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Authors

Sowell, KD
Uriu-Adams, JY
Graham, JL
et al.

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Implications of altered maternal cytokine concentrations on infant outcomes in children with prenatal alcohol exposure

K.D. Sowell[1], **J.Y. Uriu-Adams**[1], **J. Van de Water**[2], **C.D. Chambers**[3], **C.D. Coles**[4], **J.A. Kable**[4], **L. Yevtushok**[5], **N. Zymak-Zakutnya**[6], **W. Wertelecki**[3], **C.L. Keen**[1], and **the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD)**

[1]Department of Nutrition, University of California-Davis, Davis, CA, United States

[2]Division of Rheumatology, Allergy, and Clinical Immunology, Department of Internal Medicine, University of California, Davis, CA, United States

[3]Departments of Pediatrics and Family Medicine and Public Health, University of California, San Diego, La Jolla, CA, United States

[4]Departments of Psychiatry and Pediatrics, Emory University School of Medicine, Atlanta, GA, United States

[5]OMNI-Net Center, Rivne Provincial Medical Diagnostic Center, Rivne, Ukraine

[6]OMNI-Net Center, Khmelnytsky City Perinatal Center, Khmelnytsky, Ukraine

Abstract

Excessive alcohol consumption has been shown to increase serum plasma levels of numerous immune cytokines. Maternal immune activation and elevated cytokines have been implicated in certain neurological disorders (e.g. autism and schizophrenia) in the offspring. We investigated the hypothesis that elevated cytokines during pregnancy are a risk factor in women who gave birth to a child with Fetal Alcohol Spectrum Disorder (FASD) or a child with neurobehavioral impairment irrespective of prenatal alcohol exposure. Moderate to heavy alcohol-exposed (AE) (N=149) and low or no alcohol-exposed (LNA) (N=92) women were recruited into the study during mid pregnancy (mean of 19.8±5.8 weeks' gestation) in two regions of Ukraine; Khmelnytsky and Rivne. Maternal blood samples were obtained at enrollment into the study at early to mid-pregnancy and during a third-trimester follow-up visit and analyzed for plasma cytokines. Children were examined at six and/or twelve months of age and were classified as having FASD if their mothers reported alcohol use and if they had at least one standardized score (Bayley Scales of Infant Development II Mental Development Index, MDI, or Psychomotor Development Index, PDI) below 85 with the presence or absence of physical features of FASD. In multivariate analyses of maternal cytokine levels in relation to infant MDI and PDI scores in the entire sample, increases in the ratio of TNF-α/IL-10 and IL-6/IL-10 were negatively associated with PDI scores at six months (p=0.020 and p=0.036, respectively) and twelve months (p=0.043 and p=0.029,

respectively), and with MDI scores at twelve months (p=0.013 and p=0.050, respectively). A reduction in the odds ratio of having an FASD child was observed with increasing levels of IL-1β, IL-2, IL-4, IL-6, and IL-10 in early-mid pregnancy and IL-1β and IL-10 during late pregnancy. However, women that failed to increase IL-10 levels in the third trimester in order to maintain the balance of pro- and anti-inflammatory cytokines had an elevated risk of having an FASD child, specifically a significant increase in the odds ratio of FASD with every one-unit log increase in late pregnancy TNF-α/IL-10 levels (aOR: 1.654, CI: 1.096–2.495, p=0.017). These data support the concept that disruptions in the balance between pro- and anti-inflammatory cytokines may contribute to neurobehavioral impairment and alter the risk of FASD.

Introduction

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term that encompasses several alcohol-related birth defects, including Fetal Alcohol Syndrome (FAS), partial FAS, alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD) (Warren et al., 2011). Growth restriction, facial anomalies, and cognitive and behavioral impairment are abnormal birth outcomes associated with alcohol use during pregnancy (Jones and Smith, 1973; Riley et al., 2011). While FASD is widely recognized to be a major public health problem in numerous developed and developing countries, the mechanisms underlying the diverse pathologies associated with FASD are poorly understood (Warren et al., 2011). The current paper addresses the idea that one mechanism underlying some of the FASD phenotype is alcohol-induced alterations in the maternal immune system. That the consumption of alcohol can affect the immune system is well documented, with alcohol affecting both innate and adaptive immune systems (Waldschmidt et al., 2008). In particular, chronic alcohol consumption has been shown to significantly increase pro-inflammatory cytokine levels, in particular IL-1α, IL-1β, TNF-α, and IL-6, during pregnancy (Ahluwalia et al., 2000). Cytokines are small-secreted proteins that act like messengers of the immune system and modulate its responses. Cytokines have a diverse range of functions outside the immune response including neurotrophic roles that are important for the development and function of the brain (Deverman and Patterson, 2009). While cytokines are necessary for proper neurodevelopment, animal studies have shown that excessive levels of certain cytokines can pose harm to the developing central nervous system (CNS) (DeVito et al., 2000; Svinarich et al., 1998; Vink et al., 2005).

In humans, maternal infections and immune activation have been linked to the development of certain behavioral disorders in children including autism spectrum disorders (ASD) (Ashwood et al., 2011; Jones et al., 2016) and schizophrenia (Brown et al., 2004; Penner and Brown, 2007). Similarly, epidemiological studies have associated high maternal cytokines (Ashwood et al., 2011; Goines et al., 2011) in mid-gestation with an elevated risk for ASD and more specifically, a higher risk of having an autistic child with intellectual disability than without intellectual disability (Jones et al., 2016). In addition, altered cytokine and chemokine profiles have also been reported in neonatal blood samples of children with ASD (Abdallah et al., 2012; Zerbo et al., 2014).

Work with animal models has supported the concept that an excessive maternal immune activation can result in abnormal neurodevelopment in their offspring. Injections of poly(I:C) or IL-6 induced behavioral changes in mouse pups that were characteristic of those seen in ASD and schizophrenia (Smith et al., 2007). Co-injections of poly(I:C) with anti-IL-6 was able to normalize behavior. This was also observed in IL-6 knock out mice injected with poly(I:C) suggesting a specific role of IL-6 in the neurodevelopmental pathway. Lipopolysaccharide (LPS)-induced elevations of cytokines in maternal serum, amniotic fluid, and fetal brain tissue were evident up to 24 hours post-injection (Oskvig et al., 2012) and pups born to dams receiving LPS injections displayed reduced social preferences and exploration behaviors into young adulthood (Malkova et al., 2012). Overexpression of IL-10 was able to prevent behavior abnormalities seen in adult mice following LPS injection of dams (Meyer et al., 2008). However, the overexpression of IL-10 in the absence of poly(I:C) exposure was associated with behavioral defects in adulthood, suggesting that the homeostasis and balance of anti-inflammatory to pro-inflammatory cytokines is important for normal brain development. In summary, there are numerous studies that show that maternal cytokine disruption can alter offspring neurodevelopment.

