87	1.13	0.888	0.935
Type I IFN	IFNβ	0.74	0.63	0.87	<0.001	<0.001	0.75	0.63	0.89	<0.001	0.002
IL2 family	IL4	0.43	0.36	0.52	<0.0001	<0.0001	0.43	0.36	0.53	<0.0001	<0.0001
	IL15	0.63	0.53	0.75	<0.0001	<0.0001	0.63	0.53	0.76	<0.0001	<0.0001
	IL21	0.63	0.53	0.75	<0.0001	<0.0001	0.63	0.53	0.75	<0.0001	<0.0001
IL6 (gp130) cytokine family	LIF	0.80	0.69	0.93	0.004	0.007	0.81	0.70	0.94	0.004	0.008
IL12 family	IL12p40	0.76	0.66	0.88	<0.001	<0.001	0.77	0.67	0.89	<0.001	0.001
	IL12p70	0.52	0.43	0.62	<0.0001	<0.0001	0.52	0.43	0.62	<0.0001	<0.0001
	IL23	0.94	0.81	1.08	0.383	0.469	0.93	0.80	1.08	0.331	0.432
IL17 family	IL17A	0.54	0.45	0.64	<0.0001	<0.0001	0.54	0.45	0.64	<0.0001	<0.0001
	IL17F	0.61	0.51	0.72	<0.0001	<0.0001	0.60	0.51	0.72	<0.0001	<0.0001
	IL22	0.46	0.39	0.56	<0.0001	<0.0001	0.47	0.39	0.56	<0.0001	<0.0001
Type I IFN	IFNα2	0.78	0.67	0.92	0.003	0.005	0.79	0.67	0.93	0.004	0.007
Type II IFN	IFNγ	0.43	0.36	0.52	<0.0001	<0.0001	0.42	0.35	0.51	<0.0001	<0.0001
CC chemokines	CCL2 (MCP1)	0.79	0.68	0.92	0.002	0.004	0.79	0.68	0.91	0.002	0.003
	CCL7 (MCP3)	0.67	0.57	0.79	<0.0001	<0.0001	0.67	0.57	0.80	<0.0001	<0.0001
CXC chemokines	CXCL1 (GROa)	1.01	0.88	1.16	0.872	0.918	1.00	0.87	1.14	0.949	0.962
Neurotrophic/stimulating factors	CSF1 (M-CSF)	0.39	0.32	0.47	<0.0001	<0.0001	0.39	0.33	0.47	<0.0001	<0.0001
	CSF2 (GM-CSF)	0.50	0.42	0.60	<0.0001	<0.0001	0.50	0.42	0.60	<0.0001	<0.0001
Other growth factors	HGF	0.97	0.85	1.12	0.714	0.779	0.96	0.83	1.11	0.593	0.679
TNF superfamily	TNFβ (LTA)	0.56	0.47	0.67	<0.0001	<0.0001	0.57	0.47	0.68	<0.0001	<0.0001
	TRAIL	0.75	0.64	0.87	<0.001	<0.001	0.75	0.64	0.88	<0.001	<0.001
Adipose-derived hormones	Leptin	0.74	0.64	0.86	<0.0001	<0.001	0.73	0.63	0.85	<0.0001	<0.001

