SUPPLEMENTARY MATERIAL

Immune activity at birth and later psychopathology in childhood

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1. SUPPLEMENTARY MATERIALS AND METHODS

1.1. 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. Enrolment 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.

1.2. Study sample

Among the 2002 pregnant women included in the EDEN study, 95 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). A total of 1367 cord blood samples (CBS) were collected and 1255 children were followed at 5 years. Our study sample consisted of the 869 mother-child pairs for which both CBS and the behavioral outcome at 5-year-old were available.

1.2. Covariates and confounding factors

Previous studies have identified maternal, perinatal and psychosocial variables associated with behavioral abnormalities in children (De La Rochebrochard and Joshi, 2013; Galera et al., 2018; Gumusoglu and Stevens, 2019; Heikkilä et al., 2011; Noonan, 2018; Philippat et al., 2018; Philippat et al., 2019; Polańska et al., 2015; Soomro et al., 2018; van der Waerden et al., 2015). Based on these studies, and considering that many of these variables can also influence cytokine production (O'Connor et al., 2009), we included the following covariates: maternal age at delivery (years), maternal pre-pregnancy body mass index (BMI in kg/m^{-2}), smoking during pregnancy (number of cigarettes/day), caffeine intake during pregnancy (mg caffeine/day), alcohol drinking during pregnancy (mean number of glasses/week), gestational age (weeks of amenorrhea), delivery mode (vaginal, C-section), birth weight (g), birth trimester and sex (female/male), maternal and paternal education duration (years), multiparity (number of older siblings) and record of depression during pregnancy (yes/no) and symptoms of prenatal anxiety. Maternal depression was assessed at 24-28 weeks of amenorrhea, using the Center for Epidemiological Studies

Depression questionnaire (CES-D (Radloff, 1991)) and women presenting a CES-D score above a cutoff of 17 were considered as depressed. Symptoms of maternal anxiety were assessed at 24-28 weeks of amenorrhea, using the State-Trait Anxiety Inventory (STAI (Gaudry et al., 1975; Spielberger and Vagg, 1984)), the continuous STAI score was used to assess the severity of anxiety symptoms.

1.3. Assessment of the outcome: child's psychopathology at age 5

Child's behavioral outcome was assessed using the Strengths and Difficulties Questionnaire (SDQ) 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 (Goodman, 1997) and entails five subscales 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 85^{th} upper percentile and 15^{th} lower percentile respectively. Such a dichotomization was used in previous studies (Barbosa et al., 2020; Melchior et al., 2015; Philippat et al., 2017) and in studies using similar psychometric scales (Amone-P'Olak et al., 2009; Huisman et al., 2010; Schneiders et al., 2003). For conduct

problems, hyperactivity/attention disorder, peer relationship problems and prosocial behavior 4 for emotional symptoms, we used cutoffs of 4, 5, 6, 3 and 6, respectively, yielding a high-risk class accounting for 20.6%, 14.8%, 14.5%, 14.8% and 14.9% of all children respectively (**Supplementary Figure 1**).

1.3. Cytokine measurements in CBS

Umbilical CBS were collected by research midwives immediately after delivery. Immediately after birth (vaginal delivery) or after extraction of the fetus through the uterine incision (elective caesarean section), umbilical cord was doubly clamped and rinsed to prevent contamination with maternal blood. Venous cord blood was sampled between the 2 clamps, transferred to tubes and allowed to clot. Blood samples were centrifuged within 24 h post collection, CBS were collected and stored at −80°C.

CBS were thawed on ice once and assessed using kits and reagents from two independent lots. All assays were completed within a 4-week period by the same investigator, blind to the samples' class. The concentrations of 27 cytokines (chemokine (C-C motif) ligand (CCL) 2, CCL3, CCL4, CCL11, CCL17, CCL26, C-X-C motif chemokine 10 (CXCL10), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), IFN-γ, IL-1-α, IL-1ß, 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, Vascular Endothelial Growth Factor (VEGF)-A, Tumor Necrosis Factor (TNF)-α and TNF-ß) were measured using the following V-plex® multiplex immunoassays: Proinflammatory Panel 1, Cytokine Panel 1 and Chemokine Panel 1, and according to the manufacturer's instructions (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 concentrations below the lower limit of detection (LLOD), we imputed a value equal to half the LLOD value indicated by the manufacturer, as recommended for immunological measurements constrained by detection limits (Uh et al., 2008). Eight cytokines (IL-1-α, IL-2, IL-4, IL-5, IL-12p70, IL-13, GM-CSF and VEGF-A) were presenting concentrations below LLOD in more than 15% of the samples were excluded from downstream analyzes (**Table 2**). For the 19 remaining biomarkers, we assessed the quality of our measurements by retrospectively calculating 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 869 serum samples. **Supplementary Table 1** summarizes the inter-CV for each of the 19 cytokines and shows that inter-CV were below 20% within the range of cytokines concentrations comprised between the 1% and 99% percentiles in our samples. CV below 20% comply with the recommendations of the US Food and Drugs Administration (FDA) regarding validation of ligand binding assays.

1.6. Descriptive statistics

Comparison analyzes 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, without and with Benjamini & Hochberg's multiple testing correction (False Discovery Rate). Correlations between concentrations of cytokine pairs were performed using

Spearman's rho correlation coefficient rank test with Benjamini & Hochberg's multiple testing correction. Statistical significance was set at a *p*-value < 0.05.

1.7. Association studies

The methodological workflow adapted from (Barbosa et al., 2020) is summarized in **Supplementary Figure 2** and detailed hereafter. The child's outcomes, corresponding to each SDQ dimension (emotions, conduct, hyperactivity, peer problems, prosocial behavior), were each considered independently in a predictive penalized regression framework. We elected the Elastic Net framework which uses linear models with penalties to avoid overfitting, enables handling of multiple predictors and addresses the issue of multi-multicollinearity between variables (correlation). First, missing data (corresponding to NAs in **Table 1**) were imputed using the Multivariate Imputation (MI) by Chained Equations (MICE) procedure in R (Buuren and Groothuis-Oudshoorn, 2010; Kontopantelis et al., 2017), to generate 40 independent MI datasets (Graham et al., 2007). Second, non-parametric bootstrap was used for statistical inference of penalized regression (Abram et al., 2016) and involved 100 resampling for each of the 40 MI datasets. Third, the CARET (Kuhn, 2008a) and GLMNET (Friedman et al., 2010) R packages were used to implement Elastic Net penalized (or regularized) logistic regression models on the 40 x 100 samples of data leading to 4000 models (Pavlou et al., 2016). For each variable we computed the variable inclusion probability (VIP), as the percentage of the runs in which the variable's coefficient was different from 0 and therefore selected by the penalized regression, the median odd ratios (OR) and the percentile bootstrap confidence intervals (CI). In agreement with previous studies (Barbosa et al., 2020; Bunea et al., 2011), the VIP was interpreted as the posterior probability of including a variable in the model, in the absence of asymptotically valid

p-values for Elastic Net. In this study, we set a stringent VIP threshold at 85% to identify variables stably associated with behavioral dimensions. Selected variables presenting OR equals to 1 were not interpreted, as their contribution to the model was likely to be limited. Finally, the predictive capabilities of each model were estimated by computing the overall predicted accuracy using the Area Under the Receiver Operating Characteristics (AUROC), sensitivity, specificity, and positive and negative predictive values using the CARET, rms and pROC packages (Harrell, 2001; Kuhn, 2008b; Robin et al., 2011).

1.8. 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.

