Fetal Sex-Based Differences in Maternal Hormones, Angiogenic Factors, and Immune Mediators During Pregnancy and the Postpartum Period

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Problem
Several pregnancy complications have disparities based on the sex of the fetus. It is unknown whether the sex of the fetus differentially alters the maternal immune milieu, potentially contributing to the observed differences.

Method of study
Using maternal plasma collected during 38 uncomplicated pregnancies (19 males, 19 females), we compared levels of cytokines, sex hormones, and angiogenic factors throughout gestation and postpartum.

Results
Male fetal sex was associated with higher levels of proinflammatory cytokines (G-CSF, IL-12p70, IL-21, and IL-33) and angiogenic factors (PlGF and VEGF-A) compared with female fetal sex at multiple time-points. Female fetal sex was associated with higher levels of regulatory cytokines (IL-5, IL-9, IL-17, and IL-25). IL-27 increased throughout pregnancy regardless of fetal sex. There was no fetal sex-based difference in analyte concentrations at the postpartum measurement.

Conclusion
Women carrying a male fetus exhibit a more proinflammatory/proangiogenic immune milieu than women carrying a female fetus.

Introduction
Although pregnancy is known to alter the maternal immune milieu, it is unknown whether the sex of the fetus results in distinct maternal immune changes throughout the course of pregnancy. This question is relevant given that several observations of disparate outcomes in pregnancy are based on fetal sex. Women carrying male fetuses have disproportionate rates of preterm births, higher birth weights, and greater fetal mortality. A study of women diagnosed with preeclampsia found that women carrying male fetuses had a significant impairment in vasodilation in response to the potent vasodilator corticotrophin-releasing hormone (CRH). Yet others have found women carrying female fetuses had a higher risk of hypertensive disorders and asthma flares. Fetal sex-based differences in maternal adaptation to pregnancy can have long-term consequences, as seen in studies of the effects

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of fetal sex on maternal nutrient deprivation and excess.\textsuperscript{11,12} These observations suggest that differences in the maternal immune response based upon the sex of the fetus are important for both short-term and long-term health outcomes.

Recent studies have assessed mechanisms underlying fetomaternal immunity and their effects on pregnancy outcome based on fetal sex. Histological examination of placentas from babies delivered before 32 weeks found that male placentae had more inflammation, possibly as a result of a more robust maternal immune response to male fetuses.\textsuperscript{13} Pathway enrichment analyses of placental tissue mRNA revealed an increase in expression of genes involved in the immune system, inflammation, and graft-versus-host disease during gestation based on male fetal sex,\textsuperscript{14} while genes that were increased during gestation when carrying a female fetus were involved in immune regulation and included such genes as \textit{JAK1}, \textit{IL2RB}, \textit{CXCL1}, and \textit{IL1RL1}.\textsuperscript{15} In addition, mothers with mild asthma who carried a female fetus had increased placental expression of inflammatory and allergic mediators including tumor necrosis factor (TNF)-\textgreek{z}, interleukin (IL)-1\textbeta, IL-8 and IL-5 mRNA.\textsuperscript{16} Fetal sex-based immune changes have also been observed on the fetal side. Umbilical cord blood from healthy term male fetuses had greater proinflammatory responses \textit{in vitro} to lipopolysaccharide (LPS) based on measures of IL-1\textbeta and IL-6.\textsuperscript{17} While these studies represent critical observations, they are based on single time points and do not assess longitudinal changes that occur throughout the course of gestation.

On the basis of these prior observations, we hypothesized that the maternal cytokine and growth factor milieu would be more inflammatory in women carrying male versus female fetuses and that these differences would be apparent as early as the first trimester. We collected plasma from healthy, first-time mothers monthly throughout gestation and at 6 weeks postpartum. We then analyzed plasma for protein concentrations of sex hormones, cytokines, and angiogenic factors.

