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Sex modifies placental gene expression in response to metabolic and inflammatory stress

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ABSTRACT

Introduction: Metabolic stress (*e.g.*, gestational diabetes mellitus (GDM) and obesity) and infections are common during pregnancy, impacting fetal development and the health of offspring. Such antenatal stresses can differentially impact male and female offspring. We sought to determine how metabolic stress and maternal immune activation (MIA), either alone or in combination, alters inflammatory gene expression within the placenta and whether the effects exhibited sexual dimorphism.

Methods: Female C57BL/6 J mice were fed a normal diet or a high fat diet for 6 weeks prior to mating, with the latter diet inducing a GDM phenotype during pregnancy. Dams within each diet group at gestational day (GD) 12.5 received either an intraperitoneal injection of the viral mimic, polyinosinic:polycytidylic acid (poly(I:C)) or saline. Three hours post injection; placentae were collected and analyzed for changes in the expression of 248 unique immune genes.

Results: Placental immune gene expression was significantly altered by GDM, MIA and the combination of the two (GDM + MIA). mRNA expression was generally lower in placentae of mice exposed to GDM alone compared with the other experimental groups, while mice exposed to MIA exhibited the highest transcript levels. Notably, fetal/placental sex influenced the responses of many immune genes to both metabolic and inflammatory stress.

Discussion: GDM and MIA provoke inflammatory responses within the placenta and such effects exhibit sexual dimorphism. The combination of these stressors impacts the placenta differently than either condition alone. These findings may help explain sexual dimorphism observed in adverse pregnancy outcomes in human offspring exposed to similar stressors.

1. Introduction

The developmental origins of health and disease (DOHaD) framework posits that the *in utero* environment influences risk for non-communicable diseases (NCDs) for offspring throughout life [1]. Associations have been identified between stress during pregnancy and the occurrence of NCDs in offspring, such as cardiovascular disease, metabolic disorders (*e.g.*, obesity and diabetes mellitus), and neurocognitive problems [1]. Among the most prevalent maternal stressors are obesity [2], gestational diabetes mellitus (GDM) [3–5] and infection [6–8]. How these comorbidities impact fetal development and long-term health outcomes for offspring remains an open question, though it likely involves some combination of cellular/tissue damage and/or epigenetic modification [9]. This question is complicated by the

Abbreviations: HFD, high fat diet; MIA, maternal immune activation; poly(I:C), polyinosinic:polycytidylic acid; SAL, saline

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observation that some DOHaD-associated health outcomes exhibit sexual dimorphism, meaning that male and female offspring are affected differently [10,11].

The placenta is a critical organ orchestrating nutrient transport to the fetus, hormone production, maternofetal gas exchange, removal of waste, maintenance of maternofetal immune tolerance, and host defense. It is thus an important biological conduit that could mediate the non-genomic transmission of risk for NCDs [12,13]. Because the predominant tissue-specific cell of the placenta, the trophoblast, is derived from the blastocyst and is genetically fetal, placental sex is biologically congruent with that of the fetus and may influence developmental origins of disease [14–16]. Recent studies suggest that the placental transcriptome is largely driven by the fetal genome and that placental gene networks influence postnatal risk of multiple diseases [13]. Thus, sexual dimorphism in DOHaD might reflect an influence of placental sex on fetal development.

While most investigations linking antenatal stress to adverse outcomes have focused on a single stressor [17], real world experience demonstrates that human populations are routinely subjected to more than one simultaneously. For example, diabetes is often accompanied by obesity, collectively referred to as diabesity [18], a condition that has received significant attention for its impact on fetal development and developmental outcomes [4,19]. In contrast, the co-occurrence of infectious diseases and metabolic stress has received little attention [17].

Infections pose a persistent threat to human reproductive health through causing direct fetal tissue damage (*e.g.*, congenital Zika virus, cytomegalovirus or syphilis infections) or, indirectly via immune-mediated interference with normal fetal programming and/or other mechanisms such as epigenetics [20]. Maternal immune activation (MIA), as might be provoked by infections that do not cross the placenta, such as influenza, appears to impact the risk for NCDs in exposed offspring [21,22]. Many low- or middle-income countries are disproportionately affected by highly endemic infections such as tuberculosis, malaria, and HIV, but are also challenged by obesity and diabetes as societies transition from poor access to nutrition to a Western diet [23]. This is noteworthy because metabolic stressors such as these are associated, like some infections, with proinflammatory alterations within the placenta.

To advance our understanding of the extent to which metabolic and inflammatory stressors impact placental immune activation, we utilized a high fat diet-induced, pregnant mouse model of GDM, with or without acute inflammatory MIA stress [24]. The process of virus-induced immune activation was modeled using a mid-gestational challenge with the viral mimetic poly(I:C), a synthetic, double-stranded RNA agonist of Toll-like receptor 3 [25,26]. Placental gene expression for 248 genes involved in innate and adaptive immunity were evaluated because a delicate balance of immune tolerance is required for fetal development [27] and both metabolic and infectious stressors have been shown to alter the inflammatory state of the placenta [28–30]. The study design allowed us to test the hypothesis that metabolic stress or MIA or the combination of the two can induce changes in inflammatory gene expression within the placenta and tissue responses would exhibit sexual dimorphism.

2. Materials and methods

2.1. Animal procedures

Procedures were approved by the Vanderbilt Animal Care and Use Committee and conducted according to our previous protocol [24]. Briefly, C57Bl/6 J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Control fed mice received standard chow throughout the experiment (5LOD, Lab Diet, St. Louis, MO, USA) and to induce GDM, received a 60% calories-by-fat diet (58Y1, Test Diet, St. Louis, MO, USA) from 4 weeks of age throughout pregnancy. These mice are referred to as GDM mice herein and were previously shown by our group to develop a GDM phenotype compared to normal diet-fed controls [24]. At 10 weeks of age, mice were mated, and the presence of a vaginal plug marked gestational day 0.5 (GD0.5). Pregnant females were left undisturbed except for cage changes at GD9.5 and weight measurements.

GDM and controls were assigned to receive a mid-gestational intraperitoneal injection with either saline (control) or poly(I:C) as reported [24], creating 4 experimental groups: control fed saline (SAL or control), high fat fed saline (GDM), control fed poly(I:C) (MIA), and high fat fed poly(I:C) (GDM + MIA) [24]. GD12.5 was chosen as a mid-gestation timepoint, at which point pregnant females were injected intraperitoneally with either sterile saline or 20 mg/kg poly(I:C) potassium salt (Sigma Aldrich, St. Louis, MO, USA) in sterile saline, based on the weight of poly(I:C) itself. Pregnant mice were sacrificed 3 h after injection at GD12.5 during the acute phase of the inflammatory response. Sex genotyping was performed for each embryo using a previously published protocol [31]. Each of the 4 groups contained 9 pregnant females with the exception of GD12.5 MIA, which contained 8 pregnant females, making a total of 35 females. Litter parameters including litter size and statistics on number of resorptions, malformed embryos, and male: female ratios are included in Supplemental Table 1.