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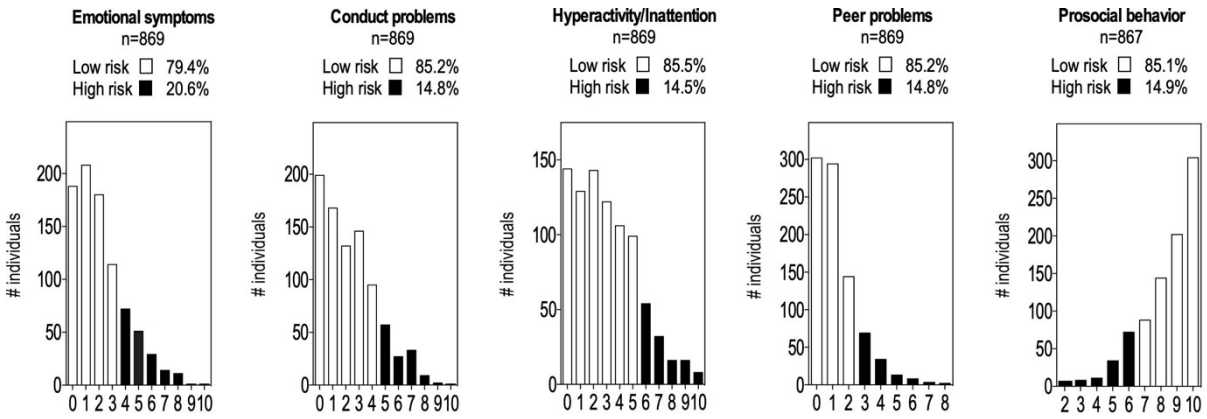
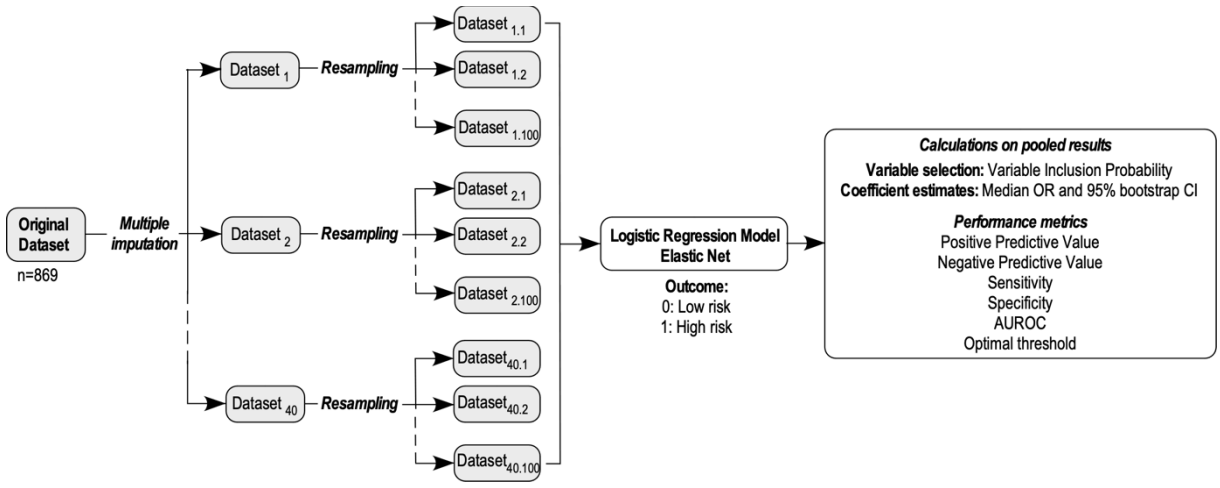
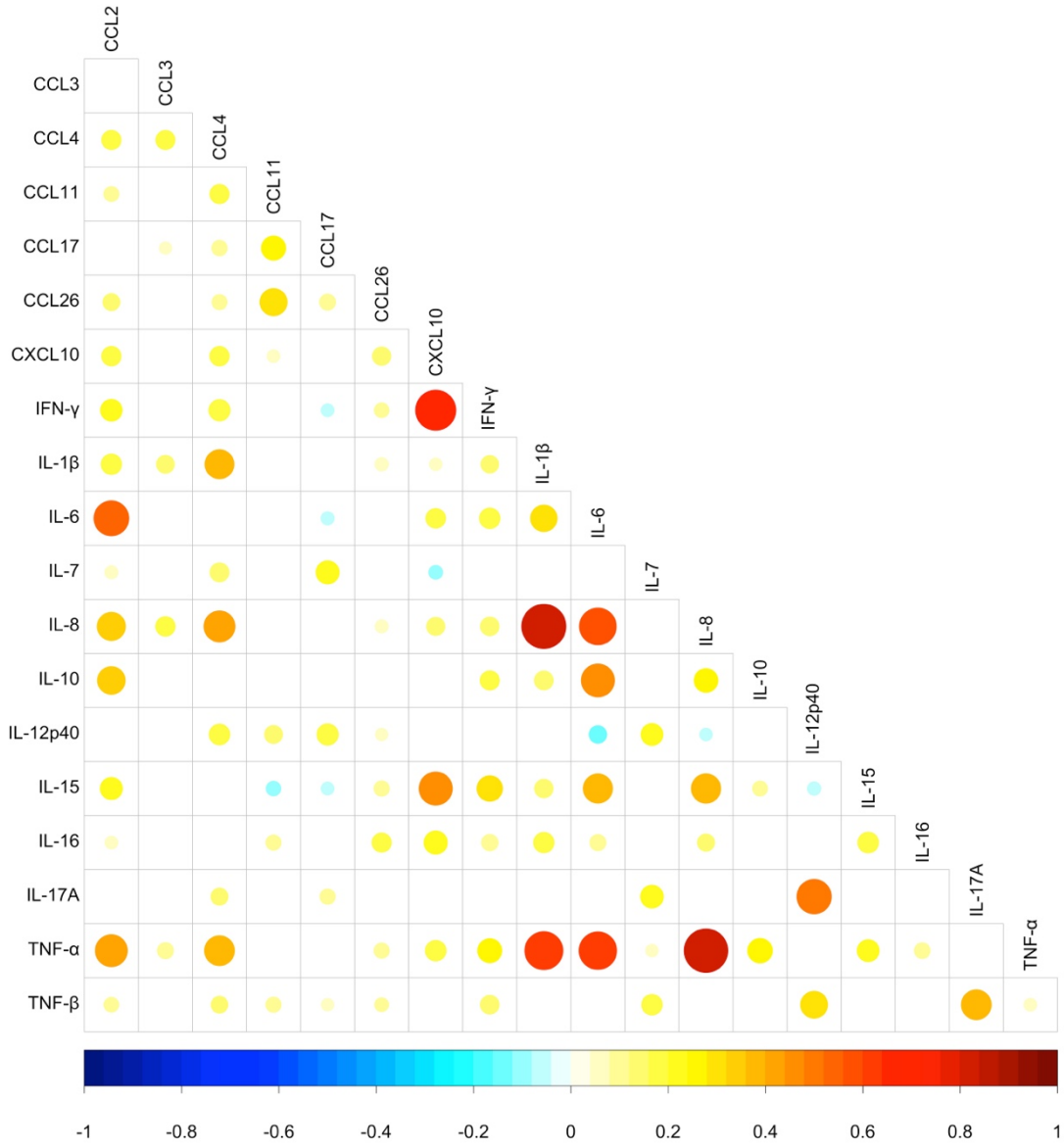
2. SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 1: Distribution of SDQ scores. Histograms show frequencies of low-risk (empty bars) and high-risk (filled bars) individuals per score in each of the indicated dimensions assessed by the SDQ.