Table 2. continued

Immune family	Immune molecule	CB: Girls (n=63 ASD, 62 controls)									
		Unadjusted model					Adjusted model[b]				
		OR	95% CI		p-value	Adj p[c]	aOR	95% CI		p-value	Adj p[c]
IL1 superfamily	IL1α	1.15	0.78	1.71	0.480	0.610	1.14	0.75	1.72	0.541	0.662
	IL1β	1.06	0.71	1.59	0.784	0.871	1.02	0.67	1.56	0.917	0.963
	IL18	1.52	1.03	2.23	0.033	0.071	1.53	1.02	2.29	0.039	0.077
	IL1RA	1.90	1.26	2.86	0.002	0.005	1.79	1.17	2.74	0.008	0.019
IL2 family	IL2	1.79	1.15	2.78	0.010	0.025	1.78	1.13	2.79	0.012	0.029
	IL7	2.96	1.92	4.56	<0.0001	<0.0001	3.13	1.95	5.04	<0.0001	<0.0001
	IL9	1.07	0.69	1.66	0.770	0.871	1.07	0.67	1.70	0.778	0.850
IL12 family	IL27	1.42	0.96	2.12	0.083	0.155	1.38	0.91	2.08	0.127	0.225
TNF superfamily	TNFα	2.94	1.87	4.64	<0.0001	<0.0001	3.24	1.95	5.36	<0.0001	<0.0001
	sFasL	1.20	0.83	1.73	0.337	0.486	1.21	0.82	1.79	0.325	0.476
IL6 (gp130) cytokine family	IL6	0.97	0.68	1.39	0.886	0.930	1.01	0.70	1.46	0.940	0.963
	IL31	0.88	0.62	1.26	0.488	0.610	0.86	0.59	1.24	0.416	0.542
Th2 type	IL5	2.20	1.41	3.45	<0.001	0.002	2.26	1.39	3.67	0.001	0.003
	IL13	2.02	1.41	2.89	<0.001	<0.001	2.28	1.54	3.39	<0.0001	<0.001
	IL10	0.84	0.57	1.23	0.375	0.510	0.86	0.58	1.28	0.458	0.572
CC chemokines	CCL5 (RANTES)	2.55	1.74	3.74	<0.0001	<0.0001	2.91	1.87	4.52	<0.0001	<0.0001
	CCL3 (MIP1a)	0.94	0.66	1.33	0.712	0.838	0.92	0.63	1.34	0.650	0.780
	CCL4 (MIP1b)	1.28	0.85	1.90	0.236	0.372	1.25	0.83	1.90	0.290	0.447
	CCL11 (eotaxin)	1.02	0.71	1.47	0.915	0.931	0.95	0.64	1.39	0.779	0.850
CXC chemokines	CXCL8 (IL8)	1.19	0.80	1.76	0.382	0.510	1.20	0.79	1.81	0.396	0.527
	CXCL9 (MIG)	0.74	0.51	1.07	0.110	0.194	0.72	0.49	1.05	0.090	0.164
	CXCL10 (IP10)	2.07	1.40	3.07	<0.001	<0.001	2.10	1.38	3.20	<0.001	0.002
	CXCL12a (SDF1)	0.87	0.62	1.22	0.423	0.552	0.85	0.60	1.21	0.367	0.502
Neurotrophic/stimulating factors	CSF3 (G-CSF)	1.34	0.94	1.90	0.108	0.194	1.33	0.91	1.95	0.144	0.246
	SCF (KITLG)	0.84	0.59	1.20	0.340	0.486	0.81	0.57	1.17	0.267	0.422
Serine protease inhibitors	Serpin E1 (PAI-1)	3.04	1.91	4.83	<0.0001	<0.0001	3.40	2.05	5.65	<0.0001	<0.0001
Cell adhesion molecules	VCAM1 (CD106)	3.76	2.44	5.81	<0.0001	<0.0001	4.12	2.55	6.67	<0.0001	<0.0001
	sICAM1 (CD54)	1.22	0.85	1.77	0.283	0.424	1.23	0.83	1.82	0.298	0.447
PDGF family/VEGF subfamily	VEGFD	1.81	1.36	2.40	<0.0001	<0.001	2.02	1.45	2.82	<0.0001	<0.001
	VEGFA	1.04	0.72	1.49	0.854	0.915	1.01	0.69	1.48	0.970	0.970
	PDGFBB	1.26	0.85	1.85	0.248	0.381	1.17	0.78	1.76	0.447	0.571
Other growth factors	EGF	1.91	1.35	2.70	<0.001	<0.001	1.91	1.33	2.75	<0.001	0.001
	FGFb	1.59	1.03	2.47	0.037	0.077	1.85	1.13	3.02	0.014	0.033
	βNGF	1.02	0.71	1.45	0.935	0.935	1.01	0.69	1.49	0.947	0.963
	TGFα	0.96	0.66	1.39	0.8230	0.8978	0.93	0.63	1.36	0.706	0.830
	TGFβ	0.62	0.41	0.93	0.022	0.049	0.58	0.38	0.91	0.017	0.037
Neurotrophic/stimulating factors	BDNF	0.98	0.69	1.38	0.899	0.930	0.94	0.65	1.36	0.752	0.850
Adipose-derived hormones	Resistin	1.06	0.74	1.51	0.743	0.858	1.04	0.72	1.51	0.825	0.884
Type I IFN	IFNβ	0.77	0.53	1.12	0.175	0.283	0.77	0.52	1.15	0.197	0.319
IL2 family	IL4	0.28	0.18	0.44	<0.0001	<0.0001	0.26	0.16	0.42	<0.0001	<0.0001
	IL15	0.50	0.32	0.77	0.002	0.004	0.47	0.29	0.75	0.002	0.004
	IL21	0.42	0.27	0.66	<0.001	<0.001	0.41	0.26	0.65	<0.001	<0.001
IL6 (gp130) cytokine family	LIF	0.67	0.48	0.94	0.020	0.046	0.66	0.46	0.93	0.019	0.041
IL12 family	IL12p40	0.71	0.50	0.99	0.043	0.085	0.67	0.47	0.95	0.024	0.050
	IL12p70	0.43	0.28	0.65	<0.0001	<0.001	0.39	0.24	0.62	0.0001	<0.001
	IL23	1.08	0.80	1.47	0.614	0.736	1.06	0.77	1.46	0.736	0.849
IL17 family	IL17A	0.38	0.25	0.57	<0.0001	<0.0001	0.35	0.22	0.54	<0.0001	<0.0001
	IL17F	0.36	0.22	0.58	<0.0001	<0.001	0.32	0.18	0.54	<0.0001	<0.001
	IL22	0.28	0.18	0.45	<0.0001	<0.0001	0.26	0.15	0.43	<0.0001	<0.0001
Type I IFN	IFNα2	0.76	0.52	1.12	0.163	0.272	0.76	0.51	1.14	0.183	0.305
Type II IFN	IFNγ	0.35	0.22	0.54	<0.0001	<0.0001	0.31	0.19	0.50	<0.0001	<0.0001
CC chemokines	CCL2 (MCP1)	0.76	0.56	1.03	0.081	0.155	0.74	0.54	1.01	0.060	0.116
	CCL7 (MCP3)	0.52	0.36	0.77	<0.001	0.003	0.50	0.33	0.75	<0.001	0.002
CXC chemokines	CXCL1 (GROa)	0.72	0.49	1.08	0.115	0.198	0.67	0.44	1.02	0.064	0.120
Neurotrophic/stimulating factors	CSF1 (M-CSF)	0.30	0.21	0.44	<0.0001	<0.0001	0.26	0.16	0.41	<0.0001	<0.0001
	CSF2 (GM-CSF)	0.38	0.24	0.61	<0.0001	<0.001	0.36	0.22	0.59	<0.0001	<0.001
Other growth factors	HGF	1.14	0.78	1.66	0.502	0.615	1.20	0.81	1.80	0.366	0.502
TNF superfamily	TNFβ (LTA)	0.35	0.23	0.54	<0.0001	<0.0001	0.34	0.21	0.53	<0.0001	<0.0001
	TRAIL	0.61	0.42	0.89	0.010	0.025	0.57	0.38	0.86	0.007	0.018
Adipose-derived hormones	Leptin	0.84	0.58	1.22	0.359	0.501	0.84	0.56	1.24	0.368	0.502