2.2. Tissue collection

Immediately after sacrifice, the gravid uterus was removed followed by the removal of each individual fetal-placental unit. The amniotic sac was removed from the placenta, at which point the decidual tissue was surgically separated from the placenta [32] and the placental tissue was flash frozen. In total, we collected 64 placentae from the saline treated group, 62 from the MIA group, 72 from the GDM group, and 67 from the combined MIA and GDM group. Twelve placentae (6 male, 6 female) were chosen for Nanostring® gene expression analysis from each experimental condition (except GDM group with 5 male, due to low quality RNA isolation, thus total 47 placentae), with 12 pregnant mice contributing 2 placentae each and 23 pregnant mice contributing one single placenta. Tissues were obtained across 8–9 pregnant dams per experimental condition [24]. All pups were measured to have crown/rump lengths between 8 mm and 9 mm. It was not possible to choose samples that were evenly distributed between the right and left uterine horn, although, all efforts were made to remain as evenly distributed while concurrently maintaining an even male to female ratio. Specific parameters for the placental samples chosen for gene expression analysis are summarized in Supplemental Table 2.

2.3. RNA isolation and Nanostring® transcriptional profiling

The entire placenta was utilized for RNA isolation. Tissue was dissociated using gentleMACS dissociator m-tubes in 1 mL TRIzol. RNA was isolated in TRIzol following manufacturer protocols (Invitrogen, Carlsbad, CA, USA). Samples then underwent a Qiagen RNeasy clean-up, following manufacturer's instructions (Qiagen, Hilden, Germany). A Thermo Scientific Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) was utilized to measure RNA concentration and purity (absorbance wavelength 260/280 values > 2 and 260/230 values > 1.5). Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was utilized to ensure RNA integrity, with all RNA integrity numbers falling between 9.5 and 10.

Purified RNA (150 ng) from each placental sample was utilized for unblinded Nanostring nCounter gene expression analysis (http://www.nanostring.com/applications/technology) using the mouse inflammation V2-panel which included 248 inflammation-related mouse genes and 5 internal reference controls to determine gene expression performed according to manufacturer's instructions.

2.4. Nanostring® gene expression analysis and data visualization

Count data produced by the n-Counter Digital Analyzer were normalized to positive and negative spike-ins as well as 5 housekeeping genes (*Cltc, Gapdh, Gusb, Hprt,* and *Tubb5)* and used for gene expression analysis. Principle Component Analysis (PCA) on the scaled gene expression data was performed and a 3-D plot using the first 3 components was generated using R package "rgl". To compare the gene expression level between different treatment groups and placental sex, a linear regression with robust standard errors (Huber-White method to account for the pregnant mouse cluster) on the treatment groups, sex, and their interactions was fitted for each gene. Estimated effects and p values for different contrasts were reported and summarized. We considered each gene as an endpoint and adjustment top values was not applied. These statistical analyses were performed using R version 3.5.0 (https://www.R-project.org).

2.5. Pathway analysis

PANTHER, Protein Analysis Through Evolutionary Relationships, classification system was utilized to classify and identify pathways perturbed in our experimental conditions based on gene expression data [33]. All genes upregulated or down regulated within a treatment segregated based upon sex were uploaded into the PANTHER online analysis program and functional pathway classification analysis was performed to determine the pathways perturbed in our treatment groups.

Pie charts represent the pathways involved in each treatment segregated by placental sex regardless of directionality of the expression change. Each pathway was color-coded so that each pathway was represented by the same color in all figures and were placed in order of abundance of genes in each pathway, from the most genes to the fewest genes implicated in the pathway, beginning with the miscellaneous category and moving clockwise around the circle. Miscellaneous represents the compilation of any pathway in which genes were differentially expressed between male and female that contained 3 or fewer implicated genes.

3. Results

3.1. Placental sex is associated with gene expression profiles in pregnant C57BL/6 mice in response to metabolic and inflammatory stress

To model placental transcriptional responses to acute inflammatory stress and determine the extent to which such changes were modified, we quantified mRNA of 248 immune response genes on GD12.5, 3 h following exposure to poly(I:C) or saline. The experimental groups were control (normal diet followed by saline injection on GD12.5), MIA (normal diet followed by poly(I:C) injection on GD12.5), GDM (high fat diet followed by saline injection on GD12.5), or the combination of GDM + MIA (high fat diet followed by poly(I:C) injection on GD12.5). We visualized the data by a PCA plot, in which each data point represents an individual placenta. The closer data points are to each other, the more closely related the transcriptional responses. Transcriptional responses varied most dramatically between mice exposed to MIA versus saline, regardless of the presence of diet-induced GDM (Fig. 1B). However, the added stress of GDM disturbed gene expression, as evidenced by the widening gaps on the PCA plot between data points (transcriptional responses of individual placentae). When mice with GDM were subjected to immune activation (MIA), the data points did not cluster as tightly, suggesting a highly variable transcriptional response compared to the other experimental conditions (Fig. 1B). A possible influence of placental sex on transcriptional profiles was suggested by PCA visualization, particularly among mice not exposed to the poly(I:C)-induced immune activation.

Possible interactions between placental sex and treatment group were assessed by linear regression, as detailed above. A heat map (Fig. 1C) displays row z-scores for the expression of genes for which the response to metabolic or inflammatory stress was significantly influenced by placental sex ($p < 0.05$). Here, it is evident that the placental transcriptional profiles of this subset of genes within each of the experimental groups (including the normal control mice) differed based upon sex. Metabolic and inflammatory stress induced unique changes in gene transcription and the greatest distance was observed between mice exposed to GDM and those exposed to GDM + MIA. The heat map reveals that on average, mRNA expression within placental tissues exposed to GDM alone was generally lower (more blue) than the other experimental groups, while mice exposed to MIA in combination with GDM (MIA + GDM) exhibited the highest transcript levels (more red) (Fig. 1C). Immune genes for which sex significantly modified the response to each treatment are summarized in Supplemental Tables 3–7. A more detailed analysis of the impact of fetal/placental sex on gene transcriptional responses to inflammatory and/or metabolic stress is presented in the sections that follow.

3.2. Placental sex influences gene expression in normal control mice

Within the placentae from normal control pregnant dams (normal diet, saline injection), gene differential expression analysis revealed that 13 genes (5.2% of the 248 total number of genes) were statistically significantly (p value < 0.05) differentially expressed comparing male to female tissues (*C1ra, Ccl4, Ccl24, Cfl1, Cxcl2, Ddit3, Ifna1, Jun, Ptgs2, Rac1, Tlr5, Tlr6, Tlr8*) based on linear regression models. Eleven of these genes were more highly expressed in male placentae, and two (*C1ra* and *Tlr5*) more highly expressed in female placental tissues. The estimated mean differences for all 13 genes are displayed in Supplemental Table 8.

3.3. Placental sex influences placental gene expression in response to metabolic stress

A comparison of inflammatory gene expression among placental tissues harvested from mice exposed to a high fat diet (GDM mice) and mice exposed to normal diet revealed that levels of 93 of 248 (37.5%) genes were statistically significantly different (Fig. 2A). Of these 93 genes, 36 were impacted by GDM with similar direction and scale in male and female placental tissues (11 induced and 25 repressed), while the significant changes in the expression of 40 inflammatory genes (9 up-, 31 down-modulated) were limited to male tissues and 17 genes (8 up-, 9 down-modulated) were significantly altered in female placentae. The identity and levels of changes for these genes are indicated in Supplemental Tables 9–12.

