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Serum cytokines associated with behavior: A cross-sectional study in 5-year-old children

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ABSTRACT

Nearly 10% of 5-year-old children experience social, emotional or behavioral problems and are at increased risk of developing mental disorders later in life. While animal and human studies have demonstrated that cytokines can regulate brain functions, it is unclear whether individual cytokines are associated with specific behavioral dimensions in population-based pediatric samples. Here, we used data and biological samples from 786 mother-child pairs participating to the French national mother-child cohort EDEN. At the age of 5, children were assessed for behavioral difficulties using the Strengths and Difficulties Questionnaire (SDQ) and had their serum collected. Serum samples were analyzed for levels of well-characterized effector or regulatory cytokines. We then used a penalized logistic regression method (Elastic Net), to investigate associations between serum levels of cytokines and each of the five SDQ-assessed behavioral dimensions after adjustment for relevant covariates and confounders, including psychosocial variables. We found that interleukin (IL)-6, IL-7, and IL-15 were associated with increased odds of problems in prosocial behavior, emotions, and peer relationships, respectively. In contrast, eight cytokines were associated with decreased odds of problems in one dimension: IL-8, IL-10, and IL-17A with emotional problems, Tumor Necrosis Factor (TNF)-α with conduct problems, C-C motif chemokine Ligand (CCL)2 with hyperactivity/inattention, C-X-C motif chemokine Ligand (CXCL)10 with peer problems, and CCL3 and IL-16 with abnormal prosocial behavior. Without implying causation, these associations support the notion that cytokines regulate brain functions and behavior and provide a rationale for launching longitudinal studies.

1. Introduction

Nearly 10% of 5-year-old children present behavioral problems and are at increased risk of developing mental disorders later in life (Egger and Angold, 2006; Ogundele, 2018). Complex interactions between genetic as well as pre- and post-natal environmental factors are thought to determine the development of conduct problems, hyperactivity/inattention, social and emotional disturbances in children (Abbott et al., 2018). For example, increased odds of behavioral, social, and emotional problems in children are associated with prenatal exposure to environmental stressors such as pollutants (Grandjean and Landrigan, 2014), maternal smoking or alcohol consumption (Polanska et al., 2015), gestational diabetes (Kong et al., 2018), maternal obesity (Sanchez et al., 2018), maternal immune activation (Gumusoglu and Stevens, 2019), parental separation, low family income, and maternal psychological distress (D'Souza et al., 2018; Noonan et al., 2018; van der Waerden et al., 2017; van der Waerden et al., 2015). In contrast, having older siblings (de La Rochebrochard and Joshi, 2013) and being breastfed (Heikkila et al., 2011) appear to be associated with lower odds of behavioral problems later in life. Although the impact of pre- and post-natal environmental factors on neurodevelopment and behavior has been recognized for many years, the underlying mechanisms remain unknown. Yet, it is commonly accepted that early life adversity impacts either directly or indirectly the child's immune system and contributes altered cytokine production (O'Connor et al., 2019; Pedersen et al., 2018).

Cytokines are small signaling molecules which act by binding to their cognate receptors at the surface of target cells. While most

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cytokines are known for their role during immune responses, they also regulate many biological functions in the body. Both pre-clinical and clinical studies have pointed to a critical role of cytokines in neurodevelopment and brain function, both in physiological and pathological conditions (Morimoto and Nakajima, 2019). Indeed, many cell types in the brain including microglia, astrocytes and neurons express cytokine receptors and can therefore be regulated by cytokines (Morimoto and Nakajima, 2019). This is the case of microglia which are brain-resident macrophages that sense and respond rapidly to alterations in brain homeostasis and integrate brain response to threats (Frank et al., 2019). Microglia also shape neuronal circuits (Morimoto and Nakajima, 2019), regulate the size of the neural precursor cell pool (Cunningham et al., 2013), and promote synaptic pruning during neurodevelopment (Filiano et al., 2016). As microglia express several receptors including those of interleukin (IL)-1β, IL-6, IL-10 and tumour necrosis factor (TNF)- α, their functions are tightly regulated by these cytokines as demonstrated in rodent models of anxio-depression (Dantzer et al., 2008; Harden et al., 2006).

Cytokines may also act directly on neurons and neurotransmission (Morimoto and Nakajima, 2019). For example, TNF- α secreted by astrocytes controls neurotransmitter receptors trafficking at the synapse, thereby directly regulating neurotransmission in the hippocampus, cortex and striatum (Beattie et al., 2002; Lewitus et al., 2014; Santello et al., 2011; Stellwagen and Malenka, 2006). *Tnf*-deficient mice display elevated hippocampal neurogenesis (Iosif et al., 2006) and TNF- α modulates survival, proliferation, and neuronal differentiation of neural progenitors (Bernardino et al., 2008). The absence of TNF- α conditions reduced fear response in mice (Zupan et al., 2017), as well as enhanced spatial memory associated with increased hippocampal neurogenesis (Liu et al., 2014a). Also, IL-15 produced by astrocytes regulates neurogenesis (Gomez-Nicola et al., 2011) and facilitates γ-aminobutyric acid (GABA)-ergic neurotransmission in the hippocampus (He et al., 2010). Furthermore, interferon (IFN)- γ promotes neuronal connectivity and influences social behavior (Filiano et al., 2016). Finally, IL-4 is essential for learning and memory processes, likely by inducing the production of brain derived neurotrophic factor (BDNF) (Derecki et al., 2010) Altogether, these studies suggested that proper levels of cytokines in the brain surroundings and in its parenchyma are required for the optimal establishment of brain circuitry and adapted behavioral responses (Filiano et al., 2015).

The blood levels of cytokines are often altered in neurodevelopmental and psychiatric disorders, supporting the notion that cytokines are involved in the etiopathology of these diseases (Dantzer, 2018; Mitchell and Goldstein, 2014). In young individuals presenting psychiatric symptoms and subjected to a task involving reward circuitry activation, the circulating levels of 41 cytokines associate with brain activation patterns (Bradley et al., 2019), suggesting that peripheral cytokines can regulate behavior. Although both preclinical and clinical studies suggested that IL-1 β, IL-4, IL-6, IL-15, IFN- γ and TNF- α, regulate neurodevelopment, behavior and brain function, whether any of these cytokines is associated with behavioral problems in children is unknown. Hence, there is a need for exploring possible associations between these specific cytokines and behavioral dimensions in children. The fact that most cytokines interact with and are regulated by other cytokines makes this issue more complex and requires a systematic approach. To identify possible associations between specific cytokines and behavioral dimensions in children, we have taken advantage of clinical data and serum samples collected from 5-year-old children enrolled in the French mother–child EDEN cohort (Heude et al., 2016). In this cross-sectional study, we have studied potential associations between 27 cytokines and five dimensions of a child's behavior: emotions, conduct, hyperactivity/inattention, peer relationships and social behavior.

2. Methods and materials

2.1. Study design

The EDEN (Etude sur les Déterminants pré- et postnataux du développement psychomoteur et de la santé de l'Enfant) mother–child cohort was set up to assess the influence of pre- and post-natal determinants on the child psychomotor development and health (Heude et al., 2016). Pregnant women were recruited before 24 weeks of gestation at the Obstetrics and Gynecology department of the University Hospitals of Nancy and Poitiers, France. Enrollment spanned over 27 months in each center. Exclusion criteria included multiple pregnancies, a known history of diabetes, the inability to speak and read French or plans to move out of the study region in the following 3 years. Among 3,758 eligible women, 2002 (53%) were enrolled in the study. During pregnancy and after birth (4, 8, 12, 24 months, 3, 4 and 5 years), sociodemographic and biomedical data on the mother and child were gathered (i) from medical records, (ii) in face-to-face interviews with the mother, and (iii) by mother's self-completed questionnaires. Participants to the EDEN study were representative of national estimates with regards to average age, proportion of unmarried couples, parental socio-demographic characteristics, offspring birth weight and prematurity rate, except for higher maternal education level (Blondel et al., 2012; Heude et al., 2016). Over the follow-up period, attrition rates were higher in families in which the mother was young, had low educational level and low income, did not live with the child's father, presented higher levels of caffeine intake, smoked tobacco, had psychological difficulties in pregnancy and whose child did not have low birth weight.

2.2. Study sample

Among the 2002 pregnant women included in the EDEN study, 1907 were still followed-up at delivery. Mother-child pairs were excluded for the following reasons: miscarriage (n = 11), *in utero* death (n = 7), abortion for medical reasons (n = 2), loss to follow-up (n = 14), moving away (n = 8), mother changing their mind (n = 51), and not meeting inclusion criteria (n = 2). Birth data were obtained from 1,899 mother-infant pairs. Among the 1907 children born in the cohort, 1255 children were still followed at 5 years. In the context of this specific study, our study sample consisted of 786 5-year-old children.

2.3. Measurements of serum cytokine levels

Venous blood was obtained from 5 to 6 (mean age 5.5)-year-old children and allowed to clot for 1 h at room temperature prior to centrifugation (1500 × g, 10 min). Serum samples were stored at −80 °C. Serum concentrations of cytokines were determined for the 786 individuals of our study sample. Serum samples were thawed on ice only once, and assayed using kits and reagents from two batches. All assays were completed within a 4 week period by the same investigator, blind to the samples' class. All assays were performed according to the manufacturer's instructions. Concentrations of Chemokine (C-C motif) ligand (CCL) 2, CCL3, CCL4, CCL11, CCL13, CCL17, CCL26, C-X-C motif chemokine 10 (CXCL10), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), IFN- γ, IL-1 α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, Tumor Necrosis Factor (TNF)- α, and TNF- β were measured using the following v-plex® multiplex immunoassays: Proinflammatory Panel 1, Cytokine Panel 1 and Chemokine Panel 1 (Meso Scale Diagnostics, Rockville, USA). On each of the 96-well plates, seven serial dilutions of standards and buffer

only (in duplicates) were run together with 80 samples (run in singlicate) on the Sector Imager 2400 plate reader (Meso Scale Diagnostics, Rockville, USA). Concentrations of biomarkers in each sample were interpolated from standard curves generated with a five-parameter logistic regression equation in Discovery Workbench 3.0 software (Meso Scale Diagnostics, Rockville, USA). For cytokine levels below the lower limit of detection (LLOD), we imputed a value equal to half the LLOD value indicated by the manufacturer. Cytokines presenting concentrations below LLOD in more than 15% of the samples (i.e. CCL26, IL-1 α, IL-1 β, IL-2, IL-12p70, IL-4, IL-5, IL-13, GM-CSF) were excluded from downstream analyses (Tab. S1). For the 18 remaining biomarkers, we assessed the quality of our measurements by retrospectively calculating intra-run and inter-runs coefficients of variations (CV), based on the concentrations obtained for each of the 7 serially diluted standards across the 11 plates used to analyze the 786 serum samples. The intra- or inter-CVs were below 20% within the range of cytokines concentrations comprised between the 1% and 99% percentiles of our sample (Tab. S2). CVs below 20% comply with the US FDA recommendations regarding validation of ligand-binding assays (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry).

2.4. Assessment of the child's outcome at 5 years

Child's behavioral outcome was assessed using the SDQ and was completed by the mother when the child was between 5 and 6 years of age (Goodman, 2001). The SDQ is a broadly used psychometric instrument (Cox et al. 1987; Goodman, 1997, 2001) and entails five scales measured by five items each: emotional symptoms (fears, worries, clingy, unhappy, somatic), conduct problems (lies, fights, tempers, steals, obedient), hyperactivity/inattention (distractible, persistent, restless, fidgety, reflective), peer relationship problems (good friend, popular, best with adults, solitary, bullied) and prosocial behavior (helps out, caring, considerate, kind to kids, shares). All items refer to the past 6 months or the current school year and are scored 0 (never), 1 (sometimes true), or 2 (certainly true). The SDQ items scored on the 3-point scale can be combined into 4 difficulties subscales (emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems) and one strength subscale (prosocial behavior). To stratify children at high-risk and low-risk for behavioral symptoms, we dichotomized the SDQ difficulties subscales and the prosocial subscale at the 85th upper percentile and 15th lower percentile respectively. Such a dichotomization was used in previous studies (Melchior et al., 2015; Philippat et al., 2017) and in studies using similar psychometric scales (de Winter et al., 2005; Huisman et al., 2008; Schneiders et al., 2003; Amone-P'Olak et al., 2009). Because SDQ scores are discrete, this led us to use cutoffs of 4, 5, 6, 3 and 6 for emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems and prosocial behavior, respectively. Using these cutoffs, the high-risk class for these behavioral problems comprised 21.5%, 16.1%, 17.4%, 14.6% and 17.3% of the children respectively (Supplementary Fig. 1). In his seminal paper, Goodman recommended to use cutoffs of 4, 3, 6, 3 and 5 to stratify children in a "normal" and a "borderline-abnormal" class for emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems and prosocial behavior, respectively. Therefore, we used the same cutoffs as those recommended by Goodman for emotional symptoms, hyperactivity/inattention and peer relationship problems. In contrast, the cutoffs of 5 and 6 that we used for conduct problems and prosocial behavior respectively were different than those recommended by Goodman. We made this decision because the cutoffs recommended by Goodman would have resulted in a high-risk class consisting of 43.9% and 7.39% of children respectively, i.e. much higher and much lower than the targeted 15th upper percentile.

2.5. Covariates and confounding factors

We selected maternal, perinatal, psychosocial and child's variables that could impact child development and/or serum cytokine levels (Fig. 1). Previous studies have identified psychosocial and clinical variables associated with altered odds of behavioral abnormalities in children (D'Souza et al., 2018; de La Rochebrochard and Joshi, 2013; Galera et al., 2015; Grandjean and Landrigan, 2014; Gumusoglu and Stevens, 2019; Heikkila et al., 2011 ; Kong et al., 2018; Noonan et al., 2018; Philippat et al., 2019; Philippat et al., 2017; Polanska et al., 2015; Sanchez et al., 2018; van der Waerden et al., 2017). Based on these studies, and considering that many of these variables can also influence cytokine production, we included several covariates. Maternal variables were: age at delivery (years), maternal pre-pregnancy body mass index (BMI in kg.m^{-2}), gestational diabetes (yes/no), alcohol drinking during pregnancy (mean number of glasses/week), smoking throughout pregnancy (number of cigarettes/day), caffeine intake during pregnancy (mg caffeine/day) and maternal urinary /cervical/vaginal infection during pregnancy (yes/no). Perinatal variables were: breastfeeding (yes/no), gestational age (weeks of amenorrhea), delivery mode (vaginal, forceps/ventosa, C-section), birth weight (kg) and birth trimester. Psychosocial variables were: parental separation during pregnancy or after birth (biological father not living with the mother and child: yes/no), maternal and paternal education duration (years), family income from pregnancy to age 5 (mean of the seven class numbers: 0, <800€/month; 1, 801-1500€/month; 2, 1501-2300€/ month; 3, 2301-3000€/month; 4, 3001-3800€/month; 5, 3801-4500€/month; 6, greater than4500€/month), financial difficulties from pregnancy to age 5 (yes/no), number of older siblings and stimulations of the child at home (mean score of three subscales of the Home Observation for Measurement of the Environment inventory (Bradley and Caldwell, 1977) at 5 years: language stimulation, academic stimulation, variety of experimentation), and record of depression (yes/no) either during pregnancy or post-pregnancy. Symptoms of maternal depression were detected using the Center for Epidemiological Studies Depression questionnaire (CES-D (Radloff, 1977)) at 24–28 weeks of amenorrhea or with the Edinburgh Postnatal Depression Scale (EPDS (Cox et al., 1987)) at 4, 8, and 12 months post-partum. Child-related variables were: allergies (yes/no), BMI (kg.m^{-2}) at age 5 and the sex (female/male). We also included the blood sampling time which could impact cytokine levels.

2.6. Univariate statistics

Comparison analyses of variables in the low- versus high-risk group based on each SDQ subscore were performed using the Wilcoxon-Mann & Whitney *U* test for numerical variables or with the Chi-square test for categorical variables, with Benjamini & Hochberg's multiple testing correction (False Discovery Rate). Correlations between concentrations of cytokine pairs were performed using Spearman's ρ correlation coefficient rank test with Benjamini & Hochberg's multiple testing correction. Statistical significance was set at a corrected p-value of < 0.05.

2.7. Association studies

An overview of the methodological workflow is presented in Supplementary Figure 2 and detailed hereafter:

2.7.1. Multiple imputation (MI)

Missing data in the original dataset were imputed using the Multivariate Imputation by Chained Equations (MICE) procedure in R (van Buuren and Groothuis-Oudshoorn, 2011). The outcome was used in the multiple imputation process, as it produces more precise estimates than standard multiple imputation. To minimize the bias introduced by a mis-specified model for imputing the missing outcomes, observations with imputed outcomes were excluded from subsequent

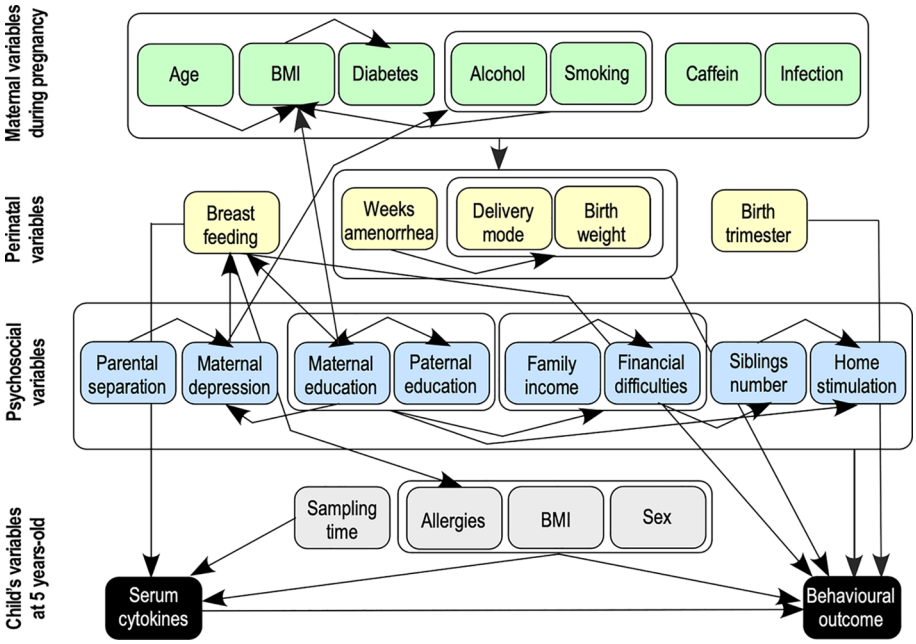

Fig. 1. Relationships between covariates, confounders, predictors and outcomes. Diagram showing possible inter-relations between selected maternal, perinatal, psychosocial and child's variables and serum cytokine levels (predictors) and/or child's behavioral outcome. Arrows depict documented or supposed influence between variables. BMI, body mass index.

analyses (Kontopantelis et al., 2017). As per the recommended procedure (Graham et al., 2007) regarding power considerations, we generated 40 independent MI datasets.

2.7.2. Resampling (R)

Traditional methods do not provide valid confidence intervals or p-values for testing the significance of penalized regression coefficients. As an alternative, the non-parametric bootstrap can be used for inference in applications of penalized regression (Abram et al., 2016). The bootstrap step involved 100 resamplings for each of the 40 MI datasets (with replacement) to create 100 different samples of each MI dataset, with the full sample size. The regression model was subsequently fitted to each of the 40x100 samples of data.

2.7.3. Variables selection using the Elastic Net

The CARET (Kuhn, 2008) and GLMNET (Friedman et al., 2010) R packages were used to implement Elastic Net penalized (or regularized) logistic regression models with the aim of performing variable selection, leading to sparser final model, in agreement with previous recommendations (Pavlou et al., 2016). The Elastic Net framework uses linear models with penalties to avoid extreme parameters that may cause overfitting while simultaneously performing variable selection and addressing the issue of multi-multicollinearity (correlation). Optimal λ and α hyperparameters for the elastic net algorithm were chosen via 10-fold cross-validation, with the optimal tuning hyperparameters values chosen to maximize the Area Under the Receiving Operator Curve (AUROC). The Elastic Net is not a deterministic process since variables associated with the outcome may not be selected in every run. For this reason, the optimization steps were repeated. In the case of the complete cases (CC) dataset, to optimize the stability of results we repeated the 10-fold cross-validation 1000 times with random dataset partitions. For MI resampling (MI-R) analyses, 40x100 models were implemented and the hyper parameters optimization step was repeated 3 times, leading to 12,000 models. For analysis of both CC and MI-R datasets, the Elastic Net was used to select a subset of variables by calculating the Variable Inclusion Probability (VIP), i.e. the percentage of times each variable was kept in the model (i.e. with an associated coefficient different from zero) out of the 1000 and 12,000 models, respectively. In the absence of asymptotically valid p-values which are (still) not available in high-dimensional regression, the VIP can be interpreted as the posterior probability of including a variable in the model and is used as a measure of the stability of the association (Bunea et al., 2011). After defining an appropriate threshold, the VIP is used to select predictors for follow-up analyses. Depending on the study, determining an appropriate threshold for the VIP can be challenging. In their landmark paper, Bunea *et al.* recommended a "conservative threshold of 50%" because their goal was "not to miss any possibly relevant predictors" (Bunea et al., 2011). However, this 50% threshold increases the risk of false positives. In this study, we set a stringent VIP threshold at 90% to identify variables stably associated with behavioral dimensions (cf. 3.).

2.7.4. Influential individuals and outliers

We sought to estimate the influence of each individual on the different models by calculating the Cook's distance. For each SDQ outcome, we considered the subset of variables selected by the Elastic net using a VIP threshold of 90% on the 40 MI $\times$ 100 bootstraps, and calculated the Cook's distance for each individual using logistic regression. As a result, we obtained for each individual the percentage of the runs in which it appeared as influential. Considering a conservative threshold of 50%, the number of influential individuals in the emotion, conduct, hyperactivity, peer problem and prosocial dimensions were 24 (out of 786), 49 (out of 785), 34 (out of 786), 54 (out of 785) and 38 (out of 784) respectively. In each dimension, all influential individuals belonged to the high-risk class. There was very little overlap between dimensions, meaning that most individuals influential in one dimension were not influential in the others. The distributions of cytokine levels were similar between influential and non-influential individuals suggesting that none of the influential individuals was an outlier in terms of cytokine levels. Since we had no objective biological ground to exclude influential individuals from the study, we decided to retain all individuals in analyses.

2.8. Ethics

The EDEN cohort received approval from the Ethical Research Committee (CCPPRB) of Bicêtre Hospital and from the French National Data Protection Agency (CNIL). Informed written consent was obtained from parents at the time of enrollment and after delivery.

3. Results

In this study, a penalized logistic regression method was used to identify cytokines whose blood levels were associated with increased or reduced risk of developing behavioral difficulties in 5-year-old children followed from birth. This population-based pediatric cohort consisted of 786 mother–child pairs from the French EDEN cohort in which mothers were enrolled at mid-pregnancy (Heude et al., 2016) and whose child's blood samples and SDQ psychometric scores were available at the age of 5 years. To identify children at high-risk of developing problems in each of the five SDQ behavioral dimensions, we dichotomized subscores at the 85th percentile for the 4 difficulties subscales of the SDQ and at the 15th lowest percentile for the prosocial strength subscore (Supplementary Fig. 1). The distribution of SDQ scores in each dimension was consistent with that of previous studies (Goodman, 1997, 2001; Melchior et al., 2015).

We assessed 786 serum samples for levels of 27 different cytokines, including several known to regulate brain function or development in animal studies and/or in humans. Nine cytokines (CCL26, GM-CSF, IL-1 α, IL-1 β, IL-2, IL-4, IL-5, IL-12p70 and IL-13) had levels below LLOD in more than 15% of the samples (Table 1) and were therefore excluded from further analyses. For the 18 remaining cytokines, intra- and inter-assay CVs were below 20%, compliant with US FDA recommendations for bioanalytical method validation regarding ligand-binding assays. We then investigated multicollinearity between these 18 cytokines using Spearman's correlation rank test with Benjamini-Hochberg's correction for multiple testing. All cytokines correlated with at least another one, and most correlated with several ones (Supplementary Table 2). For example, TNF- α correlated positively with CCL2, CCL3, CCL4, CCL11, CXCL10, IFN-γ, IL-6, IL-7, IL-10, IL-12p40, IL-15, IL-16 and IL-17A with correlation coefficients ranging from 0.1 to 0.5.

Psychosocial characteristics of the study sample were comparable to previous national estimates with the exception of maternal education level which was higher (Blondel et al., 2012; Heude et al., 2016) (Table 2). For each behavioral dimension, univariate comparison analyses between low- and high-risk groups for the 42 variables (18 cytokines, 24 covariates) are shown in Supplementary Table 2. The two groups differed for a few variables: number of older siblings for emotional symptoms; financial difficulties, home stimulation and sex for conduct problems; paternal and maternal education, maternal age, smoking during pregnancy, family income, financial difficulties for hyperactivity; paternal education, family income and financial difficulties for peer problems.

While our study sample consisted of 786 mother–child pairs, the numbers of complete cases varied from 483 to 486 depending on the SDQ subscale (Supplementary Fig. 1). We applied the Elastic Net to the MI-R and CC datasets (Supplementary Fig. 1). As a measure of the stability of the association, we used the VIP which can be interpreted as the posterior probability of including a variable in the model (Bunea et al., 2011) (cf. 2.7.3, Supplementary Table 2). To define a threshold VIP for identifying cytokines associated with behavioral problems, we relied on the VIPs of psychosocial and clinical variables already known from previous studies to be associated with child's behavior: maternal depression, breastfeeding, gestational diabetes, and number of older siblings for emotional symptoms; maternal depression, financial difficulties, home stimulation, allergies and sex for conduct problems; paternal education, financial difficulties, number of older siblings and sex for hyperactivity/inattention; gestational age, birth weight, family income, financial difficulties, home stimulations for peer problems; and sex for prosocial behavior. Corresponding VIPs ranged from 91.3% to 100% and we therefore chose a threshold VIP of 90% for identifying cytokines associated with behavioral problems (Table 3). Accordingly, IL-7 was associated with higher odds of emotional symptoms, while IL-8, IL-17A, and IL-10 were associated with lower odds (Table 3,

Table 1
Serum cytokine concentrations. The minimum (Min.), maximum (Max.), median and mean concentrations, standard deviation (SD), 5% and 95% percentiles, the LLOD, the LLOD/2 (imputed when concentrations were below LLOD) and the proportion of serum samples in which cytokine concentrations were below LLOD, are indicated for the 27 biomarkers assessed. Filled circles: variable included.

Cytokines	Min. (pg/mL)	Max. (pg/mL)	Median (pg/mL)	Mean (pg/mL)	Standard Deviation	5% percentile (pg/mL)	95% percentile (pg/mL)	LLOD (pg/mL)	LLOD/2 (pg/mL)	% <LLOD	Included in analysis
CCL2	6.057	513.080	212.507	218.771	72.081	114.200	347.200	0.090	0.045	0	●
CCL3	3.044	281.272	13.414	16.173	18.309	7.058	28.960	3.020	1.510	2.41	●
CCL4	11.892	348.647	95.392	103.228	45.050	45.370	186.500	0.370	0.185	0	●
CCL11	14.728	799.430	129.983	139.775	66.368	60.600	248.800	3.260	1.630	0	●
CCL13	9.311	251.789	75.236	79.906	41.050	25.720	160.700	1.690	0.845	0.96	●
CCL17	17.247	27741.205	291.997	414.253	1095.564	76.650	928.500	0.220	0.110	0	●
CCL26	1.809	649.825	4.953	10.347	39.870	0.885	16.240	1.770	0.885	16.99	
CXCL10	15.247	2317.137	143.598	199.533	192.408	69.780	500.800	0.370	0.185	0	●
GM-CSF	0.140	5.894	0.169	0.235	0.394	0.070	0.577	0.140	0.070	37.13	
IFN-γ	0.301	671.608	6.817	16.866	43.049	3.093	57.200	0.200	0.100	0.12	●
IL-1 α	0.091	11.450	0.045	0.214	0.686	0.045	0.815	0.090	0.045	69.10	
IL-1 β	0.040	9.624	0.020	0.141	0.553	0.020	0.568	0.040	0.020	54.92	
IL-2	0.090	11.669	0.045	0.198	0.716	0.045	0.490	0.090	0.045	53.84	
IL-4	0.020	1.784	0.010	0.029	0.099	0.010	0.070	0.020	0.010	70.26	
IL-5	0.221	26.182	0.332	0.794	1.722	0.110	2.766	0.220	0.110	39.88	
IL-6	0.061	16.787	0.422	0.704	1.098	0.132	2.340	0.060	0.030	2.64	●
IL-7	2.236	87.172	21.950	22.920	9.592	8.838	39.180	0.160	0.080	0	●
IL-8	1.778	834.001	8.759	16.337	53.524	3.685	21.970	0.040	0.020	0	●
IL-10	0.031	50.849	0.434	0.741	1.935	0.186	2.082	0.030	0.015	0.36	●
IL-12p40	9.297	690.816	227.226	241.363	91.149	121.400	415.800	0.390	0.195	0	●
IL-12p70	0.112	15.924	0.055	0.226	0.707	0.055	0.693	0.110	0.055	51.68	
IL-13	0.250	57.815	0.120	0.640	2.634	0.120	2.089	0.240	0.120	66.67	
IL-15	0.208	5.444	1.881	1.939	0.487	1.299	2.790	0.170	0.085	0	●
IL-16	13.363	1462.262	177.650	193.762	89.143	103.200	321.600	2.830	1.415	0.12	●
IL-17A	0.767	52.420	3.174	3.804	3.213	1.344	7.769	0.740	0.370	0.24	●
TNF- α	0.342	17.026	2.956	3.121	1.179	1.980	4.796	0.040	0.020	0	●
TNF- β	0.248	8.717	0.461	0.557	0.539	0.200	1.153	0.050	0.025	1.56	●

Table 2
Study population variables. BMI, body mass index; SD, standard deviation; Min., minimum; Max., maximum; N/A, non-available (N/A) values; Min., minimum; Max., maximum.

		N = 786	%	Mean (SD)	N/A (%)
Maternal variables	Age at delivery (years)			30.1 (4.8)	0
	Pre-pregnancy BMI (kg·m^{-2})			23.6 (4.6)	2.2
	Gestational diabetes		6.62		0.1
	Alcohol drinking during pregnancy (mean glasses/week)		4.96[1]	0.6 (1.5)	0.4
	Smoking during pregnancy (cigarettes/day)		21.88[2]	1.1 (2.8)	3.1
	Caffeine consumption during pregnancy (mg/day)		91.47[3]	90.6 (107.5)	1.5
	Urinary/Cervical/Vaginal infection during pregnancy		74.94[4]		0.3
Perinatal variables	Breastfeeding		68.45		0
	Gestational age (weeks of amenorrhea)			39.3 (1.7)	0
	Delivery mode	Vaginal	76.08		0.1
		Forceps/Ventosa	10.69		
		C-section	13.10		
	Birth weight (kg)			3.3 (0.5)	0
	Birth trimester	1st (Jan.–March)	23.79		0
		2nd (April–June)	24.81		
		3rd (July–Sept.)	26.59		
		4th (Oct.–Dec.)	24.81		
Psychosocial variables (Pregnancy-Age 5)	Parental separation		10.18		0.6
	Maternal depression		38.17[5]		7
	Maternal education (years)			13.8 (2.6)	0.4
	Paternal education (years)			13.1 (2.6)	7.5
	Family income	< 800 €	1.40		7.5
		801–1500 €	7.12		
		1501–2300 €	18.58		
		2301–3000 €	24.55		
		3001–3800 €	22.77		
		3801–4500 €	12.85		
		> 4500 €	11.20		
	Family financial difficulties		34.61[6]		15.3
	Number of older siblings			0.9 (1)	10.9
	Home stimulations			17.4 (2.2)	3.9
Child's variables at 5-year-old	Blood sampling time			Min.: 08:15 a.m. Mode: 09:00 p.m. Max.:16:00 p.m.	0.9
	Allergies		26.59		3.6
	BMI (kg·m^{-2})			15.4 (1.3)	0.1
	Sex	Female	44.02		0

Footnotes: [1]% of women consuming at least 3 glasses of alcohol/week during pregnancy; [2]% of women smoking during pregnancy; [3]% of women consuming caffeine during pregnancy; [4]% of women declaring at least one infection during pregnancy; [5]% of women having experienced at least one depressive episode; [6]% of families having experienced financial difficulties at least once.

Supplementary Table 3). TNF- α was associated with lower odds of conduct problems, while CCL2 was associated with lower odds of hyperactivity/inattention. IL-15 was associated with higher odds of peer problems, while CXCL10 was associated with lower odds. IL-6 was associated with increased odds of abnormal prosocial behavior, while CCL3 and IL-16 were associated with lower odds. Setting up an even more stringent threshold VIP (95%), five out of these 11 cytokines remained associated (positively or negatively) with the outcome: IL-7, IL-8, IL-10, TNF- α and CXCL10. Of note, several variables associated with altered odds of behavioral problems in the MI-R datasets were also identified when applying the Elastic Net regression model to the CC datasets (Supplementary Table 4). Thus, breastfeeding, maternal depression and number of older siblings were associated with emotional symptoms. This was also the case for maternal depression, financial difficulties, allergies, sex and TNF- α for conduct problems.

4. Discussion

To our knowledge, this cross-sectional study is the first to document associations between serum levels of individual cytokines and behavioral dimensions in 5-year-old children assessed by the SDQ. The SDQ is one the most common behavioral screening tool in children between 3 and 16 years of age (Stone et al., 2010). It has been used in large scale population-based birth cohorts such as the Copenhagen Child Cohort (CCC2000) (Niclasen et al., 2013) and the Danish National Birth Cohort (DNBC) (Elberling et al., 2010). This validated instrument allowed a large-scale study of possible associations between cytokines and behavioral outcome in young children. Because high scores in each of the SDQ subscale predict a high probability to enter a DSM IV diagnosis class later in life (Croft et al., 2015; Goodman, 2001; Russell et al., 2013; Russell et al., 2014), our results could be compared to those of studies exploring the role of cytokines in neurodevelopmental disorders. According to previous studies using other psychometric scales, scores over the 85th percentile also determine a class at high risk for clinical mental health disorders (de Winter et al., 2005; Huisman et al., 2008; Schneiders et al., 2003; Amone-P'Olak et al., 2009), thus making the possible clinical implication of our cross-sectional study more relevant from a public-health perspective.

4.1. Cytokines, behavior and neurodevelopmental disorders

High SDQ subscores for emotional symptoms are associated with DSM IV diagnoses of anxiety and depression (Goodman, 2001). In our study sample, IL-7 was associated with increased odds of emotional symptoms, while IL-8, IL-10 and IL-17A were associated with decreased odds. IL-7 is mainly known for its role in B and T lymphocyte

Table 3
Adjusted associations between selected variables and high-risk of behavioral problems. Weighted mean Odd Ratios (OR), weighted 95% Confidence Interval (CI) and Variable Inclusion Probability (VIP) for each of the variables selected by the Elastic Net in the analysis of MI-R datasets are shown. The VIP was used as a measure of the stability of an association as it can be interpreted as the posterior probability of including a given variable in the model (cf. 2.7.3 for complete definition). Only variables with VIPs above 90% are presented in Table 3. In the OR column, positive (shaded red) and negative (shaded blue) associations are indicated. Supplementary Table 3 details the results for all 42 variables included.

		Emotional symptoms			Conduct problems			Hyperactivity/Inattention			Peer relationship problems			Prosocial behavior problems		
Type	**Variable ID**	**OR**	**95% CI**	**VIP**	**OR**	**95% CI**	**VIP**	**OR**	**95% CI**	**VIP**	**OR**	**95% CI**	**VIP**	**OR**	**95% CI**	**VIP**
Maternal variables	**Age at delivery (years)**							0.854	[0.701,0.993]	91.6%						
	Gestational Diabetes	1.34	[1.012,1.853]	94.1%												
	Caffeine consumption during pregnancy (mg/day)	1.193	[1.015,1.448]	92.4%												
Perinatal variables	**Breastfeeding**	0.853	[0.709,0.991]	95.0%												
	Gestational age (weeks of amenorrhea)										0.801	[0.646,0.97]	94.4%			
	Birth weight (kg)										1.226	[1.015,1.558]	90.2%			
	Birth trimester: 1st													0.656	[0.379,0.935]	95.6%
	Birth trimester: 2nd										0.712	[0.453,0.977]	91.0%			
Psychosocial variables	**Maternal depression (Pregnancy-Age 5)**	1.171	[1.009,1.399]	94.0%	1.257	[1.051,1.535]	99.2%									
	Paternal education (years)							0.732	[0.595,0.906]	99.7%						
	Family income (Age 5)										0.802	[0.632,0.985]	93.8%			
	Family financial difficulties (Pregnancy - Age 5)				1.246	[1.026,1.561]	97.1%	1.231	[1.025,1.519]	94.8%	1.355	[1.059,1.749]	98.6%			
	Number of older siblings	0.69	[0.557,0.883]	99.7%				0.828	[0.671,0.977]	94.9%						
	Home stimulations				0.822	[0.681,0.978]	98.5%				0.853	[0.711,0.992]	91.3%			
Child's variables at 5 years	**Blood sampling time**				0.858	[0.714,0.984]	91.8%				0.822	[0.655,0.972]	96.5%			
	Allergies				1.184	[1.008,1.451]	91.1%									
	Sex:Male				1.538	[1.238,1.894]	100.0%	1.349	[1.112,1.663]	99.8%				1.296	[1.067,1.568]	99.1%
Cytokines	**CCL2**							0.825	[0.667,0.981]	93.3%						
	CCL3													0.839	[0.65,0.987]	90.7%
	CXCL10										0.788	[0.604,0.962]	96.6%			
	IL-6													1.201	[1.004,1.452]	90.1%
	IL-7	1.239	[1.052,1.477]	98.7%												
	IL-8	0.84	[0.69,0.974]	97.0%												
	IL-10	0.751	[0.537,0.962]	98.4%												
	IL-15										1.2	[1.014,1.488]	91.9%			
	IL-16													0.853	[0.688,0.995]	90.6%
	IL-17A	0.886	[0.742,0.992]	91.0%												
	TNF-α				0.756	[0.569,0.96]	98.0%									

development (Dahl et al., 2014), and IL-8 is a chemotactic factor for neutrophils, basophils, and T lymphocytes (Baggiolini and Clark-Lewis, 1992). Noteworthy, increased serum levels of IL-7 have been reported in patients with Major Depressive Disorder (Dahl et al., 2014), and plasma levels of IL-8 correlated negatively with symptoms of anxiety (Janelidze et al., 2015). IL-10 is known for its ability to inhibit the secretion of pro-inflammatory cytokines (Saraiva et al., 2019). *Il10*-deficient mice exhibit depressive-like symptoms and IL-10 treatment mitigates IL-1β-induced anxiety and sickness behavior in adult male rats (Mesquita et al., 2008; Munshi et al., 2019). Our data are therefore in keeping with earlier reports suggesting a detrimental role for IL-7 and a beneficial role for IL-8 and IL-10 in mood disorders. The situation for IL-17A is less clear. IL-17A is involved in the clearance of extracellular bacteria, the maintenance of mucosal homeostasis and the control of autoimmunity (McGeachy et al., 2019). Th17 cells, the principal immune cell types that produce IL-17A, are more abundant in the blood of depressive patients compared to healthy controls (Beurel and Lowell, 2018). In mice, IL-17A and Th17 cells promote depressive-like behaviors (Beurel et al., 2018; Nadeem et al., 2017). In contrast, mice deficient in IL-17 exhibit increased hippocampal neurogenesis, excitatory synaptic transmission and intrinsic neuronal excitability (Liu et al., 2014b). Therefore, whether and how IL-17A regulates neurodevelopment and brain function remain to be further investigated.

Conduct problems in early childhood are associated with an increased risk of conduct disorder, oppositional defiant disorder or other disruptive behavioral disorders (Goodman, 2001). We show that TNF- α is associated with lower odds of conduct problems in 5-year-old children. TNF- α is mainly known as a pro-inflammatory cytokine that plays a critical role in the early steps of immune responses directed to bacteria (Holbrook et al., 2019). In the brain, TNF- α also appears to have non-immune roles. Indeed, glial TNF- α has been identified as a modulator of synaptic strength (Beattie et al., 2002; Lewitus et al., 2014; Stellwagen and Malenka, 2006) and TNF- α modulates the survival, proliferation, and neuronal differentiation of neural progenitors (Bernardino et al., 2008). These data, together with ours, suggest that TNF- α is required for optimal brain functioning.

The hyperactivity/inattention SDQ subscore captures key symptom domains for a DSM IV diagnosis of ADHD (Goodman, 2001). In our study, CCL2 was associated with increased odds of hyperactivity/inattention in children aged 5. CCL2 is a chemokine mainly known for this ability to recruits inflammatory monocytes to inflamed tissues (Deshmane et al., 2009). To our knowledge, CCL2 has not been associated with ADHD before. However, given the critical role of CCL2 in the proinflammatory process and its reported association with ASD (Han et al., 2017), our results may suggest a link between inflammation and hyperactivity/inattention.

Peer problems are observed in ASD patients (Goodman, 2001). In our study, serum CXCL10 in 5-year-old children was associated with lower odds of peer problems. CXCL10 is a chemoattractant for monocytes and T cells (Karin and Razon, 2018), but it also mediates sickness behavior and cognitive dysfunctions after viral infection in mice (Blank et al., 2016). Our results are in keeping with an earlier report showing lower serum levels of CXCL10 in ASD patients compared to neurotypical controls, and a positive association of CXCL10 with social behaviors (Shen et al., 2016). In contrast, serum IL-15 was associated with higher odds of peer problems. IL-15 is a member of the IL-2 family of cytokines whose signaling pathways are at the crossroads between innate and adaptive immunity, it is also produced by astrocytes and neural progenitors, regulates neurogenesis and has anti-depressive effects in mice (Pan et al., 2013). More studies are required to investigate the role of IL-15 in social behavior.

A low SDQ prosocial subscore is a good predictor of ASD diagnosis in children (Russell et al., 2013; Russell et al., 2014). Here we show that the pro-inflammatory cytokine IL-6 is associated with increased odds of abnormal prosocial behavior in 5-year-old children. Several studies have pointed out a crucial role for IL-6 in the development of autistic-like behaviors in mice, and notably deficits in social interaction (Smith et al., 2007; Wu et al., 2017). Increased IL-6 levels have been observed in children with ASD, as compared to neurotypical controls (Saghazadeh et al., 2019), further supporting a role for IL-6 in the modulation of social behavior. In contrast, we show that CCL3, a potent pyrogenic chemoattractant for macrophages, monocytes and neutrophils (Maurer and von Stebut, 2004) is associated with decreased odds of abnormal prosocial behavior in our study. ASD patients exhibited lower levels of CCL3, which was positively associated with social behaviors (Shen et al., 2016). Our results thus support a relationship between CCL3 and social behavior. In our study, IL-16, a cytokine shown to recruit and activate CD4-expressing cells, including subpopulations of T cells, monocytes, eosinophils, and dendritic cells (Glass et al., 2006), was associated with decreased odds of abnormal prosocial behavior in 5-year-old children. Treatment of murine neuronal cultures with IL-16 modulates neuronal excitability and synaptic activity, suggesting that IL-16 is involved in neurotransmission control (Hridi et al., 2019). It is difficult to reconcile the previous findings with those of a recent study showing that ASD patients have higher frequencies of IL-16 expressing blood mononuclear cells compared to neurotypical controls (Ahmad et al., 2019). Further studies are warranted to decipher the possible links between IL-16 and social behavior.

4.2. Possible mechanisms

Since association does not imply causation, it remains to be demonstrated whether peripheral cytokines directly contribute to modulate brain functions and behavior. Although evidence for a causal relationship is lacking in humans, animal studies have pointed to several pathways by which peripheral cytokines could impact brain functions. First, peripheral cytokines could impact brain function indirectly either by activating peripheral afferent neural fibers (Zanos et al., 2018) or by binding to their cognate receptors on meningeal and lymphatic epithelial cells, eventually inducing the production of cytokines or other immune mediators such as prostaglandins and nitric oxide that will diffuse or be transported to the brain parenchyma (Dantzer, 2018; Pollmacher et al., 2002). Alternatively, cytokines can be transported from the periphery to the brain *via* the circumventricular organs, the brain-blood-barrier or the CNS lymphatic system, as demonstrated for TNF- α, IL-1- β and CCL2 (Banks, 2015; Norris and Kipnis, 2019). In the brain parenchyma, these cytokines could directly regulate both neurodevelopment and neurotransmission. For example, TNF- α promotes neurogenesis (Bernardino et al., 2008; Golan et al., 2004) and synaptic scaling (Beattie et al., 2002; Lewitus et al., 2014; Santello et al., 2011; Stellwagen and Malenka, 2006). Also, astrocytic IL-15 controls neurogenesis (Gomez-Nicola et al., 2011) and modulates GABAergic neurotransmission (He et al., 2010).

Pro-inflammatory cytokines such as TNF- α or IL-6 can have a deleterious effect on brain functions and behavior when their level is increased by several folds as the result of severe brain injury or infection (Pollmacher et al., 2002; Quan, 2014). However, this study as well as others using cytokine-deficient mice suggest that a negative deviation from baseline concentration could also be detrimental to brain functions and behavior. The impact of cytokines on brain functions appears complex and intertwined, its negative or positive valence depending on both their nature and their relative abundance towards other cytokines (Becher et al., 2017). In this context, it is tantalizing to speculate that baseline levels of specific cytokines are required for optimal brain functions and that deviating from these levels either in one direction of the other is detrimental.

4.3. Penalized regression for datasets with high-dimensionality

One originality of this study is the use of the Elastic Net penalized regression framework, a robust methodology adapted to datasets with high-dimensionality of data, which has good performance when the

number of events per variable (EPV) is low, performs variable selection and computes estimates, while addressing the issue of multicollinearity among variables (Bunea et al., 2011; Pavlou et al., 2016). While Elastic Net was first used to analyze omics data in genetic studies (Waldmann et al., 2013), it has been recently applied to neuroimaging (Abram et al., 2016; Bunea et al., 2011) and epidemiological data (Lu and Petkova, 2014; Philippat et al., 2019; Vasquez et al., 2016; Walter and Tiemeier, 2009). Following variable selection, it is a common practice to apply classical statistical inference, e.g. computing coefficients estimates, confidence intervals and p-values, on the same datasets. This may result in misleading conclusions because the statistical inference theory assumes data to be generated from a known model, and not that the model is selected based on the data used for inference (Leeb and Potscher, 2006). For this reason, we chose to readily interpret the Elastic Net penalized odd-ratios, keeping in mind that we could underestimate the size of the variable effect on the outcome. Consequently, we did not compute confidence intervals and p-values but rather used the weighted mean and weighted 95% CI of the odd-ratios and used the VIP as an estimate of the stability of the reported associations. As the size of epidemiological datasets tends to increase, we believe that the Elastic Net should be used more often for both variable selection and coefficients estimation, especially when there is no *a priori* or compelling evidence for selecting a small set of these variables. In previous studies, maternal depression and emotional distress appeared to be risk factors for emotional problems (D'Souza et al., 2018), while having older siblings could be protective (de La Rochebrochard and Joshi, 2013). Also, being a male is a risk factor for neurodevelopmental disorders (Davis and Pfaff, 2014) and low family income/financial difficulties are risk factors for abnormal socioemotional development of the child (Noonan et al., 2018). We consistently found these covariates to be associated with difficulties in at least one dimensions of behavior. The later observations lend support to the pertinence of our variable selection process.

4.4. Limitations

Our study has several limitations. First, and although we have adjusted for many covariates known or suspected to impact cytokine levels or behavioral outcome, we did not include other potential confounders which were not collected during the course of the EDEN study. This is for example the case for medications, infections and intestinal microbiota composition at the time of blood sampling. Second, the association coefficients and odds ratios that we computed using the Elastic Net method may underestimate the effect size of the variables on the behavioral outcomes because these are shrunken by the method. Therefore, these coefficients must be interpreted with caution. Third, the current study relies on serum samples collected at the age of 5 and is therefore based on cytokines measured at a single time point and in a sole biological matrix, serum. Therefore, we could not investigate the stability of our results overtime and over other biological matrices derived from peripheral blood, such as plasma. Fourth, despite our study sample being within the national estimates for most socioeconomic factors (Blondel et al., 2012; Heude et al., 2016), maternal level of education in the EDEN cohort was higher and may limit the generalization of our findings. Lastly, our results have not yet been replicated in an independent mother–child cohort, which would be warranted to validate our findings.

5. Conclusions and prospects

This study is the first to identify blood cytokines associated with specific behavioral problems in children. Serum levels of cytokines suspected or known to play a role in neurodevelopment and brain functions, such as IL-6, IL-10, IL-15, IL-17A, and TNF- α, were indeed associated with behavioral outcome in children. Furthermore, blood levels of cytokines poorly studied in this context – including IL-7, IL-8, IL-16 and the chemokines CCL2, CCL3 and CXCL10 – were associated with behavioral outcome in children. Further validation of the associations between cytokine levels and behavior is necessary, including analysis of other features, such as childhood infections and use of steroids. On another topic, several psychosocial variables previously associated with behavioral outcome in children were confirmed in our study sample. It would be interesting to investigate the association between these variables, and more specifically maternal depression, parental separation and poor socioeconomic status, with cytokine levels. Such a study may help unravel the mechanisms whereby early life adversity contributes altered cytokine production and lifelong psychiatric disorders (O'Connor et al., 2019; Pedersen et al., 2018). Whether the associations disclosed by this study reflect causal relationships, have prognostic value, or are merely phenomenological, would require longitudinal studies initiated prior to diagnosis of neurodevelopmental disorders and ideally pursued several years after clinical disease onset.

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Appendix A. Supplementary data

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