We considered a cytokine to be significantly associated with risk of autism spectrum disorder (ASD) if it satisfied adjusted odds ratio (aOR) >1.5 or <0.667 and false discovery rate (FDR) adjusted p value < 0.05. Green denotes levels positively associated with ASD. Blue denotes levels negatively associated with ASD. Maternal mid-gestation (MMG) $n = 854$. Cord blood (CB) $n = 793$.

aOR adjusted odds ratio, *CI* confidence interval.
[a]Adjusted for maternal age, gestational age at MMG blood sample collection, illnesses (fever, infection, inflammatory, autoimmune, allergic disorders), emotional distress scores (SCL-5), and use of non-NSAID antipyretic medications (e.g., acetaminophen) in pregnancy up until sample acquisition.
[b]Adjusted for maternal age, maternal illnesses during pregnancy (fever, infection, inflammatory/autoimmune/allergic disorders), maternal emotional distress scores, and use of non-NSAID antipyretic medications in pregnancy.
[c]Adj *p* is the FDR adjusted *p* value using Benjamini–Hochberg procedure controlling the FDR at 0.05 level.

(AUROC) values and receiver operating characteristic (ROC) curves. Data analysis was implemented using MATLAB and Statistics Toolbox release 2013a (MathWorks, Inc., Natick, MA), R version 3.6.3 (RStudio, Inc., Boston, MA), and IBM SPSS Statistics for Windows, version 24.0. All *p* values were two-tailed.

RESULTS

Subject characteristics

Table 1 summarizes the maternal and child characteristics for the MMG and CB study samples. In the MMG and CB analyses, mothers of ASD boys were younger than mothers of control boys ($p = 0.001$ for both MMG and CB analyses). Birth year was differently proportioned between ASD boys and control boys in both MMG and CB analyses ($p < 0.001$). Distribution of birth year was also different between ASD girls and control girls in MMG analysis ($p = 0.021$) and showed a nonsignificant trend in CB ($p = 0.058$). Gestational age at birth for ASD boys was more likely to be outside of the 37–41 gestational week window as compared with control boys ($p = 0.041$ for MMG boys, $p = 0.005$ for CB boys). These parameters did not differ for girls in either MMG or CB analyses.

ASD is associated with altered cytokine profiles

We used unadjusted and adjusted logistic regression models to test for ASD association with levels of cytokines in MMG and CB samples (Table 2). We considered a cytokine to be significantly associated with risk of ASD if it satisfied the following criteria with respect to control samples: (1) adjusted OR (aOR) >1.5 or <0.667 and (2) FDR adjusted *p* value < 0.05. An OR of 1.5 (or the reciprocal ~0.667) is roughly equivalent to a Cohen's effect size $d = 0.224$ [49], and Cohen's $d = 0.2$ was proposed as an indicator of a small effect size [50]. Table 2 reports the sex-stratified unadjusted OR and aOR of each cytokine, together with their associated 95% CI, crude *p* value, and FDR adjusted *p* value, in MMG and CB samples, respectively. We also rebuilt the adjusted logistic models with further adjustments for the year of birth and found no significant changes in the results (Supplementary Table 3).

In the MMG dataset, in comparison with male controls, male ASD subjects had significantly higher levels of interleukins IL1β, IL1RA, IL2, IL5, and IL13; TNFα; CCL5; CXC chemokines CXCL8 and CXCL10; and vascular, growth and stimulating factors Serpin E1, VCAM1, VEGFD, EGF, and CSF3 (adjusted $p < 0.0001$). Levels of interleukins IL4, IL12p40, and IL22; IFNγ; CCL2; and growth and stimulating factors HGF, CSF1, and CSF2 were significantly reduced (adjusted $p < 0.0001$). aORs ranged from 1.51 (EGF) to 2.63 (TNFα) with a mean of 1.92. The mean aOR was calculated as the natural exponential of the average absolute log-odds associated with the significant analytes (aOR >1.5 or <0.667, and FDR adjusted *p* value < 0.05). Compared with female controls, female ASD subjects had significantly higher levels of interleukins IL1α, IL1β, IL1RA, IL2, IL5, IL7, IL9, IL10, IL13, IL27, and IL31; TNF superfamily factors TNFα and sFasL; IFNβ; CC chemokines CCL3, CCL4, CCL5, CCL7, and CCL11; CXC chemokines CXCL8, CXCL9, CXCL10, and CXCL12a; and vascular, growth and stimulating factors Serpin E1, VCAM1, sICAM1, VEGFD, VEGFA, PDGFBB, EGF, FGFb, βNGF, TGFα, TGFβ, BDNF, CSF3, and SCF (adjusted $p < 0.0001$ to $p = 0.049$). Levels of IL4, IL12p40, IL22, IFNα2, IFNγ, CCL2, CSF1, and CSF2 were reduced (adjusted $p < 0.0001$ to $p = 0.033$). The mean effect size for a significant case–control analyte difference was aOR = 3.03 in girls.

In the CB dataset, in comparison with male controls, male ASD subjects had significantly higher levels of IL7, TNFα, Serpin E1, VCAM1, and EGF (adjusted $p < 0.0001$). Levels of interleukins IL4, IL5, IL12p70, IL17A, IL17F, IL21, and IL22; IFNγ, TNFβ, CSF1, and CSF2 were reduced (adjusted $p < 0.0001$). Compared with female controls, female ASD subjects had significantly higher levels of interleukins IL1RA, IL2, IL5, IL7, and IL13; TNFα; CCL5; CXCL10; and vascular and other growth factors Serpin E1, VCAM1, VEGFD, EGF, and FGFb (adjusted $p < 0.0001$ to $p = 0.033$). Levels of interleukins IL4, IL12p40, IL12p70, IL15, IL17A, IL17F, IL21, IL22, and LIF; TNF superfamily factors TNFβ and TRAIL; CCL7; IFNγ; and growth and stimulating factors TGFβ, CSF1, and CSF2 were reduced (adjusted $p < 0.0001$ to $p = 0.050$). As in the MMG dataset, despite having a lower sample size, female subjects had larger effect sizes (mean aOR = 2.47) than male subjects (mean aOR = 1.89).

