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Altered maternal immune networks are associated with adverse child neurodevelopment: Impact of alcohol consumption during pregnancy

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Abstract

Cytokines and chemokines are potent modulators of brain development and as such, dysregulation of the maternal immune system can result in deviations in the fetal cytokine balance, altering the course of typical brain development, and putting the individual on a “pathway to pathology”. In the current study, we used a multi-variate approach to evaluate networks of interacting cytokines and investigated whether alterations in the maternal immune milieu could be linked to alcohol-related and alcohol-independent child neurodevelopmental delay. This was achieved through the measurement of 40 cytokines/chemokines from maternal blood samples collected during the second and third trimesters of pregnancy. Importantly, during the second trimester we identified network enrichment in levels of cytokines including IFN- γ , IL-10, TNF- β , TNF- α , and CRP

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associated with offspring neurodevelopmental delay. However, as elevations in levels of these cytokines have previously been reported in a wide range of neurodevelopmental disorders including autism spectrum disorder and schizophrenia, we suggest that this cytokine profile is likely not disorder specific, but rather may be an indicator of neurodevelopmental delay in general. By contrast, distinct clusters of activated/inhibited cytokines were identified based on maternal alcohol consumption and child neurodevelopmental outcome. Specifically, cytokines including IL-15, IL-10, MDC, and members of the VEGF sub-family were highest in alcohol-consuming mothers of children with neurodevelopmental delay and were identified in both network analyses and examination of individual cytokines, whereas a differential and unique cytokine profile was identified in the case of alcohol-independent child neurodevelopmental delay. We propose that the current findings could provide a critical step towards the development of early biomarkers and possibly interventions for alcohol-related neurodevelopmental delay. Importantly, the current approach could be informative for understanding mechanisms linking maternal immune system dysfunction and adverse child outcomes in a range of other neurodevelopmental disorders.

Keywords

Cytokines; Neurodevelopment; Pregnancy; Immune; Alcohol; Fetal Alcohol Spectrum Disorders

1. Introduction:

The immune system has been recognized as a type of sensory system that “samples” the external environment, making it uniquely vulnerable to environmental influences (Dietert, 2014). Importantly, the immune system also plays a critical role in development, as cytokines/chemokines are potent modulators of brain development, mediating processes including neurogenesis, neuronal migration, synaptogenesis, synaptic pruning, angiogenesis, myelination and apoptosis (Bajetto et al., 2001; Bessis et al., 2005; Deverman and Patterson, 2009; Huleihel et al., 2004; Jakovcevski et al., 2009; Smith et al., 2007; Stephan et al., 2012). As such, adverse exposures during sensitive periods of development can result in deviations in the cytokine balance, altering the course of typical brain development, putting the individual on a “pathway to pathology” (Bauman et al., 1997; Bilbo and Schwarz, 2009; Bodnar et al., 2016; Danese and McEwen, 2012; Ganguly and Brenhouse, 2015; Meyer et al., 2009; Miller et al., 2011; Rainekei et al., 2017; Raison et al., 2006).

While activity of the maternal immune system is dampened in order to protect the developing fetus, maternal immune system perturbations can result in increased cytokine production, with cytokines capable of crossing the placenta and affecting the fetus (Aaltonen et al., 2005; Zaretsky et al., 2004). Cytokines can also act on the placenta to stimulate downstream elaboration of the maternal cytokine signal in the fetal compartment (Hauguel-de Mouzon and Guerre-Millo, 2006; Jonakait, 2007). As a result, dysregulation of the maternal immune system due to disturbances including infection, drug exposure, or autoimmune disorders, has the potential to disrupt maternal-fetal interactions and thus the finely tuned fetal cytokine balance. Indeed, clinical and preclinical studies have demonstrated that inappropriate maternal immune activation/regulation is implicated in the etiology of a number of neurodevelopmental disorders including autism spectrum disorders

(ASD) (Goines et al., 2011; Jones et al., 2017), schizophrenia (Fatemi and Folsom, 2009; Meyer et al., 2009), cerebral palsy (Yoon et al., 1997), and other neuropsychiatric disorders (Buka et al., 2001). As such, many research groups have begun to examine the maternal inflammatory milieu with the goal of identifying possible immune markers that may be predictive of child outcome. Despite significant progress, distinct immune signatures have yet to be consistently linked to individual disorders. For example, both elevated (Brown et al., 2014) and depressed (Zerbo et al., 2016) maternal C-reactive protein (CRP) levels during gestation have been associated with ASD in the child. TNF- α levels have been found to be either not different (Brown et al., 2004) or elevated (Buka et al., 2001) in mothers of individuals later diagnosed with schizophrenia. Altered maternal TNF- α may also be important in ASD, with reports of higher TNF- α in cases of more severe ASD (Jones et al., 2017). Thus, with discrepancies between research groups and biomarker overlap among disorders, additional approaches, moving beyond the assessment of subsets of individual cytokines is necessary for the elucidation of disorder-specific immune signatures. Support for a broader approach, evaluating cytokines not only as individual markers but also in the context of the complex inflammatory milieu, can be found in evaluating cytokine dynamics. Cytokines do not operate independently but rather act in concert with each other to orchestrate a complex, coordinated response (Becher et al., 2017). As such, it may be possible to identify cytokine “hubs”, or critical modulators of cytokine responses, as well as clusters or networks of interacting cytokines that, together, are associated with specific diseases/disorders (Becher et al., 2017; Broderick et al., 2010; Ho et al., 2017). This network approach, examining how coordinated groups of cytokines differ across disease states, could result in a more comprehensive and robust immunological profile that, when complemented by evaluation of independent cytokine levels, may allow for better resolution among disorders.

Parallel to both ASD and schizophrenia, maternal immune disturbances have been posited to occur with alcohol consumption during pregnancy, and similarly, to be associated with neurodevelopmental delay in the offspring (Ahluwalia et al., 2000). Chronic alcohol consumption is generally associated with increased proinflammatory cytokine levels (Crews et al., 2006) and evidence also points to elevation in proinflammatory cytokine levels when alcohol is consumed during pregnancy (Ahluwalia et al., 2000). However, how alcohol consumption during pregnancy affects maternal immune networks, and the consequences for the developing fetus, remain unknown.

Here, we investigate whether the maternal immune milieu is impacted by alcohol consumption and whether immune networks in pregnancy can be identified and used to predict neurodevelopmental outcome in the child. Specifically, we evaluated a wide range of cytokines/chemokines and related factors during the second and third trimester of pregnancy in women who had a child with or without neurodevelopmental delay. We utilized both standard analyses of individual cytokines, as well as analyses of networks of interacting cytokines resulting in a broader investigation of immunological profiles than in previous studies. Importantly, due to our ability to identify one agent (alcohol) potentially responsible for the maternal immune disturbances, the comparison between alcohol-related and alcohol-independent neurodevelopmental delay in our study could be informative in beginning to understanding mechanisms linking maternal immune system dysfunction and adverse child

outcomes in a range of other neurodevelopmental disorders, including schizophrenia and autism spectrum disorder, where causative factors remain elusive.

2. Methods and Materials

2.1 Maternal cohort

Subjects were drawn from a larger cohort of women enrolled from 2007–2012 in a longitudinal study conducted at two recruitment sites (Rivne and Khmelnytsky oblasts) affiliated with the OMNI-Net Ukraine Birth Defects Prevention Program as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD.org) study. A description of cohort recruitment methods has been published previously (Chambers et al., 2014; Coles et al., 2015; Mattson et al., 2010). Women coming in for routine prenatal visits were screened using standard questions on alcohol consumption and other exposures, demographics, and pregnancy history. Women were eligible for enrollment in the alcohol consuming group if they reported moderate to heavy alcohol consumption in the periconceptual period, defined as at least weekly binge-drinking episodes (5+ drinks), or at least five occurrences of 3–4 standard drinks, or at least ten occurrences of 1–2 standard drinks, either in the month of conception or in the most recent month of pregnancy (Coles et al., 2015). Women were enrolled in the low/no alcohol consumption contrast group if they met the screening criteria of no binge episodes, minimal or no alcohol in the month around conception, and no drinking in the most recent month of pregnancy (Barr and Streissguth, 2001). All women were offered information regarding the risks of alcohol consumption during pregnancy. Study participants were also asked to report prescription and over-the-counter medication use in pregnancy. Of note, women in this sample did not report use of anti-inflammatory drugs.

All participants provided informed consent and study protocols were approved by Institutional Review Boards at the Lviv National Medical University, the University of California San Diego, and the University of British Columbia.

2.2 Blood collection

Maternal blood samples were collected during regular prenatal visits, twice during gestation, with the first blood draw occurring during the second trimester (at recruitment) and the second blood draw at a follow-up appointment during the third trimester of pregnancy (Table 1). Appointments were scheduled throughout the day to accommodate the clinic schedules. Study participants were not required to fast prior to sample collection, nor was toxicology screening for current drug and alcohol use performed during the prenatal visits. Samples were collected by venipuncture, using EDTA-coated tubes, centrifuged, and plasma aliquoted and stored at -80°C .

2.3 Child outcomes

Data on child sex, weight, length, and gestational age were collected after delivery. Children received a dysmorphology examination to evaluate the physical features of Fetal Alcohol Spectrum Disorders (FASD). Neurodevelopment was assessed by the Bayley Scales of Infant Development, Second Edition (BSID-II) at 6 and 12 months (Bayley, 1993) utilizing

the Mental Development Index (MDI; early cognitive/language development) and the Psychomotor Development Index (PDI; fine and gross motor skills). Children scoring <85 on either the MDI or PDI were classified as having neurodevelopmental delay. All scores were standardized by age and corrected for prematurity (<37 weeks).

2.4 Selection of the sample

For the current analysis, women were selected from the overall longitudinal cohort if they provided two blood samples and had a child with a completed dysmorphological examination and neurobehavioral testing. Stratification of the maternal samples took into account child outcomes in the Bayley assessment. For the moderate to heavy alcohol consumption group, this resulted in: A/TD: alcohol-consuming mother, typically developing child (n=22) and A/ND: alcohol-consuming mother, neurodevelopmental delay in the child (n=35). For the low-no consumption group, mothers were similarly stratified, resulting in: C/TD: control mother, typically developing child (n=60) and C/ND: control mother, neurodevelopmental delay in the child (n=35).

2.5 Cytokine assays

Plasma cytokine levels were measured using Meso Scale Discovery (MSD) V-PLEX Human Biomarker 40-Plex kit (K15209D-1, MSD, Rockville, MD). Plates were read using a MESO QuickPlex SQ120. Data were analyzed using the MSD Discovery Workbench software v. 4.0. For the lower limit of detection and unabbreviated cytokine list, see Supplementary Table 1.

2.6 Statistical modeling

Group comparisons of maternal and child characteristics were performed using the Kruskal-Wallis test (continuous variables), the Fisher's Exact test (categorical variables), or the Chi-Square test (SES and smoking status). Cytokine levels below the detection limit of the assay were assigned a value of zero. In addition, the following cytokines were undetectable in >10% of the samples and were thus excluded from the analyses (with the exception of the heatmaps) – GM-CSF (37.8% undetectable), IL-5 (41.8% undetectable), Eotaxin-3 (CCL26; 12.2% undetectable), IL-12p70 (87.8% undetectable), IL-13 (68.8% undetectable), IL-1 β (90.1% undetectable), IL-2 (20.1% undetectable), IL-4 (96.1% undetectable). To examine the relationship between maternal cytokine levels and child neurodevelopmental outcomes, linear regression models were fit for each cytokine as the response variable, and with exposure group, gestational age, and group x gestational age interaction (slope) as predictors. Regression models were also adjusted for maternal marital status, BMI, socioeconomic status, smoking, parity, child sex, and study site. Analyses were stratified by trimester, and cytokine levels were Box-Cox transformed to satisfy regression modeling assumptions. *P*-values were considered significant at *p* 0.05, and trends (*p*<0.07) were examined. Outliers (± 3.5 SD from the mean) were removed, as appropriate. Due to the exploratory nature of the analyses, no correction for multiple comparisons was made.

In order to highlight and compare overall cytokine patterns across groups, cytokine levels were first compared using heatmaps built on z-scored data (averaged across group). Next, constrained principal component analysis (CPCA) was performed to identify networks of

interacting cytokines and to determine the degree of involvement/importance of these networks among groups. Briefly, CPCA combines multivariate multiple regression and principal component analysis (PCA) into a unified framework, allowing for the identification of networks (components) that are specifically predictable from the independent variables (Takane, 2001, 1991). CPCA involves first regressing the matrix of dependent variables (cytokines) to the independent variables (group), resulting in a matrix of predicted scores reflecting the variance in cytokine measures that is predictable by group membership, referred to as the predictable variance. The second step in CPCA consists of a PCA on the predicted scores, which reveals the cytokine networks that are affected by group membership. PCA is a data reduction technique that uses information about the dominant patterns of inter-correlations among a set of variables to reduce these variables into a smaller number of components that best explain the variance in the dataset. PCA solutions were separately rotated using Varimax with Kaiser normalization, and the number of components extracted was determined using scree plots (Cattell and Vogelmann, 1977). In order to determine the degree to which group membership impacted the cytokine networks, correlations were computed between the variables coding groups and the component scores from each of the extracted components. Separate CPCA's were conducted on samples collected during the second and third trimesters.

All statistical analyses were performed using R statistical software (R Core Team 2013), with the exception of the CPCA, which was performed using Matlab (R2014b, Massachusetts, USA). An *a priori* power analysis was computed using G*Power (3.1.9.3, Heinrich-Heine-University Düsseldorf). To achieve a large effect size in the multiple regression model ($f^2=0.35$) at a power of 0.80 and with a Bonferroni correction to the α to account for multiple comparisons ($\alpha = 0.000156$), the total sample size was estimated at 102 individuals (actual sample size $N=152$).

3. Results

3.1 Sample characteristics

Maternal: Groups did not differ in age, gestational age (enrollment), education, gravidity, parity, pre-pregnancy body mass index (BMI), or multi or prenatal vitamin use (Table 1). However, a higher proportion of women in the A/ND compared to the C/TD group were recruited at the Khmelnytsky site ($p=0.013$). Groups differed in socioeconomic status (SES) with lower SES in the A/ND compared to the C/TD group ($p=0.006$). *Post-hoc* analysis of marital status revealed a trend ($p=0.068$) for a higher proportion of single/separated/divorced subjects in the A/ND compared to C/TD group. In addition, the proportion of subjects reporting current smoking was higher in the alcohol-consuming groups (A/TD, A/ND) compared to low/unexposed groups (C/TD, C/ND) ($p<0.001$). As group membership was defined, in part, by alcohol consumption, as expected, alcohol consumption variables were different across groups. Importantly, however, level of alcohol consumed did not differ between the two alcohol-consuming groups (A/TD vs. A/ND).

Child: Examination of child characteristics revealed group differences for key features of FASD including: height, palpebral fissure size $<10^{\text{th}}$ centile, smooth philtrum and thin

vermilion border, (Table 2); however, *post-hoc* analyses failed to reach significance with the exception of a higher proportion of children with a thin vermilion border in the A/ND compared to the A/TD group ($p=0.031$). Finally, as group membership was also dependent on neurodevelopment of the child, MDI and PDI scores were lower with neurodevelopmental delay (A/ND, C/ND), compared to typically developing (A/TD, C/TD) groups ($p<0.001$).

3.2 Maternal cytokine levels: analysis of individual cytokines

Analysis of individual cytokines during the second trimester revealed increased IL-15 and IL-10 in the A/ND group compared to controls (C/TD and C/ND; Table 3). Trends were also observed for MDC (CCL22) and TARC (CCL17; p 's=0.058), with increased levels of both cytokines in the A/ND compared to the C/TD group and for TARC only, a trend for increased levels in the C/ND ($p=0.064$) and A/TD ($p=0.053$) compared to the C/TD group (Table 3). During the third trimester, VEGF-D levels were higher in alcohol-consuming (A/TD, A/ND) compared to C/ND, and lower in the C/ND compared to the C/TD group. In addition, there was a trend ($p=0.066$) for CRP, with pairwise comparisons revealing lower CRP levels in A/ND compared to all other groups (A/TD, C/TD, C/ND).

Differences in the cytokine slopes were also investigated (Table 4, Figure 1) with a trend ($p=0.060$) for differential slope patterns in IFN- γ during the second trimester, and differences in TNF- β and MIP-1 α (CCL3) slopes during the third trimester. Specifically, for both IFN- γ and TNF- β , differential slopes were detected between alcohol-consuming groups – with typical neurodevelopment (A/TD), cytokines *decreased* as gestation progressed, with an opposite pattern of *increasing* cytokines through gestation associated with neurodevelopmental delay (A/ND). In addition, the IFN- γ slope also differed by neurodevelopmental outcome in the control group, with increasing IFN- γ over gestation associated with typical neurodevelopment (C/TD) and concomitant decreasing IFN- γ associated with neurodevelopmental delay (C/ND). Finally, MIP-1 α slope differences were detected in the C/TD group, compared to the alcohol-consuming groups – MIP-1 α increased as gestation progressed in controls (C/TD) but decreased in both alcohol-consuming groups (A/TD, A/ND).

3.3 Maternal global cytokine profiles

Overall, the cytokine signatures of the four groups were distinct (Figure 2). During the second trimester, the majority of the cytokines were highest (in red) in the A/ND group. Importantly, while there is was overlap between the A/ND and A/TD groups, the overall cytokine patterns differed, with a subset of cytokines detected at higher levels in the A/ND group. In addition, unique cytokine signatures were identified in the two groups with neurodevelopmental delay (A/ND, C/ND) – cytokines that were high with alcohol exposure were generally lowest in control mothers. During the third trimester, average cytokine levels were highest in the groups with neurodevelopmental delay (C/ND, A/ND), with minimal overlap of those at the highest levels between these groups. Similar to the second trimester, differential cytokine signatures between the two groups with neurodevelopmental delay (C/TD and A/TD) can also be identified. Generally, however, differences in the average

cytokine profiles among groups appear to be most marked in the second trimester and less distinct in the third trimester.

3.4 Maternal cytokine networks

For the CPCA, Figure 3 illustrates which cytokines are activated (in red), or inhibited (in green), and how network involvement differs among groups. In addition, for each network, the strength ($|r|$) and the significance (p value) of the correlation for each group is indicated.

Second Trimester: Network 1 was defined as the *Alcohol-Exposure Network* as the cytokines belonging to this network [IL-15, MCP-1 (CCL2), IL-10, MIP-1 β (CCL4), VEGF-D, TNF- α , sFlt-1, sVCAM-1, and MCP-4 (CCL13)] were activated with alcohol consumption (A/TD, A/ND) and inhibited in controls (C/TD, C/ND), with the exception of CRP, which, showed the opposite pattern (Figure 3). Network 2 was defined as the *Exposure/Neurodevelopmental Delay Network* as a differential pattern was detected in the C/TD group, compared to all other groups; this network (TARC, IFN- γ , TNF- β , and MDC) was inhibited in the C/TD group and activated in all other groups. Finally, network 3 was defined as the *Vulnerability Network* as this network [MDC, sICAM-1, IP-10 (CXCL10), PIGF, Eotaxin, and IL-10] was activated in the groups where child neurodevelopmental delay occurred (C/ND, A/ND) but inhibited in the A/TD group. Furthermore, this was the only network that allowed for the dissociation of typical neurodevelopment from neurodevelopmental delay, following maternal alcohol consumption (A/TD, A/ND). Of note, this network was not associated with the C/TD condition.

Third Trimester: Network 1 was defined as the *Neurodevelopmental Delay Network* as cytokines contributing to this network (IL-15, MDC, sICAM-1, Eotaxin, TARC, SAA, IL-17A, and TNF- β) were activated in groups with neurodevelopmental delay (C/ND, A/ND) and inhibited in groups with typical neurodevelopment (C/TD, A/TD). Network 2 was described as the *Alcohol-Independent Delay Network* as contrasting patterns of cytokine activation were detected between the C/ND and the other three groups. Specifically, in the C/ND group, IFN- γ , CRP, IL-6, and bFGF were activated and VEGF-D, sVAM-1, and MIP-1 β were inhibited, with the opposing pattern detected in C/TD, A/TD, and A/ND (Figure 3). Taken together, the combination of both networks in the third trimester allowed for specific resolution between the A/ND and the C/ND groups, i.e., delay associated with the effects of alcohol vs. delay due to other factors but showed overlap between the typically developing groups (C/TD, A/TD), independent of alcohol exposure. Thus, for the third trimester, a distinct cytokine signature can be described for the groups with child neurodevelopmental delay but not in the case of typically developing children.