Alcohol consumption during pregnancy occurs within all socioeconomic groups, ages, and education levels. Despite the best intentions and efforts, some women continue to drink during pregnancy. A better understanding of the mechanisms underlying alcohol teratogenicity could lead to novel intervention approaches that reduce the risk for FASD in high-risk pregnancies, and/or mitigate its consequences in the child. In the current study, we examined the relationship between maternal alcohol consumption, maternal immune profile and risk of FASD in the offspring.

Method

Study Design

As part of a prospective cohort study, pregnant women were enrolled during an antenatal appointment at one of two western Ukrainian sites; Rivne Provincial Medical Diagnostic Center and the Khmelnystsky City Perinatal Center. Site locations were members of the Omni-Net Birth Defects Prevention Program and populations were diverse in socioeconomic status and general health. This study was done as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorder (CIFASD). The CIFASD is supported by the United States National Institute on Alcohol Abuse and Alcoholism, and it is a multidisciplinary initiative conducted in several countries throughout the world (www.CIFASD.org). The primary goals of the CIFASD are to better characterize the spectrum of physical and neurodevelopmental outcomes resulting from prenatal alcohol exposure and to develop better diagnostic, prevention and treatment approaches for FASD, including the investigation of maternal nutrition as a possible permissive or protective factor. The methods and scope of CIFASD studies have been described in more detail elsewhere (Arenson et al., 2010; Mattson et al., 2010).

Women who came in for a routine prenatal visit to one of the two centers were eligible for screening into the study (Figure 1). Women were screened by a trained study nurse about their alcohol consumption and invited to participate in the study if they responded positively

for at least weekly binge-drinking episodes (5+ drinks), at least five episodes of 3–4 drinks, or at least 10 episodes of 1–2 drinks either in the month around conception or during the most recent month of pregnancy; these women were classified into the moderate/heavy alcohol exposed (AE) group. As controls, a group of low level or non-drinking women was enrolled into the low/no alcohol exposure (LNA) group if they reported no binge episodes, minimal or no alcohol in the month around conception, and no drinking in the most recent month of pregnancy. Those women who agreed to participate signed an informed consent document approved through institutional review boards at the University of California, San Diego, and the Lviv Medical University in Ukraine. Study participants were randomized into one of three groups to receive: 1) standard of care in Ukraine (women are advised to take prenatal supplements but they are not provided), 2) provision of a daily multivitamin mineral (MVM) supplement (Theravit® - a standard prenatal supplement made in the USA and available in Ukraine) or 3) the same daily MVM supplement plus an additional 750 mg choline supplement. The addition of a choline supplement was included into the study due to its reported ability to mitigate the effects of prenatal alcohol exposure in rat pups born to alcohol-consuming dams (Thomas et al., 2009; Thomas et al., 2004). Supplements were provided to participants free of charge during the enrollment and subsequent study visits. More information on recruitment can be found in (Chambers et al., 2014).

Maternal Interview

At enrollment (mean 19.8±5.8 weeks' gestation), the participants completed a one-hour in-person interview to obtain information on their demographics i.e. maternal and paternal age, pre-pregnancy body mass index (BMI), education, socioeconomic status, alcohol and substance use (including tobacco use), pregnancy characteristics (parity, gravidity), with a follow-up visit and interview in the third trimester (~32 weeks gestation). Current weeks' gestation was based on maternal report of first day of last menstrual period and adjusted if necessary based on standard ultrasound dating. Day-to-day alcohol consumption was collected on the number, volume, and type of alcoholic drink consumed in a typical week around conception and during the most recent two weeks using a timeline follow-back method (Sokol, 1983). Data were converted into average ounces of absolute alcohol per day (AAD) and average ounces of absolute alcohol per drinking day (AADD) for each time period. One ounce of absolute alcohol is equivalent to two standard drinks. Women were asked about their supplement use and whether they were currently taking a single or multiple vitamin/mineral (MVM) supplement at enrollment. After confirmation of supplement name, those women reporting MVM use at enrollment were considered "MVM use prior to enrollment," whether or not they were assigned to the MVM intervention arms. Those women taking single nutrient supplements (typically iodine or folate) or no supplements, but who were enrolled into the MVM supplement intervention arms of the study, were considered "MVM use after enrollment." Women not randomized into the MVM supplement arms and who reported only single nutrient use or were not taking any supplements at enrollment were classified as "No MVM."

Infant Outcomes

Birth outcomes and infant growth information were obtained from medical records. In the first year of life, infants underwent standard blinded dysmorphological physical

examinations performed by trained local geneticists on 1–3 occasions. On each occasion, the child was evaluated for physical features of FASD and growth using a standard checklist. Locally trained psychologists used the Bayley Scales of Infant Development II (BSID-II) (Bayley, 1993) to perform blinded neurobehavioral assessments at approximately six and/or twelve months of age. The BSID-II measures current mental and psychomotor development by providing two gestational-age and sex standardized scores: a mental development index (MDI) and a psychomotor development index (PDI), both indexes are standardized to a mean score of 100 with a standard deviation of 15. Infants were separated into groups by alcohol exposure and neurodevelopmental outcomes. Since it is still unknown why prenatal alcohol exposure results in FASD and/or poor neurodevelopmental outcomes in some but not all children, we have separated the AE infants by FASD classification and BSID-II scores in order to detect differences within this group that may increase their susceptibility to alcohol's teratogenicity. A cutoff value of one standard deviation (15 standard score points) below the standardized mean of 100 on BSID-II was used to define developmental delay (DD). A one standard deviation cutoff has been used in the CIFASD consortium in several of our previous publications (Balaraman et al, 2016; Montag AC et al, 2016) as a reasonable cutoff for "delay" in infancy. A classification of FASD was given to infants if there was reported maternal alcohol consumption as defined for the AE group and at least one BSID-II score below 85 at six or twelve months of age, with and without physical features of FASD (short palpebral fissures, smooth philtrum, or thin vermillion border of the upper lip; head circumference at or below the 10^{th} centile, and growth deficiency on weight and/or height) (Mattson et al., 2010; Montag et al., 2016). We took this approach in the Consortium due to the need to apply classification criteria at an early age. As these children progress through childhood, research reclassification will be required (just as it might in clinical practice). Within the LNA exposure group, children were classified as developmentally delayed (DD) if they had at least one BSID-II score below 85 at six or twelve months. All other children were considered to have normal development (ND).

Sample Selection

A total of 149 women were identified in the AE group with children that underwent neurobehavioral assessment and for whom plasma samples were available for cytokine analysis. In addition to investigating the association of maternal cytokine levels on infant outcomes in alcohol-exposed children, a subset of subjects within the LNA group were selected to test whether maternal cytokines are associated with DD in children who were not prenatally exposed to alcohol. To achieve this, 47 of the LNA subjects who had ND children and 45 of the subjects who had DD children were identified for cytokine analysis.

Cytokine analysis

A 25 ml sample of blood was collected from participants into EDTA-treated tubes following completion of enrollment and at the ~32 week interview. The sample was centrifuged at 1500g, for 10 min, at 4°C, and the plasma was aliquoted into tubes and frozen at -80°C until shipped to the U.S. and analyzed. Plasma samples were measured for IL-1β, TNF-α, INF-γ, GM-CSF, IL-4, IL-2, IL-6, IL-8, and IL-10 concentrations (pg/ml) using human multiplexing bead immunoassays (Luminex®, BioRad, Hercules, CA). For cytokine values that fell below the limit of detection (LOD), a value of LOD/2 was assigned prior to log

transformation to normalize their distributions. Log transformed values were used in all statistical analyses; actual concentration values are provided in the tables. Due to the large number of samples (>80%) that fell below the LOD for INF-γ, INF-γ was transformed into a categorical variable of non-detectable/detectable and only included in logistic regression analyses.

Statistical Analysis

Subjects were divided into one of four groups based on maternal alcohol consumption (AE or LNA) and neurodevelopment of the child (ND and DD for the LNA group, and ND or FASD for the alcohol-exposed group). Frequencies and percentages were used to describe characteristics of the participants by alcohol exposure/neurodevelopment outcome groups. Comparisons between groups were performed by chi-square tests for independence for categorical variables and general linear model for continuous variables adjusted with Tukey post-hoc test for multiple comparisons. Mean differences in cytokine concentration by group were analyzed by general linear model with Tukey post-hoc analysis. Linear regression was used to investigate the effect of cytokine concentration in early/mid and late pregnancy on BSID-II scores. Covariates evaluated for the models were maternal age, paternal age, maternal education, socioeconomic status (SES), pre-pregnancy BMI, parity, gravidity, MVM use, maternal smoking (cigarettes per day at enrollment), study site, gestational week at blood draw, child sex, gestational age at birth, birth weight, and birth length. Covariates were included into the linear regression model and remained in the model if their association with outcome had a $p<0.10$. Logistic regression was performed on FASD risk (yes or no) in the alcohol-exposed groups and DD risk (yes or no) in the LNA groups for each cytokine concentration, adjusted for covariates that were selected for their association with outcome at $p<0.10$. Adjusted odds ratios and 95% confidence intervals were calculated from the logistic regression models and used as an approximation of the adjusted odds ratios and their 95% confidence intervals. Missing values for covariates resulted in exclusion of subjects on a case-by-case basis in each analysis. A two-sided p-value <0.05 was considered to be statistically significant. All analyses were conducted using IBM SPSS Statistics, Version 23 for Mac OS X, Armonk, New York.

Results

Subject characteristics

A total of 419 samples were analyzed for plasma cytokine concentrations from 241 women (149 AE and 92 LNA) with 178 women providing a blood sample both at enrollment and the third trimester study visit. Of the women who provided one blood sample, 25 women provided only an early/mid pregnancy sample and 38 provided only a late pregnancy sample. Ninety children in the AE group were classified with FASD. Forty-five children in the LNA group were classified as DD, with at least one BSID-II score below 85 at approximately six or twelve months of age. BSID-II scores at ~six months were available for 43 children in the LNA-ND group, 41 in the LNA-DD group, 51 in the AE-ND group, and 76 in the AE-FASD group. BSID-II scores at ~twelve months were completed on 46 children in the LNA-ND, 44 in the LNA-DD group, 46 in the AE-ND group, and 79 in the AE-FASD group.

By design in the sample recruitment criteria, women in the AE groups consumed more alcohol at conception and enrollment than women in the LNA group. Mothers that met the AE criteria for alcohol consumption during their pregnancy, regardless of whether their child was ND or FASD, were more likely to be current smokers and smoked more cigarettes per day at enrollment than mothers in the LNA groups. Comparing within the AE group, mothers with FASD children were more likely from Khmelnystsky, and they had fewer years of formal education and were of lower socioeconomic status than AE-ND mothers (Table 1). In comparison to the normally developing children (LNA-ND), AE-FASD mothers had fewer years of formal education, lower socioeconomic status, and were less likely to be in the supplement arm of the study or take a MVM supplement on their own. Comparing women with children who scored below 85 on BSID-II, women in the AE-FASD group had fewer years of formal education, were of lower socioeconomic status, and were less likely to take a MVM supplement or receive one from the study than LNA-DD mothers. Of the mothers with normally developing children, the mothers that consumed alcohol at moderate to heavy levels (AE-ND) had less formal education than mothers with LNA use.

There was no difference in the proportion of male and female infants born between groups. Infants with FASD had on average a shorter gestational age at birth than comparison infants (LNA-ND). In addition, AE-FASD infants had on average lower birth weights and lengths, as well as the lowest MDI scores at ~six months than all other groups. DD and FASD infants had lower scores on all BSID-II scores at ~six and ~twelve months when compared to ND infants.

Maternal Cytokine Levels

The cytokine concentrations for IL-1β and IL-6 in early/mid pregnancy (Table 2) were significantly higher ($p=0.010$ and $p=0.010$, respectively) in the AE-ND group compared to the AE-FASD mothers. In late pregnancy (Table 2), IL-10 concentrations were significantly higher in the AE-ND mothers than those in the AE-FASD group ($p=0.049$). No differences were observed between mothers in the LNA groups. With regard to supplement use, there were no differences in cytokine levels in early/mid or late pregnancy when separated by MVM use or intervention group (data not shown).

Relationships between maternal cytokine levels and infant neurodevelopmental scores

In multivariate analyses of cytokine levels in relation to MDI and PDI at six months irrespective of alcohol exposure, there was a significant association between early/mid pregnancy IL-4 levels after adjustment for socioeconomic status, site, child sex, and gravidity (Table 3). Specifically, for every one unit log increase in IL-4 levels in early/mid pregnancy, MDI scores increased 3.385 points ($p=0.013$) and PDI scores increased 4.971 points ($p=0.010$) at six months. Higher early/mid and late pregnancy IL-10 levels were associated with improved PDI scores (2.032 points; $p=0.037$ and 1.812 points; $p=0.044$ per one unit log increase, respectively) at six months after adjustment for the above-named factors. Analyzing twelve month MDI and PDI scores, every one-unit log increase in late pregnancy IL-1β levels was associated with a 3.262-point increase in MDI score ($p=0.049$) after adjustment for paternal age, socioeconomic status, and MVM supplement use. A

1.343-point increase in MDI (p=0.036) and a 1.635-point increase in PDI (p=0.020) scores were associated with every one-unit log increase in GM-CSF levels in late pregnancy.

To test whether an imbalance in cytokine homeostasis was a predictor variable of BSID-II scores, ratios of pro-inflammatory:anti-inflammatory cytokines were computed (Table 3). No assocation was found for IL-1β:IL-10 ratios on BSID-II scores. However, interestingly, a late pregnancy increase in the ratios of IL-6 and TNF-α to IL-10 had a negative impact on BSID-II scores at both six and twelve months of age. A one-unit increase in late pregnancy ratio of IL-6:IL-10 was associated with a 2.429-point (p=0.036) and 2.525-point (p=0.029) decrease in PDI scores at six and twelve months, as well as a 1.951-point decrease in twelve-month MDI scores (p=0.050). Similarly, an increase in the ratio of late pregnancy TNF-α:IL:10 had a significantly negative impact on PDI scores at six months (2.097-point decrease; p = 0.020) and both MDI at twelve months (1.908-point decrease; p = 0.013), and PDI scores at twelve months (1.811-point decrease; p-value = 0.043).

Associations between maternal cytokine levels and FASD risk

Logistic regression was performed to analyze the impact of cytokine levels on risk for DD (classified as one MDI or PDI score below 85 at either six or twelve months) in the infants of the LNA mothers and for the risk of FASD in the infants of the AE mothers. There were no significant associations between cytokine levels in early/mid or late pregnancy and risk of developmental delay in the LNA exposure groups (data not shown). In contrast, early/mid pregnancy IL-1β, IL-2, IL-4, IL-6, and IL10 levels were significantly associated with a decreased adjusted odds ratio for FASD in children born to AE mothers, after adjustment for site and SES (Figure 2); there was a 72.6% (p=0.001), 39.9% (p=0.016), 59.6% (p=0.042), 59.1% (p=0.001), and 36.9% (p=0.038) decrease in odds ratio for every one unit log increase in the respective cytokine levels in early/mid pregnancy. A significantly lower adjusted odds ratio (aOR) for FASD was also observed for late pregnancy IL-1β (aOR: 0.359, CI: 0.166–0.777, p=0.009), and IL-10 (aOR: 0.524, CI: 0.346–0.792, p=0.002) levels, after adjustment for site and SES. TNF-α concentrations had no effect on risk for FASD, however, after taking into account IL-10 levels, increasing levels of the ratio of TNF-α to IL-10 led to a 64.2% increased adjusted odds ratio (CI: 1.108–2.433, p=0.013) for FASD in moderate to heavy alcohol-using mothers. An increased adjusted odd ratio for FASD in alcohol-exposed children was also observed for IL-1β:IL-10 (aOR: 1.413, CI:0.959–2.082, p=0.080) and IL-6:IL-10 (aOR: 1.506, CI:0.952–2.384,p=0.080); however, both failed to reach statistical significance.

Discussion

Although there is still some debate, the immune system during pregnancy generally shifts from a Th1 to a Th2 environment in order to ensure fetal acceptance while protecting the mother against infection (Chaouat, 2007). While the underlying mechanism(s) connecting immune dysfunction and altered neurodevelopment are not fully understood, evidence of altered cytokine profile and elevation of specific cytokines are indicated in neurological impairment. Cytokines are present in the fetal brain and have neurotropic functions that are responsible for normal CNS development and function (Deverman and Patterson, 2009). For

example, IL-1β is involved in the survival and growth of neuronal and glial cells, more specifically astrocytes, a key immune cell in brain development and formation of neuronal circuits (Giulian et al., 1988). TNF-α is responsible for inducing programed cell death of excess motor neurons, important for the pruning of neuron circuits, as well as for regulating synaptic plasticity (Barker et al., 2001; Sedel et al., 2004; Stellwagen and Malenka, 2006). IL-6 plays a role in neuronal growth, survival, and functionality, and can be secreted in response to both IL-1β and TNF- α (Wagner, 1996).

Dysregulation and impaired immune cell function, cytokine release, and the fetal immune response are all proposed mechanisms that contribute to the occurrence of FASD. When radial glia cells, the precursor cells to astrocytes, are exposed to ethanol, it results in decreased proliferation and a subsequent reduction in the number of astrocytes and neurons in the brain (Rubert et al., 2006). Altered neuronal survival and synaptic plasticity was observed after cortical neurons were incubated with astrocytes from rats prenatally exposed to ethanol (Pascual and Guerri, 2007). In the current study, moderate to heavy alcohol use during pregnancy resulted in higher plasma cytokines throughout pregnancy (Table 2), largely driven by the increases seen in AE-ND mothers. Ahluwalia et al (2000) reported no differences in plasma cytokine concentration with moderate alcohol use (less than 60 drinks per month) but found chronic alcohol consumption (more than 60 drinks per month) was significantly associated with higher plasma concentrations of TNF-α, IL-1β, and IL-6 in mothers, as well as in cord blood at delivery (Ahluwalia et al., 2000). Vink et al. described peptide fragments generated from Activity Dependent Neurotrophic Factor as neuroprotective in that they prevented learning abnormalities in offspring with prenatal alcohol exposure and ameliorated alcohol-induced increases in embryo/decidua TNF-α and IL-6 levels in a mouse model (Vink et al., 2005), thus strengthening the concept of a cytokine involvement in FASD.