Sensitivity analyses

We then restricted analysis in the MMG datasets to subjects who had mid-gestational sample collections within ±28-day window from the dates of return of the MoBa questionnaires. Three sensitivity analyses were explored for CB samples: one restricting the study population to subjects not born by Caesarean section, one restricted to subjects whose mothers did not experience preeclampsia or eclampsia during the pregnancy, and one restricted to subjects with gestational ages between weeks 37 and 41 of gestation. Each of these sensitivity analyses revealed similar estimations as in the main analysis (Supplementary Table 4).

Assessment of the 60-plex immune signature panel as a biomarker for ASD

We developed five predictive models using an 80% randomly selected training set of subject cytokine profiles: Lasso without interaction terms, Lasso with interaction terms, RF, XGBoost, and Model Average. We tested model performance in the remaining 20% of subject cytokine profiles. The test sets included 70 male cases and 70 male controls, and 12 female cases and 12 female controls.

For male subjects in MMG dataset, the Model Average was the best performing classifier and significantly out-performed RF ($p = 0.002$). All other pairwise comparisons between models were not significant. Lasso without interaction terms distinguished ASD cases from controls with an AUROC value of 0.833 (95% CI, 0.753, 0.891); Lasso with interaction terms produced an AUROC value of 0.829 (95% CI, 0.749, 0.888); RF and XGBoost yielded AUROC values of 0.786 (95% CI, 0.663, 0.855) and 0.816 (95% CI, 0.739, 0.874), respectively; Model Average separated cases from controls with an AUROC value of 0.848 (95% CI, 0.774, 0.901) (Fig. 2a). The confusion matrix of the best performing classifier (Model Average) is shown in Supplementary Table 5. When the Model Average was focused on the subjects with predictive probability greater than 0.85 ($n = 28$), the true positive rate was 89.29%; those with predictive probability greater than 0.90 ($n = 2$) were all true positives. We also measured the importance of each cytokine in the predictive models using Bootstrapping with 1000 iterations in the training set [51]. IL1RA, TNFα, CCL2, CXCL1, and Serpin E1 were