Supplementary Figure 2: Methodological workflow of statistical analysis.



Supplementary Figure 3: Correlation analysis of the serum levels of 19 cytokines in cord blood samples. Heatmap of the pairwise Spearman's rank rho correlation coefficient between all cytokine pairs. Spearman's rho coefficients are color-coded and proportional to dot area. Only significant correlations are displayed (adjusted p-value < 0.05).

Supplementary Table 1: Inter-coefficients of variations (CV) for the immunoassays of the 19 cytokines retained in the analysis. CV were computed based on the calculated concentrations of the 7 standards assayed on each of the 11 96-well plates (5 from Lot A and 6 from Lot B), used to process all the samples. To estimate cytokine concentration ranges in samples, the 1-99%, 5-95%, and 10-90% percentiles of concentration are indicated. The standards covering the range of concentrations measured our samples are shaded in green. CV above 20% are shaded in grey.

Available as Excel file

Supplementary Table 2: Characteristics of mother-child pairs from the EDEN cohort according to the low- and high-risk class in each dimension. For each variable, sample size (n), minima (Min.), mean, median and maxima (Max.), as well as standard deviation (SD) are indicated for the low-risk and high-risk groups. P-values correspond to statistical univariate analysis for low-risk versus high-risk groups comparisons using Wilcoxon-Mann-Whitney test and Chi-square's exact test for numerical and categorical variables respectively, with and without Benjamini & Hochberg's multiple test correction (FDR). Statistical significance was set at a p-value below 0.05. Significant p-values are bolded.

Available as Excel file

Supplementary Table 3: Adjusted associations between variables and psychopathology at 5 years. Data show median Odds Ratios (ORs), 95% Confidence Interval (95% CI) and Variable Inclusion Probability (VIP) for each variable computed by the Elastic Net. VIP above 85% are shaded in grey.

Available as Excel file