Materials and methods

Patient Eligibility and Blood Sample Collection

Informed consent was obtained from all patients, and this study was approved by the Mayo Clinic Institutional Review Board, Rochester, Minnesota. Women who were pregnant for the first time were eligible for this study. Women were excluded if they had a known immunodeficiency or autoimmune disease, had undergone a solid organ transplant, had a prior pregnancy termination or miscarriage, were smokers, had hemoglobin levels <9 g/dL, or had undergone fertility treatments. During the study, 10 milliliters (mL) of peripheral blood were collected from each woman at the time of their first prenatal visit (typically 8 weeks from their last menstrual period), every 4 weeks thereafter, and at 6 weeks postpartum (\pm 2 weeks for each of time point). Blood was collected in sodium heparin tubes and separated for plasma and peripheral blood mononuclear cells (PBMCs) over Ficoll-Paque (GE Healthcare) according to the manufacturer’s instructions within 18 hr of collection. Samples were collected during 2011 and 2012. Plasma was stored at -80 degrees C until thawing for laboratory analyses.

Laboratory Assays

Hormone concentrations were quantified by enzyme-linked immunosorbent assays (ELISA). ELISA kits for progesterone, estradiol, estrone, and prolactin were purchased from ALPCO (Salem, NH, USA), and an ELISA kit for CRH was purchased from Novatein Biosciences (Woburn, MA, USA). Assays were performed according to manufacturer’s instruction. ELISAs were read at 450 nm on a plate reader, and concentrations were calculated using a 6-point standard curve.

MagPlex\textsuperscript{\textregistered} Multiplex kits (Millipore, Billerica, MA, USA) were used to determine concentrations of cytokines and angiogenic factors. The Th17 cytokine MagPlex\textsuperscript{\textregistered} kit was used to quantify interferon(IFN)-\textgamma, IL-10, (C-C motif) (CC) ligand 20, IL-12p70, IL-13, IL-15, IL-17A, IL-22, IL-9, IL-1\textbeta, IL-33, IL-2, IL-21, IL-4, IL-23, IL-5, IL-6, IL-17E/IL-25, IL-27, IL-31, TNF-\textgreek{z}, TNF-\textbeta, and IL-28A. The Angiogenesis MagPlex\textsuperscript{\textregistered} kit was used to quantify epidermal growth factor (EGF), angiopoietin (Ang)-2, granulocyte colony-stimulating factor (G-CSF), bone morphogenetic protein (BMP)-9, endoglin, endothelin-1, leptin, fibroblast growth factor (FGF)-1, FGF-2, follistatin, IL-8, hepatocyte growth factor (HGF), heparin-binding (HB)-EGF, placental growth factor (PIGF), vascular endothelial growth factor (VEGF)-A, VEGF-C, and VEGF-D. Immunoassays were performed according to manufacturer’s instruction; however, five washes were utilized instead of two, and samples...
were run in wash buffer instead of sheath fluid. Completing more washes and running data collection on samples in wash buffer was recommended by the company’s support team, and we found it to increase bead counts. Multiplex assays were measured using Luminex xPONENT technology (Austin, TX, USA). Concentrations were calculated using MILLIPLEX Analyst 5.1 software (Vigene Tech, Carlisle, MA, USA). All samples were measured in duplicate and averaged to determine concentration.

Statistical Analysis

Each woman had samples analyzed on the same plate to minimize plate-to-plate variability. Analytes that were below the detection limit were changed to a concentration of 0, and analytes that were beyond the upper range of detection were assigned the highest detectable limit as described previously. For statistical analysis, samples were divided into gestational intervals: 0 = ≤8 weeks, 1 = 9–12 weeks, 2 = 13–17 weeks, 3 = 18–22 weeks, 4 = 23–27 weeks, 5 = 28–32 weeks, 6 = 33–37 weeks, 7 = delivery (14 days prior to 24 hr after), and 8 = 6 week postpartum. Data were excluded if a sampling occurred twice during a gestational interval to ensure independence of all values (47 of 310 samples were not included). Kruskal–Wallis tests were utilized to assess differences in protein concentrations of analytes across gestational intervals in the entire cohort. Significance was declared at 0.1 for preliminary analysis of differences across gestational intervals in the entire cohort. Longitudinal differences across gestational intervals based on fetal sex were determined using Kruskal–Wallis tests, and significance was declared at 0.001 to account for multiple comparisons. To analyze differences between women caring male versus female fetuses at each individual gestational interval, Wilcoxon rank-sum tests were conducted, and significance was declared at 0.001 to account for multiple comparisons. Statistical analysis was completed using JMP software version 9 (SAS, Cary, NC).

Pathway Analysis and Enrichment

Cytokines and angiogenic factors that exhibited significant differences according to fetal sex and gestational interval increased or decreased based on fetal sex and gestational interval were analyzed to determine their role in transcription factor networks and biological processes. Network analysis was completed using MetaCore (New York, NY, USA), and pathway enrichment was completed using Ingenuity Pathway Analysis (IPA) (Redwood City, CA, USA). Only interactions based on human data were included. Targets that were significantly different from one sex to the other were matched using comparison analysis. In the pathway enrichment analysis, Fisher’s exact test was used to calculate P-values that represent the probability of the biological function in our data set being due to chance alone. Differentially expressed proteins with a P-value ≤ 0.01 were used to determine differences in the canonical pathways based on the sex of the fetus. Analysis settings included direct and indirect comparisons and a relaxed search filter to maximize possible interactions between proteins.

Results

Patient Demographics

Fifty healthy primigravid women between 18 and 35 years of age consented to participate in the study. Twelve women were excluded from the analysis because of miscarriage (3), voluntary withdrawal (2), or incomplete data collection defined as completing fewer than 50% of study blood draws (7). Of the 38 women available for analysis, 19 carried a male fetus, and 19 carried a female fetus. The mean age of the cohort of 38 women was 28.2 years (range 19–34). There were no significant differences in age between women carrying male fetuses versus female fetuses (average 27.7 versus 28.7 years, respectively; P = 0.434).

Variation Based on the Sex of the Fetus In Maternal Cytokines, Growth Factors, Hormones, and Angiogenic Factors

Several notable fetal sex-based differences in maternal cytokines and growth factors were observed during gestation (Fig. 1). Levels of proangiogenic factors and Th1 cytokines were greater in women carrying a male fetus (versus female); specifically, women carrying a male fetus had higher levels of BMP-9, endothelin-1, FGF-2, follistatin, G-CSF, HB-EGF, HGF, IL-12p70, IL-21, IL-33, PlGF, prolactin, and VEGF-A in over 50% of gestational intervals. In addition, women carrying a male fetus had significantly higher levels of angiopoietin-2, BMP-9, follistatin, HB-EGF, HGF,
PlGF, and IL-12p70 during the first gestational interval (≤8 weeks). Among women carrying a female fetus, cytokines indicative of a Th2 type immune response were observed; specifically, women carrying a female fetus had higher levels of IL-5, IL-9, IL-17A, and IL-17E/IL-25 in over 50% of gestational intervals. Analytes that were not reported were not significantly different between women carrying a male versus female fetus at any time point. Notably, there were also no significant differences between women carrying a male or female fetus at the 6-week postpartum visit; however, only 15 of 38 women (40.5%) had their postpartum blood draw.

For the entire cohort, plasma levels of estradiol, progesterone, estrone, and IL-27 increased throughout gestation (Fig. 2), with little variability between the women in early gestation and postpartum. IL-27 levels peaked at around 33–37 weeks before returning to low levels after delivery. Plasma levels of sex hormones and IL-27 levels did not differ by fetal sex. Among women pregnant with a female fetus, angiopoietin-2, endoglin, and follistatin levels

**Fig. 1** Summary of analytes that showed significant variability based upon the sex of the fetus at different gestational intervals. Median analyte concentrations are plotted on the Y axis (log-transformed in a and b, and linear scale in c, d, e). Gestational intervals are plotted on the X axis, with separation between women carrying female versus male fetuses. Statistical significance was declared at $P = 0.001$ due to multiple comparisons with this analysis, and comparisons between females versus males that reach significance at each gestational time point is indicated with an asterisk (*).
were significantly different longitudinally throughout pregnancy (Fig. 3, \( P < 0.0001 \)). These levels peaked at gestational intervals 2, 7, and 4, respectively. Several of the cytokines and growth factors that showed different patterns of increase based upon fetal sex have known functions in immunity and angiogenesis (Fig. 4).

