THE INTERSECTION OF MATERNAL INFLAMMATORY STRESS AND THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

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To my greatest accomplishments

My daughters, Ilyse and Natalie

&

To my fiancé Jason

The one and only true love of my life

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LIST OF ABBREVIATIONS

ASC ..... apoptosis-associated speck-like protein containing CARD

ASD ..... autism spectrum disorder

BMI ..... body mass index

CI ..... confidence interval

Ct ..... detection cycle threshold

CTR ..... control

CXCL ..... C-X-C chemokine ligand

CXCR ..... C-X-C chemokine receptor

DAMP ..... danger associated molecular pattern

DNA ..... deoxyribose nucleic acid

DOHaD ..... developmental origins of health and disease

dsRNA ..... double-stranded ribonucleic acid

ERK ..... extracellular signal-regulated kinase

Fe ..... iron

FFA ..... free fatty acid

FFPE ..... formalin fixed paraffin embedded

GD ..... gestational day

GDM ..... gestational diabetes mellitus

H&E ..... Hematoxylin and eosin stain

HBC ..... hofbauer cell

Hct ..... hematocrit

HFD ..... high fat diet

HFM ..... high fat fed poly(I:C) treated

HFS ..... high fat fed saline treated

ICD ..... International Classification of Diseases

IFN ..... interferon

IL ..... interleukin

IRF ..... Interferon regulatory factor

JNK ..... c-Jun N-terminal kinase

LPS ..... lipopolysaccharide

MAPK ..... Mitogen-activated protein kinase

MCP ..... monocyte chemoattractant protein

MetaC ..... metabolic cocktail

MHC ..... major histocompatibility complex

MIA ..... maternal immune activation

MyD88 ..... myeloid differentiation primary response 88

NCD ..... non-communicable disease

NFκB ..... nuclear factor kappa B

NLR ..... nucleotide-oligomerization domain-like receptors

OGTT ..... oral glucose tolerance test

OR ..... odds ratio

PAMP ..... pathogen associated molecular pattern

PCA ..... principle component analysis

PM ..... placental macrophage

poly(I:C) ..... polyinosinic-polycytidylic acid

PRR ..... pattern recognition receptor

qPCR ..... quantitative polymerase chain reaction

RANTES ..... regulated on activation, normal T cell expressed and secreted factor

RNA ..... ribonucleic acid

Rs-LPS ..... *Rhodobacter sphaeroides* lipopolysaccharide

SAL ..... control fed saline treated

SD ..... synthetic derivative

SEM ..... standard error of the mean

STAT ..... signal transducer and activator of transcription

STZ ..... streptozotocin

T2D ..... type-2 diabetes

TGF ..... transforming growth factor

Th1 ..... T helper lymphocyte type 1

Th17 ..... T helper lymphocyte type 17

Th2 ..... T helper lymphocyte type 2

TIR ..... Toll/IL-1R homology

TLR ..... toll-like receptor

TNF ..... tumor necrosis factor

TRIF ..... TIR-domain-containing adapter-inducing interferon-β

CHAPTER 1

Introduction

The prenatal environment is influenced by a number of factors; the most important of which being the maternal environment. There is a growing body of evidence suggesting that the maternal and intrauterine milieu, through the action of inflammatory mediators, hormones, and nutrient availability may permanently change the health of the fetus (Barker, 1995, 2004). This work will focus on two possible environmental stressors, both of which have the potential to influence fetal development and programming; gestational diabetes mellitus (GDM) and maternal immune activation (MIA).

Gestational diabetes mellitus is the most common metabolic disorder during pregnancy and causes considerable morbidity, mortality, and long-term complications for both mother and child ("Gestational diabetes mellitus," 2004; Monteiro, Norman, Rice, & Illanes, 2016). A major barrier to reducing the incidence and burden of GDM is a lack of clarity regarding the underlying pathophysiological mechanisms and environmental factors that contribute to these complications. Aberrant systemic inflammation is associated with a multitude of metabolic disorders, including GDM and maternal obesity (Gregor & Hotamisligil, 2011). Maternal immune activation (MIA), another prenatal insult, occurs as the result of an infection during pregnancy and activates that maternal immune system (Howerton & Bale, 2012). Several epidemiological as well as clinical studies have shown associations between infection and excessive inflammation during pregnancy and an increased risk for developing neurocognitive disorders such as autism and schizophrenia in offspring (Buka et al., 2001; M. Cannon, P. B. Jones, & R. M. Murray, 2002; Gardener, Spiegelman, & Buka, 2009). The aberrant inflammation caused by GDM and maternal obesity is

also gaining evidence as a possible cause for the same neurocognitive disorders (M. Cannon et al., 2002; Xiang et al., 2015). In order for progress to be made in the prevention and treatment of these disorders it is important to identify both the biological and environmental factors underlying disorder pathogenesis.

The research put forth in this document will focus primarily on the interactions of metabolic stress and immune activation. More specifically the impact of GDM on placental structure, inflammation-associated risk factors during pregnancy, pathological alterations within the fetal brain and placenta as a result of inflammation, and how maternal inflammation influences placental cellular structure. The role of sexual dimorphism will also be addressed as it relates to the placental response to metabolic stress and maternal immune activation.

The Placenta: Anatomy, function, and importance

The placenta is a transient, but complex organ, acting as the link between the mother and the developing fetus. This maternal-fetal structure acts as the interface between the uterine mucosa and the extraembryonic tissues of the developing fetus. It plays important roles throughout pregnancy, maintaining intrauterine homeostasis, mediating the selective exchange of nutrients, waste, gases, and other modulatory factors across the maternal/fetal interface (Lo & Frias, 2017). The placenta also orchestrates and maintains maternal/fetal immune tolerance and acts in an endocrine fashion by producing key modulatory hormones while separating the fetus from a possibly adverse or harmful maternal environment (Lo & Frias, 2017).

The placenta is composed of both maternally and fetally-derived cells (Huppertz, 2008). The uterus and maternally-derived decidua contain large numbers of maternal leukocytes such as uterine natural killer cells, compromising roughly 30-40% of the total cells in the first trimester

decidua, macrophages, remaining stable at 10% to 15% of the total cells within the decidua throughout pregnancy, and dendritic cells all of which display a unique uterine and placental phenotype (Hunt, Petroff, & Burnett, 2000; Schumacher & Sharkey, 2018). The presence of these cells and their proper contextual phenotype is essential for successful implantation and a subsequent successful and healthy pregnancy. Maternal blood supplied from the spiral arteries and fetal blood coming in from the umbilical arteries exchange waste and nutrients within an area known as the labyrinth zone in mice or the chorionic villous in human. Separating the maternal and fetal endothelial cells is a layer of trophoblast cells that function to control maternal-fetal exchange, to prevent the direct mixing of maternal and fetal blood circulations, and to coordinate maternal-fetal tolerance (Hsiao & Patterson, 2012). In order for proper placentation to occur increased vascular expansion is crucial. This is made possible through the invasion of fetal trophoblasts into the decidua and the expansion of maternal spiral arteries into the villous core. This vascular remodeling is orchestrated with the help of maternal leukocytes in order to ensure sufficient blood supply for the growing fetus. (Harris et al., 2010; Hazan et al., 2010; Moffett & Loke, 2006).

Although mouse and human pregnancies are similar in their hemochorial mode of placentation and the accumulation of uterine natural killer cells within the decidua during placentation, they differ in many ways. The obvious is that mouse pregnancies last approximately 20 days where as human gestation takes 9 months. Another significant difference is the architecture of the labyrinth zone and villous core of the placenta where fetal-maternal exchange occurs. Within the mouse placenta, the placental trophoblasts do not invade into the decidual arterioles as deeply and invasively as they do in the human placenta. In humans however, placental trophoblasts are much more intrusive and actually temporarily replace

maternal endothelial cells stretching all the way to the smooth muscle of the uterus creating a much more vascular environment (Erlebacher, 2013). These differences account for some of the differential conclusions when comparing mouse models of maternal-fetal outcomes to human outcomes. Despite these differences, the relationship between mouse and human studies can reveal promising insights in to pathophysiological mechanisms driving an array of human pregnancy complications.

Maternal-fetal tolerance is essential to a successful pregnancy. Thus, a delicate balance of fetal immune tolerance, without overt maternal immunosuppression, is required for the survival of the developing fetus (T. Jansson & Powell, 2007). The maternal-fetal interface utilizes multiple unique mechanisms to ensure maternal tolerance to the developing fetus. The maternal decidua plays a seminal role in orchestrating systemic immunological tolerance of the developing embryo (Arck & Hecher, 2013). Some of the multiple mechanisms of tolerance include fetal trophoblast cell microchimerism, trophoblast shedding in order to desensitize maternal circulating cells to paternal alloantigens, limited MHC expression on trophoblasts, and dendritic cell entrapment which limits their migration from the decidua to the uterine lymph nodes (Aluvihare, Kallikourdis, & Betz, 2004; Erlebacher, 2013; Makhseed et al., 2001). Any significant disruption in fetal immune tolerance can cause fetal rejection and/or impaired placental function, which has devastating effects on pregnancy success.

The placenta, although transient in nature, is a dynamic organ paramount to the interrelationship between mother and fetus. The studies presented in this work are important in order to expand our understanding of how environmental stressors such as maternal inflammation caused by infectious agents and GDM and metabolic stress affect placental health

therefore increasing our understanding of how these processes can be modified to improve fetal outcome.

Placental macrophages

Macrophages function as a first line of antimicrobial defense. As an abundant leukocyte population within the placenta, totaling ~20% of the total leukocytes within the decidua, macrophages play an important role at the maternal-fetal interface as initiators and targets of immune modulation (Erlebacher, 2013). Placental macrophages (PMs) consist of both fetally-derived macrophages, known as Hofbauer cells (HBC), which are found in the villous core, and maternally-derived decidual macrophages, which reside in the maternal decidua. HBCs have been characterized, in healthy pregnancies, as having an anti-inflammatory profile, characterized by high cell surface expression of the receptors CD163, CD209, and CD206 and increased secretion of IL-10 and transforming growth factor (TGF)-β (Gustafsson et al., 2008; Svensson et al., 2011; Z. Tang et al., 2011). Furthermore, the DNA methylation pattern of HBCs is consistent with an anti-inflammatory phenotype (S. Y. Kim et al., 2012). Placental macrophages are known to perform various functions including matrix remodeling during placental development, maintaining fetal tolerance, host defense, and the initiation of parturition (Erlebacher, 2013). The documented anti-inflammatory phenotype and importance of placental macrophages in maintaining immune tolerance suggest that these cells not only protect against infection, but also play a regulatory role within the placenta.

Macrophages within the placenta and decidua play various pivotal roles during pregnancy; from conception, to implantation, to parturition. The importance of the macrophage begins at insemination. The introduction of seminal fluid into the uterus induces the production

of various cytokines and chemokines into the stromal compartment recruiting innate immune cells including macrophages, dendritic cells, and granulocytes (Schjenken, Glynn, Sharkey, & Robertson, 2015). The newly recruited macrophages allow for successful implantation by clearing away debris, infectious agents, and excess sperm that may have been introduced during insemination (De, Choudhuri, & Wood, 1991). It was recently shown that the ablation of $CD11b^+$ macrophages in mice resulted in implantation failure stressing their importance in the successful origination of pregnancy (Care et al., 2013). Human decidual macrophages have been shown to localize in close proximity to invading trophoblasts early in pregnancy suggesting their potentially important role in successful invasion and placentation (Helige et al., 2014). As pregnancy progresses, the macrophage takes on other roles such as the secretion of immunosuppressive molecules and the presentation of reproductive antigens to T-cells contributing to maternal-fetal tolerance (Schumacher & Sharkey, 2018). Lastly, the initiation of parturition is partially induced by a macrophage phenotype switch from that of an anti-inflammatory phenotype, which sustains pregnancy, to a pro-inflammatory phenotype characterized by the release of pro-inflammatory cytokines and mediators such as prostaglandin E_2 (Nagamatsu & Schust, 2010). Numerous studies have concluded that macrophages play an important role throughout pregnancy from insemination through parturition.

The varied, yet critical, functions that the macrophage displays within the context of the placenta make its understanding a difficult and overwhelming endeavor in the context of both healthy and diseased states. Yet, given the pleiotropic nature of macrophages in essentially all organ systems, they are a natural candidate to study for their importance in maintaining a healthy pregnancy and regulating the maternal-fetal interface. These studies highlight how

environmental stressors have the ability to influence basic macrophage function and phenotype in an already established pregnancy.

Macrophages and their role in iron homeostasis

Iron is critical for the function of many systems, including the immune system. Macrophages are involved in iron homeostasis and couple this iron cycling to activation status. Tissue resident macrophages are responsible for roughly 80% of the iron (Fe) availability within the body on a daily basis making them critical to iron homeostasis and availability (Andrews, 2008). The role of the macrophage in Fe homeostasis is essential as mammals do not have the ability to secrete excess Fe in a regulated fashion. As a way to reduce high extracellular Fe levels macrophages sequester excess Fe. The sequestration of high levels of extracellular Fe can lead to phenotypic changes within the macrophage leading to the release of pro-inflammatory mediators (Cairo, Recalcati, Mantovani, & Locati, 2011). Macrophage polarization and inflammatory status are associated with differential expression patterns of genes related to Fe storage, uptake, and release. Pro-inflammatory macrophages increase expression of proteins associated with Fe storage while decreasing the expression of heme importers and Fe exporters. In contrast, anti-inflammatory macrophages increase the expression of heme importers and Fe exporters in order to decrease the intracellular storage of Fe (Cairo et al., 2011).

Iron-cycling in macrophages involves multiple proteins. Fe can enter the macrophage through multiple different mechanisms. Fe bound to transferrin is taken up into cells through the transferrin receptor (TfrC). Extracellular heme complexes with hemopexin in the plasma and is taken in through CD91. Hemoglobin bound to its plasma carrier haptoglobin is internalized through CD163. Within the endosome, heme-bound Fe is released through the action of heme-

oxygenase 1 (Hmox1) then transported out of the endosome through Nramp1 and DMT1. Iron can be stored within the macrophage in the form of ferritin (Fth1 and Ftl) or can be exported through the iron exported ferroportin (Fpn).

CD163 is a hemoglobin scavenger receptor exclusively expressed on macrophages. CD163 is a signal-inducing receptor that scavenges hemoglobin through the endocytosis of hemoglobin-haptoglobin (Hb:Hp) complexes (Kristiansen et al., 2001). The pro-inflammatory mediators IFN-γ, TNF-α, and Lipopolysaccharide (LPS), a gram negative bacterial cell wall component, suppress the expression of CD163 while IL-6 and IL-10 increase its expression (Buechler et al., 2000). The binding of the Hb:Hp complex to CD163 initiates a heme-oxygenase-dependent anti-inflammatory signaling pathway eliciting IL-10 secretion initiating a positive feedback loop ultimately resulting in increased expression of CD163 and heme-oxygenase-1 (Landis, Philippidis, Domin, Boyle, & Haskard, 2013; Philippidis et al., 2004). CD163 also exists in a soluble form (sCD163) (Etzerodt, Maniecki, Moller, Moller, & Moestrup, 2010; Moller, Nielsen, Maniecki, Madsen, & Moestrup, 2010). sCD163 is a biomarker for a number of inflammation related diseases. Recent evidence has correlated elevated levels of sCD163 to such conditions as GDM (Bari et al., 2014). The role of macrophages in iron-cycling and homeostasis has been described within the liver, spleen, and adipose tissue; however, no such mechanism has been investigated in the placenta. The significance of how iron and iron storage within the placenta change in response to maternal stressors will be investigated in this work.

Iron is transferred to the infant from maternal circulation by way of the placenta. It has been reported that the presence of maternal stressors, such as psychological stress, can influence the exchange of micronutrients across the placenta. It was reported that infants born to mothers

who suffered stress during pregnancy developed iron deficiency anemia soon after birth (Lubach & Coe, 2006). In rodent models, low levels of iron in neonates is associated with decreased numbers of macrophages, decreased natural killer cell activity, and an impairment in antibody formation all leading to increased susceptibility to neonatal infection (Hallquist, McNeil, Lockwood, & Sherman, 1992; Kochanowski & Sherman, 1985). Due to the importance of macrophages in iron cycling, storage, and availability and the evidence that this micronutrient is known to play a role in fetal outcomes, we investigated how maternal stress influences iron in the placenta.

Developmental origins of health and disease

The developmental origins of health and disease (DOHaD) framework posits that the gravid *in utero* environment influences the risk for non-communicable diseases (NCDs) for offspring throughout life (Eriksson, 2016). The fundamental belief of DOHaD is that hormonal, metabolic, inflammatory, as well as nutritional factors during specific and sensitive stages impact fetal health and development (Lucas AA 1994, Barker DJ 2004, Warner MJ 2010). *In utero* development sets the stage for the lifelong health of offspring. But sometimes stressors can adversely impact fetal development in such a way that the health of the offspring is put at risk. It is possible that this programming may be advantageous from an evolutionary stand point, but may also have long-term detrimental outcomes for society as a whole. By no means does the DOHaD framework rule out the role of genetics in the development of NCDs, but rather incorporates the idea that prenatal stressors can modify genetic susceptibility further enhancing or decreasing already programmed genetic predisposition. Many studies exploring the DOHaD framework have centered on neurocognitive outcomes in offspring, probably because brain

development seems to be very sensitive to the in utero environment. These two outcomes will be further introduced in the following sections.

Strong associations have been identified between various stressors during pregnancy and the occurrence of NCDs, including cardiovascular disease, metabolic disorders (*e.g.*, obesity and diabetes mellitus), and neurocognitive problems (Eriksson, 2016). In a study conducted in Scotland, maternal obesity during pregnancy was associated with the increased risk of premature death of the offspring in adulthood (Reynolds RM, 2013). An analysis of the Helsinki birth cohort study revealed compelling evidence that a high maternal body mass index (BMI) is associated with an increased risk of cancer, cardiovascular disease, and type-2 diabetes (T2D). Interestingly, the association with T2D was stronger in female offspring than male (Eriksson JG, 2014). Evidence suggests that many commonly occurring diseases that have a substantial impact on society and long-term health appear to have some basis in the prenatal environment to which they were exposed.

Studies are increasing in number and specificity to identify links between maternal morbidity during pregnancy and the risk for developing disease later in life. The studies presented in this work were developed under the DOHaD framework under the hypotheses that maternal inflammatory stressors during pregnancy influence fetal development.

Maternal inflammation modifies the fetal environment and adverse outcome risk

There are obvious sources of inflammation such as prenatal infection and autoimmune disease, and there are the more complex sterile forms of inflammatory states such as obesity and gestational diabetes (Berk et al., 2013). Many low- or middle-income countries are disproportionately affected by highly endemic infections such as tuberculosis, malaria, and HIV,

but are also challenged by GDM and obesity as societies transition from poor access to nutrition to a Western food culture (Popkin, Adair, & Ng, 2012). This is noteworthy because metabolic stressors such as these are associated, like some infections, with pro-inflammatory alterations within the placenta. The placenta functions in a protective manner as well, protecting the developing fetus from a possibly adverse maternal environment. For example, in cases of nutritional-deprivation, the placenta maintains fetal growth and development by sacrificing itself through autophagy in an attempt to save the fetus (Alwasel, 2010; Broad and Keverne, 2011). Placental adaptations to an adverse maternal environment are to increase the likelihood of offspring survival in the short-term, however, these adaptations can lead to adverse long-term effects. One possible long-term effect of these placental adaptations is the development of psychiatric disorders and developmental delays in the offspring.

Psychiatric disorders affect a large portion of the world's population (Kessler et al., 2009). Psychiatric disorders are highly heritable. Schizophrenia has an estimated heritability of between 30 – 70% (Light et al., 2014; Sullivan, Kendler, & Neale, 2003; Wray & Gottesman, 2012) and in the case of autism spectrum disorder (ASD), heritability estimates are around 50% (Sandin et al., 2014), thus making genetics a factor in disease incidence. Genetic factors, however, are insufficient in entirely explaining the incidence of psychiatric disorders.

Excess inflammation leads to pathologic alterations contributing to psychiatric disorder development and progression later in life (Muller, 2014; Saetre et al., 2007). Both genetic and environmental risk factors contribute to an altered inflammatory state, working in tandem to influence the pathological basis for such psychiatric disorders (Horvath & Mirnics, 2014). Environment-associated risk factors modify genetic risk by altering brain development and function (Jaffee & Price, 2007; Klengel & Binder, 2015). Important neurodevelopmental stages,

such as *in utero*, are especially sensitive to environmental perturbations (M. H. Johnson, Jones, & Gliga, 2015; Meredith, 2015; Roth & Sweatt, 2011). Both schizophrenia and autism have been associated with numerous *in utero* disruptions, including prenatal infection (Atladóttir et al., 2010; Buka et al., 2001), and gestational diabetes (M. Cannon et al., 2002; Gardener et al., 2009; Xiang et al., 2015). This latter fact raises an important question concerning the convergence of such metabolic and infectious stressors on neurocognitive development and long term outcomes in offspring.

Fetal and placental sex modifies disease risk and pregnancy outcomes

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 (Di Renzo, Picchiassi, Coata, Clerici, & Brillo, 2015; Rosenfeld, 2015; Sedlmeier et al., 2014). In fact, recent studies suggest that the placental transcriptome is largely driven by the fetal genome and that placental gene networks influence postnatal risk of multiple human diseases (Peng et al., 2017). Thus sexual dimorphism in DOHaD might reflect an influence of placental sex on fetal development.

While the majority of investigations linking antenatal stress to adverse outcomes in offspring have focused on a single type of stress (such as metabolic stress or infection) (J. A. Goldstein, S. A. Norris, & D. M. Aronoff, 2017), real world experience demonstrates that human populations are routinely subjected to more than one fetal stressor at a time. For example, diabetes is often accompanied by obesity, collectively referred to as diabesity (Sims et al., 1973), a condition that has received significant attention for its impact on fetal development and developmental outcomes (Desoye & van Poppel, 2015; Tomar et al., 2015). In contrast, the co-

occurrence of infectious diseases and metabolic stress has received little formal attention (J. A. Goldstein et al., 2017).

Gestational diabetes

One in ten pregnancies are complicated by glucose intolerance (DeSisto, Kim, & Sharma, 2014; Perkins, Dunn, & Jagasia, 2007). GDM is the most common metabolic disorder during pregnancy with a mean prevalence in North America and the Caribbean of 7 %. It is defined as glucose intolerance newly diagnosed in the second or third trimester of pregnancy ("2. Classification and Diagnosis of Diabetes," 2017). GDM occurs as a result of a breakdown in normal glucose utilization during pregnancy. The placenta produces human chorionic somatomammotropin that inhibits peripheral uptake of glucose by the mother while stimulating the secretion of insulin by the fetus. In healthy pregnancies, some level of insulin resistance is required to preferentially transfer nutrients to the fetus, however, in cases of GDM, a lack of β-cell hyperplasia leads to an exacerbated state of insulin resistance (Perkins et al., 2007). GDM not only affects levels of circulating glucose and insulin within the mother, but also circulating systemic inflammatory mediators. For example, circulating TNF-α levels are increased in patients with GDM when compared to healthy controls (Radaelli, Varastehpour, Catalano, & Hauguel-de Mouzon, 2003). Both placental and subcutaneous adipose tissue explants exhibited higher levels of TNF-α release when incubated in the presence of glucose (Coughlan, Oliva, Georgiou, Permezel, & Rice, 2001), suggesting that both the placenta and adipose tissue are responsible for increased secretion of TNF-α under high glucose conditions.

Type 2 diabetes and GDM are often found in conjunction with maternal obesity (K. E. Martin, Grivell, Yelland, & Dodd, 2015; Whiteman et al., 2015). Both GDM and T2D are known

to increase the risk of complications such as macrosomia, hypoglycemia, hypocalcemia, and hyperinsulinemia from islet cell hyperplasia at birth (Frias, Frias, Frias, & Martinez-Frias, 2007; Perkins et al., 2007). Fetal hypoxia, fetal brain-iron deficiency, and respiratory distress syndrome can also occur as a result of diabetes during pregnancy due to the fact that the placenta shows signs of increased hypoxia and is unable to provide the increased oxygen delivery required for the increased metabolic functioning (Georgieff, 2006). GDM, T2D, and obesity are often accompanied by an increase in circulating levels of free-fatty acids (FFA) and characterized by low-grade inflammation endotoxemia (Catalano, 2002; Lappas, 2011; Winzer, 2004; Wolf, 2004). Multiple studies have demonstrated that these FFA byproducts of diabesity constitute metabolic danger signals and have the ability to alter the activation status of the NLRP3 inflammasome leading to changes in the inflammatory environment (L'Homme et al., 2013; Vandanmagsar et al., 2011; Wen et al., 2011; Y. Yan et al., 2013).In addition to the metabolic related consequences of GDM, this condition is also associated with the dysregulation of multiple inflammatory pathways (Abell, De Courten, Boyle, & Teede, 2015). Gestational diabetes has also been associated with increased leptin, IL-6, and TNF-α as well as decreased adiponectin (insulin-sensitizing adipokine) levels midgestation (Gao, Yang, & Zhao, 2008; Kautzky-Willer et al., 2001; Kinalski, Telejko, Kuzmicki, Kretowski, & Kinalska, 2005; Nergiz et al., 2014; Ortega-Senovilla et al., 2011; Park et al., 2013; Vitoratos et al., 2001). Similar to and sometimes in conjunction with maternal obesity, gestational diabetes leads to an increased production of reactive oxygen species by hyperglycemia-induced increased electron transport in mitochondria (Nishikawa et al., 2000). In addition, placenta from gestationally diabetic mothers exhibit gene expression patterns and DNA methylation consistent with a pro-inflammatory phenotype, highlighting the potential role for placental inflammation in negative fetal outcomes

(Enquobahrie, Williams, Qiu, Meller, & Sorensen, 2009; Rong et al., 2015). Taken together, gestational diabetes induces maternal and placental changes consistent with a shift to a pro-inflammatory state and an increase in hypoxic conditions within the placenta.

Gestational diabetes induces changes in placental characteristics

In cases of GDM, evidence suggests that within the placenta the number of macrophages, $CD68^{+}CD14^{+}$ cells, increases compared to control groups (Yu et al., 2013). Diabetes during pregnancy has been shown to induce a pro-inflammatory phenotype in placental macrophages, as characterized by an increase in the expression of IL-1β and CCR7 and a decrease in the anti-inflammatory markers CD163, CD209, and IL-10 (Sisino et al., 2013). Another recent study, however, concluded that HBCs maintain their anti-inflammatory phenotype even in the presence of GDM (Schliefsteiner et al., 2017). T-cell directed immunoregulation is skewed in patients with GDM, as evidenced by reduced development and suppressive capacity of regulatory $CD4^{+}$ T cell (T_{reg}) populations (Pendeloski et al., 2015). Thus, there is ample evidence suggesting that the presence of GDM influences phenotypic properties of the placenta, although some controversy regarding the impact of GDM on the inflammatory potential of placental macrophages and the overall inflammatory state of the placenta exists.

Gestational diabetes is associated with increased levels of maternal serum ferritin. Serum ferritin is an iron containing protein that functions as an intracellular iron storage compound (Charlton, Jacobs, Torrance, & Bothwell, 1963). In cases of iron-overload, ferritin can be excreted from macrophages in an apo-form into serum (Cohen et al., 2010). Multiple studies have demonstrated that GDM is accompanied by an increase in serum ferritin levels (Afkhami-Ardekani & Rashidi, 2009; Amiri et al., 2013; Chen, Scholl, & Stein, 2006). These findings

suggest that GDM may be accompanied by, or influenced by, an increase in intracellular iron levels within macrophages.

Literature regarding iron supplementation during pregnancy and risk of GDM is unclear. Prospective cohort studies have been performed to determine if iron intake during pregnancy correlates with an increased incidence of GDM. The reports thus far have been conflicting and correlative at best. One study reported a 2-fold higher incidence of GDM, hypertension, and metabolic syndrome in iron-supplemented women compared to controls while a different study concluded that high iron intake increased the risk of GDM for only a small subset of individuals (Bo et al., 2009; Helin et al., 2012). Neither of the studies looked at iron supplementation throughout the course of pregnancy and results were mostly based upon patient surveys.

Interestingly, one identifying characteristic of placental macrophages is the high expression of the anti-inflammatory marker CD163, which is a scavenger receptor known to be involved in the clearance of hemoglobin-haptoglobin complexes and iron uptake (Svensson et al., 2011). The role of CD163 in iron recycling and handling is especially of interest in GDM as iron-containing proteins, iron availability, and levels of CD163 differ between women with GDM and non-diabetic pregnancies (Afkhami-Ardekani & Rashidi, 2009; Amiri et al., 2013; Bo et al., 2009; Chen et al., 2006; Helin et al., 2012; Rawal et al., 2017). Women with GDM exhibit signs of elevated iron stores, as estimated by an increase in maternal serum, hepcidin, and ferritin levels (Amiri et al., 2013; Chen et al., 2006; Rawal et al., 2017); all of which have been shown to play a possible role in the development of GDM. Two independent studies have shown that antenatal iron supplementation, leading to increased levels of maternal iron, increased the risk of GDM and was also associated with glucose impairment and hypertension throughout gestation suggesting a link between imbalanced iron homeostasis and GDM (Bo et al., 2009; Helin et al.,

2012). Although a relationship has been established between GDM and maternal iron levels, the causal nature of this interaction and the extent to which it influences placental health and fetal outcome has yet to be well established.

Maternal obesity and GDM: their impact on placental biology and fetal development

Maternal obesity, excessive gestational weight gain, and other maternal metabolic disorders have been shown to have a significant impact on the physiology of the offspring (Maftei et al., 2015; Olson, Strawderman, & Dennison, 2009; Stice, Yokum, Burger, Epstein, & Small, 2011). Obesity is characterized by a low-grade inflammatory state (Friis et al., 2013) and leads to adipocyte hyperplasia and an increased secretion of pro-inflammatory adipokines and cytokines. Consequently, macrophage and T lymphocyte populations in adipose tissue increase, gut permeability decreases, and the gut microbiome is altered (Castanon, Luheshi, & Laye, 2015). Of these obesity related biological factors, many are known to mediate risk to the developing fetus including excessive nutrient availability (e.g. glucose), circulating hormones (e.g. insulin, leptin), and inflammatory cytokines (Rivera, Christiansen, & Sullivan, 2015). The placenta is not immune to the effects of maternal obesity and as a result shows significant reduction in vascular function (N. Jansson et al., 2013; Stewart et al., 2007). An increase in maternal BMI is also associated with placental phosphorylation of NFκB and increases IL-1β levels, suggesting changes in placental inflammatory potential (Aye et al., 2014; Martino et al., 2016).

Uncontrolled diabetes in conjunction with obesity leads to increased adiposity and hyperglycemia increasing signs of oxidative stress and systemic inflammation (Guest, Gao,

O'Connor, & Freund, 2007). When obese women become pregnant, the inflammatory burden further increases as made evident by increases in maternal C reactive protein, IL-6, IL-1β, and MCP-1 midgestation compared to non-obese pregnant women (Christian & Porter, 2014; Friis et al., 2013). In the context of predisposing factors, such as maternal obesity and an unhealthy diet, gestational diabetes often inevitably develops (K. E. Martin et al., 2015; Whiteman et al., 2015). Pregnancy itself induces an insulin resistant maternal state allowing for increased glucose availability for the fetus (Salzer, Tenenbaum-Gavish, & Hod, 2015). Increased maternal glucose and free fatty acid production to accommodate the developing fetus combined with heightened inflammation exacerbate an already heightened state of insulin resistance as a consequence of pregnancy (Shoelson, Lee, & Goldfine, 2006). Most pregnant females are able to compensate for this increase in insulin resistance with pancreatic beta cell hyperplasia, hypertrophy and increased insulin production, leading to a hyperinsulinemic, normoglycemic state (Pasek & Gannon, 2013). However, when the factors propagating insulin resistance cannot be overcome by maternal compensation, gestational diabetes often develops (Georgieff, 2006). For the developing fetus, this translates into increases in both inflammatory (e.g. cytokines) and metabolic (e.g. insulin, leptin, glucose, free fatty acids) factors that either act at the placenta or freely cross the placenta to disrupt normal development (Silverman, Rizzo, Cho, & Metzger, 1998).

Models of gestational diabetes

There are numerous rodent models of gestational diabetes. Many utilize a drug-induced streptozotocin (STZ) model of diabetes both during gestation and in the absence of pregnancy. STZ destroys pancreatic beta cells therefore producing a type 1 diabetic-like phenotype (López-

Soldado & Herrera, 2003). Although genetic models inhibiting maternal beta cell proliferation exist and are shown to produce a gestational diabetic phenotype, these mutations are rarely if ever found in gestationally diabetic women and were therefore not chosen to be utilized in our studies (Pasek & Gannon, 2013). Even though other animal models are often used and known to induce a state of maternal hyperglycemia, the high fat diet induction of gestational diabetes is the most clinically relevant model of gestational diabetes for our studies.

It is well established that a high fat diet produces obesity in rodents. Most researchers utilize a 32% to 60% fat by calorie diet producing a steady increase in weight over time without overt nutritional deficiencies (Pasek & Gannon, 2013). As a result of this high fat diet, adult female offspring from high fat fed dams exhibit increases in inflammation characterized by increases in IL-1β and TNFα levels (Kang, Kurti, Fair, & Fryer, 2014). Adult offspring of gestationally diabetic mothers, females in particular, present with various phenotypes including increased weight, hyperinsulinemia, hyperglycemia, hypertension, and higher serum levels of triglycerides and C reactive protein (Elahi et al., 2009; Liang, Oest, & Prater, 2009; Plagemann et al., 1998). Increases in the expression and/or activity of cytokines and proteins related to hypoxic response and oxidative stress have also been reported (Chandna et al., 2015; Lian, Dheen, Liao, & Tay, 2004; Melo et al., 2014; X. Tang, Qin, Xie, & He, 2015; Wu et al., 2015). As a result of a maternal high fat diet, the placenta exhibited signs of hypoxia and inflammation (Jones et al., 2009; Li, Chen, & Li, 2013; Liang, DeCourcy, & Prater, 2010).

Maternal obesity, even in the absence of diagnosed diabetes, is a state of metabolic dysfunction and exhibits signs of excessive inflammation, which contribute to insulin resistance, an increased risk for the development of diabetes, and further excessive inflammation (Abell et al., 2015). The ongoing presence of inflammation in association with the presence of metabolic

dysfunction induces a positive feedback in which the two conditions promote the presence and intensity of one another. Rodent models of obesity exhibit signs of hyperglycemia and hyperinsulinemia both of which are known to increase the risk of developing gestational diabetes (Gallou-Kabani et al., 2007; Rivera et al., 2015). This knowledge not only makes the use of a high-fat induced model of gestational diabetes an effective one, but a physiologically relevant one as well for studying its effects on placental and fetal development.

Gestational diabetes influences fetal neurodevelopment

Although the link between gestational diabetes and neurocognitive disorders is not as well documented as with maternal immune activation, there is growing evidence indicating that diabetes and obesity during gestation negatively affect neurodevelopment. With regards to autism and maternal diabetes, a meta-analysis performed by Xu and colleagues found significant pooled odds ratios of 1.48 (95% CI: 1.25 – 1.75) and 1.72 (95% CI: 1.24-2.41) for 3 cohort and 9 case-control studies, respectively (Xu, Jing, Bowers, Liu, & Bao, 2014). Furthermore, schizophrenia risk has been documented as increased with diabetes during gestation, with a meta-analysis by Mary Cannon finding a significant odds ratio of 7.76 (95% CI: 1.37 – 43.9) (Mary Cannon, Peter B. Jones, & Robin M. Murray, 2002; Van Lieshout & Voruganti, 2008). Similarly, a study of 1051 offspring from 315 Finnish families found a 1.66-fold increase risk for developing schizophrenia with gestational diabetes exposure (Wegelius et al., 2011). Although limited, a possible link between gestational diabetes and neurocognitive disorders has been established. In order to further evaluate the causative nature of this association and possible mechanisms involved further studies are necessary.

Maternal immune activation

Inflammation when appropriate is protective, but aberrant inflammation is not. Inflammation during critical developmental time windows can produce long-lasting changes in both immune and brain function (Coe & Lubach, 2003; U. Meyer, Yee, & Feldon, 2007). Many different environmental stressors, including infections, gestational diabetes, obesity, and altered nutrition have the potential to influence critical stages of development (Berk et al., 2013; Fagundes, Glaser, & Kiecolt-Glaser, 2013). Perturbations within the intrauterine environment have the potential to induce long lasting and significant consequences on immune function. Infections traditionally pose a persistent threat to human reproductive health through 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 (Bobetsis et al., 2007). The later indirect cause can be referred to as maternal immune activation (MIA). MIA as might be provoked by viral infections that do not cross the placenta, such as influenza or medically-treated HIV, appears to impact the risk for NCDs in exposed offspring (Adams Waldorf & McAdams, 2013; Zager et al., 2015). Perhaps one of the most dramatic impacts maternal immune activation can have on pregnancy and fetal development is preterm birth and early pregnancy loss. Preterm birth can lead to a multitude of complications for the offspring including an increase in the occurrence of allergies and asthma (Fergusson, Crane, Beasley, & Horwood, 1997; Kent et al., 2016; Pekkanen, Xu, & Jarvelin, 2001)

MIA can be modeled in rodents through multiple different scenarios. The two most common include the use of polyinosinic:polycytidylic acid (poly(I:C)) and the administration of LPS during midgestation inducing immune activation between gestational day (GD) 6 and 14 in

rodents. Both LPS and poly(I:C) are associated with a pro-inflammatory phenotype in the offspring (Garay, Hsiao, Patterson, & McAllister, 2013; Mandal et al., 2013; Onore, Schwartzer, Careaga, Berman, & Ashwood, 2014). Poly(I:C) is a synthetic double stranded RNA (dsRNA) that activates an acute antiviral immune response. dsRNA is recognized by TLR3, leading to an innate immune response that includes NFκB activation and pro-inflammatory cytokine expression (Urs Meyer, 2014; U. Meyer & Feldon, 2012). As with viral infection, poly(I:C) induces acute fever, sickness behavior, and weight loss usually resolving within 1 to 2 days (Patterson, 2009). The administration of poly(I:C) produces an effective antiviral response stimulating maternal immune activation in the absence of live pathogens mimicking an *in utero* viral infection (U. Meyer, Feldon, Schedlowski, & Yee, 2006; U. Meyer et al., 2007) (U. Meyer & Feldon, 2012). The ability of poly(I:C) to produce a consistent viral-like maternal response makes it a valuable tool in maternal immune activation research.

Viral infections and maternal immune activation are linked to psychiatric disorders

Since many psychiatric disorders are thought to have developmental origins, much research has focused on the importance of prenatal and early postnatal inflammation-associated events. It has long been known that *in utero* infections can alter the fetal brain, ranging from mild anomalies to severe deficits. Multiple studies have demonstrated that active maternal infections such as herpes simplex virus, cytomegalovirus, rubella, and toxoplasmosis can lead to childhood intellectual disabilities and learning difficulties (Hagberg & Kyllerman, 1983; H. M. Meyer, Jr., 1969). Recent events make this association all the more important since the emergence of Zika virus as the newest viral threat to the developing fetus. The most frequently studied association between maternal infections and neurocognitive disorders is influenza

(O'Callaghan, Sham, Takei, Glover, & Murray, 1991; Simanek & Meier, 2015; Zerbo et al., 2015). The earliest studies of *in utero* infection and psychiatric risk observed increases in schizophrenia diagnosis after influenza epidemics. Groups in Wales, England, and Finland reported increases in schizophrenia risk in those born a few months after the 1957 A2 influenza pandemic (Mednick, Machon, Huttunen, & Bonett, 1988; O'Callaghan et al., 1991). Study results investigating other influenza outbreaks and schizophrenia risk associations, however, show inconsistent results. Some studies find a relationship between second trimester infections and increased schizophrenia diagnosis (J. Adams et al., 1993; Barr, Mednick, & Munk-Jorgensen, 1990; Kendell & Kemp, 1989; Kunugi et al., 1995; Limosin, Rouillon, Payan, Cohen, & Strub, 2003; Sham et al., 1992; Takei, O'Callaghan, Sham, Glover, & Murray, 1993; Takei et al., 1994) while other groups find no association (Grech, Takei, & Murray, 1997; Kendell & Kemp, 1989; Mino, Oshima, Tsuda, & Okagami, 2000; Morgan et al., 1997; Susser, Lin, Brown, Lumey, & Erlenmeyer-Kimling, 1994; Takei, Murray, Sham, & O'Callaghan, 1995). Conflicting results have also been found for other infections during pregnancy such as diphtheria, pneumonia, measles, varicella, mumps, and poliovirus (Cahill, Chant, Welham, & McGrath, 2002; O'Callaghan et al., 1994; Suvisaari, Haukka, Tanskanen, Hovi, & Lonnqvist, 1999; Torrey, 1988; Watson, Kucala, Tilleskjor, & Jacobs, 1984).

Comparable to schizophrenia, autism has been associated with maternal infections during pregnancy. Rubella and other viral pathogens such as cytomegalovirus as well as some bacterial infections in the second trimester have been associated with a diagnosis of autism in the offspring (Atladóttir et al., 2010; Chess, 1977; Libbey, Sweeten, McMahon, & Fujinami, 2005; Sweeten, Posey, & McDougle, 2004; Yamashita, Fujimoto, Nakajima, Isagai, & Matsuishi, 2003; Zerbo et al., 2015).

Similar to viral infections, prenatal poly(I:C) has demonstrated molecular, structural, and behavioral abnormalities reminiscent of psychiatric disorder pathology in the offspring in rodent models (Winter et al., 2009). Prenatal poly(I:C) treatment also has effects on immune development which may play a role in the progression of neurodevelopmental disorders. After treatment, high levels of many inflammatory markers are increased in the fetal brain, including IL-1β, IL-13, and MCP-1 (Arrode-Bruses & Bruses, 2012). In addition to changes in the brain, offspring usually present with a generalized, systemic pro-inflammatory phenotype. MIA via poly(I:C) in a mouse induces immune changes that last long after the initial insult. These long-lasting immune perturbations could potentially contribute to the dysfunction observed throughout the postnatal period in MIA offspring.

Metabolic stress, inflammation, and inflammasomes

A strong foundation of evidence exists linking metabolic disorders and inflammation. With recent discoveries suggesting that chronic inflammation plays an essential role in the pathogenesis of many metabolic driven diseases. Highly conserved across species both the immune and metabolic responses to a variety of stimuli are essential for survival. In order to maintain a physiologically homeostatic environment proper communication between these two essential pathways is vital. A hallmark of chronic inflammation is increased levels of circulating inflammatory markers such as cytokines and chemokines which have been implicated in promoting the progression of chronic inflammation in metabolic disease. As the incidence of gestational diabetes rises, it is important that we seek to understand the immunological and metabolic processes responsible for its progression and how these processes affect one another

within the context of disease. Understanding the cellular responses to such perturbations could lead to the development of new or repurposed therapeutic and preventative treatments.

The NLRP3 Inflammasome

Pattern recognition receptors (PRRs) were first characterized for their ability to recognize microbial signals and mediate host immune responses to microbial insults. It is becoming ever more apparent that the PRRs have the ability to not only recognize and respond to microbial insults, but to respond to various endogenous danger signals such as those that may arise as a consequence of metabolic disease. Several different families of PRRs have since been discovered including the primarily membrane-bound toll-like receptors (TLRs) and c-type lectin receptors (Takeuchi & Akira, 2010). Another important family of PRRs is the nucleotide-oligomerization domain-like receptors (NLRs). The NLRs encompass a large family of intracellular proteins that respond to microbial patterns as well as changes in cellular homeostasis. NLR family is evolutionarily conserved in both plants and animals, although unlike the TLRs which are found in Drosophila, NLRs are unique to higher eukaryotes (Ting & Davis, 2005).

The NLR family of PRRs contains 22 members with broad ranging and often times divergent functions. The most extensively studied of the NLR sub-families are those proteins known to be involved in the formation of large multimeric complexes known as inflammasomes (Martinon, Burns, & Tschopp, 2002). The best characterized of these inflammasomes is that which is formed around NLRP3. Activation of the NLRP3 inflammasome induces oligomerization of NLRP3 leading to the recruitment of ASC (apoptosis-associated speck-like protein containing CARD), which in turn, forms large structures leading to the recruitment of pro-caspase-1. Autocatalytic cleavage of pro-caspase-1 into the p10 and p20 caspase-1 subunits

allows for the formation of active caspase-1 as the two subunits form hetero-tetramers which are then able to convert pro-IL-1β and pro-IL-18 into their bioactive secreted forms (C. A. Dinarello, 2009; Wilson et al., 1994). The importance of IL-1β and its possible role in metabolic stress during pregnancy will be discussed further in the following sections.

NLRP3 activation occurs in two steps. The strict regulation of inflammasome activation is important to restrict aberrant activation which may lead to possible damage of surrounding tissues through an unnecessary and excessive inflammatory response. Signal 1 is a priming step and is required for complete activation. This initial priming can be mediated through various pathways such as PRRs, cytokine receptors, or other factors known to activate NF-κB. This priming step is critical as it produces a pool of pro-IL-1β and increases NLRP3 to functional levels (Bauernfeind et al., 2009; Franchi, Eigenbrod, & Nunez, 2009). The second signal is the activating signal and can vary in nature from microbial signals, toxins, crystalline substances, and ATP released from dead or dying cells (Stutz, Golenbock, & Latz, 2009).

The NLRP3 inflammasome is important in the context of metabolic disease as it has the ability to be activated by a number of different byproducts of metabolic disease resulting in a state of sterile inflammation (Agostini et al., 2004; Mariathasan et al., 2004; Meylan, Tschopp, & Karin, 2006; Miao et al., 2006). This is in contrast to other NLR inflammasomes such as NLRC4/NAIP and NLRP1 which have a more restricted repertoire of agonists (L. C. Hsu et al., 2008; Newman et al., 2010). Due to this fact, the NLRP3 inflammasome is recognized as a sensor of metabolic dysregulation. For example, ER stress, as seen in patients with T2D, activates the NLRP3 inflammasome resulting in the release of IL-1β (Menu et al., 2012). In mice, studies inducing obesity through the administration of a high fat diet (HFD) have shown that expression of inflammasome components ASC, Caspase-1, and NLRP3 are elevated in

adipose tissue macrophages and isolated adipocytes, which can also produce IL-1β, further promoting obesity (Koenen et al., 2011; Stienstra, Tack, Kanneganti, Joosten, & Netea, 2012; Stienstra et al., 2011). Further evidence outlining the importance and possible role for the NLRP3 inflammasome in the context of metabolic stress will be outlined in later chapters.

The importance of IL-1β

The IL-1 family of cytokines are important regulators of inflammation. It has multiple functions including acting as a co-stimulatory molecule of T lymphocytes, inducing a humoral Th2 immune response, and promoting Th17 cell differentiation (Charles A. Dinarello, 2009; V. J. Johnson, Yucesoy, & Luster, 2005; Sutton, Brereton, Keogh, Mills, & Lavelle, 2006). IL-1 can also increase adhesion molecule expression on mesenchymal and endothelial cells, promote angiogenesis and tumor metastasis, and stimulate myeloid differentiation (Dinarello, 2007; Charles A. Dinarello, 2009; Voronov et al., 2003). The IL-1 family of cytokines includes the IL-1α and IL-1β isoforms, IL-1 receptors 1 and 2, IL-1 receptor accessory protein, and IL-1 receptor antagonist. IL-1α is expressed constitutively in most cell types whereas IL-1β is mostly produced by immune cells (Berda-Haddad et al., 2011). Biological activity is controlled at both the transcriptional and post-translational levels of processing. IL-1β is produced in an inert pro-peptide form that must be cleaved by enzymes such as caspase-1 or neutrophil serine proteases (Guma et al., 2009; Joosten et al., 2009). Upon binding of biologically active IL-1, the IL-1 receptor heterodimerizes, interacts with IL-1 receptor accessory protein, and promotes pro-inflammatory signaling through a myeloid differentiation factor 88 (MyD88)-dependent pathway to activate NF-κB and multiple stress-activated kinases such as JNK and ERK (Palomo, Dietrich, Martin, Palmer, & Gabay, 2015). The IL-1 receptor antagonist is usually released by the same

immune cells that release IL-1, dampening excess IL-1 signaling via competitive antagonism (Dripps, Brandhuber, Thompson, & Eisenberg, 1991; Schreuder et al., 1997). IL-1 receptor 2 is expressed on immune cells and other cytokine targets decreasing IL-1 signaling by acting as decoy receptor (Gabellec, Griffais, Fillion, & Haour, 1996; McMahan et al., 1991). IL-1 receptor 2 expression is induced by anti-inflammatory factors whereas pro-inflammatory cytokines induce IL-1 receptor 1 expression (Colotta et al., 1993; P. Martin et al., 2013; Orlando et al., 1997).

Interleukin-1β is a pro-inflammatory cytokine that is produced by activated monocytes and macrophages in response to stimuli acting as an important regulator of systemic inflammation. It is the primary cause of both chronic and acute inflammation in response to injury, infection, and other immunological challenges (Dinarello, 1998). As mentioned previously, IL-1β is produced in its inactive precursory form, pro-IL-1β p35, which requires cleavage into its active p17 form by IL-1β-converting enzyme, also known as caspase-1 (Cerretti et al., 1992; Thornberry et al., 1992).

As an important regulator of inflammation, it is not surprising that IL-1β has been shown to play a role in metabolic disorders such as T2D and obesity. Both IL-1β and IL-18 have been shown to be risk factors for T2D (Spranger et al., 2003; Thorand et al., 2005). IL-1β has been linked to the possible development and progression of T2D through multiple mechanisms. Through increasing the expression of other inflammatory mediators, via IL-1R signaling, IL-1β can initiate the expression of IL-18 and IL-33 families of cytokines initiating a self-amplifying cytokine system (Arend, Palmer, & Gabay, 2008). IL-1β also induces cell stress pathways which have been linked to the progression of T2D (Cardozo et al., 2005; Verma & Datta, 2010). High glucose and high free fatty acid levels, as seen in T2D and often times obesity, have been shown to directly increase inflammatory responses and IL-1β production (Boni-Schnetzler et al., 2009).

Multiple studies have focused on the identification of endogenous and exogenous ligands responsible for activating the inflammasome in murine models of T2D. Multiple danger associated molecular patters (DAMPs) including islet amyloid polypeptide and glucose itself have been associated with increased NLRP3 inflammasome activation in T2D to name just a few (Maedler et al., 2002; Masters et al., 2010).

The role of Toll-like receptors in metabolic disease

One of the most studied and best characterized of the PRR families is the TLR family. TLRs are responsible for sensing invading pathogens as well as endogenous DAMPs both extracellularly and within endosomes and lysosomes intracellularly (Akira, 2006). Ten different TLRs have been identified in humans while 12 have been identified in mice and are characterized by N-terminal leucine-rich repeats, a transmembrane region, and a cytoplasmic Toll/IL-1R homology (TIR) domain (Kawai & Akira, 2007). TLRs differ not only in their localization within the cell, but also in their ability to recognize specific molecular patterns whether exogenous in nature such as microbial components or endogenous in nature. Some of the relevant TLRs will be introduced in brief. TLR2, has the widest specificity, and senses various gram-positive as well as gram-negative bacterial and fungal components when in complex with other TLRs, TLR1/TLR2 or TLR6/TLR2, the heterodimer recognizes triacyl and dicyl lipoproteins (Aliprantis et al., 1999; Iwaki et al., 2002; Krutzik et al., 2003; Lien et al., 1999; Opitz et al., 2001; Takeuchi et al., 2001; Yoshimura et al., 1999). When stimulated, TLR2 induces pro-inflammatory cytokine production. TLR5 is highly expressed in the small intestine and recognizes flagellated bacteria. In response, dendritic cells induce differentiation of naïve T cells in to Th17 and Th1 cells (Uematsu et al., 2008). TLR3, TLR7, TLR8, and TLR9 all

recognize bacterial and viral nucleic acids as well as endogenous nucleic acids (Akira, 2006). The nature of the nucleic acid and the downstream effects vary depending on the TLR. TLR4, was the first human TLR to be identified, in complex with MD2, recognizes lipopolysaccharide (LPS) (Medzhitov, 2001; Poltorak et al., 1998) and is also involved in the recognition of viruses (Imai, 2008).

TLR signaling can be divided into two main groups dependent upon the TIR domain-containing adaptor molecules recruited to the TLR upon activation. The most common signaling pathway is through the adaptor protein MyD88 (Takeuchi et al., 2001). Upon pathogen associated molecular pattern (PAMP) recognition MyD88 is recruited to the TIR domain of the TLR initiating the subsequent kinase cascade that triggers the downstream activation of NF-κB and MAPK pathways resulting in an inflammatory response (Akira, 2003). TLR3 and TLR4, however, can signal in a MyD88 independent fashion through another adaptor protein, TRIF. TRIF, although also responsible for NF-κB activation, also phosphorylates IRF-3 resulting in an anti-viral response (Akira, 2003; Yamamoto et al., 2002).

The ability to sense a bacterial or viral infection at the maternal fetal interface is essential to a successful and healthy pregnancy. *In vivo* models of bacterial infections during pregnancy in multiple animal models have shown that live or heat-killed bacterial products can trigger preterm birth (Elovitz, Wang, Chien, Rychlik, & Phillippe, 2003). PRRs, more specifically TLRs, have been implicated in the pathogenesis of infection-associated preterm birth (Ilievski, Lu, & Hirsch, 2007; Wang & Hirsch, 2003). These PRRs are expressed throughout the immune system as well as on non-immune cells, especially within the pregnant uterus, thus increasing their capacity to initiate an inflammatory response. For example, amniotic epithelial cells possess TLR2 and TLR4 that recognize and respond to bacterial threats (K. M. Adams, Lucas, Kapur, & Stevens,

2007; Y. M. Kim et al., 2004). In cases of chorioamnionitis, infection of the fetal membranes, TLR4 expression is upregulated (Y. M. Kim et al., 2004). Within the decidua, term decidual immune cells express TLR2 and TLR4 (Y. M. Kim et al., 2004). Decidual stromal cells also express TLRs. Term decidua express all 10 at the transcript level (Krikun et al., 2007) while expression at the protein level is gestation dependent with TLR2 and TLR4 being exressed during the first trimester and all 10 show expression at term (Canavan & Simhan, 2007; Krikun et al., 2007). The TLRs with the decidua are active and mount an inflammatory response upon activation with their known ligands (Canavan & Simhan, 2007; Krikun et al., 2007; Simhan, Chiao, Mattison, & Caritis, 2008). The majority of research regarding pattern recognition in at the maternal fetal interface has focused on the placenta. Normal term placentae express all 10 TLRs (Chuang & Ulevitch, 2000; Zarember & Godowski, 2002). Like within the decidua, the expression of these changes throughout gestation in a time dependent fashion. In first trimester placental tissues, villous cytotrophoblasts and extravillous trophoblasts express high levels of TLR2 and TLR4, however, the outermost syncytiotrophoblasts do not express any TLRs (Abrahams et al., 2004). These outer most fetal cells are in direct contact with maternal blood and the lack of TLR expression is more than likely a method of immune tolerance. This expression pattern continues into the second trimester (Rindsjo, Holmlund, Sverremark-Ekstrom, Papadogiannakis, & Scheynius, 2007). By the time the pregnancy has reached the third trimester, TLR2 and TLR4 expression can be found in the villous cytotrophoblasts, extravillous trophoblasts, and the syncytiotrophoblasts (Holmlund et al., 2002; Kumazaki, Nakayama, Yanagihara, Suehara, & Wada, 2004; Rindsjo et al., 2007). This altered PRRs expression pattern may reflect the changes in placental function as gestation proceeds. TLR expression may be just

another way that the placenta orchestrates the delicate balance between tolerance of the fetal allograft and maintaining defense against pathogens.

As is many aspects of the immune system, TLR expression and function is altered in response to systemic inflammation and metabolic stress. Although a link between TLR4 and metabolic stress has been established, little work specifically looking at gestational diabetes has been completed. A case-control study looking at 30 females with GDM and 32 healthy pregnant females reported that both TLR4 expression levels in peripheral blood monocytes and serum TNF-α levels were increased in females with GDM compared to controls (Xie, Jin, & Zhu, 2014). Additionally they found a positive correlation between serum TNF-α levels and blood monocyte TLR4 expression in all females. This indicates that TLR4 expression may be involved in the pathogenesis of gestational diabetes although the causal relationship cannot be interrogated. In a model of diet-induced obesity in mice, similar results were found. Mice lacking TLR4 and wild-type mice were fed either a normal chow diet or a HFD for 8 weeks at which point their thoracic aorta was removed. Samples from wild-type mice on a HFD showed increased markers of vascular inflammation, NF-κB expression, and impaired insulin sensitivity compared to normal chow mice. The $TLR4^{-/-}$ mice, however, showed no signs of vascular inflammation or impaired insulin responsiveness as a result of HFD despite comparable body fat mass implicating a role for TLR4 in diet-induced vascular inflammation and insulin resistance (F. Kim et al., 2007). Using a diet-induced model of T2D, Lu, *et al*. reported that mice who received *Rhodobacter sphaeroides* lipopolysaccharide (rs-LPS), a TLR4 antagonist, had an attenuated presence of atherosclerotic lesions compared to non-rs-LPS treated mice. Furthermore, rs-LPS treated mice displayed a reduction in the number of infiltrating monocytes and macrophages into atherosclerotic lesions when present (Lu, Zhang, Li, Lopes-Virella, &

Huang, 2015). