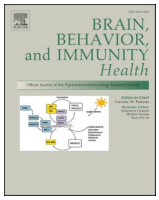
Brain, Behavior, & Immunity - Health 40 (2024) 100832

Contents lists available at ScienceDirect

Brain, Behavior, & Immunity - Health

journal homepage: www.editorialmanager.com/bbih/default.aspx



Maternal immune response during pregnancy and neurodevelopmental outcomes: A longitudinal approach

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ARTICLE INFO

Keywords:
Intrauterine stress
Cytokines
Neurodevelopment
Longitudinal study
Biomarkers
Maternal distress

ABSTRACT

Background and objectives: The neurodevelopment of the offspring is suggested to be influenced by the maternal immune system's responses throughout pregnancy, which in turn is also vulnerable to maternal psychosocial stress conditions. Therefore, our main goal was to investigate whether maternal peripheral immunological biomarkers (IB) during two stages of gestation are associated with distinct neurodevelopmental trajectories in the first two years of life. As a second goal, we also explored the association between maternal distal (childhood) and proximal (gestation) stressful experiences and the immunological markers assessed during pregnancy.
Methods: Maternal childhood trauma, depressive and anxiety symptoms, and peripheral IB (IFNγ, IL-10, IL1β, IL6, IL8, TNFα, EGF, IL13, IL17, IL1Ra and IL4) were measured at baseline (8–16 weeks of pregnancy) and at 30 weeks of pregnancy in 160 women. The participants had the blood samples collected from two randomized clinical trials conducted by the same team and methods in the same community. A Principal Component Analysis (PCA) was implemented to create meaningful composite variables that describe the cytokines joint variation. Finally, linear mixed-effects modeling was used to investigate the influence of inflammatory biomarkers, maternal childhood trauma, anxiety, and depressive symptoms on Bayley's III scores trajectories.
Results: The IB profile during the 3rd trimester of pregnancy predicted the offspring's neurodevelopmental trajectories in the first two years of life. The components derived from PCA were important predictors and captured different immune responses, reflecting both pro- and anti-inflammatory states. Maternal stressful experiences did not correlate with the immunological markers. Although not a reliable predictor alone, maternal psychosocial stress at the 1st trimester of pregnancy interacted with the mother's immune response while predicting the neurodevelopmental scores during the first two years of life.
Conclusions: Our results underscore the importance of the maternal immune response during pregnancy in shaping the neurodevelopmental trajectory of the offspring. Additionally, we observed that the maternal distress at the early stages of pregnancy has an incremental effect on the neurodevelopmental outcome but depends upon the immune response.

1. Introduction

The immune system and inflammatory responses during pregnancy

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https://doi.org/10.1016/j.bbih.2024.100832
Received 9 April 2024; Received in revised form 23 July 2024; Accepted 28 July 2024
Available online 3 August 2024

Abbreviations

BAI -	Beck Anxiety Inventory
BDI -	Beck Depression Inventory
BMI -	Body mass index
CRP	C-Reactive Protein
CTQ -	Childhood Trauma Questionnaire
EGF -	Epidermal growth factor
EPDS -	Edinburgh Postpartum Depression Scale
IL:	Interleukin
IFN-γ	Interferon gamma
IB	Immunological biomarkers
IQ	Intelligence quotient
PCA	Principal Component Analysis
RCT	Randomized Clinical Trial
STAI	State-Trait Anxiety Inventory
Th	T Helper
TNF	Tumor necrosis factor
Treg	T Regulatory

present specific signatures to protect the developing fetus and ensure a healthy brain development (Werneburg et al., 2017; Zengeler and Lukens, 2021). Insults during gestation, such as viral infections and psychosocial stress, can disrupt the immune system orchestrated regulation (DeRosa et al., 2023; Greenbaum et al., 2023). It has been suggested that cytokines can cross the fetus' blood brain barrier (Muldoon et al., 2013), mediating diverse processes such as neurogenesis, controlling the switch to gliogenesis, migration, axon pathfinding, differentiation, and survival (Bourgognon and Cavanagh, 2020; Dafny et al., 1985; Werneburg et al., 2017). However, little is known about the role of immunological markers during pregnancy to predict the trajectory of human neurodevelopment.

It is well recognized that a healthy pregnancy is associated with a humoral immunity enhancement and a downregulation of cell-mediated immunity (Gao et al., 2023; Martinez et al., 2022; Wegmann et al., 1993). In animal models, innate immune signals can increase the risk of worse obstetric and neurodevelopmental outcomes (Kalish et al., 2021; Kowash et al., 2022; Money et al., 2018). Using immunological biomarkers (IB), animal models have identified maternal immune activation (MIA) as a link between maternal infection and aberrant offspring brain and behavior development (Aria et al., 2020; Xiao et al., 2021). Reinforcing that, the presence of autoimmune diseases increases the risk of neurodevelopmental disorders (He et al., 2022). In humans, child intelligence quotient (IQ) score was previously reported as associated with cytokines levels during gestation (Dozmorov et al., 2018). Also, mean plasma interleukin-6 (IL-6) concentrations in different time points during human gestation, predicted functional connectivity and working memory at two years of age, suggesting that this could explain executive dysfunctions later in life(Rudolph et al., 2018). Moreover, using a panel of peripheral cytokines in the beginning to mid-gestation, associations between specific cytokines, child IQ score, cognitive flexibility and inhibitory control and attention at four years old has been described (Dozmorov et al., 2018).

The immune system presents plastic features and has been associated with psychosocial stressors such as depressive and anxiety symptoms, which can influence neurodevelopmental results as well (Lugo-Candelas et al., 2023; Meaney, 2018; Nolvi et al., 2023). A recent study found IL-1B and IL-6 maternal levels positively associated with depressive symptoms' severity across pregnancy (Sha et al., 2022). Furthermore, alterations in hypothalamic–pituitary-adrenal (HPA) axis activity indexed by diurnal cortisol slopes has been described as a potential pathway linking psychosocial stress (anxiety/depression) to inflammation (Knight et al., 2021). Specifically, mother's childhood trauma has been associated with immune-related alterations in pregnancy physiology, increasing concentrations of IL-6 and C-Reactive Protein (CRP), both pro-inflammatory health mediators (Finy and Christian, 2018; McCormack et al., 2021). Both obstetric and neurodevelopmental outcomes have been reported when the mother experienced a traumatic event during her childhood, (Keenan-Devlin et al., 2023).

Additionally, the timing and the type of stress exposure and immune response throughout the pregnancy seems to matter regarding the outcome (Hall et al., 2023). In mice models, the effects of maternal immune challenge between middle and late gestation periods were dissociable in terms of fetal brain cytokine responses to maternal inflammation and the pathological consequences in brain and behavior (Meyer et al., 2006). Indeed, epidemiological data suggest that maternal infection during early-to-mid human pregnancy is more likely to be associated with long-term abnormalities in offspring behavior and brain development (Allswede et al., 2020). Furthermore, fetus response to gestational adversity seems to depend on the sex, and it has been linked with sex biased neurodevelopmental disorders, such as autism spectrum disorder and schizophrenia (DiPietro and Voegtline, 2017; Hall et al., 2023). Recent studies support the hypothesis that following early life stress separate mechanisms, including epigenetic signatures, are associated with developmental and behavioral outcomes in male and female offspring (V. Euclydes et al., 2022; Hui et al., 2020). Specifically, in animal models, the adverse effects of MIA were suggested to be mediated by neuron-microglia interaction in a sex specific manner (Ardalan et al., 2019; Chamera et al., 2020).