4. Discussion

Here, for the first time, evaluation of levels of a wide range of cytokines identified maternal immune signatures indicative of child risk or resilience. We identified maternal cytokine profiles that could be linked to alcohol-related neurodevelopmental delay, which included second trimester elevations in IL-15, IL-10, MDC, TARC observed across both network analyses and examination of individual cytokines. Cytokine profiles indicative of alcohol-

independent neurodevelopmental delay were also identified and included third trimester elevations in commonly reported cytokines such as IL-6, CRP, and IFN- γ . Taken together, by evaluating cytokines both individually and within networks, our results provide a richer and more functionally relevant understanding of the maternal prenatal immunological milieu and its impact on the fetus than can occur with evaluation of individual cytokines alone, and suggest that maternal immune profiles might serve as a novel biomarker with potential for early identification of at risk children.

Network analysis of samples collected during the second trimester allowed for complete resolution among experimental groups, with distinct clusters of cytokines activated/inhibited based on maternal alcohol consumption and/or child neurodevelopmental outcome. Specifically, cytokines levels were highest in alcohol-consuming mothers of children with neurodevelopmental delay (A/ND, with activation of the *Alcohol Exposure, Exposure/Neurodevelopmental Delay, and Vulnerability Networks*) and lowest in control mothers of typically developing children (C/TD, with inhibition of the *Alcohol Exposure and Exposure/Neurodevelopmental Delay Networks*). Importantly, we also significantly extend the literature by further demonstrating that alcohol consumption results in specific patterns of alterations in the maternal immunological milieu, with activation of the *Alcohol Exposure Network*, regardless of child outcome (A/TD, A/ND). In addition, the two alcohol-consuming groups could be distinguished based on differential involvement of the *Vulnerability Network*, which was activated in the case of children with neurodevelopmental delay but inhibited with typical neurodevelopment. As such, we propose that, following alcohol-exposure, dampening of this cytokine network may underlie, at least in part, child resiliency. Finally, the two groups with child neurodevelopmental delay (C/ND, A/ND) could also be distinguished from each other during the second trimester with this network approach – control mothers showed inhibition, whereas alcohol-consuming mothers showed activation, of the *Alcohol Exposure Network*.

Network analysis during the third trimester, by contrast, allowed for the identification of a *Neurodevelopmental Delay Network*, activated in association with both alcohol-related and alcohol-independent neurodevelopmental delay (C/ND and A/ND), as well as an *Alcohol-Independent Delay Network* showing predominant activation in control mothers of children with neurodevelopmental delay (C/ND). Importantly, unlike the second trimester, complete immunological resolution among all groups was not possible. During the third trimester, parallel cytokine network activation/inhibition was detected between the two typically developing groups (C/TD, A/TD). This highlights the importance of considering gestational timing when probing for “windows of vulnerability” both in the current study and in studies of other neurodevelopmental disorders. Based on our dataset, samples collected during the second trimester may be more sensitive in uncovering cytokine biomarkers/signatures, as we show differential network involvement across all groups. By the third trimester, by contrast, resiliency patterns appear to predominate, with all mothers of typically developing children showing the same pattern of cytokine network activation/inhibition.

Network analysis further revealed that a number of cytokines were altered in a consistent manner with alcohol-related neurodevelopmental delay. IL-15, TARC, MDC, sVCAM-1, TNF- β , MIP-1 β VEGF-D, and Eotaxin levels were all elevated, and CRP levels were

dampened, in the A/ND group, across both second and third trimesters. This profile suggests that these cytokines may represent critical “hubs” within the network, or key contributors responsible for alcohol-induced alterations in the fetal neurodevelopmental trajectory. In addition, it is important to note that the majority these cytokines (67%) were also identified in the univariate analyses of cytokine levels and slope across gestation. Taken together, this highlights important underlying parallels between analyses of individual cytokines and network analyses, providing strong support for the network approach, which is more powerful and more physiologically relevant in providing insight into global cytokine activity.

Interestingly, of the maternal cytokines populating immune networks associated with alcohol-related and alcohol-independent neurodevelopmental delay, there was also some overlap with commonly reported cytokines associated with other neurodevelopmental disorders. Specifically, elevations in IFN- γ , IL-10, and TNF- β , which were detected in the C/ND and A/ND groups, as well as TNF- α in the A/ND group, and IL-6 in the C/ND group, parallel results from other related fields. Elevated maternal IFN- γ (Goines et al., 2011; Jones et al., 2017) and elevated amniotic fluid IL-10 and TNF- β (Abdallah et al., 2013) have been previously associated with ASD. In addition, increased TNF- α has been linked to ASD (Abdallah et al., 2013), CP (Yoon et al., 1997), and schizophrenia (Buka et al., 2001) diagnoses. We also detected consistently lower CRP levels in the A/ND group across pregnancy, a finding that overlaps with a study of immune markers of ASD (Zerbo et al., 2016) and may represent a marker of maternal immune system dysregulation (Zerbo et al., 2016) rather than a disorder-specific marker. Finally, elevated maternal IL-6 has also been reported in ASD (Jones et al., 2017), developmental delay (Goines et al., 2011) and other childhood psychiatric disorders (Abdallah et al., 2013), with elevated amniotic fluid IL-6 linked to cerebral palsy (Yoon et al., 1997). Based on the similarities in cytokine profiles between alcohol-related and alcohol-independent neurodevelopmental delay and other neurodevelopmental disorders, we propose that this cytokine profile may be an indication of neurodevelopmental delay in general, rather than being part of a disorder-specific immune signature. In addition, the overlap between disorders also points to the importance of evaluating a broader range of immunological markers, moving beyond the narrow range of typically evaluated cytokines, in order to identify consistent and unique immune signatures. Corroborating this suggestion, a recent investigation examining a subset of individual cytokines in women consuming alcohol failed to detect differences between controls (low/no alcohol exposure) and alcohol consuming mothers of children with neurodevelopmental delay (Sowell et al., 2018), lending further support to the importance of evaluating a broad range of immunological markers and utilization of more functionally-relevant analytic approaches.

The role of clusters or networks of cytokines and their normal ranges during pregnancy remains an evolving area of research. This highlights a key limitation in our interpretation of the alterations observed in cytokine networks – while our data highlight key networks, that when enriched or dampened can be associated with either risk or resilience, knowledge of the functions that can be ascribed to members of these networks stem mainly from investigations of individual cytokines. While this speaks to the importance of continuing to evaluate the potential effects of elevations/decreases in individual cytokines, it also highlights the importance of contextualizing these effects within broader cytokine networks.

Importantly, as the field moves towards evaluation of a wider range of immune parameters (Jones et al., 2017), network approaches and other data reduction strategies should be adopted, so that our functional understanding of how groups of cytokines interact is advanced.

To this end, we probed our network analysis for unique cytokine clusters sharing common functions. This resulted in the identification of members of the VEGF sub-family, including VEGF-D, sFlt-1 (soluble VEGF receptor-1), and PlGF that were all activated in cytokine networks associated alcohol-related neurodevelopmental delay. As a whole, overexpression of the VEGF sub-family members has been linked to disease (Saharinen et al., 2004; Taylor et al., 1997). Of relevance, VEGF-D stimulates lymphangiogenesis (Veikkola et al., 2001) and plays a key role in angiogenesis, with its receptor, VEGFR-3, localized to capillaries of fetal neuroendocrine tissue (Partanen et al., 2000). Thus, in alcohol-exposed pregnancies, elevations in VEGF-D may have a subsequent impact on development of blood supply to these tissues. This is supported by evidence of alterations in the cortical vascular network in alcohol-exposed fetuses (Jegou et al., 2012). Importantly, while altered levels of VEGF sub family members have been detected previously in alcohol-exposed placentae and fetuses (Jegou et al., 2012; Lecuyer et al., 2017), our data point to the predictive potential of maternal VEGF levels, and levels of related factors, in identifying at risk children.

Of the cytokines beyond those in the VEGF sub-family that we identified as potential critical hubs or key contributors responsible for alcohol-induced alterations in the fetal neurodevelopmental trajectory (IL-15, TARC, MDC, sVCAM-1, TNF- β , MIP-1 β , VEG-D and Eotaxin), very little is known regarding the function or impact of these cytokines, particularly from a network perspective, during pregnancy. IL-15 was consistently elevated in alcohol-consuming mothers of children with neurodevelopmental delay, both in the univariate analyses and identified in the alcohol-exposure network (second trimester) and neurodevelopmental delay network (third trimester). Notably, IL-15 was among a group of cytokines shown to be elevated in maternal sera in a rodent model of maternal viral infection (Arrode-Bruses and Bruses, 2012), with such models known to impact neurodevelopment in the offspring [reviewed in (Solek et al., 2018)]. Furthermore, maternal elevations in IL-15 have been previously reported following alcohol exposure in a mouse model. As IL-15 plays a significant role in activating the Janus kinase (Jak) 1 pathway, which is expressed throughout the CNS, the finding of elevated IL-15 in the A/ND and A/TD groups has important implications for neurodevelopment of the offspring (Roberson et al., 2012). The cell adhesion molecule sVCAM-1 was also identified in the alcohol-exposure network (second trimester) and while evidence from alcohol-exposure models is absent, sVCAM-1 has been shown to be elevated with pre-eclampsia (Lyll et al., 1994) and due to the presence of VCAM-1 on endothelial cells, elevations in sVCAM-1 are commonly associated with endothelial cell damage. VCAM-1 expression is also regulated by the cytokine milieu (Thornhill and Haskard, 1990) and mediates adhesion of immune cells to the endothelium (Elices et al., 1990). Thus, while the pathological significance of elevated VCAM-1 is currently unknown, this may be an indication of suboptimal uterine conditions. Clearly, additional work is required to examine the role of elevations in IL-15, sVCAM-1, and other cytokines in the context of the broader cytokine milieu and to probe for mechanisms that could underlie the impact of these cytokines on neurodevelopment. However, our novel

findings, and data from others in this emerging field indicate that this will to be a promising area of future research.

Finally, while the current study identified differential cytokine profiles that were associated with alcohol-related or alcohol-independent neurodevelopmental delay, it is important to consider the possible limitations of the study design. Firstly, while elevations in cytokine levels were identified in maternal plasma, the efficacy of transplacental transfer of cytokines was not evaluated. Currently, the literature remains unclear, with animal studies providing contradictory information regarding the transfer of cytokines between mother and fetus (Aaltonen et al., 2005; Dahlgren et al., 2006; Gayle et al., 2004; Zaretsky et al., 2004). Based on these inconsistent findings, it is likely that cytokine passage to the fetus may depend on the cytokine in question, the gestational time point under consideration, and other biological and environmental variables, with evidence also pointing to additional mechanisms that would allow for the maternal cytokine signal (in the absence of the cytokine itself) to pass to the fetus (Jonakait, 2007). Thus, while our results linking maternal cytokine profiles with neurodevelopmental outcomes in the offspring provide important evidence for the developmental impact of alterations in the maternal immune milieu, future work is required to examine the degree of transfer/elaboration of the cytokine signal between mother and fetus. Secondly, the study sample examined represents only a small subset of the overall longitudinal Ukraine cohort. The current sample was limited to subjects with complete datasets (two blood samples provided, dysmorphology and neurobehavioral testing of the child); however, it is acknowledged that study attrition is expected to be higher in alcohol-consuming women. This is consistent with the well documented increase in study dropout rates for individuals with a range of substance use problems (Claus et al., 2002; de Graaf et al., 2000). As a result, follow-up and replication studies in additional cohorts will be required to further validate the current findings.

In conclusion, the current data are novel and important, and represent a significant step forward in the FASD field. Despite increasing efforts in promoting awareness, prenatal alcohol exposure represents the leading preventable cause of birth defects and neurodevelopmental disabilities (Williams et al., 2015); the global prevalence of alcohol consumption during pregnancy remains as high as 9.8% (Popova et al., 2017). As a result, the worldwide prevalence of FASD is estimated at 1 – 5% in the general population (May et al., 2018) and as high as 1–20% in certain vulnerable populations (Ospina and Dennett, 2013; Williams et al., 2015). Despite the significant number of individuals affected by alcohol exposure *in utero*, therapeutic intervention strategies remain limited. To this end, our ability to identify alterations in maternal cytokine networks that can be associated with neurodevelopmental delay in their children, provides information that could be utilized as a critical step towards the development of biomarkers for early identification of at risk children who could then be targeted for early intervention. In addition, this work highlights the possible therapeutic potential of immunomodulatory agents in intervention and treatment for FASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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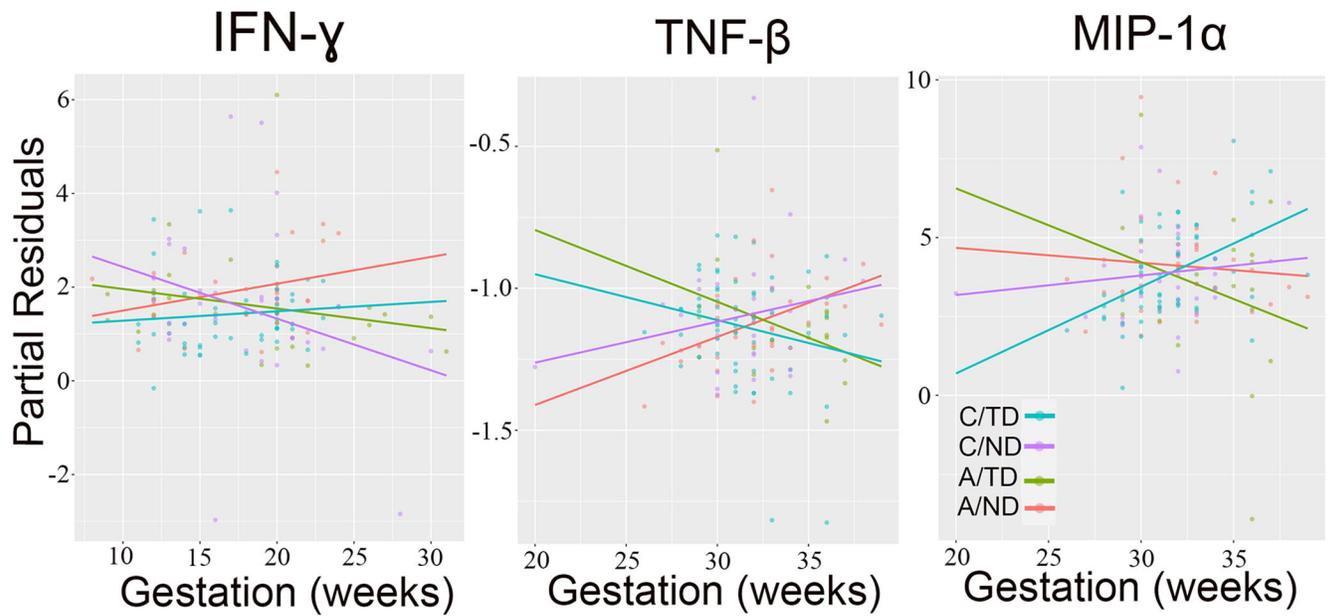


Figure 1.

Graphical representation highlighting cytokine slopes differences from the regression analyses. Significant group comparisons are listed in Table 4.

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60);

C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35);

A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35).

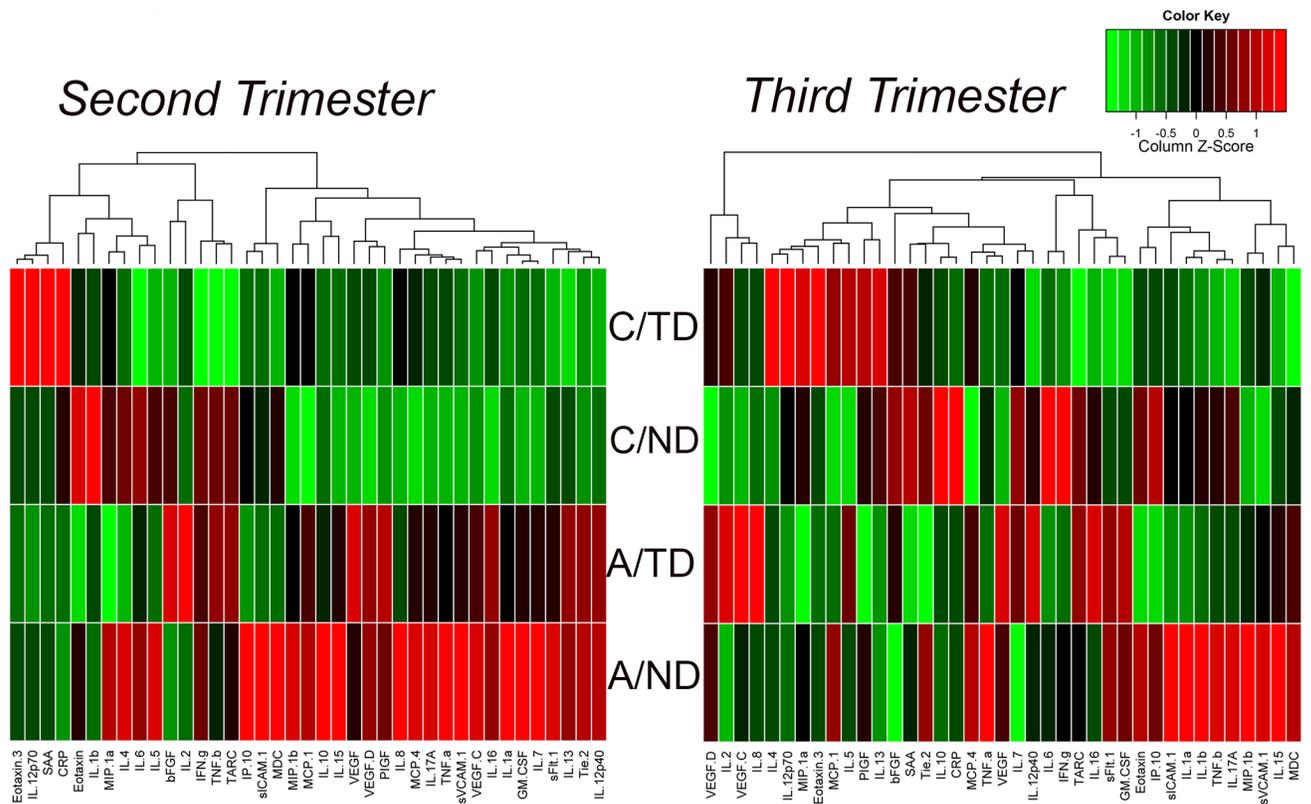


Figure 2:

Heatmaps showing the overall cytokine profile during the second and third trimesters. Rows represent groups (C/TD, C/ND, A/TD, A/ND), as indicated, and columns represent mean cytokine levels (z-scored data), for each group. Colors demonstrate deviations from the mean of zero, as indicated in the colour key

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60); C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35); A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35).

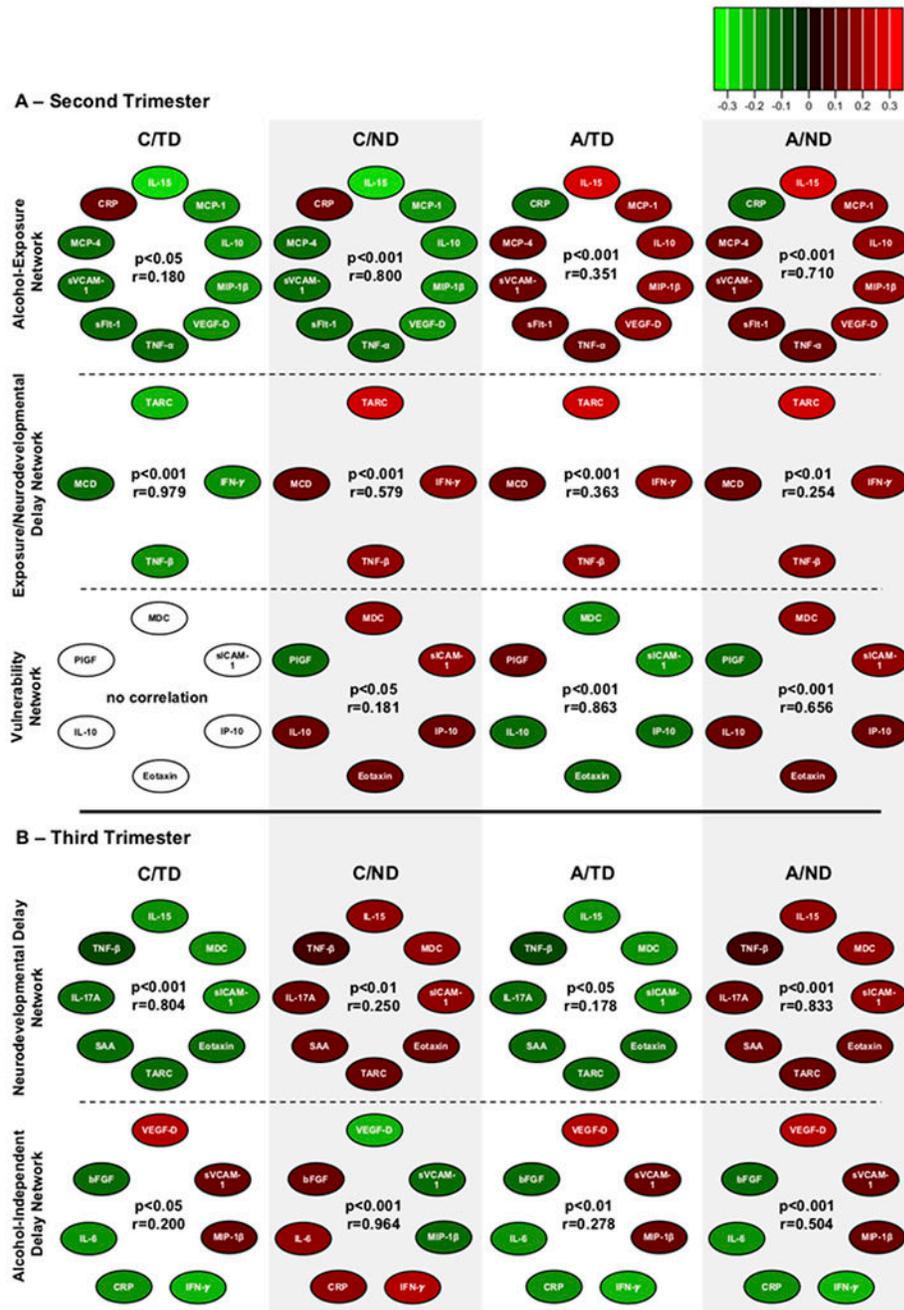


Figure 3: Cytokines contributing to the immune networks during the second (A) and third (B) trimesters. Network membership was defined based on component loadings from the constrained principle component analysis (CPCA). Activated cytokines are indicated in red, and inhibited cytokines indicated in green, with colour gradation depicting the value of the component loading. For each network, the strength ($|r|$) and the significance (p -value) of the correlation between groups (C/TD, C/ND, A/TD, A/ND) and component scores, is

indicated. The cytokine network indicated in white (*Vulnerability Network*) was not significantly correlated with the C/TD condition only.

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60);

C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35);

A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35).

Table 1:

Maternal characteristics

Variable	C/TD (n = 60)	C/ND (n = 35)	A/TD (n = 22)	A/ND (n = 35)	P value	
Maternal age at enrollment	26.47 ± 5.04	26.09 ± 4.56	24.45 ± 4.70	26.40 ± 5.78	0.485 ¹	
Gestational age at enrollment (weeks)	17.70 ± 5.04	17.75 ± 4.83	19.32 ± 7.38	17.84 ± 4.51	0.871 ¹	
Recruitment site	Khmelnysky	8.3% (5)	14.3% (5)	9.1% (2)	34.3% (12)	0.012 ²
	Rivne	91.7% (55)	85.7% (30)	90.9% (20)	65.7 (23)	
Marital status	Married or cohabitating	96.7 (58)	97.1 (34)	90.9 (20)	80.0% (28)	0.022 ²
	Single/divorced	3.3% (2)	2.9% (1)	9.1% (2)	20.0% (7)	
Education level	Less than high school	5.0% (3)	5.7% (2)	9.1% (2)	17.1% (6)	0.080 ²
	High school or equivalent	33.3% (20)	48.6% (17)	50.0% (11)	51.4% (18)	
	Some college	61.7% (37)	45.7% (16)	40.9% (9)	31.4% (11)	
Socio-economic status category (Hollingshead score)	1 (high): 55–66	6.7% (4)	5.7% (2)	18.2% (4)	2.9% (1)	0.002 ³
	2: 40–54	40.0% (24)	22.9% (8)	18.2% (4)	20.0% (7)	
	3: 30–39	36.7% (22)	48.6% (17)	36.4% (8)	42.9% (15)	
	4: 20–29	16.7% (10)	20.0% (7)	22.7% (5)	11.4% (4)	
	5 (low): 8–19	0.0% (0)	2.9% (1)	4.5% (1)	22.9% (8)	
Gravidity	> 1	61.7% (37)	65.7% (23)	40.9% (9)	48.6% (17)	0.178 ²
	1	38.3% (23)	34.3% (12)	59.1% (13)	51.4% (18)	
Parity	> 0	50.0% (30)	57.1% (20)	27.3% (6)	34.3% (12)	0.070 ²
	0	50% (30)	42.9% (15)	72.7% (16)	65.7% (23)	
Pre-pregnancy body mass index (BMI)	21.73 ± 3.71	21.79 ± 2.94	21.99 ± 3.55	21.50 ± 2.83	0.935 ¹	
Smoking status	Current smoker	3.3% (2)	0.0% (0)	18.2% (4)	28.6% (10)	< 0.001 ³
	Never smoked	88.3% (53)	79.4% (27)	22.7% (5)	31.4% (11)	
	Quit after realized pregnant	0.0% (0)	8.8% (3)	36.4% (8)	31.4% (11)	
	Quit before pregnancy	8.3% (5)	11.8% (4)	22.7% (5)	8.6% (3)	
Vitamin use pre-enrollment	No	40.0% (24)	37.1% (13)	40.9% (9)	40.0% (14)	0.055 ²
	Yes	51.7% (31)	54.3% (19)	59.1% (13)	48.6% (17)	
	Missing	8.3% (5)	8.6% (3)	0.0% (0)	11.4% (4)	
Vitamin use post-enrollment	No	40.0% (24)	37.1% (13)	40.9% (9)	40.0% (14)	0.979 ²
	Yes	51.7% (31)	54.3% (19)	59.1% (9)	48.6% (17)	
	Missing	8.3% (5)	8.6% (3)	0.0% (0)	11.4% (4)	
Gestational age at 1 st blood draw (weeks)	17.27 ± 3.57	17.69 ± 4.48	18.95 ± 6.75	17.80 ± 4.49	0.752 ¹	
Gestational age at 2 nd blood draw (weeks)	31.52 ± 2.58	31.46 ± 3.00	33.45 ± 2.82	32.03 ± 2.99	0.051 ¹	
Ounces of alcohol/day at time of conception	0.00 ± 0.01	0.00 ± 0.03	0.57 ± 0.22	0.62 ± 0.63	< 0.001 ¹	
Ounces of alcohol/drinking day at conception	0.01 ± 0.10	0.03 ± 0.21	1.75 ± 1.07	1.75 ± 1.26	< 0.001 ¹	

Variable	C/TD (n = 60)	C/ND (n = 35)	A/TD (n = 22)	A/ND (n = 35)	P value
Ounces of alcohol/day two weeks prior to enrollment	0.00 ± 0.00	0.00 ± 0.01	0.06 ± 0.14	0.14 ± 0.46	< 0.001 ¹
Ounces of alcohol/drinking day two weeks prior to enrollment	0.00 ± 0.00	0.02 ± 0.10	0.29 ± 0.53	0.57 ± 0.96	< 0.001 ¹

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60); C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35); A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35). Continuous variables are reported as average value ± standard deviation. Categorical variables are reported as percentage of group followed by the N in parentheses.