Pathway analysis revealed that male and female placentae exhibited perturbations in multiple shared pathways. Perturbations in the Wnt signaling pathway and pathways governing cytoskeletal regulation by Rho GTPases were unique to male placentae exposed to GDM (Fig. 2B). Female placentae saw unique differences within the Insulin/IGF/MAPK pathway (Fig. 2C).

3.4. Placental sex influences gene expression in response to acute exposure to immune activation (MIA)

Comparison of inflammatory gene expression among placental tissues harvested from mice exposed to a normal diet followed by poly (I:C) injection (MIA) with mice exposed only to a normal diet demonstrated male and female placentae tended to respond differently to MIA exposure. Expression levels of 114 genes (of 248; 46%) showed statistically significant changes with MIA exposure (Fig. 3A). Of these 114 genes, 52 were similarly impacted by MIA in male and female placental tissues (39 induced and 13 repressed), while the significant changes in the expression of just 17 inflammatory genes (5 up-, 12 down-modulated) were limited to male tissues and 45 genes (33 up-, 12 down-

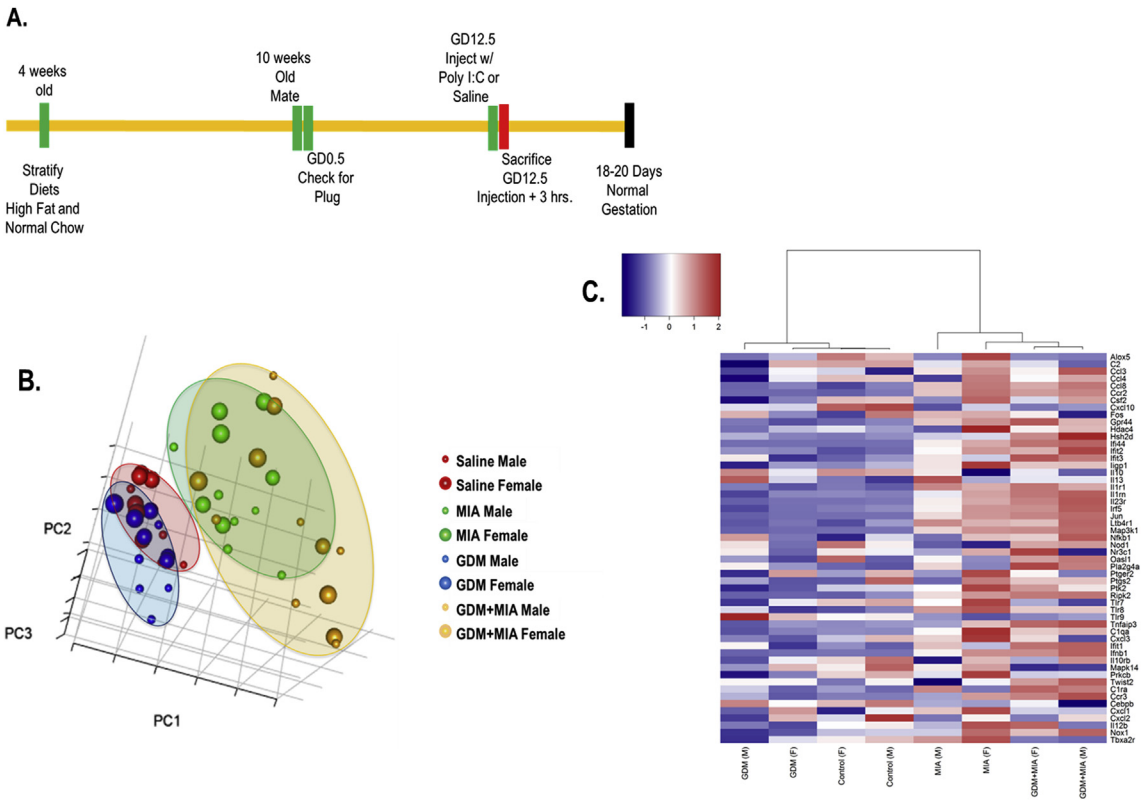

Fig. 1. Visual comparison of gene expression profiles for male and female placentae exposed *in utero* to gestational diabetes mellitus (GDM), maternal immune activation (MIA) or both GDM and MIA. Control animals received normal chow and saline injections. (A) Experimental timeline highlighting the treatment and sample collection conditions. (B) Principal component analysis (PCA) of transcription data of 248 endogenous mouse inflammation genes in mouse placenta. Each data point represents a single placental sample. Experimental groups as indicated, with small symbols for male and large symbols for female placental specimens. (C) Heat map displays row z-scores for the expression of genes for which the response to metabolic or inflammatory stress was significantly influenced by placental sex ($p < 0.05$). Heat map was generated under R version 3.5.1.

modulated) were significantly altered in female placentae. The identity and level of change (induced, repressed) of all of these genes are indicated in (Supplemental Tables 13–16).

Pathway analysis revealed that MIA induced significant changes in four pathways within female placentae that were not altered in male tissues: FAS signaling, Huntington disease pathway, insulin/IGF/MAPK pathway, and the apoptosis pathway. Although male and female placentae exhibited perturbations in multiple shared pathways there were no pathways unique to male placentae that were not also changed in female placentae as a result of maternal MIA exposure (Fig. 3B and C).

3.5. Placental sex influences gene expression in response to exposure of both GDM and MIA

We next evaluated the impact of sex on inflammatory gene expression among placental tissues harvested from mice exposed to a high fat diet (GDM) and poly(I:C) injection (MIA) with mice exposed to a normal diet followed by saline injection. These analyses demonstrated response differences between male and female tissues as expected from the above results. Expression levels of 123 genes (of 248; 37.5%) showed statistically significantly alteration after GDM and MIA exposure (Fig. 4A; Supplemental Tables 17–20). Of these 93 genes, 74 were similarly impacted by GDM + MIA in male and female placental tissues (57 induced and 17 repressed), while the significant changes in the expression of 21 inflammatory genes (7 up-, 14 down-modulated) were limited to male tissues and 28 genes (17 up-, 11 down-modulated) were significantly altered in female placentae.

Gene pathway analysis revealed 2 pathways, P53 feedback pathway and Wnt signaling pathway, significantly altered and unique to male placentae in GDM + MIA-exposed dams. Female placentae exhibited perturbations in multiple shared pathways and 2 unique pathways, TGF-beta signaling and Insulin/IGF/MAPK pathways (Fig. 4B and C).

4. Discussion

Both antenatal metabolic stress and infection pose tremendous risks to fetal health and development, risks that might be transmitted through actions within the placenta. The present study newly demonstrates that both metabolic stress and immune activation, or the combination of the two, perturb immune gene expression in the placenta. Furthermore, such effects exhibit sexual dimorphism.

A wealth of data supports the association between maternal stressors during pregnancy and adverse outcomes in offspring [34–36]. What has not been frequently studied is the co-occurrence of multiple stressors and their ability to converge on fetal development thereby influencing health outcomes for the next generation [17]. Our group recently demonstrated the unique impact of combined maternal stressors (GDM and MIA) on the fetal brain [24]. Equally important as the impact on fetal brain development is the study of the placenta and the use of fetal sex as a biological variable. Increasingly, the placenta is being viewed as a key mediator of DOHaD-related sexual dimorphism [37,38]. Placental sex is largely fetal (with exception of maternally-derived cells) [15]. That sex influences placental biology is evidenced by findings that male, rather than female, placentae are generally more responsive to changes in the maternal environment [15,39–41].