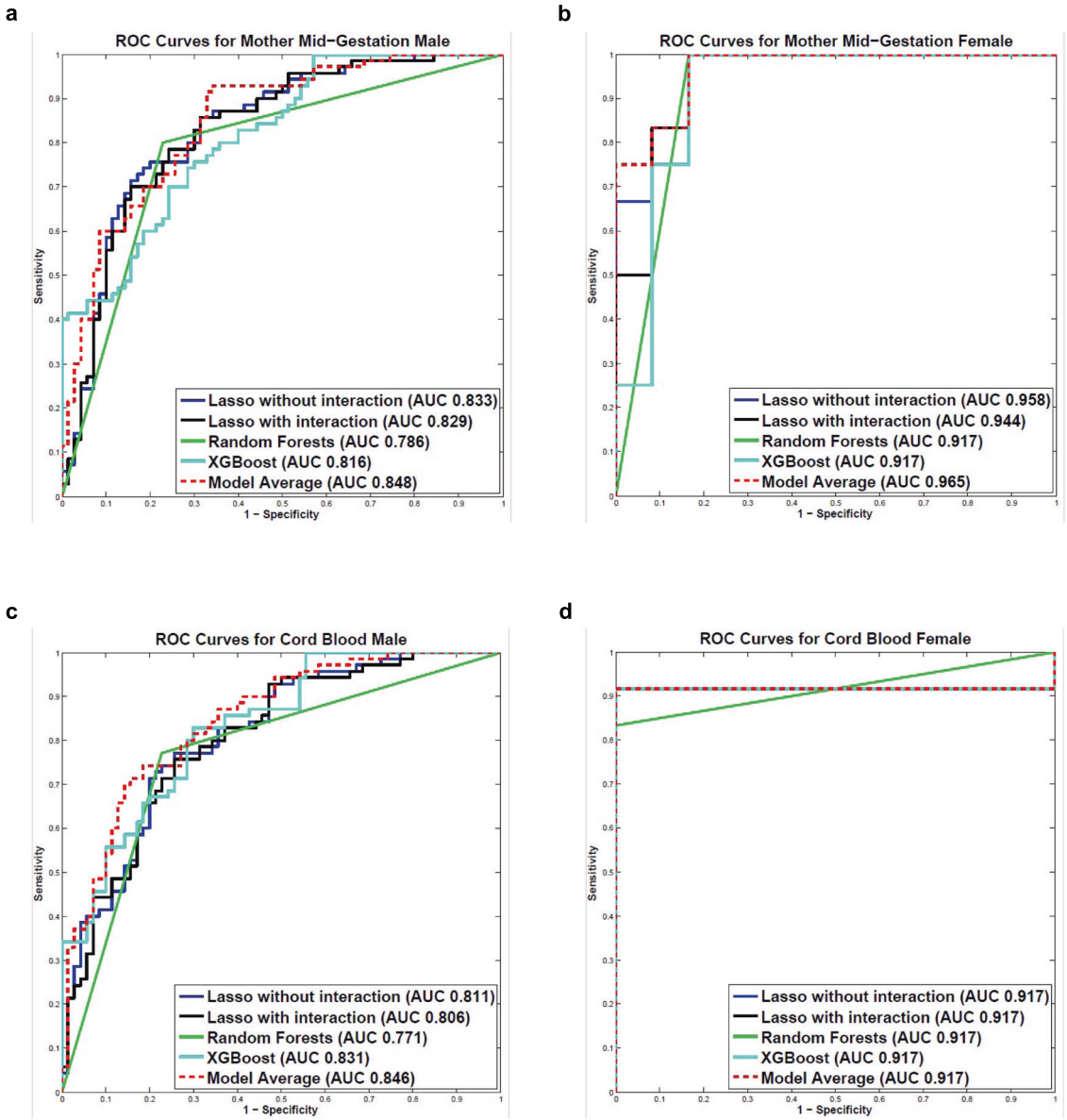

Fig. 2 Autism spectrum disorders (ASDs) predictive modeling. a ROC curves for mother mid-gestation male. **b** ROC curves for mother mid-gestation female. **c** ROC curves for cord blood male. **d** ROC curves for cord blood female. Five models were built to predict ASD outcome using the 60-plex immunoassay: Lasso without interaction terms, Lasso with interaction terms, Random Forests, XGBoost, and Bayesian Model Averaging (Model Average). Models were built and evaluated within each sample type and sex, separately. The models were first trained in the 80% randomly selected training set using ten-fold cross-validation, and the remaining 20% of the study population was used as the independent test set to validate model performance. The predictive performance of the five models in the test set was evaluated using area under the receiver operating characteristic curve (AUROC) values and receiver operating characteristic (ROC) curves.

ranked in top 10 in Lasso without interactions, RF, and XGBoost (Supplementary Table 6).

For female subjects in MMG dataset, all five models distinguished ASD cases from controls with AUROC values greater than 0.9. Lasso without and with interaction terms yielded AUC values of 0.958 (95% CI, 0.801, 0.992) and 0.944 (95% CI, 0.749, 0.990), respectively; both RF and XGBoost produced an AUROC value of 0.917 (RF 95% CI, 0.532, 0.986; XGBoost 95% CI, 0.666, 0.984); Model Average separated cases from controls with an AUROC value of 0.965 (95% CI, 0.823, 0.994) (Fig. 2b). None of the pairwise comparisons between models were significant. The confusion matrix of the best performing classifier (Model Average) is shown in Supplementary Table 5. When we focused on the subjects with predictive probability greater than 0.80 ($n = 10$), the true positive rate reached 100%. IL1RA, TNFα, CCL2, CSF1, and Serpin E1 were ranked in top 10 in all the models in which we measured feature importance using 1000 iterations of Bootstrapping in the training set (Supplementary Table 6).