While a positive effect of the intervention groups (MVM alone and MVM+choline) on MDI scores of alcohol-exposed males at 6 months of age (Coles et al., 2015) and improved neurophysiological encoding and memory in MVM+choline alcohol-exposed infants (Kable et al., 2015) have been shown using data from the same study, we did not observe an effect of the intervention or MVM use on cytokine concentrations in the present study. Early pregnancy levels of IL-4 and IL-6, as well as late pregnancy levels of IL-10, GM-CSF, and IL-1β, were positively associated with neurological outcomes at 6 and 12 months of age and/or reduced the risk of FASD in children prenatally exposed to moderate to heavy amounts of alcohol. While some increase in cytokines are necessary for neurodevelopment and repair, these results do contrast the current model that increased cytokine levels generated from maternal immune activation is associated with neurological disorders. Elevated levels of IL-6, TNF-α, IL-4, IL-10, and INF-γ in maternal and offspring circulation have been associated with ASD (Abdallah et al., 2012; Goines et al., 2011; Jones et al., 2016). More specifically, higher mid-gestation levels of TNF- α, IL-1α, IL-1β, and IL-6 have been associated with a higher risk of ASD with intellectual disability compared to ASD without intellectual disability (Jones et al., 2016). This could suggest that cytokine levels in FASD are reflective of a different neuronal response to exposure than other neurological disorders such as protection from the neurologic damage inflicted by alcohol.

We report that maternal TNF-α and IL-6 levels alone were not significantly associated with neurobehavioral impairment, but when levels of IL-10 were taken into account, our data show that an increasing ratio of TNF-α:IL-10 and IL-6:IL-10 during late pregnancy had a negative impact on neurological development at six and twelve months of age. In addition, increasing the TNF-a:IL-10 ratio in late pregnancy resulted in a 64.2% increased risk of FASD in alcohol-exposed children. These findings suggest that while individual cytokine concentrations may positively impact neurodevelopment, when children are prenatally exposed to alcohol the balance between IL-6 and TNF-α to IL-10 is crucial to outcome.

Although its role is still unclear, the regulatory cytokine IL-10 is expressed in fetal brain tissue as early as the first trimester (Mousa et al., 1999) and is elevated in normal, uncomplicated pregnancy (Holmes et al., 2003). Meyer et al injected poly(I:C) to simulate a viral infection into pregnant transgene dams with IL-10 overexpressing macrophages and found that overexpression of IL-10 mitigated rises in TNF-α and the subsequent ratios of IL-1β, IL-6, and TNF-α in the fetal brain (Meyer et al., 2008). Prenatal poly(I:C) exposure resulted in offspring behavior abnormalities, however these effects were reversed in those offspring over-expressing IL-10 (Meyer et al., 2008). Unexpectedly, over-expressing IL-10 in the absence of maternal immune activation was associated with behavior abnormalities similar to wild-type offspring exposed to poly(I:C) (Meyer et al., 2008). We suggest that the results from Meyer et al. support our current findings that the balance of anti- and pro-inflammatory cytokines may be key in healthy neurodevelopment beyond the elevation of individual cytokine levels.

In our study the risk for DD in children from LNA mothers was not affected by alterations in maternal cytokine concentrations in early/mid or late pregnancy (data not shown). Varner et al. measured umbilical cord serum IL-8, IL-1β, and TNF-α at delivery and found no association with neurodevelopmental delay, defined as BSID-II score below 70, in children at 2 years of age (Varner et al., 2015). Although we observed a relationship between maternal alcohol consumption and cytokine concentrations on our classification of FASD, we did not observe an association of maternal cytokine levels and DD in the absence of moderate to high levels of maternal alcohol use. This is similar to the study by Jones et al. where the maternal mid-gestational serum cytokine levels did not differ from the general population controls (Jones et al., 2016).

The current study has a number of strengths and limitations. To our knowledge, this is the first report that describes maternal cytokines as a potential risk factor for FASD. Our study documented detailed information on quantity and frequency of alcohol throughout pregnancy as well as a wide range of other covariates. An additional strength of our study was the availability of blood samples from two time points allowing us to better delineate the effect of cytokine at different stages of pregnancy. However, information regarding recent or current infection was not available for illnesses, like influenza or colds, and thus we were unable to control for recent or current infections when analyzing samples. Our study population consisted entirely of Caucasian females reducing the ability to translate our results to other race/ethnic groups. Future work should be expanded to include a larger sample in different geographic and ethnic populations. Some children classified as FASD in this analysis may be ultimately "misclassified", e.g., may ultimately have no developmental

concerns (as happens in clinical practice), we would expect that such misclassification would only serve to bias the estimates of associations toward the null.

Despite these limitations, we report that disruptions in maternal cytokines profiles, mainly the balance between anti- and pro-inflammatory cytokines, during pregnancy can alter the risk of FASD. Maternal alcohol consumption was associated with an increase in cytokine levels throughout pregnancy, however, subjects that failed to elevate IL-10 levels in the third trimester in order to maintain the balance of pro- and anti-inflammatory cytokines had an elevated risk of having a child with FASD. Although the exact mechanism(s) through which cytokines contribute to FASD remain unknown, cytokines are known to influence fetal neurodevelopment. The possibility that disruptions in the cytokine anti- and pro-inflammatory homeostasis during pregnancy may have different effects on FASD warrants further investigation.

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Highlights

- Early/mid pregnancy levels of IL-1β and IL-6, as well as late pregnancy IL-10 levels, were elevated in alcohol-exposed women with normally developing children compared to alcohol-exposed women with FASD children.
- Increases in the ratio of maternal TNF-α/IL-10 and IL-6/IL-10 were negatively associated with PDI scores at six months and twelve months and with MDI scores at twelve months.
- Increasing levels of IL-2, IL-4, IL-6, and IL-10 in early-mid pregnancy and IL-1β and IL-10 during late pregnancy was associated with decreased odd ratios of having a child with FASD when the mother consumed moderate/heavy amounts of alcohol.
- Increasing levels of the ratio of pro-inflammatory TNF-α to anti-inflammatory IL-10 in moderate to heavy alcohol-using mothers was associated with an increased odd ratio of having a child with FASD.

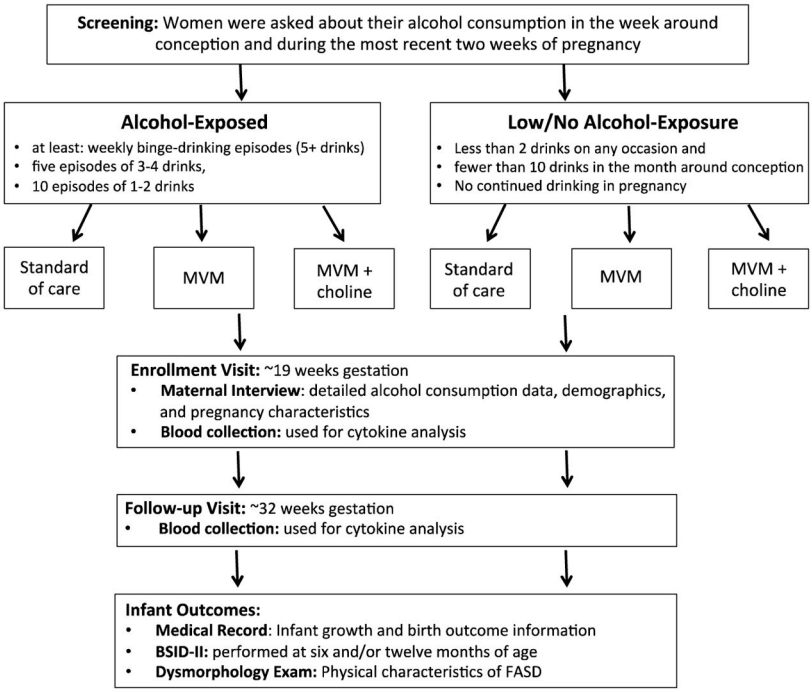

Figure 1.
Study Design

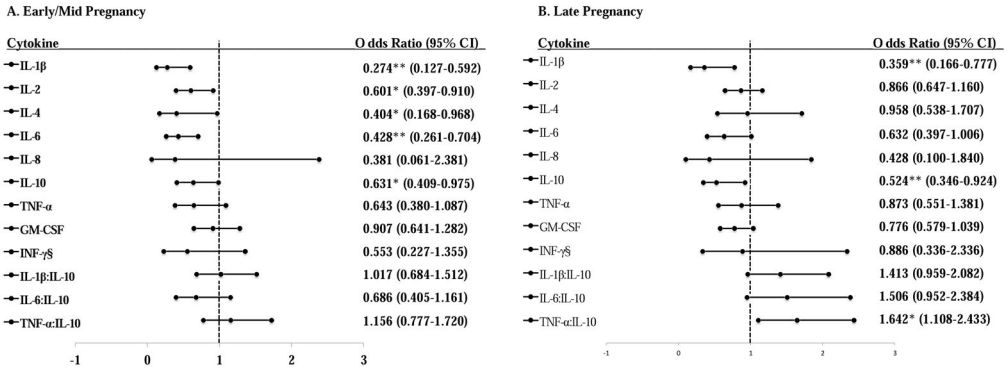

Figure 2.

Adjusted odds ratios for FASD diagnosis in alcohol-exposed children with increasing maternal cytokine levels in (A) early/mid pregnancy and (B) late pregnancy. Increasing maternal levels of IL-1β (p=0.001), IL-2 (p=0.016), IL-4 (p=0.042), IL-6 (p=0.001), and IL-10 (p=0.042) in early/mid pregnancy, and late pregnancy IL-1β (p=0.009) and IL-10 (p=0.002) decreased the risk of FASD in children. Ratios of IL-1β:IL-10 (p=0.080) and IL-6:IL-10 (p=0.080) in late pregnancy failed to reach significance, however, there was an increased risk of FASD as TNF- α:IL-10 (p=0.013) increased. Logistic Regression model are adjusted for site and SES. §Detectable / Non-detectable (Ref group) cytokine classification was used for INF-γ levels *p-value < 0.05 ** p-value < 0.01

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Table 1

Characteristics of mothers by alcohol use and child neurodevelopment status

	Low/No Alcohol Normal Development (LNA-ND) N=47	Low/No Alcohol Developmental Delay (LNA-DD) N=45	Alcohol Exposed/ Normal Development (AE-ND) N=59	Alcohol Exposed/ FASD (AE-FASD) N=90	*P*-Value
Maternal/Paternal Characteristics					
Gestational week at enrollment visit	18.60±0.63[a]	18.80±0.88[ab]	21.98±0.94[b]	20.51±0.72[ab]	0.020
Gestational week at 2nd study visit	32.07±0.41	31.38±0.26	32.62±0.37	32.26±0.30	0.141
Site, n (%)					
Rivne	22 (46.8)	25 (55.6)	38 (64.4)	37 (41.1)	0.038
Khmelnytsky	25 (53.2)	20 (44.4)	21 (35.6)	53 (58.9)	
Maternal age, years	26.6±0.64	26.31±0.76	25.68±0.76	25.92±0.59	0.816
Maternal educational level, n (%)					
Less than 9 years	0 (0)	0 (0)	1 (1.7)	0 (0)	
9 years (uncompleted high school diploma)	0 (0)	1 (2.2)	5 (8.5)	18 (20)	
High school diploma /vocational or trade school;	16 (34)	23 (51.1)	26 (44.1)	53 (58.9)	0.001
College degree or unfinished university education	6 (12.8)	2 (4.4)	12 (20.3)	6 (6.7)	
University graduate	25 (53.2)	19 (42.2)	15 (25.4)	13 (14.4)	
Paternal age, years	28.51±0.66	28.2±0.81	29.41±0.75	31.1±0.87	0.045
Pre-pregnancy BMI	21.9±0.62	21.98±0.55	22.74±0.62	22.07±0.42	0.694
Parity	0.55±0.12	0.76±0.16	0.76±0.13	0.81±0.11	0.562
Gravidity	1.92±0.17	2.31±0.22	2.46±0.17	2.12±0.17	0.179
SES Categorical[§], n (%)					
1	4 (8.5)	5 (11.1)	6 (10.2)	3 (3.3)	
2	22 (46.8)	13 (28.9)	16 (27.1)	14 (15.6)	
3	15 (31.9)	17 (37.8)	25 (42.4)	32 (35.6)	<0.0005
4	6 (12.8)	9 (20)	7 (11.9)	24 (26.7)	
5	0 (0)	0 (0)	5 (8.5)	16 (17.8)	
Supplement Use					

	Low/No Alcohol Normal Development (LNA-ND) N=47	Low/No Alcohol Developmental Delay (LNA-DD) N=45	Alcohol Exposed/ Normal Development (AE-ND) N=59	Alcohol Exposed/ FASD (AE-FASD) N=90	*P*-Value
Supplement Intervention, n (%)					
Standard of care	24 (51.1)	17 (37.8)	34 (57.6)	51 (56.7)	
MVM supplement group	9 (19.1)	18 (40.0)	11 (18.6)	10 (11.1)	0.01
MVM supplement + choline group	14 (29.8)	10 (22.2)	14 (23.7)	29 (32.2)	
MVM supplement use, n (%)					
No MVM	14 (29.8)	9 (20)	25 (42.4)	41 (45.6)	
MVM use prior to enrollment	13 (27.7)	17 (37.8)	17 (28.8)	29 (32.2)	0.037
MVM use after enrollment	20 (42.6)	19 (42.2)	17 (28.8)	20 (22.2)	
Maternal Alcohol Use and Smoking					
Absolute alcohol use at conception, oz.					
AAD	0.0023±0.0023	0.0039±0.0039	0.59±0.07^{b}	0.71±0.08^{b}	<0.0005
AADD	0.02±0.02^{a}	0.03±0.03^{a}	1.6±0.15^{b}	2.00±0.28^{b}	<0.0005
Absolute alcohol use at enrollment, oz.					
AADXP	0.00±0.00^{a}	0.001±0.001^{a}	0.16±0.06^{b}	0.21±0.04^{b}	<0.0005
AADDXP	0.00±0.00^{a}	0.01±0.01^{a}	0.77±0.14^{b}	0.88±0.11^{b}	<0.0005
Maternal Smoking Status, n (%)					
Never smoker	42 (91.3)	38 (84.4)	20 (33.9)	25 (28.1)	
Past smoker; quit before pregnant	3 (6.5)	3 (6.7)	5 (8.5)	9 (10.1)	<0.0005
Past smoker; quit after pregnant	0 (0.0)	4 (8.9)	22 (37.3)	25 (28.1)	
Current smoker	1 (2.2)	0 (0.0)	12 (20.3)	30 (33.7)	
Cigarettes smoked per day at enrollment	0.09±0.09^{a,c}	0.00±0.00^{a}	1.36±0.48^{b,c}	2.71±0.53^{b}	<0.0005
Infant Characteristics					
Child's sex, n (%)					
Male	25 (53.2)	32 (71.1)	31 (52.5)	47 (52.2)	0.163
Female	22 (46.8)	13 (28.9)	28 (47.5)	43 (47.8)	
Gestational age at birth, weeks	39.92±0.14^{a}	39.39±0.31^{ab}	39.46±0.26^{ab}	38.64±0.27^{b}	0.005
Birth Weight, g	3401.92±54.13^{a}	3323.56±82.44^{a}	3312.2±71.60^{a}	2968.81±72.62^{b}	<0.0005

	Low/No Alcohol Normal Development (LNA-ND) N=47	Low/No Alcohol Developmental Delay (LNA-DD) N=45	Alcohol Exposed/ Normal Development (AE-ND) N=59	Alcohol Exposed/ FASD (AE-FASD) N=90	*P*-Value
Birth Length, cm	52.19±0.27[a]	51.53±0.44[a]	51.93±0.35[a]	49.82±0.48[b]	<0.0005
Bayley Scale of Infant Development II					
6 Months					
MDI	94.49±1.17[a]	87.68±1.31[b]	94.96±0.74[a]	83.2±1.07[c]	<0.0005
PDI	96.07±1.06[a]	82.59±1.88[b]	96.61±1.19[a]	79.79±1.32[b]	<0.0005
12 Months					
MDI12	95.44±1.08[a]	81.64±1.59[b]	95.59±1.26[a]	79.03±1.31[b]	<0.0005
PDI12	101.94±1.32[a]	92.32±2.04[b]	104.02±1.48[a]	87.05±1.72[b]	<0.0005

[§]Socioeconomic status: based on Hollingshead score calculated from maternal and paternal education and occupation; 1 is highest score and 5 is lowest (Hollingshead and Redlich, 2007). Missing values were as follows for each group: 2 in the AE-FASD group and 1 in AE-ND group for paternal age; 1 in the AE-FASD group for MVM use; 1 in the LNA-DD and AE-FASD group for SES, 1 in the LNA-DD, AE-FASD, and AE-ND group for pre-pregnancy BMI. FASD is broadly classified as reported maternal alcohol consumption, as defined for the AE group, and at least one BSID-II score below 85 at six or twelve months of age, with and without physical features of FASD

[a,b]Means in a row with different superscript letters differ with a p value <0.05.

Table 2

Comparison of plasma cytokine levels throughout pregnancy in mothers with low/no alcohol (LNA) and moderate/heavy alcohol consumption (AE) with normal developing (ND), developmentally delayed (DD), or children with FASD.

A. Early/mid pregnancy (< 26 weeks gestation)

Cytokine (pg/ml)	LNA-ND N=46	LNA-DD N=41	AE-FASD N=72	AE-ND N=45
IL-1β	0.98±0.30^{ab}	0.63±0.12^{ab}	0.83±0.26^{a}	1.18±0.26^{b}
IL-2	2.45±0.88^{a}	3.72±1.61^{ab}	4.20±1.20^{ab}	66.62±56.42^{b}
IL-4	0.32±0.04	0.23±0.04	0.34±0.08	0.60±0.15
IL-6	3.07±1.07^{a}	3.62±1.37^{a}	4.32±1.25^{a}	40.55±32.82^{b}
IL-8	5.28±0.64	4.98±0.42	5.59±0.37	6.14±0.41
IL-10	6.08±1.77	8.74±3.86	10.25±2.57	82.43±66.47
TNF-α	4.93±1.40	5.54±2.57	15.20±9.49	95.54±84.32
GM-CSF	53.34±9.38	42.66±8.97	53.26±8.52	62.53±10.90

B. Late pregnancy (≥ 26 weeks gestation)

Cytokine (pg/ml)	LNA-ND N=47	LNA-DD N=38	AE-FASD N=79	AE-ND N=51
IL-1β	0.86±0.16	0.56±0.08	1.13±0.31	1.16±0.23
IL-2	3.36±1.03	4.03±1.37	3.19±0.86	53.96±45.49
IL-4	0.21±0.03	0.19±0.04	0.84±0.38	0.61±0.23
IL-6	2.90±0.75	3.99±1.30	4.24±1.08	29.60±23.80
IL-8	8.01±3.05^{ab}	4.24±0.27^{a}	5.45±0.32^{ab}	6.53±0.61^{b}
IL-10	6.42±1.48^{ab}	11.29±4.38^{ab}	9.59±2.31^{a}	60.77±45.16^{b}
TNF-α	3.05±0.90	3.62±1.44	13.54±6.29	74.96±69.12
GM-CSF	55.21±9.81	59.10±12.56	54.06±9.15	81.39±12.46

General Linear model results are presented as unadjusted mean ± SEM. (A) maternal cytokine levels in early/mid pregnancy, defined as prior to 26 weeks gestation (B) maternal cytokines levels in late pregnancy, defined as 26 weeks gestation or later.

^{a,b}Means in a row with different superscript letters differ with a p value <0.05.

Table 3

Results of multivariate linear regression on BSID-II at 6 and 12 months of age in relation to maternal cytokine levels in early/mid and late pregnancy.

	MDI at 6 Months				PDI at 6 Months			
	Early/Mid Pregnancy N=183		Late Pregnancy N=189		Early/Mid Pregnancy N=183		Late Pregnancy N=189	
Maternal Log Cytokine Levels	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value
IL-1β	**1.280**	0.270	**2.043**	0.099	**1.544**	0.351	**2.932**	0.088
IL-2	**0.378**	0.582	**0.015**	0.975	**1.076**	0.271	**0.639**	0.338
IL-4	**3.385** *	0.013	**−1.359**	0.166	**4.971** *	0.010	**0.582**	0.670
IL-6	**0.435**	0.544	**−0.236**	0.757	**1.493**	0.143	**0.494**	0.642
IL-8	**−2.626**	0.372	**−2.833**	0.244	**−1.715**	0.683	**3.589**	0.287
IL-10	**0.484**	0.480	**0.572**	0.379	**2.032** *	0.037	**1.812** *	0.044
TNF-α	**1.252**	0.147	**−0.647**	0.430	**1.824**	0.138	**−0.435**	0.702
GM-CSF	**0.133**	0.812	**0.251**	0.601	**0.887**	0.264	**1.163**	0.080
Ratios								
IL- 1β:IL-10	**−0.037**	0.956	**−0.011**	0.986	**−1.460**	0.130	**−0.947**	0.279
IL-6:IL-10	**0.144**	0.871	**−1.242**	0.139	**−1.115**	0.378	**−2.429** *	0.036
TNF-α:IL-10	**−0.274**	0.674	**−0.985**	0.130	**−0.797**	0.391	**−2.097** *	0.020

	MDI at 12 months				PDI at 12 months			
	Early/Mid Pregnancy N=180		Late Pregnancy N=190		Early/Mid Pregnancy N=180		Late Pregnancy N=190	
Maternal Log Cytokine Levels	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value	Estimated Coefficient	*P*-value
IL-1β	**2.051**	0.229	**3.262** *	0.049	**−0.565**	0.770	**2.196**	0.255
IL-2	**0.413**	0.682	**−0.341**	0.597	**1.883**	0.098	**−0.677**	0.367
IL-4	**0.992**	0.653	**0.454**	0.734	**0.864**	0.730	**−0.656**	0.672
IL-6	**1.038**	0.324	**−0.334**	0.746	**1.775**	0.136	**−1.073**	0.369
IL-8	**0.874**	0.837	**2.216**	0.502	**−1.031**	0.830	**−3.846**	0.315
IL-10	**0.056**	0.954	**1.163**	0.167	**0.671**	0.544	**1.075**	0.272
TNF-α	**0.367**	0.777	**−1.955**	0.082	**1.345**	0.359	**−1.908**	0.145
GM-CSF	**0.438**	0.542	**1.343** *	0.036	**0.476**	0.593	**1.635** *	0.020

	MDI at 12 months				PDI at 12 months			
	Early/Mid Pregnancy N=180		Late Pregnancy N=190		Early/Mid Pregnancy N=180		Late Pregnancy N=190	
Maternal Log Cytokine Levels	**Estimated Coefficient**	***P*-value**	**Estimated Coefficient**	***P*-value**	**Estimated Coefficient**	***P*-value**	**Estimated Coefficient**	***P*-value**
Ratios								
IL-1β:IL-10	**0.540**	0.555	**−0.320**	0.702	**−0.754**	0.468	**−0.502**	0.605
IL-6:IL-10	**1.195**	0.306	**−1.951**	0.050	**1.219**	0.357	**−2.525** *	0.029
TNF-α:IL-10	**0.121**	0.890	**−1.908** *	0.013	**0.073**	0.941	**−1.811** *	0.043

Results of linear regression on BSID-II scores at 6 and 12 months presented as estimated coefficients indicating the change in corresponding MDI and PDI scores with one unit increase in maternal log cytokine concentration. MDI and PDI scores at 6 months were adjusted for site, SES, absolute alcohol per day at conception, gravidity, and child sex. Results from MDI and PDI scores at 12 months were adjusted for SES, absolute alcohol per day at enrollment, MVM supplement use, and paternal age.

* $p < 0.05$