At baseline we found that the transcription of some immune genes differed significantly between male and female placentae (Supplemental Table 8). While only 13 genes were significantly differentially expressed between male and female tissues, it was interesting that several chemokines (*Ccl4*, *Ccl24*, *Cxcl2*), pathogen

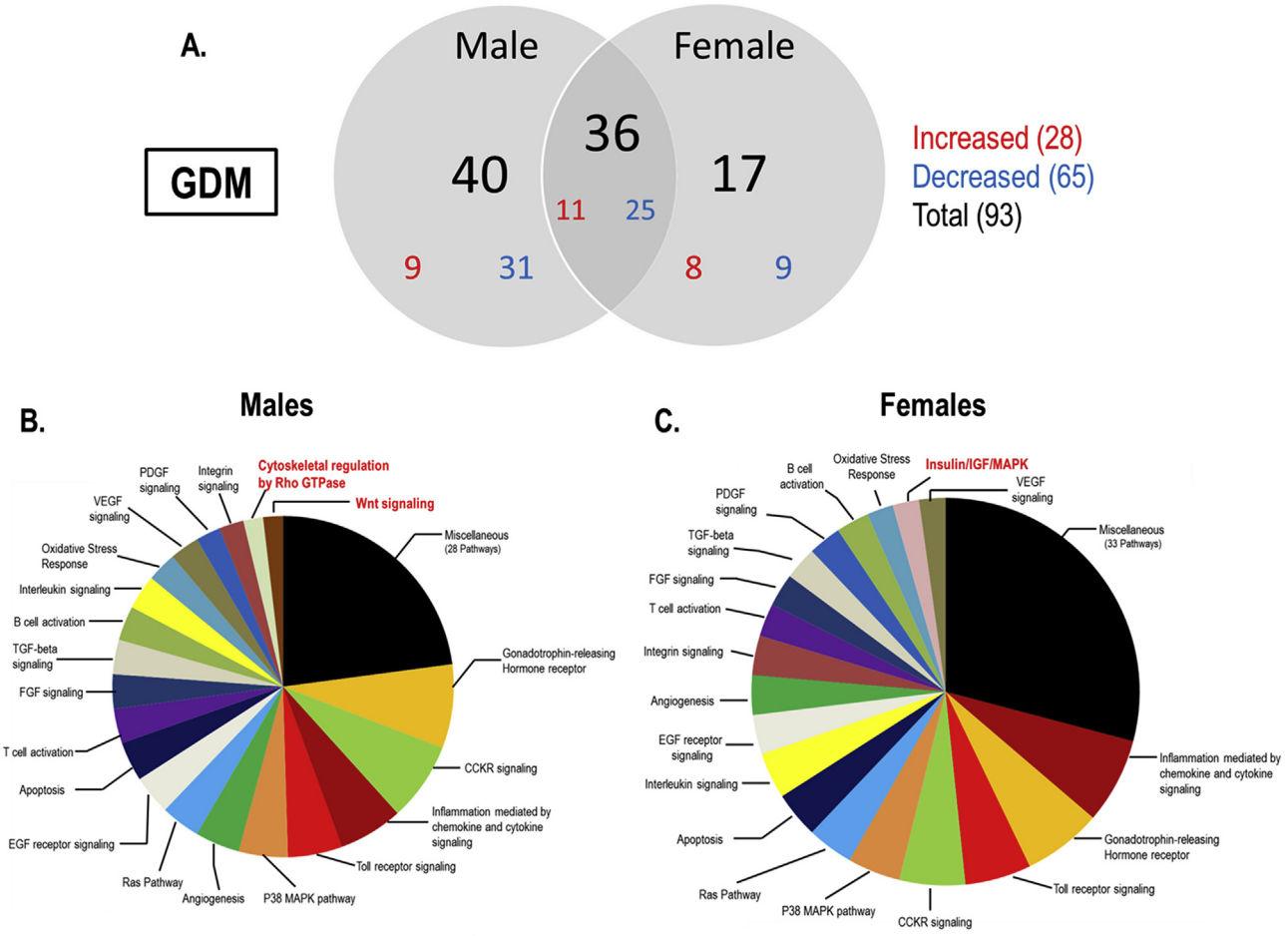

Fig. 2. Sex-associated changes in gene expression in the placenta induced by diet-induced gestational diabetes mellitus (GDM) compared to the saline treated, normal-diet controls. (A) Number of genes whose expression levels were statistically-significantly impacted by GDM compared to control ($p < 0.05$). Large black numbers indicate total number of genes altered regardless of directionality. Red numbers indicate number of genes whose expression levels increased due to GDM and blue numbers indicate the number of genes who expression levels decreased as a result of GDM. (B and C) Pathways perturbed in GDM irrespective of change in gene expression directionality. All pathways which contained ≤3 genes were grouped together in the miscellaneous category. Pathways listed in red bold print are unique to that sex. Data are displayed for male (B) and female (C) placentae separately. Number of genes included in this figure were statistically-significantly impacted by the indicated treatment ($p < 0.05$). See methods for detailed description.

recognition receptors (*Tlr5, Tlr6, Tlr8*) and the prostaglandin-generating, inducible cyclooxygenase-2 (*Ptgs2*) were among these. The male-specific increase in *Ptgs2* expression is interesting given that prostaglandins are critically important in labor and sexual dimorphism has been observed in the incidence of preterm labor [42]. Also, cyclooxygenase inhibitors were shown to have sex-specific effects in modifying the inflammatory effects of antenatal stress in the placenta and improving behavioral outcomes in male offspring in a mouse model of environmental psychological and physical stressors [43]. Similar to our data, Sood and colleagues [44] examined gene expression patterns in 19 human placentas from successful full-term pregnancies using microarray analysis and found significant differences between male and female tissues within the villus parenchyma. In fact, immunoregulatory genes such as *JAK1*, *IL2RB*, *Clusterin*, *LTBP*, *CXCL1*, and *IL1RL* were found to have sexually dimorphic expression patterns [44]. A more recent study of late first trimester placentae from humans also found sex differences in the transcriptome that included a set of 18 autosomal genes, suggesting sex-specific physiology impacts non-sex related genes [45].

The TLR3 dsRNA ligand poly(I:C) is commonly used to model viral infection [46], most notably to define the impact of MIA on fetal brain development and neurocognitive function in offspring [24,46]. We examined changes in placental gene expression 3 h following exposure to this stimulus because previous research has shown that even this soon after injection pregnant mice show significant behavioral and systemic inflammatory changes mimicking infection [24,47,48]. Antenatal poly(I:C) exposure has been shown to differentially alter behaviors in male vs. female mouse offspring [49]. To our knowledge, this is the first study that has examined changes in numerous inflammatory genes within the placenta provoked by poly(I:C) in a sex-specific manner. Hsiao and Patterson investigated the impact of MIA on placental gene expression in a small number of prespecified inflammatory genes in C57BL/6 J mice finding that inflammatory gene expression was markedly induced 3 h following poly(I:C) exposure [50]. This study however did not examine sex-specific differences in gene expression.