Previously, in the *Primeiros Laços* study, a randomized clinical trial evaluating a nurse home visiting program designed to support adolescent mothers, we showed that differentially methylated positions from two genes, mediated the association between the maternal intervention and the cognitive domain at 12 months of age. STXBP6 is involved with vesicle trafficking and signaling, has been associated with dendritic protein localization in neurons and PF4 and encodes a member of the CXC chemokine family that initiates the secretion of cytokines (V.L.V. Euclydes et al., 2022). As presented before, studies investigating the relationship between inflammatory biomarkers and cognitive developmental outcomes early in life, have been the main focus of this research area, but fewer studies have started looking into other neurodevelopment domains (Kelly et al., 2022). Here, we combined two prospective studies with pregnant adolescents to investigate whether childhood trauma, gestational anxiety and depression, biomarkers of maternal immune system, in the first and third gestational trimester can predict the cognitive, language and motor domains trajectories in the first 2 years of life, considering sex and intervention group. n addition to incorporating a broader range of neurodevelopmental and immune markers, our study differentiates between childhood exposures and those during gestation. We examine how varying timing of exposure affects neurodevelopmental outcomes at 3, 6, 12, and 24 months of age, and analyze the consistent impacts of these exposures over time.

2. Methods

2.1. Participants

This study uses data from all individuals that have blood samples available from two randomized clinical trials "Primeiros Laços" (RCT 1 - NCT02807818, and RCT 2- NCT04362098). Primeiros Laços was a RCT conducted to test the efficacy of home visiting intervention for adolescent mothers living in a poor urban area of São Paulo, Brazil. delivered by trained nurses. The two studies were conducted by the same team, using similar methods in the same community, but with different sample sizes (RCT 1 with N = 80; RCT 2 with N = 167). The inclusion criteria were: first pregnancy, age between 14 and 19 years old, low socioeconomic status (classes C, D, and E according to the Brazilian Economic Classification Criteria [ABEP] scale), pregnancy between 8 and 16 weeks and living in the western region of São Paulo. The study was approved

by the ethics boards of the University of São Paulo Medical School and the Municipal Health Secretariat of São Paulo and informed consent was obtained for all participants. Participants were assessed by blind interviewers at 8–16 weeks of pregnancy (baseline), 30 weeks of pregnancy, and 3, 6, 12, and 24 months of infant's age. Nurses visited participants: (a) biweekly during gestation and from 2 to 20 months of child's age, (b) weekly during the first and last month of pregnancy, and the puerperium, and (c) monthly from 21 to 24 months of infant's age. The details of these procedures, and human subject's approval from affiliated institutions, have been described elsewhere (Alarcão et al., 2021; Fatori et al., 2021).

2.2. Maternal cytokine profile

Maternal peripheral blood was collected in EDTA tubes in two timepoints: at baseline (8–16 weeks of pregnancy) and at 30 weeks of pregnancy. The samples were centrifuged for 10 min at 3000×*g* for plasma separation. Plasma aliquots were stored at −80 °C until the time of the cytokines analysis. The cytokines IFN-γ (Interferon gamma), IL-10, IL-1β, IL-6, IL-8, TNF-α (Tumor necrosis factor-alpha), EGF (Epidermal growth factor), IL-13, IL-17, IL-1Ra and IL-4 were quantified by Luminex assay (MAGPIX® System – MAGPX15021702; Millipore #HCYTOMAG-60K - Human Cytokine/Chemokine Magnetic Bead Panel). The concentration of the analytes for the samples was calculated using the best-parameter logistic fit curve generated for each analyte from the seven standards using Milliplex™ Analyst software. Results were expressed as pg/mL for samples and analyzed according to the manufacturer instructions (ViageneTech, Carlisle, MA, USA) according to the manufacturer's instructions.

2.3. Assessment of anxiety and depression symptoms during pregnancy and maternal childhood trauma

To assess psychosocial stress exposure, we evaluated depression and anxiety during pregnancy in the first and third gestational trimesters. The RCT 1 measured anxiety and depressive symptoms using the Portuguese language version of the Beck Anxiety Inventory (BAI) (Beck et al., 1988a) and Beck Depression Inventory (BDI) scales (Beck et al., 1988b), respectively. While for the RCT 2, the anxious and depressive symptoms were assessed using the Portuguese version of the State-Trait Anxiety Inventory (STAI) (Skapinakis, 2014), Edinburgh Postpartum Depression Scale (EPDS) (Cox et al., 1987), respectively.

To assess childhood trauma, the Portuguese version of the Childhood Trauma Questionnaire (CTQ) was used covering five subscales: emotional abuse, physical abuse, sexual abuse, emotional neglect, and physical neglect (Grassi-Oliveira et al., 2006).

2.4. Assessment of childhood neurodevelopmental outcomes

The cognitive, language and motor neurodevelopment domain were assessed at 3, 6, 12 and 24 months of age, using the Portuguese language version of Bayley Scales of Infant and Toddler Development III (BSID-III) (Bayley, 2006), considered standard of world reference for assessing the neurodevelopment of children between 1 and 42 months of age. A total of 154, 130, 103, and 135 infants were assessed at 3, 6, 12 and 24 months of age, respectively.

2.5. Statistical analysis

2.5.1. Exposure to depression and anxiety symptoms during pregnancy

As the instruments capturing anxiety and depression symptoms diverged between the two studies, the available data were integrated using the Equipercentile Linking method(Albano, 2016) with an equivalent-group design. The "equate" package in R software version 4.1.3 was used for this purpose. Spearman correlation was used to evaluate the monotonic association between mother childhood trauma, depression and anxiety symptoms, and inflammatory markers expression during pregnancy while avoiding bias due to non-normality.

Out of the total sample covering both studies (N = 247), only individuals not affected by missing data (23 participants in RCT 1) were used (N = 224). As shown in Fig. 1, they were all used in the RCTs integration, and to assess mother psychosocial stress variables (anxiety and depression) and trauma correlation. Of the initial 224 participants, only 193 had maternal cytokines measures and were used to perform cytokines and chemokines profile and further correlation analysis. Only 188 had the infant's biological sex information (Supplemental Table 1). This subset was used to assess male/female differences and create the neurodevelopment mixed-effect models.

Equipercentile linking was performed to integrate the anxiety and depression measurements from RCT1 (NTC02807818; N = 57) and RCT2 (NCT04362098; N = 167), which were the only instruments that differed between studies. The individual BDI scores were converted to their equivalent EPDS scores, while the BAI scores were converted to their equivalent STAI-State scores. Loglinear smoothing was used to reduce irregularities in the score frequency distribution. The equipercentile linking procedure quality was assessed and presented in the Supplemental Figs. 1 and 2.

2.5.2. Cytokines patterns and dimensionality reduction

The concentration of systemic inflammatory markers reflects the state and response of the immune system to various contexts and stimuli. Cytokines, as pieces of this biological system, interact and coordinate their expression, resulting in covariance patterns. To address the redundancy of correlated cytokines and create independent variables, dimensionality reduction methods were employed. These methods aim to produce new variables that capture patterns in the co-expression of functionally related immunological markers. Cytokines were log-transformed prior to analysis. To deal with zero values, we utilized half of the minimum detectable concentration (MinDC) value for each analyte, as documented by HCYTOMAG-60K panel manufacture assay of sensitivities. Principal Component Analysis (PCA) with *varimax* rotation was used with the *psych* R package (Revelle, 2023). *Varimax* rotation aims to maximize the variance of the squared loadings of each independent component. This method tends to produce interpretable variables with standardized factor loadings close to 0 or 1. Unlike standard PCA, it also helps to balance the explained variance across the new independent variables, lessening a funneling effect towards the first component.

2.5.3. Neurodevelopmental trajectories

To investigate immunological biomarkers, maternal childhood trauma, anxiety, and depression influence on cognitive, language and motor BSID-III scores we employed linear mixed-effects modeling using the lme4 package (Bates et al., 2015). This method allows us to create models that predict neurodevelopmental scores, accommodating the nested structure of repeated measures. The models allow us to estimate the linear effects of hypothesized predictors while assessing the impact of time and other potential confounding variables. Prior to the model creation the numerical predictors were scaled by their standard deviation and mean centered.

$$NS_{s,t} = \mu_0 + \sum_{k=1}^{p} + \beta_k x_{s,k} + \beta_s t + \varepsilon_{s,t} \quad \text{(Equation 1)}$$

In Equation (1) we have the mixed-effect model specification. The model includes the following elements: the dependent variable, *Neurodevelopment Score* (NS), which represents the individual cognitive, language or motor domain measures for each infant (*s*) at each time point (*t*); the fixed mean score (μ_0), which represents the samples expected score and relates to the expected normative neurodevelopment; the set of *p* independent variables with fixed effects (β_k) are represented in the summation, which encompass variables of interest such as cytokines,

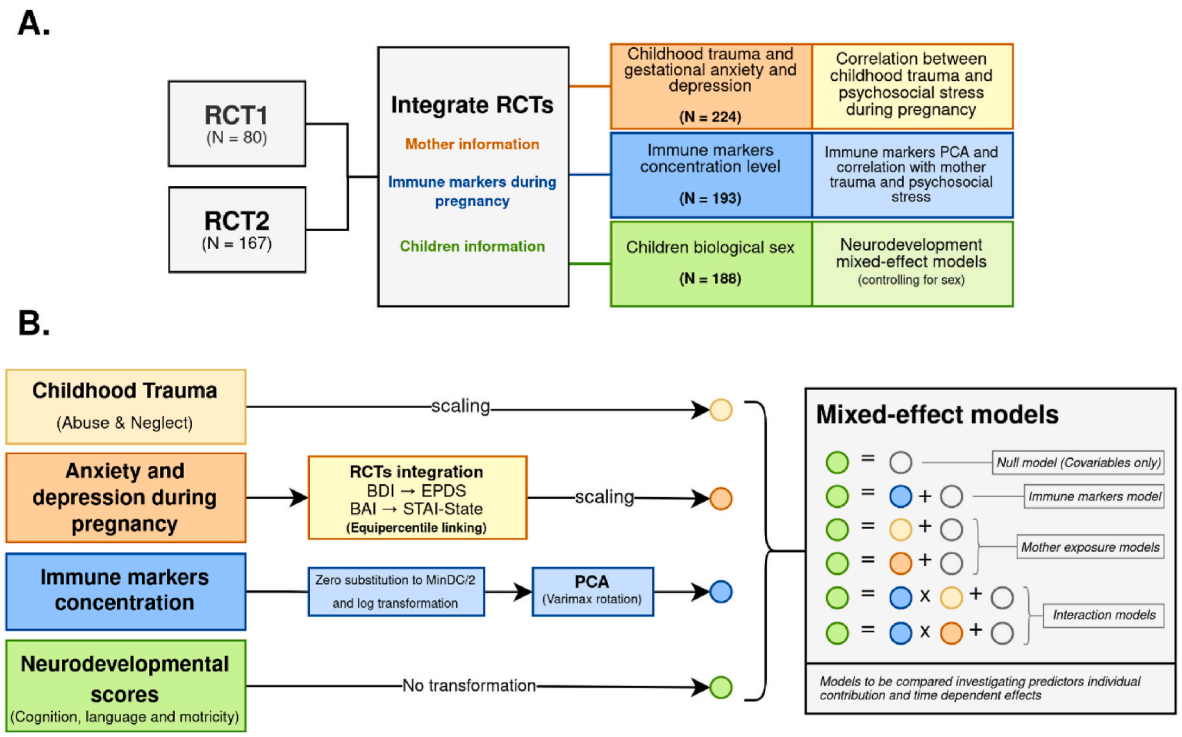

Fig. 1. (A) Illustrative scheme of the sample size after integrating both studies and the analysis in which three sample subsets were examined. The sample size varies depending on the available information. (B) Scheme illustrating general analysis workflow for the mixed-effect model (MinDC = Minimum detectable concentration).

trauma, anxiety and depression, but also includes a set confounder variables - discussed in the following paragraph; the gaussian distributed time related random effect (β_s) that varies for each infant; and finally the error term (ε). The models include the following variables: assigned sex at birth, RCT group (control or intervention), and RCT study (RCT 1 or RCT 2). While maternal age might influence inflammatory processes and offspring neurodevelopmental outcomes, it was not included as it was one of the RCT inclusion criteria (adolescents). Although gestational age would also be an essential confounding variable, unfortunately, no information about it was available in the dataset. A model comparison approach, utilizing the *likelihood ratio test*, was implemented to assess the maternal trauma, psychosocial stress, and immune factors potential as predictors of the child's neurodevelopment. The null model was defined as the simpler model, using only confounding variables along with the time variable, to predict BSID-III cognitive, language, and motor scores. These models were then compared to more complex ones, incorporating the hypothesized predictors to evaluate their predictive power. To estimate confidence intervals of estimates, the final models underwent bootstrapping, using the performance package (Lüdecke et al., 2021), with a thousand repetitions to estimate the confidence intervals of their parameters, including the variables coefficient and the Nakagawa's R-squared (Nakagawa and Schielzeth, 2013). Marginal and conditional R-squared were estimated. The former captures the proportion of variance explained by fixed-effect variables in the model, while the latter includes the variance explained by the random-effect variables. The DHARMa package was employed to evaluate the mixed models assumptions, considering the distribution of their residuals.

3. Results

3.1. Gestational anxiety and depression data integration

The anxiety and depression score distribution for the integrated data shows a higher median score at the first measure (1st trimester of pregnancy) with a reduction in subsequent assessments (Supplemental Fig. 3). The mother's expected anxiety and depression score values at early pregnancy stages are within the interval that indicates a moderate anxiety, but also close to a possible depression, given the STAI-State and EPDS scales reference guides (Pye, 2014; Skapinakis, 2014).

3.2. Boys/girls and case/control sample used in the mixed-effect model

The final sample used in our mixed-effect models consists of 188 mothers/infants. Their characteristics are detailed in Supplemental Table 1. We utilized the non-parametric Wilcoxon rank-sum test to assess differences between girls and boys (Supplemental Table 1) and between the control and intervention RCT groups (see Supplemental Table 2). No significant differences were observed in mothers' cytokine expression patterns and infant neurodevelopment scores. However, a difference in maternal trauma experiences was noted between girls and boys, although not significant after correction. Additionally, as expected, at birth girls seem smaller in size and weight compared to boys.

3.3. Immunological biomarkers patterns and correlation with mother trauma and psychosocial stress

The IB in the first and third trimesters of pregnancy exhibit a similar correlation pattern. This correlation is reflected in the PCA factor loadings shown in Supplemental Table 3. Cytokines concentration measured in maternal blood during these two time points did not show substantial changes (Supplemental Fig. 4). The PCA results for both trimesters indicate the presence of a similar cytokines coexpression structure, which is consistent with the lack of cytokines concentration change.

In the first trimester two PCA components explained 68% of the eleven immunological markers' variance, while in the third trimester the first two PCA explained 62% of the same cytokines variance. By examining the standardized factor loadings at baseline or 30w, we identified PC1 as a composite factor of IL13, IL10, IL8, IL6, and IL4, while PC2 represented the composite factor of IFNγ, IL17a, IL1b, and TNFα, although with slight differences for the last two (see

Supplemental Table 3). Similarly, this pattern is observed in a PCA capturing the differences between concentrations in the third trimester and the first trimester of pregnancy (Supplemental Table 4). This suggests that, as a coordinated system, an important portion of cytokine variation is shared among related cytokines. In the estimated principal components two groups of markers can be identified coordinating their variance during pregnancy (i.e., PC1 with IL13, IL10, IL8, IL6, and IL4; PC2 with IFNγ, IL17a, TNFα, and IL1b). Although, EGF and IL1RA markers seem to operate more independently of these groups.

Cytokine expression profiles in the first and third trimesters of pregnancy were uncorrelated to the mother's psychosocial stress variables depression or anxiety during the same period (see Supplemental Fig. 5). However, further analysis with a multiple linear regression model accounting for anxiety and depression joint effect on the PC2 factor captured an association between PC2 and depression scores at the first trimester (Supplemental Table 5). Additionally, PC1 values for the same time show a weak negative correlation with the mother's childhood history of emotional abuse, $r(191) = -0.27$, $p < 0.001$, and physical abuse, $r(222) = -0.19$, $p = 0.009$. PC1 exhibits a weaker and less significant negative correlation with the mother's childhood experience of neglect. In additional analysis, we observed that depressive and anxiety symptoms during and after pregnancy were positively correlated with all types of abuse and neglect evaluated in the study (Supplemental Fig. 6).

3.4. Neurodevelopment models

The mean composite score values for each time-point (3 months, 6 months, 12 months, and 24 months) were found to be similar in all neurodevelopment domains (Fig. 2). This implies a stable pattern of expected scores over time, with mean values ranging between 93 and 101 for the cognitive domain, between 90 and 98 for the language domain, and between 95 and 101 for the motor domain. The consistency close to the score 100 is anticipated, given that the composite score is obtained by normalizing individual measures against infants' scores from a normative sample of the same age. This normative sample, used as a reference, exhibits a composite score distribution of $N(\mu = 100, \sigma^2 = 15^2)$, suggesting that the study sample does not significantly deviate from the normative sample neurodevelopment.

We hypothesized that mother childhood trauma, psychosocial stress (anxiety and depression), and cytokines factors could partially account for the variance around the expected neurodevelopment scores. With this assumption in mind, first we evaluated, in distinct models, the independent linear contribution of each predictor, comparing, when applicable, different times of exposure (Supplemental Tables 6, 7, and 8). For instance, we compared models for the cytokines in the 1st and 3rd trimester of pregnancy, and the psychosocial stress variables at 1st trimester, 3rd trimester, and in the postnatal setting.

Cytokines principal components emerge as the most influential variable in our neurodevelopment models compared to the other predictors. Especially when looking at the cytokine concentration at the 3rd trimester of pregnancy (Supplemental Table 6 and Supplemental Fig. 7). The model described in Table 1 allows us to identify different effects operating on neurodevelopment scores when comparing the independent contribution of the principal components. PC2 (capturing IFNγ, IL17a, TNFα, and IL1b) appears to exert a negative effect on neurodevelopment, observed across all BSID-III domains, while PC1 (capturing IL13, IL10, IL8, IL6, and IL4) seems to have a positive effect, identified only for the cognitive domain.

Mother psychosocial stress variables and childhood trauma did not perform significantly better as independent predictors when compared to the null model (see Supplemental Tables 7 and 8 and Supplemental Figs. 8 and 9). However, higher anxiety levels during pregnancy, particularly in the 3rd trimester, seem to predict better language scores (see Supplemental Fig. 8). On the other hand, depression seems to have a negative influence, although not statistically significant, when predicting motricity scores.

We hypothesized that cytokine patterns during pregnancy could also interact with both mother psychosocial stress variables and childhood trauma when predicting neurodevelopment scores. To explore this hypothesis, we assessed the interaction terms contribution in separate models, comparing the impact at different times of exposure when interacting with the 3rd trimester cytokine PCs.

For the interaction models, we conduct a three-level comparison (Supplemental Tables 9 and 10). Firstly, we compared the null model with the models in which the cytokine principal components were added. Given the varying sample sizes in psychosocial stress measures across different time points (1st trimester, 3rd trimester, and postnatal), separate null models were created covering only complete cases. This was done to ensure that the models being compared were constructed using the same set of observations, differing only with the addition of new predictor variables. Secondly, we compared the independent cytokine models with the models in which the interaction terms were added - considering trauma or depression scores. Finally, for the psychosocial stress interaction models we further compare the first interaction model with a more complex model where we added the anxiety interaction with the cytokines factors besides the depression interaction.

Mothers' psychosocial stress interacts with cytokine patterns when predicting neurodevelopment scores (see Table 2). Notably, adding the cytokines factor interactions with the psychosocial stress experienced at