For male subjects in CB dataset, Lasso without interaction terms distinguished ASD cases from controls with an AUROC value of 0.811 (95% CI, 0.730, 0.872); Lasso with interaction terms produced an AUROC value of 0.806 (95% CI, 0.724, 0.868); RF and XGBoost yielded AUROC values of 0.771 (95% CI, 0.669, 0.862) and 0.831 (95% CI, 0.756, 0.887), respectively; Model Average separated cases from controls with an AUROC value of 0.846 (95% CI, 0.771, 0.899). The ROC curves of the five models are shown in Fig. 2c. The Model Average was the best performing classifier and significantly out-performed Lasso with interaction terms ($p = 0.028$) and RF ($p < 0.001$). The confusion matrix of the best performing classifier (Model Average) is shown in Supplementary Table 5. When the

Model Average was focused on the subjects with predictive probability greater than 0.85 ($n = 16$), the true positive rate was 93.75%; those with predictive probability greater than 0.90 ($n = 4$) were all true positives. IL4, CSF1, and Serpin E1 were ranked top 10 in Lasso without interactions, RF, and XGBoost (Supplementary Table 6).

For female subjects in CB dataset, all five models distinguished ASD cases from controls with an AUROC value of 0.917. For RF, the 95% CI was (0.532, 0.986). For the other four models, the 95% CI was (0.565, 0.989). The ROC curves for the five models are shown in Fig. 2d. When we focused on the subjects with predictive probability greater than 0.80 ($n = 10$), the true positive rate reached 100%. IL4, TNFα, VEGFD, CSF1, VCAM1, and IL22 were ranked in top 10 in all the models wherein we measured feature importance (Supplementary Table 6).

DISCUSSION

We and others have shown that maternal fever and infection during pregnancy are associated with an increased risk of ASD [12, 52, 53]. Research in animal models has also shown that gestational exposure to IL-6 or IL-17 or triggers of innate immunity such as double stranded nucleic acid and lipopolysaccharide cause neurodevelopmental damage reminiscent of ASD. To explore potential links between ASD and maternal immune activation during gestation, we leveraged the resources of the ABC, a nationwide birth cohort in Norway that prospectively collected questionnaire data and plasma samples. We identified subjects with ASD through the national patient registry and used MMG plasma and CB to ask three questions: (1) Was systemic maternal inflammation during gestation associated with increased risk of ASD? (2) Do cytokine profiles in maternal MMG plasma or CB differ by sex in ASD? (3) Could we identify biomarkers in CB that might be used to guide early identification of children at risk for ASD?

Cytokine profiles of MMG plasma, collected at 17–21 weeks gestation from mothers of both boys and girls who received a diagnosis of ASD, were consistent with systemic inflammation. In both boys and girls, we found elevations in a wide range of molecules associated with inflammation including IL1β, IL1RA, IL2, TNFα, and CCL5 (RANTES). We also found sex-specific effects despite the difference in sample sizes between boys and girls. The repertoire of elevated proinflammatory cytokines, chemokines, and adhesion molecules was larger in girls than in boys (37 versus 14). Furthermore, the effect sizes were also larger in girls than in boys (mean aOR 3.03 versus 1.92). Differences between cases and controls were less pronounced in CB with respect to the number of molecules that were elevated and the effect sizes. Levels of only five analytes were elevated in CB of ASD boys: IL7, TNFα, Serpin E1, VCAM1, and EGF. Four of the five analytes elevated in CB (TNFα, Serpin E1, VCAM1, and EGF) were also elevated in MMG plasma. Levels of 13 analytes were elevated in girls: all five that were elevated in CB of ASD boys plus IL1RA, IL2, IL5, IL13, CCL5 (RANTES), EGF, and FGFb. As in MMG analyses, effect sizes in CB were larger in girls than in boys (mean aOR 2.47 versus 1.89). Levels of IL-4, IL22, IFNγ, and growth factors CSF1 (MCSF) and CSF2 (GMCSF) were reduced in MMG and CB plasma in both ASD boys and ASD girls. A Venn diagram that indicates significant case/control alterations in cytokines present in MMG and CB samples from male and female ASD subjects is shown in Supplementary Fig. 1.