Pathway analysis in the MIA model demonstrated the most hits in inflammation-related pathways such as TLR signaling, interleukin signaling, and cytokine and chemokine signaling in both male and female placentae. The female placentae exhibited a higher degree of differential regulation in the insulin/IGF/MAPK pathway, indicating that genes within this pathway may be important to the female response to inflammatory stress. Although few studies have focused on this pathway specifically, one study of 987 healthy singleton pregnancies found that cord-blood from females had increased concentrations of insulin-like-growth factors (IGF)-1 and IGFBP-3 compared to males [51]. The IGF axis has been reported to be differentially regulated in a sex-dependent manner in other inflammatory related diseases during pregnancy such as asthma [52]. The role for the IGF axis within the

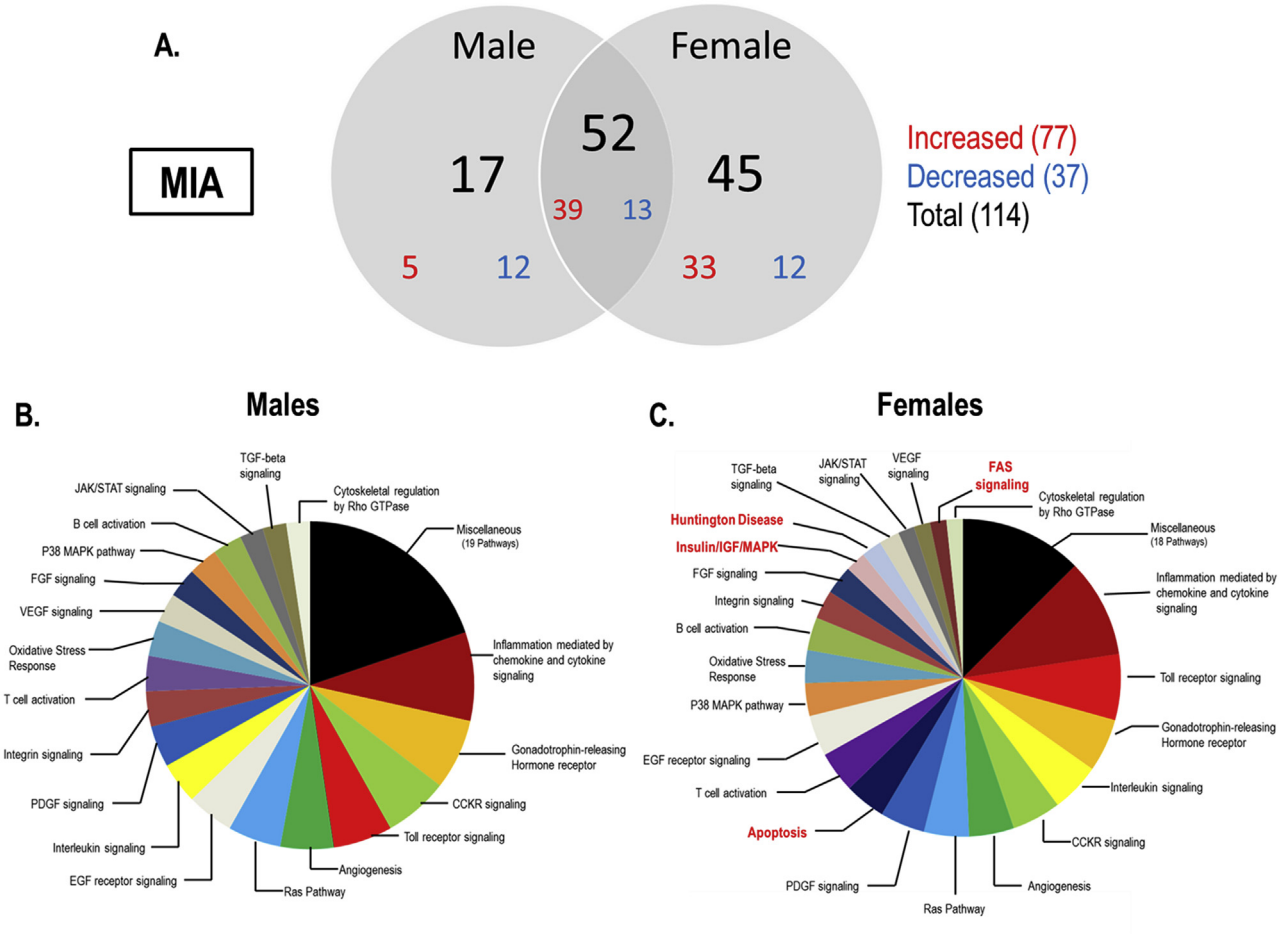

Fig. 3. Sex-associated changes in gene expression in the placenta induced by maternal immune activation (MIA) compared to the saline treated, normal-diet controls. (A) Number of genes whose expression levels were statistically-significantly impacted by MIA compared to control ($p < 0.05$). Large black numbers indicate total number of genes altered regardless of directionality. Red numbers indicate number of genes whose expression levels increased due to MIA and blue numbers indicate the number of genes who expression levels decreased as a result of MIA. (B and C) Pathways perturbed in MIA irrespective of change in gene expression directionality. All pathways which contained ≤3 genes were grouped together in the miscellaneous category. Pathways listed in red bold print are unique to that sex. Data are displayed for male (B) and female (C) placentae separately. Number of genes included in this figure were statistically-significantly impacted by the indicated treatment ($p < 0.05$). See methods for detailed description.

placenta has not been well-established although its possible importance in the sex-dependent response to inflammatory insults should be investigated further.

Both obesity during pregnancy and GDM are associated with chronic systemic inflammation and have been implicated in provoking placental inflammation [29,53]. The impact of obesity on placental inflammation appears to affect male and female tissues differently [54] and mice fed a high fat diet have been found to have divergent patterns of gene expression in male vs. female placentae [35]. GDM has not, to our knowledge, been associated with sex differences in placental gene expression. Our results show sexual dimorphism in GDM-induced inflammation-related gene expression changes within the placenta. Pathway analysis revealed two pathways more highly changed in males; the cytoskeletal regulation by Rho GTPase and the Wnt signaling pathways. Neither of these pathways have been previously implicated in sex-dependent placental gene expression alterations as a result of GDM. Similar to MIA, the female placentae showed a higher degree of change in the Insulin/IGF/MAPK signaling pathway. Thus, reiterating the possible importance of this pathway in sex-driven gene expression changes within the placenta as a result of inflammatory stress. Studies have interrogated the IGF axis as it relates to fetal development and outcomes in maternal asthma, however not in the context of GDM [55].

A fascinating aspect of our study was the combination of GDM and MIA. A total of 123 genes within the placenta were significantly different compared to control in the combination of GDM and MIA and 74 of these genes were shared between male and female placentae. The insulin/IGF/MAPK pathway was more significantly changed in female placentae compared to male, similar to the GDM and MIA conditions alone. In addition to the insulin/IGF/MAPK pathway, the TGF-β signaling pathway was more significantly changed in female placentae. TGF-β has been implicated in supporting maternal-fetal immune tolerance [56] so disturbing its expression could have important consequences. Like what we observed in GDM alone, the Wnt signaling pathway was more significantly altered in male placentae. In addition, the p53 feedback loop pathway was more significantly altered in males. Alterations in the p53 feedback loop pathway have been implicated in increased levels of apoptosis within the placenta in the context of preeclampsia [57] although no link to GDM or MIA has been established. Our analyses highlight both the number of pathways that are perturbed and the degree to which GDM and MIA influence gene expression patterns within those pathways in a sex-dependent manner as depicted by the change in appearance of the pie graphs for each treatment group (Figs. 2–4). An interesting finding was that while GDM had more male-predominant gene expression changes, MIA affected more the females, while, in general, GDM + MIA did not exhibit such a distinct effect. It will be important for future studies to establish how sex influences these pathways. It is also interesting that while both GDM and MIA impacted gene expression in placental tissues, the

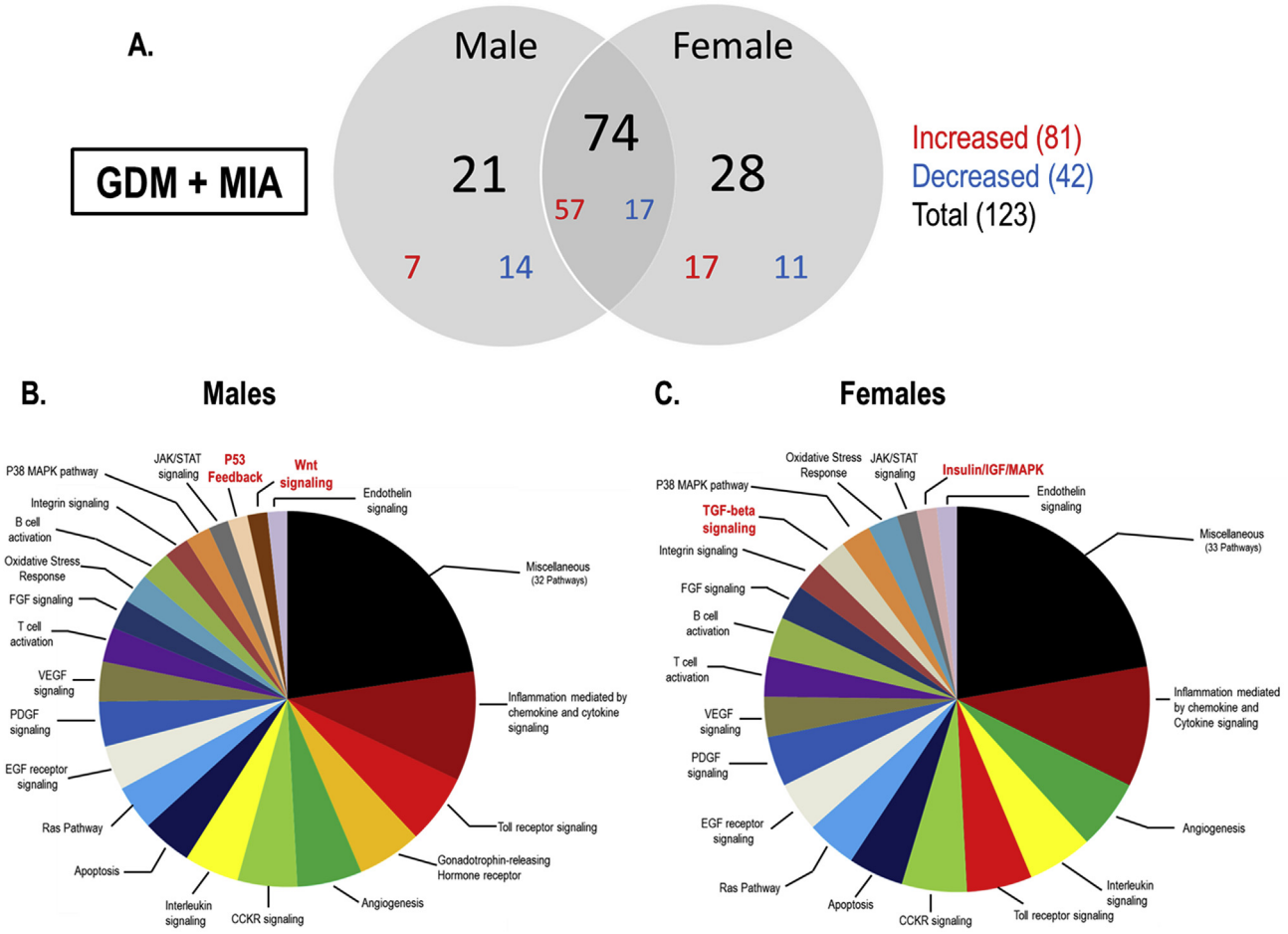

Fig. 4. Sex-associated changes in gene expression in the placenta induced by the combination of maternal immune activation and diet-induced gestational diabetes (GDM + MIA) compared to the saline treated, normal-diet controls. (A) Number of genes whose expression levels were statistically-significantly impacted by the combination of GDM and MIA compared to control ($p < 0.05$). Large black numbers indicate total number of genes altered regardless of directionality. Red numbers indicate number of genes whose expression levels increased due to GDM + MIA and blue numbers indicate the number of genes who expression levels decreased as a result of GDM + MIA. (B and C) Pathways perturbed in GDM + MIA irrespective of change in gene expression directionality. Pathways listed in red bold print are unique to that sex. All pathways which contained ≤3 genes were grouped together in the miscellaneous category. Data are displayed for male (B) and female (C) placentae separately. Number of genes included in this figure were statistically-significantly impacted by the indicated treatment ($p < 0.05$). See methods for detailed description.

magnitude of the effect of poly(I:C) exposure was greater than that induced by the metabolic stress of the high fat diet, which was best evidenced in the PCA plot in Fig. 1.

Limitations of our study are important. Gene expression was assessed using a preselected set of 248 immune genes (compiled by the commercial vendor after querying several public databases for inflammation-related genes), which introduces bias and limits conclusions regarding other functions of the placenta, such as nutrient transport or metabolism. It is possible that a larger set of genes would yield different results. Our GDM model was generated with a high fat diet, which itself could produce inflammation in the absence of diabetes [58]. We used this model because mice consistently exhibited enhanced glucose intolerance and hyperinsulinemia following a glucose challenge only while pregnant [24]. The use of poly(I:C) to provoke a systemic inflammatory response mimicking a viral infection during pregnancy has advantages, including the lack of live virus that might cross the placenta and a more consistent, controlled inflammatory response. However, a viral mimetic lacks the complexities of host-pathogen interactions, thus limiting generalizability. The use of a mouse model is an important caveat, given differences between human and mouse placentae [59]. Our investigations did not examine associations between placental gene expression and behavioral or other outcomes in offspring, which awaits future study. Another limitation is a lack of understanding of the exact mechanisms whereby these antenatal stressors, singly and in combination, impact gene expression. Whether through epigenetic or other modifications, such mechanisms await future studies. We attempted to remove decidual tissue from placental tissue surgically but cannot rule out maternal contamination of placental specimens. Despite these limitations, our work sheds new light on the convergence of stressors at the maternal-fetal interface that deserves ongoing attention.

This work suggests that common antenatal stressors impact immune gene expression within the placenta and appear to interact. Placental sex can influence the relationship between stress and immune homeostasis, supporting a placental role in the sexual dimorphism observed in human clinical studies of DOHaD-related health outcomes. Future studies should continue to model multiple stressors and pay heed to sex-related effects.

Conflicts of interest

There is no conflict of interest to disclose for any of the authors of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.02.008.