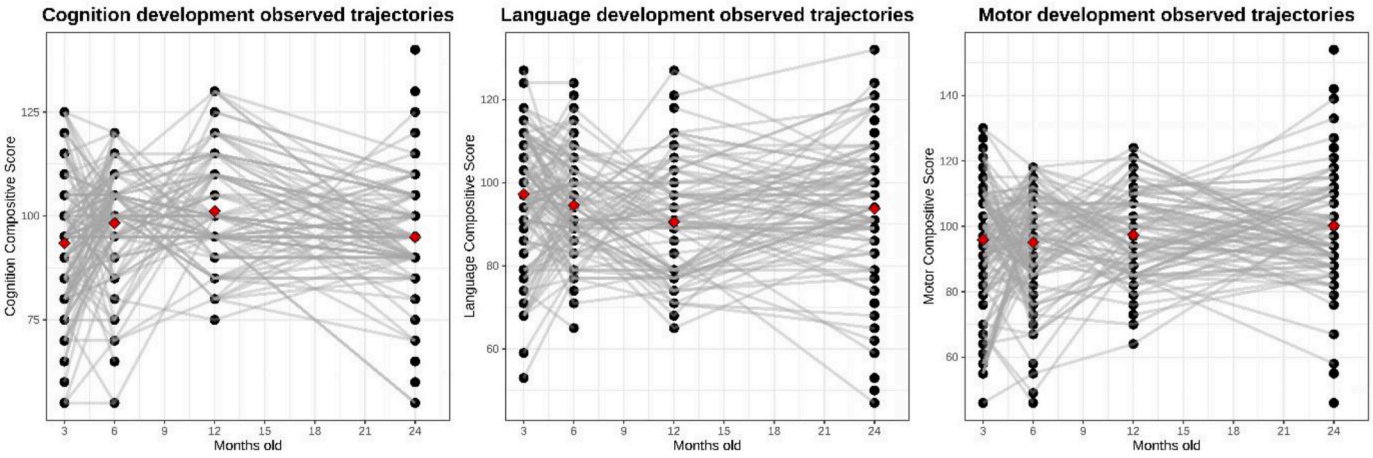

Fig. 2. Developmental trajectories during the first 2 years of life. Red dot represents mean values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Summary statistics for the repeated measures model with cytokines' independent fixed-effects on infant's neurodevelopment scores. Models' predictors coefficient estimates (*Estimate*), estimates confidence intervals 95% (*CI*), predictors p-values (*p*) and model R-squared (Marginal R^2 and Conditional R^2) were estimated through bootstrap with 1000 repetitions; ICC = Intraclass Correlation Coefficient; τ = random-effect between-subject variance.

Predictors	Cognition			Language			Motricity		
	Estimate	*CI*	*p*	*Estimate*	*CI*	*p*	*Estimate*	*CI*	*p*
Main effect									
(Intercept)	101.92	98.2–105.4	**<0.001**	92.34	88.86–95.81	**<0.001**	94.43	90.61–98.43	**<0.001**
PC1 3rd trimester	1.35	0.01–2.69	**0.050**	0.14	−1.36–1.56	0.846	0.59	−0.93–2.2	0.490
PC2 3rd trimester	−2.11	−3.51–−0.85	**<0.001**	−2.45	−3.82–−1.1	**<0.001**	−2.44	−4.05–−0.98	**<0.001**
Covariables									
Time [month]	−0.18	−0.36–0.01	*0.074*	−0.10	−0.27–0.07	0.222	0.27	0.07–0.46	**0.004**
RCT study [RCT2]	−4.24	−7.26–−1.42	**0.004**	4.85	1.42–7.54	**<0.001**	−1.26	−4.77–2.17	0.436
RCT group [Intervent.]	2.11	−0.41–4.74	*0.084*	1.76	−0.85–4.20	0.184	2.71	−0.61–5.67	*0.094*
Sex [F]	−2.13	−4.69–0.31	0.102	−1.44	−3.93–1.07	0.298	−2.67	−5.44–0.47	*0.100*
Random Effects									
σ^2	184.07			141.85			207.14		
$\tau_{11\ (Subject\ x\ Time)}$	0.04			0.16			0.14		
ICC	0.05			0.21			0.13		
Marginal R^2	0.051, CI (0.015–0.146)			0.080, CI (0.034–0.171)			0.050, CI (0.016–0.137)		
Conditional R^2	0.094, CI (0.034–0.290)			0.270, CI (0.118–0.413)			0.173, CI (0.035–0.361)		
N_{ID}	188			188			188		
Observations	511			511			512		

Table 2
Summary statistics for the model with cytokines' fixed effects interacting with mother depression score while predicting the infant's neurodevelopment scores. Models predictors coefficient estimates (*Estimate*), estimates confidence intervals 95% (*CI*), predictors p-values (*p*) and model R-squared (Marginal R^2 and Conditional R^2) were estimated through bootstrap with 1000 repetitions; ICC = Intraclass Correlation Coefficient; τ = random-effect between-subject variance.

Predictors	Cognition			Language			Motricity		
	Estimate	*CI*	*p*	*Estimate*	*CI*	*p*	*Estimate*	*CI*	*p*
Main effect									
(Intercept)	101.77	98.1–105.4	**<0.001**	91.79	88.32–95.32	**<0.001**	93.96	90.19–98.11	**<0.001**
PC1 3rd trimester	1.31	−0.02–2.70	*0.056*	−0.07	−1.54–1.35	0.952	0.47	−1.09–2.05	0.562
PC2 3rd trimester	−2.11	−3.50–−0.86	**<0.001**	−2.51	−3.86–−1.17	**<0.001**	−2.46	−4.07–−1.02	**<0.001**
Depression 1st trim.	−0.44	−1.59–0.80	0.512	−0.32	−1.55–0.82	0.608	−1.64	−2.99–−0.14	**0.034**
Interaction terms									
Depression:PC1	−0.78	−2.05–0.52	0.266	0.73	−0.62–2.05	0.290	−0.90	−2.33–0.55	0.214
Depression:PC2	−1.42	−2.70–0.07	*0.062*	−1.99	−3.33–−0.55	**0.006**	−2.30	−3.89–−0.83	**0.004**
Covariables									
Time [month]	−0.53	−1.08–0.03	*0.072*	−0.30	−0.80–0.20	0.224	0.80	0.22–1.37	**0.004**
RCT study [RCT2]	−3.63	−6.64–−0.80	**0.010**	5.50	2.51–8.36	**<0.001**	−0.28	−3.69–3.05	0.892
RCT group [Intervent.]	2.05	−0.44–4.71	*0.094*	2.04	−0.51–4.48	0.140	2.69	−0.44–5.62	*0.078*
Sex [F]	−2.41	−5.03–0.03	*0.052*	−1.23	−3.79–1.18	0.362	−2.71	−5.57–0.38	*0.082*
Random Effects									
σ^2	183.11			140.89			202.24		
$\tau_{11\ (Subject\ x\ Time)}$	0.34			1.40			1.20		
ICC	0.04			0.20			0.13		
Marginal R^2	0.062, CI (0.024–0.156)			0.098, CI (0.041–0.216)			0.081, CI (0.063–0.391)		
Conditional R^2	0.104, CI (0.047–0.303)			0.279, CI (0.124–0.419)			0.201, CI (0.033–0.173)		
N_{ID}	188			188			188		
Observations	511			511			512		

the first trimester of pregnancy improved the model across all neurodevelopmental domains (Supplemental Table 9). When considering the interaction effect for anxiety and depression in distinct models, only the depression interaction with cytokines were significant. However, when they were included together in a more comprehensive model, where we assess the independent effect of both anxiety and depression interactions, we do observe a significant interaction between anxiety and the cytokines PC1 factor (Table 3).

We observed that while prenatal maternal psychosocial stress alone does not have any significant effect on offspring neurodevelopment it can accentuate cytokines' effects. For instance, mothers' depression scores at baseline interact with PC2 in a way that lower than average depression did not change PC2 effect, but as the score increases the PC2 negative impact on neurodevelopment also increases (Fig. 3). Beyond this effect, when anxiety at baseline is included in the model, with an independent interaction with PC1 and PC2, we can see that different anxiety and depression profiles can also affect PC1 positive contribution to the neurodevelopment scores (Table 3 and Supplemental Fig. 11). While an increase in depression scores has the potential to reduce PC1 effect on neurodevelopment, anxiety can increase it (see Fig. 4).

Trauma, on the other hand, did not interact with cytokines in the same manner as mothers' psychosocial stress. A significant interaction was observed only when predicting language scores (Supplemental Fig. 12 and Supplemental Table 10). Cytokines PC1 factor interacts with all trauma measures (physical neglect/abuse and emotional neglect/abuse) with a positive coefficient. This result is interesting as PC1, in other models and in this specific interaction model, did not predict language scores independently. It is also noteworthy that, while predicting motricity scores, we observe a similar consistency between interaction estimates across all trauma measures, although not

Table 3
Summary statistics for the model with cytokines' fixed-effects interacting with mother psychosocial stress while predicting infant's neurodevelopment scores. Model's predictors coefficient estimates (*Estimate*), estimates confidence intervals 95% (*CI*), predictors p-values (*p*) and model R-squared (Marginal R^2 and Conditional R^2) were estimated through bootstrap with 1000 repetitions; ICC = Intraclass Correlation Coefficient; τ = random-effect between-subject variance.

Predictors	Cognition			Language			Motricity		
	Estimate	*CI*	*p*	*Estimate*	*CI*	*p*	*Estimate*	*CI*	*p*
Main effect									
(Intercept)	102.16	98.5–105.7	**<0.001**	92.16	88.73–95.61	**<0.001**	94.38	90.62–98.45	**<0.001**
PC1 3rd trimester	1.57	0.22–3.01	**0.024**	0.18	−1.40–1.64	0.806	0.66	−0.92–2.24	0.434
PC2 3rd trimester	−1.62	−3.08–−0.32	**0.012**	−2.11	–3.64 to −0.65	**0.012**	−2.20	−3.89–−0.56	**0.012**
Anxiety 1st trim.	0.29	−1.02 – 1.6 0	0.656	1.07	−0.32–2.33	0.124	−0.31	−1.79–1.22	0.704
Depression 1st trim.	−0.72	−2.04–0.63	0.322	−0.79	−2.10–0.55	0.242	−1.68	−3.21–−0.09	**0.034**
Interaction terms									
Depression:PC1	−1.95	−3.34–−0.48	**0.014**	−0.18	−1.85–1.44	0.842	−2.06	−3.79–−0.39	**0.020**
Depression:PC2	−1.64	−3.17–0.01	*0.052*	−2.03	−3.56–−0.40	**0.014**	−2.42	−4.12–−0.68	**0.004**
Anxiety:PC1	2.37	0.86–3.88	**<0.001**	1.65	0.10–3.38	**0.036**	2.37	0.58–4.18	**0.014**
Anxiety:PC2	0.54	−1.03–2.02	0.484	0.11	−1.47–1.65	0.880	0.20	−1.44–1.97	0.794
Covariables									
Time [month]	−0.18	−0.36–0.00	*0.064*	−0.10	−0.27–0.07	0.216	0.27	0.07–0.46	**0.004**
RCT study [RCT2]	−3.92	−6.9–−1.04	**0.004**	5.36	2.47–8.27	**<0.001**	−0.70	−4.02–2.59	0.714
RCT group [Intervent.]	2.19	−0.26–4.88	*0.072*	2.04	−0.52–4.41	0.132	2.93	−0.31–5.92	*0.064*
Sex [F]	−2.43	−5.01–−0.02	**0.050**	−1.30	−3.82–1.13	0.338	−2.75	−5.58–0.35	*0.080*
Random Effects									
σ^2	181.29			141.51			200.09		
$\tau_{11\ (Subject\ x\ Time)}$	0.03			0.15			0.13		
ICC	0.04			0.19			0.13		
Marginal R^2	0.082, CI (0.038–0.193)			0.109, CI (0.050–0.234)			0.098, CI (0.045–0.204)		
Conditional R^2	0.118, CI (0.056–0.308)			0.279, CI (0.130–0.436)			0.217, CI (0.086–0.403)		
N_{ID}	188			188			188		
Observations	511			511			512		

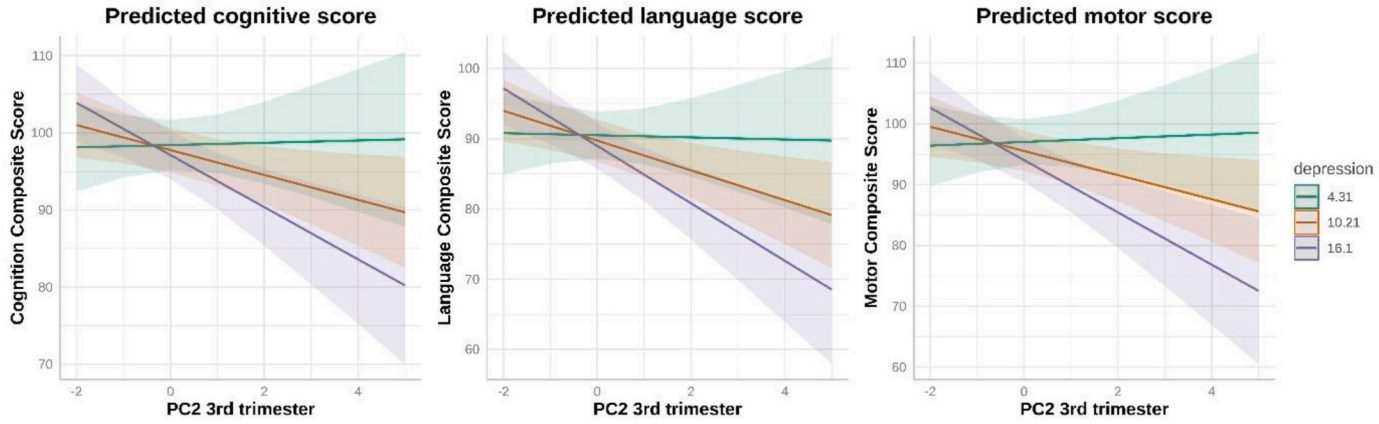

Fig. 3. The relationship between cytokines factor, maternal depression scores at baseline, and child neurodevelopmental outcomes. This represents the incremental effect of depression when interacting with PC2 beyond the independent negative effect of PC2. Reference values for depression were set at the mean scores (10.21). High and low scores, represented by the mean score plus and minus standard deviation, respectively, were used to assess the influence of varied maternal depressive contexts and cytokine expression patterns on the predicted neurodevelopmental scores.

significant (Supplemental Fig. 12). In this case, PC2 may interact with trauma, but with a negative coefficient.

Besides the mother's cytokines, trauma and psychosocial stress effect on neurodevelopment score prediction we also identified a significant effect of the covariates. In different mixed-effect models the study variable (RCT), which encodes the RCTs that originated each observation (NCT02807818 and NCT04362098), was able to detect a bias in the cognitive and language domain, but not for motricity. It's important to notice that identifying the bias in our models is also controlling for its influence on the predictors estimated effects. Furthermore, the consistency in results across domains demonstrates that most of our findings are unaffected by this specific bias.

The infant sex at birth, although not significant across most of the models, presented a consistent effect across all domains. Always pointing towards a slightly lower neurodevelopment score in girls compared to boys. Similarly, the intervention variable, which encodes the subjects who participated in the nurse home visiting program, consistently presented a positive effect, indicating a slightly higher neurodevelopment score for the children of mothers who received the intervention.

Time was also an important covariate for motricity. Time here should be understood as the children's age at the BSID-III assessment. This covariable was included in our models as a fixed effect and was able to capture the infant's age constant effect on the neurodevelopment score. While predicting motricity, time was a significant predictor with a positive coefficient. Considering that the estimated intercept value for motricity was below the expected normative score of 100, this result might suggest an overall catch-up trend.

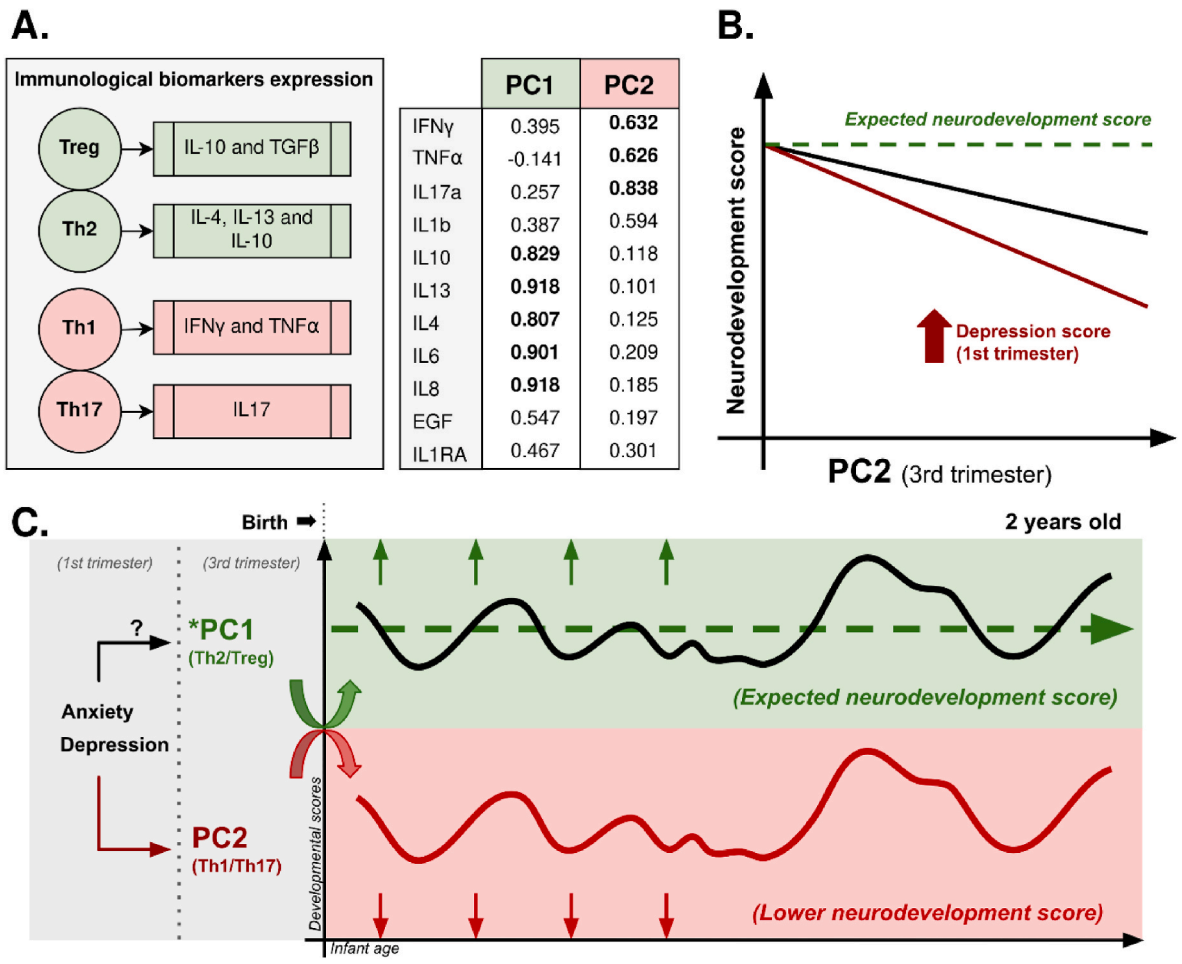

Fig. 4. Illustrative figure representing our main findings. **(A)** We have side by side the T Helper (Th) and T Regulatory (Treg) signature expression (Saito et al., 2010) and the 3rd trimester PCs factor loading values. **(B)** Example graph for the linear relationship between neurodevelopmental scores and PC2 factor, highlighting the additional role of depression. **(C)** Illustration showing that prenatal maternal immune imbalance, coupled with psychosocial stress, can shift the offspring neurodevelopmental trajectory with a persistent effect for the first two years of life. *PC1 factor exhibits a positive effect while predicting cognitive score, although its positive effects on the other domains seem to depend on the mother's psychosocial state - it interacts in distinct ways with anxiety and depression.

4. Discussion

Immunological biomarkers (IB) were predictors for cognitive, language and motricity scores during the first two years of life. Given the complex nature of immune molecules interactions throughout gestation and their high correlation, instead of investigating individual cytokines, we approached them using PCA analysis to find composite factors that could represent their coordination. The two factors that explained the IB variation also demonstrated distinct contributions to the neurodevelopmental scores' prediction. Additionally, psychosocial stress interacts with these factors to increase or decrease the IB effect on neurodevelopment.

Cytokines PCA analysis have been previously used to show that maternal inflammation indices over pregnancy were associated with inflammation in cord blood at birth(Ross et al., 2016). Here the IB factors identified and their effect on neurodevelopment reflect common cytokines profiles described in pregnancy success or failure (Guzeloglu-Kayisli et al., 2009). In PCA analysis we identified PC1 as a factor explained by the joint variation of IL13, IL10, IL8, IL6, and IL4, while PC2 by IFNγ, IL17a, TNFα, and IL1b. The differences observed between the cytokine factors is interesting as it suggests specific immune signatures that relate to T Helper (Th) and T Regulatory (Treg) T-cells phenotypes. Traditionally, the Th1/Th2 paradigm (Wegmann et al., 1993) recognizes Th2-biased response (IL-4, IL-6, IL-13 and IL-10) as a mechanism to promote maternal tolerance toward the fetus and maintain pregnancy, while Th1 cytokines (IL-2, IFNy, and TNF) are associated with lower fetus survival and higher rates of spontaneous abortion. However, the current understanding of maternal immunological tolerance suggests the need to expand the Th1/Th2 paradigm to cover for the Th17/Treg contribution (Figueiredo and Schumacher, 2016; Sakaguchi et al., 2008). The Th17 cells produce the proinflammatory cytokine IL-17, playing a role in host defense by inducing inflammation to combat pathogenic infections (Amatya et al., 2017; Hemdan et al., 2010). Conversely, Treg cells are responsible for producing anti-inflammatory factors such as IL-10 and TGF-B, regulating the Th17 inflammatory response(Sakaguchi et al., 2008). The delicate balance between the different phenotype activities aims to provide host protection against infection while simultaneously maintaining and supporting the developing fetus (Krop et al., 2020). Based on our PCA results, we can suggest PC1 as a factor capturing Th2 and Treg activity, while PC2 represents Th1 and Th17 activity. Our models indicate that Th1/Th17 activity during the third trimester of pregnancy is the most predictive of unfavorable neurodevelopmental outcomes (cognitive, language and motricity), whereas Th2/Treg response during the same period appears to be associated with better cognitive development.

Depression and anxiety during gestation is recognized as a risk factor for neurodevelopmental impairments and may have a bidirectional association with the mother's immune system (Martinez et al., 2022; Naudé et al., 2022). Here, we also approached distal maternal stress, through childhood trauma exposure, understanding that via

immunological alterations it could modify neurodevelopmental trajectories. Proximal distress was measured as anxiety and depression symptoms, during the beginning and the end of gestation. It is known that peripheral inflammatory molecules have the potential to influence the integrity of the blood-brain barrier (BBB) and impact human behavior(Daneman and Prat, 2015; Dion-Albert et al., 2022). During gestation, despite the control mechanism involving the placenta, it is known that the fetus brain can also be targeted, and neurodevelopmental routes altered. Rodent models and human studies have provided evidence that exposure to heightened maternal inflammation during pregnancy can cross the placental barrier and harm fetus developing brain (Gibson et al., 2023; Woods et al., 2023; Zhou et al., 2023). However, even in non-infectious conditions, such as depression, low-grade inflammation was observed in a quarter of 13541 depressive patients and half of them presented mildly elevated CRP levels (Osimo et al., 2019), additionally, even not at thresholds to diagnosis criteria, the presence of depression symptoms in gestational could impact neurodevelopment (Fitzgerald et al., 2021). Here we found that maternal childhood trauma showed a positive correlation with depression and anxiety symptoms experienced during pregnancy, reinforcing previous findings already described in the literature (Corona et al., 2022). However, in most of our model's maternal childhood trauma and psychosocial stress weren't significant predictors for neurodevelopment scores when considered independently of IB. Still psychosocial stress and mother's childhood trauma independently interact with IB in different models. This result might not exclude that distal distress may shape the mother's stress response in early stages of pregnancy contributing to immunological balances during gestation and its effect on neurodevelopment.

Therefore, beyond the fact that we found an independent association between immunological biomarkers and neurodevelopmental trajectories, interestingly, the inclusion of the depressive symptoms improved the explanation of the model for our outcomes. Our results showed that a significant part of psychosocial effect on offspring neurodevelopment depends on maternal cytokines profile during pregnancy. Conversely, the cytokines have effects independent of mother psychosocial stress. An increase in depression scores coupled with a more inflammatory state led to lower scores on language, motricity, and, although not as significant as the others, also for cognitive scores. Additionally, we observed that this interaction specifically happens when we look at the cytokines pattern at the third trimester of pregnancy and the depression symptoms at the first trimester, suggesting a time relevant response.

When we assessed anxiety and depression independent interactions with IB factors we observed contrasting effects on neurodevelopment, especially when looking at the Th2/Treg factor. This suggests that besides the initially observed Th2/Treg effect on cognition, its positive effects for the other neurodevelopment domains depends on mothers' psychosocial stress profile at baseline with differences between depression and anxiety symptoms. Interesting differences in psychosocial stress profiles have also been described on the microbiota shift during gestation and its possible consequences (Rajasekera et al., 2024). The study design and models discussed in this article have strengths and limitations. For instance, an important strength here is the variables availability and a reasonable sample size considering the longitudinal nature of this study. We had data on maternal childhood trauma, cytokines expression, and psychosocial stress measured throughout pregnancy and the neurodevelopmental assessment covering the infant's first two years of life. Furthermore, this data is interesting considering that most studies involving IB investigate a pathological condition, and here we observe that even in the absence of a known pathology characterized by inflammatory states, attenuated signatures of the immune system can have implications for neurodevelopment. Besides this rich dataset, we also implemented mixed-effect models to estimate mothers' exposure fixed-effects on offspring neurodevelopmental scores. Seeking to capture robust and persistent effects influencing the child neurodevelopment trajectory from 3 months to 24 months old. Although, as a trade-off we may have missed important time-dependent effects, as some variables might only predict changes in neurodevelopment scores observed during specific developmental time-windows. Also, a noticeable bias was introduced as a result of increasing the sample size through RCT studies integration, evident in our models as the *Study* covariate effect. This might suggest unexpected differences between studies but with minor implications for our results and models. Additionally, as the study sample comprises adolescent mothers from poor socioeconomic backgrounds, our results, especially for the full interaction model (Table 3), may lack external validity, not being generalizable for other demographics. Finally, the absence of pre gestational mother BMI and age in our models is also a limitation, as prematurity could be an important underlying factor connecting the negative effects of Th1/Th17 at the third trimester of pregnancy and the lower neurodevelopmental scores, and pre gestational mother BMI could alter IB patterns.

In summary, IB in maternal peripheral blood predicts the offspring's overall neurodevelopmental trajectories in the first 2 years of life. Importantly, we found that this association is beyond individual cytokines, but through factors that capture immunological phenomena. These factors were associated with a more pro- or anti-inflammatory state important to the balance between the different phenotype activities to provide host protection against infection while simultaneously maintaining and supporting the developing fetus. Additionally, maternal psychosocial stress variables at early pregnant stages interacted with the IB factors increasing the prediction of the infant's neurodevelopmental domains of language, motor, and cognition.

Funding/support

This work is supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001 and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grants 2018/18560–6, 2018/21934-5 and 2016/22455-8). Grand Challenges Canada (GCC), Fundação Maria Cecília Souto Vidigal (0722-03). V.E., C. G., C.C and C.I.S.B., were supported by FAPESP #2020/15590-1, #2020/16376-3, #2021/006079, #2021/00485-0, #2023/01343-0, respectively. H.B. and A.M. were supported by CNPq #310823/2021-8. CNPq #310823/2021-8 and #312746/2021-0, respectively.

CRediT authorship contribution statement

Veronica Euclydes: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Caio I.S. Braga:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Gisele Gouveia:** Writing – review & editing, Project administration, Investigation. **Raquel C.R. Martinez:** Resources, Investigation. **Caroline Camilo:** Writing – review & editing, Investigation. **Sergio N. Simões:** Formal analysis. **David C. Martins-Jr:** Formal analysis. **Lislaine Fracolli:** Investigation. **Adriana Argeu:** Investigation. **Alexandre Ferraro:** Investigation. **Alicia Matijasevich:** Writing – review & editing, Investigation. **Daniel Fatori:** Writing – review & editing, Formal analysis, Data curation. **Euripedes C. Miguel:** Investigation. **Guilherme V. Polanczyk:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition. **Helena Brentani:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

We assure that all procedures from the study "*Maternal immune response during pregnancy and neurodevelopmental outcomes: a longitudinal approach*" were performed in compliance with relevant laws and institutional guidelines. The present study was approved by the Ethics

Committee of the University of São Paulo Medical School (ref: 052/15), the University Hospital of the University of São Paulo, and by the Sao Paulo Municipal Health Department. Signed informed consent was given to participants and their primary caregivers. All methods were performed in accordance with the relevant guidelines and regulations. The study was registered at clinicaltrial.gov (Registration NCT02807818- date: June 21, 2016 and NCT04362098 – date: April 24, 2020). No relevant changes to methods, design or outcomes after trial commencement were made. The full study protocol can be sent upon request.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2024.100832.

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