Pathway Analysis of Fetal Sex-Based Variations in Maternal Proteins During Pregnancy

Using Metacore, we analyzed potential pathways under similar transcriptional control based on the sex of the fetus (Fig. 5). Pathway analysis indicated that the majority of proteins that increased among women carrying a male fetus are under transcriptional regulation of NF-κB, SP1, c-Jun, and CREB1 (Figs S1 and S2). The proteins that increased among women carrying a female fetus are predominantly regulated by transcription factors NFAT and STAT-3 (Fig. S3). Utilizing Ingenuity Pathway Analysis, we compared biological processes based on involvement of cytokines and angiogenic proteins that were statistically significant based on our earlier analysis. Prolactin was excluded from analysis because it is increased by each sex at one gestational interval. The threshold defined as a fold change greater than 2.5, and the association between data and the canonical pathway was measured by Fisher’s exact test \( (P \leq 0.05) \). In the majority of biological processes, proteins that were increased among women carrying a male fetus were highly associated with signaling, development, and inflammatory response pathways (Fig. S4). However, proteins that were increased among women carrying a female fetus were enhanced in pathways important for hematological disease, humoral (Th2) immune responses, and inflammatory disease.
Discussion

Male fetuses have disproportionate rates of preterm births, higher birth weights, and greater fetal mortality. In our cohort of 38 women, we show that the maternal hormonal and immune milieu undergoes many changes during pregnancy that vary based on fetal sex. The hormonal and immune changes among women carrying a male fetus were characterized by increases in levels of proinflammatory cytokines and proangiogenic growth factors, while the hormonal and immune changes among women carrying a female fetus were characterized by increases in the expression of regulatory cytokines. These findings may explain the disparate pregnancy outcomes based on fetal sex and are consistent with our hypothesis that women carrying a male fetus would have a cytokine and growth factor milieu that is biased toward a Th1 inflammatory response as compared to women carrying a female fetus.

Compared to women pregnant with a female fetus, women carrying a male fetus had higher levels of inflammatory cytokines at multiple time
points during gestation. Specifically, male fetuses were associated with higher levels of IL-12p70, IL-21, IL-33, and G-CSF in maternal plasma during pregnancy, with many of these proteins increased above females as early as 6 weeks post-conception. These cytokines are typical of a proinflammatory, Th1 T cell response. IL-12p70, produced by monocytes, is involved in the differentiation of naïve T cells into Th1-biased T cells. IL-12p70 has previously been shown to increase in pregnant women with preeclampsia. In neonatal immunity, IL-21 drives a Th1 response that is hypothesized to

Fig. 4 Summary of cytokines and growth factors that showed differences based specifically on the sex of the fetus through multiple time points during pregnancy, along with predominant function based upon current literature. This is an oversimplified representation of the function of these cytokines and growth factors, as many of these factors, especially IL-17 family members, may have different context-dependent roles.

Fig. 5 Summary of transcription factor regulation of proteins showing different maternal levels based on fetal sex. Cytokines and angiogenic factors upregulated during gestation with a male fetus predominantly utilize transcription factors NF-κB, c-Jun, SP-1 and CREB-1, while female fetuses predominantly utilize NF-AT and STAT3.
decrease infectious disease susceptibility of newborns. IL-33 is an IL-1 family member involved in innate immunity and inflammation. This immune milieu with high levels of IL-12, IL-21, and IL-33 could account for disparate outcomes in prenatal infectious disease. In addition, human miscarriage is associated with a Th1-biased, proinflammatory response at the maternal-fetal interface. Although not addressed in this study, one could hypothesize that there may be a higher incidence of miscarriages when women are carrying a male fetus versus a female. Further studies must be completed to test this hypothesis.

Angiogenesis is required during placentation to supply essential nutrients and oxygen to the developing fetus. An appropriate balance between proangiogenic and anti-angiogenic factors is essential to develop adequate, but not pathologically aggressive, placental invasion. Adding an additional layer of complexity, many angiogenic factors have immunomodulating properties; therefore, we considered the role of angiogenesis proteins along with recognized immunoregulatory molecules. We found that angiogenic growth factors such as PlGF and VEGF-A were increased in the blood of women carrying a male fetus, where higher levels of inflammatory cytokines were observed. PlGF has also been associated with adaptation to inflammation in other settings (e.g., sepsis), suggesting that its relative increase with male fetuses may also be adaptive. Pregnancy complications, such as ectopic pregnancies and threatened abortions, correlate with a decrease in PlGF and an increase in VEGF-A expression in plasma. PlGF has been suggested as a biomarker for preeclampsia as reduced levels of this growth factor occur weeks before clinical onset of disease.

Levels of BMP-9, HB-EGF, and HGF, factors also involved in angiogenesis, were also increased in pregnancies with male fetuses. BMP-9 binds to activin receptor-like kinase (ALK1) with high affinity which effectively inhibits angiogenesis. However, in combination with transforming growth factor (TGF)–β, a cytokine expressed at high levels in the placenta, BMP-9 binding to ALK1 improved the angiogenic response of endothelial cells. HB-EGF production is regulated by estradiol and progesterone in the endometrium prior to conception and blocking it negatively affected uterine receptivity during implantation. Syncytiotrophoblasts and extravillous trophoblasts express HGF, which is embryonic lethal when mutated in murine models. Taken together, our results suggest that higher levels of inflammatory cytokines coupled with higher levels of proangiogenic growth factors observed with male fetuses could reflect the normal adaptation to a greater immunologic challenge for the mother compared with that induced by female fetuses.

In our cohort, female fetuses induced a type 2-like regulatory maternal response with increased levels of IL-5, IL-9, IL-17, and IL-25. Research into the role of IL-5 in pregnancy is limited, but an IL-5 knockout mouse model showed that this cytokine regulates eosinophilia and endometrial tissue remodeling. Although little has been published on IL-9 in human pregnancy, it has been shown to promote T-cell proliferation of CD4+ cells, and induce a Th17 autoimmune response. IL-17 is a proinflammatory cytokine that has an important role in the pathogenesis of chronic inflammation, autoimmune diseases and pregnancy through the induction of anti-inflammatory cytokines IL-6 and IL-10. In healthy pregnancies, IL-17 levels in maternal serum have been reported to increase throughout gestation, with the highest levels of expression during the third trimester. Others have found that IL-17 producing T cells do not change in peripheral blood during normal human pregnancies, only in the decidua. Levels of IL-17E/IL-25 have not been documented during pregnancy to our knowledge, but the cytokine is known to initiate a type 2 immune response through the alternative activation of macrophages and expansion of innate lymphoid cells. Lymphocyte effector functions guided by nuclear factor of activated T cells (NFAT) may be in part responsible for the maternal response to fetal sex. Based on numerous prior experimental results, successful pregnancy has previously been characterized as a type 2 immune-biased phenomenon. Our results appear consistent with that view for women carrying a female fetus.

At the 6-week postpartum visit, we saw no significant differences between women carrying a male fetus versus women carrying a female fetus, which increases the probability that our results are due to fetal sex and not individual variation. However, only 15 of the 37 (40.6%) women on our study came in for their postpartum blood draw. Because of the nature of designing studies with a vulnerable (and often very busy) population, our sample size was limited,
and it was difficult to achieve compliance for monthly blood draw. Even with these limitations, our study is one of the first to look at maternal immune based differences longitudinally throughout pregnancy based on the sex of the fetus. We cannot rule out that some differences in maternal cytokine/mother. The immunologic changes are more extensive chronic graft-versus-host disease has been observed in male recipients of female hematopoietic cell transplants (HCT). More differences in outcomes in female-to-male transplants are often related to minor histocompatibility antigens involving the Y chromosome, but additional sex-based differences in immune response (e.g., B7-H1-dependent regulatory T-cell responses in females) are possible. A deeper understanding of female-to-male alloimmune reactions may help identify new molecular targets for sex-based immune modulation.

In conclusion, we have found that the maternal immune milieu undergoes many changes throughout gestation. Several of these changes appear to be divergent based on the sex of the fetus: male fetuses are associated with an increase in the levels of proinflammatory cytokines and proangiogenic growth factors, while female fetuses are associated with an increase in the levels of proinflammatory cytokines and proangiogenic growth factors. These results suggest that tolerance induction programs may be divergent based upon fetal sex, although these results do not define the mechanism by which such adaptation occurs. Future mechanistic studies should aim for a deeper understanding of fetomaternal tolerance as it relates to fetal sex.
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Transcriptional regulation of proteins increased in pregnancies with male fetuses, part 1.

**Figure S2.** Transcriptional regulation of proteins increased in pregnancies with male fetuses, part 2.

**Figure S3.** Transcriptional regulation of proteins increased in pregnancies with female fetuses. Pathway analysis shows that extracellular proteins which are increased in the systemic maternal immune system due to a female fetus have shared transcriptional regulation through STAT3 and NF-AT.

**Figure S4.** Top biological processes (Ingenuity Pathway Analysis) of proteins that differ between male and female fetuses during pregnancy.