¹Kruskal-Wallis Rank Sum Test

²Fisher's Exact Test

³Chi-Square Test; *p*-values in **Bold** are statistically significant.

Table 2:

Child characteristics

Variable		C/TD (n = 60)	C/ND (n = 35)	A/TD (n = 22)	A/ND (n = 35)	P value
Child Sex	Male	53.3% (32)	68.6% (24)	40.9% (9)	45.7% (16)	0.139 ²
	Female	46.7% (28)	31.4% (11)	59.1% (13)	54.3% (19)	
Height < 10 th percentile	No	95.0% (57)	85.7% (30)	100.0% (22)	77.1% (27)	0.013 ²
	Yes	5.0% (3)	14.3% (5)	0.0% (0)	22.9% (8)	
Weight < 10 th percentile	No	88.3% (53)	82.9% (29)	95.5% (21)	82.9% (29)	0.481 ²
	Yes	11.7% (7)	17.1% (6)	4.5% (1)	17.1% (6)	
Occipital-frontal circumference < 10 th percentile	No	95.0% (57)	91.4% (32)	90.9% (20)	82.9% (29)	0.273 ²
	Yes	5.0% (3)	8.6% (3)	9.1% (2)	17.1% (6)	
Smooth philtrum	No	91.7% (55)	82.9% (29)	100% (22)	77.1% (27)	0.036 ²
	Yes	8.3% (5)	17.1% (6)	0.0% (0)	22.9% (8)	
Thin vermilion border	No	80.0% (48)	85.7% (30)	95.5% (21)	62.9% (22)	0.020 ²
	Yes	20.0% (12)	14.3% (5)	4.5% (1)	37.1% (13)	
Palpebral fissure < 10 th percentile	No	90.0% (54)	91.4% (32)	95.5% (21)	74.3% (26)	0.021 ²
	Yes	8.3% (5)	8.6% (3)	0.0% (0)	25.7% (9)	
	Missing	1.7% (1)	0.0% (0)	4.5% (1)	0.0% (0)	
Age at 1 st exam (months)		6.84 ± 1.31	6.46 ± 0.88	6.88 ± 1.41	6.60 ± 1.25	0.720 ¹
Mental development index (1 st exam)		95.42 ± 5.58	88.32 ± 10.13	96.44 ± 5.12	86.07 ± 12.43	< 0.001 ¹
Psychomotor development index (1 st exam)		99.28 ± 7.87	82.93 ± 12.79	100.38 ± 9.37	82.40 ± 15.11	< 0.001 ¹
Age at 2 nd exam (months)		13.23 ± 1.43	13.27 ± 2.15	13.06 ± 1.24	12.67 ± 1.49	0.139 ¹
Mental development index (2 nd exam)		98.73 ± 7.77	82.60 ± 9.95	98.88 ± 10.18	80.44 ± 11.52	< 0.001 ¹
Psychomotor development index (2 nd exam)		104.56 ± 8.24	90.20 ± 9.64	107.31 ± 10.85	87.26 ± 15.67	< 0.001 ¹

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60); C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35); A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35). Continuous variables are reported as average value ± standard deviation. Categorical variables are reported as percentage of group followed by the N in parentheses.

¹Kruskal-Wallis Rank Sum Test

²Fisher's Exact Test; p-values in **Bold** are statistically significant.

Maternal cytokine levels stratified by child outcome

Table 3:

Cytokine	Second Trimester					Third Trimester							
	p-value	A/ND vs. C/TD	A/TD vs. C/TD	C/ND vs. C/TD	A/ND vs. C/ND	A/TD vs. A/TD	A/ND vs. A/TD	p-value	A/ND vs. C/ND	A/TD vs. C/TD	C/ND vs. C/TD	A/ND vs. C/ND	A/TD vs. A/TD
IL-15	* (0.021)	* (0.011)	—	—	** (0.009)	—	—	—	—	—	—	—	—
IL-10	* (0.050)	* (0.014)	—	—	* (0.026)	—	—	—	—	—	—	—	—
MDC	# (0.058)	** (0.010)	—	—	—	—	—	—	—	—	—	—	—
TARC	# (0.058)	** (0.006)	# (0.053)	# (0.064)	—	—	—	—	—	—	—	—	—
VEGF-D								* (0.021)	—	—	* (0.046)	** (0.009)	** (0.004)
CRP								# (0.066)	* (0.020)	—	—	* (0.049)	* (0.034)

Overall p-values are presented in the case of significant effects/trends from in the regression analyses. Highlighted cells represent significant *post-hoc* comparisons. Red highlight denotes increased cytokine levels in the comparison group, green highlight denotes decreased cytokine levels in the comparison group. Non-significant p-values are not reported

* : $p \leq 0.05$

** : $p \leq 0.01$

: trend, $0.05 < p \leq 0.07$

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60); C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35); A/TD: alcohol-consuming mother, typically developing child (n=22); A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35).

Table 4:

Maternal cytokine slopes stratified by child outcome

Cytokine	p-value	Comparisons						Model Estimates (pg/mL)			
		A/ND vs. C/TD	A/TD vs. C/TD	C/ND vs. C/TD	A/ND vs. C/ND	A/TD vs. C/ND	A/ND vs. A/TD	C/TD	C/ND	A/TD	A/ND
<i>Second Trimester:</i>											
IFN- γ	# (0.060)	—	—	* (0.045)	# (0.051)	—	* (0.032)	0.020	-0.11	-0.042	0.057
<i>Third Trimester:</i>											
TNF- β	* (0.015)	—	—	—	—	—	* (0.039)	-0.016	0.015	-0.025	0.024
MIP-1 α	* (0.015)	* (0.014)	** (0.007)	—	—	—	—	0.275	0.062	-0.233	-0.047

Overall p-values are presented in the case of significant slope differences from in the regression analyses, with *post-hoc* p-values indicated significant group comparisons. Model estimates indicate, for each group, the degree of cytokine change (pg/mL) associated with a one week increase in gestational timing (blue: positive; yellow: negative). Graphical representation highlighting differential slopes between groups is presented in Figure 1.

* : $p \leq 0.05$

** : $p \leq 0.01$

: trend, $0.05 < p \leq 0.07$

Abbreviations: C/TD: control mother (low/no alcohol), typically developing child (n=60); C/ND: control mother (low/no alcohol), neurodevelopmental delay in the child (n=35); A/TD: alcohol-consuming mother, typically developing child (n=22), A/ND: alcohol consuming mother, neurodevelopmental delay in the child (n=35).