Two of the proinflammatory cytokines with the largest effect sizes in MMG plasma of ASD boys and girls were IL1RA and TNFα. IL1RA is a member of the large, tightly regulated IL1 cytokine family comprised of cytokines, cytokine receptors, and modulating factors. It has potent antagonist effects with respect to IL1 signaling, achieved at least in part through the strength of its binding to IL1R1, which has affinity exceeding that of IL1 [54]. Although we did not find significantly elevated levels of IL-6 in MMG or CB plasma of ASD boys or girls, IL1RA may serve as a marker for elevation at an earlier time point. Infusion of IL-6 into normal human volunteers, the same molecule implicated by Patterson et al. in mediating maternal immune activation in the placenta and brain in rodent models of ASD, resulted in elevated levels of IL1RA. TNFα is expressed by activated monocytes/macrophages, NK and T cells, as well as endothelial cells and fibroblasts. It has myriad functions including caspase-dependent apoptotic cell death, and activation of NFκB, which in turn promotes expression of proinflammatory cytokines [55, 56]. TNFα is elevated in serum and cerebrospinal fluid of children with ASD [57, 58] and in neonatal blood spots of children subsequently diagnosed with ASD [59].

The observation that proinflammatory cytokine expression is more pronounced in MMG than CB plasma is consistent with earlier work in the ABC wherein we found increased ASD risk with maternal exposure to fever in the first or second versus the third trimester [12], and to the presence of high titers of antibodies to herpes simplex virus type II [60]. Work with this same cohort showed a trend toward increased risk in women with documented influenza infection and a prospectively collected report of influenza like illness [61]. We cite these studies not to implicate a specific infection in the pathogenesis of ASD but rather to point to a period of vulnerability during gestation when exuberant immune responses may interfere with central nervous system development.

Cytokines are important as growth factors as well as mediators of inflammation. Neuropoietic cytokines, including IL6 and TGFβ (elevated in MMG ASD girls but not in MMG boys), orchestrate fate switching and differentiation of neurons, astrocytes, and oligodendrocytes [62]. CSF1, reduced in MMG and CB of ASD boys and girls, has been implicated in microglial and neuronal development and function [63, 64]. CSF1-depleted mice have reduced numbers of Purkinje cells, disordered cerebellar architecture, and deficits in both motor function and social memory [65].

The use of machine learning predictive models allowed us to distinguish ASD cases from controls with high accuracies in the randomly selected test sets in both sample types (MMG and CB) and in both sexes (boys and girls). The AUROC values for MMG boys ranged from 0.786 to 0.848; for MMG girls, the AUROC values ranged from 0.917 to 0.965; the AUROC values for CB boys ranged from 0.771 to 0.846; all five models yielded an AUROC value of 0.917 for CB girls. The Model Average was the best performing model. When focused on subjects with predictive probabilities above 0.9, the true positive rates reached 100%. To our knowledge, there are no reported examples of comparably robust predictions where independent test sets were used for model evaluations. TNFα, Serpin E1, and CSF1 were constantly top ranked. Activation of danger-associated molecular pattern and Toll-like receptor pathways could lead to increased expression of TNFα, Serpin E1, VCAM1, and IL1β [9, 66–69]. Dysregulation of these molecules may have implications for angiogenesis during brain development and have been invoked in ASD pathogenesis [23, 69, 70].

In summary, our results provide robust evidence of immune dysregulation in mothers as early as 17–21 weeks gestation and in CB of neonates later diagnosed with ASD. Future work focused on identification of genetic factors in and environmental triggers of immune activation has the potential to lead to strategies for mitigating ASD risk.

Strengths and limitations

Among the strengths of this study are its sourcing of eligible cases and controls from a large population-based study; inclusion of multiple methods for case ascertainment and validation of ASD diagnoses; ongoing diagnostic follow-up through child age 6–16 years; use of plasma samples derived from venous CB collected,

handled, processed, and stored under quality-controlled study procedures; methods for optimization and QC of laboratory assays and their data; and cautious statistical approaches, including adjustment for multiple comparisons (conceptualizing the risk of Type I errors) and the use of three different machine learning algorithms (Random Forests, Lasso with and without interaction terms) to assess convergence across classifiers. Autism is less common in girls; thus, our sample size was smaller for girls than boys. A larger sample size in the female cohort might have allowed us to find more significant associations. Nonetheless, the power is similar in the female and male cohorts because the effect size is larger in the female cohort than the male cohort (Supplemental Table 7).

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AUTHOR CONTRIBUTIONS

CS, ES, MB, MH, PM, and WIL developed the experimental design. MH directed cytokine assays. XC directed statistical analyses. WIL and XC wrote the manuscript. CS, ES, MB, MH, PM, PS, SM, TR-K, and WIL contributed to the data analysis, edited, and approved the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Ezra Susser or W. Ian Lipkin.

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