References

[1] J.G. Eriksson, Developmental Origins of Health and Disease - from a small body size at birth to epigenetics, Ann. Med. 48 (6) (2016) 456–467.
[2] B. Brenseke, M.R. Prater, J. Bahamonde, J.C. Gutierrez, Current thoughts on maternal nutrition and fetal programming of the metabolic syndrome, Journal of pregnancy 2013 (2013) 368461.
[3] R.M. Breyer, C.K. Bagdassarian, S.A. Myers, M.D. Breyer, Prostanoid receptors: subtypes and signaling, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 661–690.
[4] A.S. Tomar, D.S.P. Tallapragada, S.S. Nongmaithem, S. Shrestha, C.S. Yajnik, G.R. Chandak, Intrauterine programming of diabetes and adiposity, Current Obesity Reports 4 (4) (2015) 418–428.
[5] J. Yan, H. Yang, Gestational diabetes mellitus, programing and epigenetics, J. Matern. Fetal Neonatal Med. 27 (12) (2014) 1266–1269.
[6] C.L. Howerton, T.L. Bale, Prenatal programing: at the intersection of maternal stress and immune activation, Horm. Behav. 62 (3) (2012) 237–242.
[7] C. Monk, M.K. Georgieff, E.A. Osterholm, Research review: maternal prenatal distress and poor nutrition - mutually influencing risk factors affecting infant neurocognitive development, JCPP (J. Child Psychol. Psychiatry) 54 (2) (2013) 115–130.
[8] M. Rupérez, R. González, S. Maculuve, L. Quintó, E. López-Varela, O. Augusto, A. Vala, A. Nhacolo, E. Sevene, D. Naniche, C. Menéndez, Maternal HIV infection is an important health determinant in non-HIV-infected infants, AIDS 31 (11) (2017) 1545–1553.
[9] E.F. Sutton, L.A. Gilmore, D.B. Dunger, B.T. Heijmans, M.F. Hivert, C. Ling, J.A. Martinez, S.E. Ozanne, R.A. Simmons, M. Szyf, R.A. Waterland, L.M. Redman, E. Ravussin, Developmental programming: state-of-the-science and future directions-Summary from a Pennington Biomedical symposium, Obesity 24 (5) (2016) 1018–1026.
[10] J.S. Gilbert, M.J. Nijland, Sex differences in the developmental origins of hypertension and cardiorenal disease, Am. J. Physiol. Regul. Integr. Comp. Physiol. 295 (6) (2008) R1941–R1952.
[11] M. Gannon, R.N. Kulkarni, H.M. Tse, F. Mauvais-Jarvis, Sex differences underlying pancreatic islet biology and its dysfunction, Molecular metabolism 15 (2018) 82–91.
[12] L. Konkel, Lasting impact of an ephemeral organ: the role of the placenta in fetal programming, Environ. Health Perspect. 124 (7) (2016) A124.
[13] S. Peng, M.A. Deyssenroth, A.F. Di Narzo, L. Lambertini, C.J. Marsit, J. Chen, K. Hao, Expression quantitative trait loci (eQTLs) in human placentas suggest developmental origins of complex diseases, Hum. Mol. Genet. 26 (17) (2017) 3432–3441.
[14] G.C. Di Renzo, E. Picchiassi, G. Coata, G. Clerici, E. Brillo, Is there a sex of the placenta? Journal of Pediatric and Neonatal Individualized Medicine (JPNIM) 4 (2) (2015) e040246.
[15] C.S. Rosenfeld, Sex-specific placental responses in fetal development, Endocrinology 156 (10) (2015) 3422–3434.
[16] E.M. Sedlmeier, S. Brunner, D. Much, P. Pagel, S.E. Ulbrich, H.H. Meyer, U. Amann-Gassner, H. Hauner, B.L. Bader, Human placental transcriptome shows sexually dimorphic gene expression and responsiveness to maternal dietary n-3 long-chain polyunsaturated fatty acid intervention during pregnancy, BMC Genomics 15 (2014) 941.
[17] J.A. Goldstein, S.A. Norris, D.M. Aronoff, DOHaD at the intersection of maternal immune activation and maternal metabolic stress: a scoping review, Journal of developmental origins of health and disease 8 (3) (2017) 273–283.
[18] E.A.H. Sims, E. Danforth Jr., E.S. Horton, G.A. Bray, J.A. Glennon, L.B. Salans, Endocrine and metabolic effects of experimental obesity in Man1 A2 - GREEP, ROY O, Proceedings of the 1972 Laurentian Hormone Conference, Academic Press, Boston, 1973, pp. 457–496.
[19] G. Desoye, M. van Poppel, The feto-placental dialogue and diabesity, Best Pract. Res. Clin. Obstet. Gynaecol. 29 (1) (2015) 15–23.
[20] Y.A. Bobetsis, S.P. Barros, D.M. Lin, J.R. Weidman, D.C. Dolinoy, R.L. Jirtle, K.A. Boggess, J.D. Beck, S. Offenbacher, Bacterial infection promotes DNA hypermethylation, J. Dent. Res. 86 (2) (2007) 169–174.
[21] K.M. Adams Waldorf, R.M. McAdams, Influence of infection during pregnancy on fetal development, Reproduction 146 (5) (2013) R151–R162.
[22] A. Zager, J.P. Peron, G. Mennecier, S.C. Rodrigues, T.P. Aloia, J. Palermo-Neto, Maternal immune activation in late gestation increases neuroinflammation and aggravates experimental autoimmune encephalomyelitis in the offspring, Brain Behav. Immun. 43 (2015) 159–171.
[23] B.M. Popkin, L.S. Adair, S.W. Ng, Global nutrition transition and the pandemic of obesity in developing countries, Nutr. Rev. 70 (1) (2012) 3–21.
[24] K.M. Money, T.L. Barke, A. Serezani, M. Gannon, K.A. Garbett, D.M. Aronoff, K. Mirnics, Gestational Diabetes Exacerbates Maternal Immune Activation Effects in the Developing Brain, (2017).
[25] L. Shi, S.H. Fatemi, R.W. Sidwell, P.H. Patterson, Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring, J. Neurosci. 23 (1) (2003) 297–302.
[26] L. Shi, N. Tu, P.H. Patterson, Maternal influenza infection is likely to alter fetal brain development indirectly: the virus is not detected in the fetus, Int. J. Dev. Neurosci. 23 (2–3) (2005) 299–305.
[27] J. Trowsdale, A.G. Betz, Mother's little helpers: mechanisms of maternal-fetal tolerance, Nat. Immunol. 7 (3) (2006) 241–246.
[28] S. Altmae, M.T. Segura, F.J. Esteban, S. Bartel, P. Brandi, M. Irmler, J. Beckers, H. Demmelmair, C. Lopez-Sabater, B. Koletzko, S. Krauss-Etschmann, C. Campoy, Maternal pre-pregnancy obesity is associated with altered placental transcriptome, PLoS One 12 (1) (2017) e0169223.
[29] P. Pantham, I.L. Aye, T.L. Powell, Inflammation in maternal obesity and gestational diabetes mellitus, Placenta 36 (7) (2015) 709–715.
[30] E.L. Johnson, R. Chakraborty, HIV-1 at the placenta: immune correlates of protection and infection, Curr. Opin. Infect. Dis. 29 (3) (2016) 248–255.
[31] M.M. Jacobs, R.L. Fogg, R.B. Emeson, G.D. Stanwood, ADAR1 and ADAR2 expression and editing activity during forebrain development, Dev. Neurosci. 31 (3) (2009) 223–237.
[32] M. Arenas-Hernandez, E.N. Sanchez-Rodriguez, T.N. Mial, S.A. Robertson, N. Gomez-Lopez, Isolation of Leukocytes from the Murine Tissues at the Maternal-Fetal Interface, JoVE : JoVE 99 (2015) e52866.
[33] H. Mi, X. Huang, A. Muruganujan, H. Tang, C. Mills, D. Kang, P.D. Thomas, PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements, Nucleic Acids Res. 45 (D1) (2017) D183–d189.
[34] C.M. Reynolds, M.H. Vickers, C.J. Harrison, S.A. Segovia, C. Gray, Maternal high fat and/or salt consumption induces sex-specific inflammatory and nutrient transport in the rat placenta, Physiological reports 3 (5) (2015).
[35] A. Gabory, L. Ferry, I. Fajardy, L. Jouneau, J.D. Gothie, A. Vige, C. Fleur, S. Mayeur, C. Gallou-Kabani, M.S. Gross, L. Attig, A. Vambergue, J. Lesage, B. Reusens, D. Vieau, C. Remacle, J.P. Jais, C. Junien, Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta, PLoS One 7 (11) (2012) e47986.
[36] D.W. Kim, S.L. Young, D.R. Grattan, C.L. Jasoni, Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse, Biol. Reprod. 90 (6) (2014) 130.
[37] A. Andres, H.R. Hull, K. Shankar, P.H. Casey, M.A. Cleves, T.M. Badger, Longitudinal body composition of children born to mothers with normal weight, overweight, and obesity, Obesity (Silver Spring, Md 23 (6) (2015) 1252–1258.
[38] A. Gabory, T.J. Roseboom, T. Moore, L.G. Moore, C. Junien, Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics, Biol. Sex Differ. 4 (1) (2013) 5.
[39] T.L. Bale, The placenta and neurodevelopment: sex differences in prenatal vulnerability, Dialogues Clin. Neurosci. 18 (4) (2016) 459–464.
[40] V.L. Clifton, Sexually dimorphic effects of maternal asthma during pregnancy on placental glucocorticoid metabolism and fetal growth, Cell Tissue Res. 322 (1) (2005) 63–71.
[41] B.R. Mueller, T.L. Bale, Sex-specific programming of offspring emotionality after stress early in pregnancy, J. Neurosci. : the official journal of the Society for Neuroscience 28 (36) (2008) 9055–9065.
[42] P.E. Verburg, G. Tucker, W. Scheil, J.J. Erwich, G.A. Dekker, C.T. Roberts, Sexual dimorphism in adverse pregnancy outcomes - a retrospective Australian population study 1981-2011, PLoS One 11 (7) (2016) e0158807.
[43] S.L. Bronson, T.L. Bale, Prenatal stress-induced increases in placental inflammation and offspring hyperactivity are male-specific and ameliorated by maternal antiinflammatory treatment, Endocrinology 155 (7) (2014) 2635–2646.
[44] R. Sood, J.L. Zehnder, M.L. Druzin, P.O. Brown, Gene expression patterns in human placenta, Proc. Natl. Acad. Sci. U.S.A. 103 (14) (2006) 5478–5483.
[45] T.L. Gonzalez, T. Sun, A.F. Koeppel, B. Lee, E.T. Wang, C.R. Farber, S.S. Rich, L.W. Sundheimer, R.A. Buttle, Y.I. Chen, J.I. Rotter, S.D. Turner, J. Williams 3rd, M.O. Goodarzi, M.D. Pisarska, Sex differences in the late first trimester human placenta transcriptome, Biol. Sex Differ. 9 (1) (2018) 4.
[46] S. Reisinger, D. Khan, E. Kong, A. Berger, A. Pollak, D.D. Pollak, The poly(I:C)-induced maternal immune activation model in preclinical neuropsychiatric drug discovery, Pharmacol. Ther. 149 (2015) 213–226.
[47] M. Mandal, R. Donnelly, S. Elkabes, P. Zhang, D. Davini, B.T. David, N.M. Ponzio, Maternal immune stimulation during pregnancy shapes the immunological phenotype of offspring, Brain Behav. Immun. 33 (2013) 33–45.
[48] S.E. Smith, J. Li, K. Garbett, K. Mirnics, P.H. Patterson, Maternal immune activation alters fetal brain development through interleukin-6, J. Neurosci. : the official journal of the Society for Neuroscience 27 (40) (2007) 10695–10702.
[49] I.C. Xuan, D.R. Hampson, Gender-dependent effects of maternal immune activation on the behavior of mouse offspring, PLoS One 9 (8) (2014) e104433.
[50] E.Y. Hsiao, P.H. Patterson, Activation of the maternal immune system induces endocrine changes in the placenta via IL-6, Brain Behav. Immun. 25 (4) (2011) 604–615.

[51] M.P. Geary, P.J. Pringle, C.H. Rodeck, J.C. Kingdom, P.C. Hindmarsh, Sexual dimorphism in the growth hormone and insulin-like growth factor axis at birth, J. Clin. Endocrinol. Metab. 88 (8) (2003) 3708–3714.
[52] V.L. Clifton, N.A. Hodyl, V.E. Murphy, W.B. Giles, R.C. Baxter, R. Smith, Effect of maternal asthma, inhaled glucocorticoids and cigarette use during pregnancy on the newborn insulin-like growth factor axis, Growth Hormone IGF Res. : official journal of the Growth Hormone Research Society and the International IGF Research Society 20 (1) (2010) 39–48.
[53] J.E. Hsu, T.A. Jones, Time-sensitive enhancement of motor learning with the less-affected forelimb after unilateral sensorimotor cortex lesions in rats, Eur. J. Neurosci. 22 (8) (2005) 2069–2080.
[54] S.M. Leon-Garcia, H.A. Roeder, K.K. Nelson, X. Liao, D.P. Pizzo, L.C. Laurent, M.M. Parast, D.Y. LaCoursiere, Maternal obesity and sex-specific differences in placental pathology, Placenta 38 (2016) 33–40.
[55] V.L. Clifton, Review: sex and the human placenta: mediating differential strategies of fetal growth and survival, Placenta 31 (Suppl) (2010) S33–S39.
[56] J. Alijotas-Reig, E. Llurba, J.M. Gris, Potentiating maternal immune tolerance in pregnancy: a new challenging role for regulatory T cells, Placenta 35 (4) (2014) 241–248.
[57] A.N. Sharp, A.E. Heazell, D. Baczyk, C.E. Dunk, H.A. Lacey, C.J. Jones, J.E. Perkins, J.C. Kingdom, P.N. Baker, I.P. Crocker, Preeclampsia is associated with alterations in the p53-pathway in villous trophoblast, PLoS One 9 (1) (2014) e87621.
[58] D. Zhou, Y.X. Pan, Pathophysiological basis for compromised health beyond generations: role of maternal high-fat diet and low-grade chronic inflammation, J. Nutr. Biochem. 26 (1) (2015) 1–8.
[59] A. Malassine, J.L. Frendo, D. Evain-Brion, A comparison of placental development and endocrine functions between the human and mouse model, Hum. Reprod. Update 9 (6) (2003) 531–539.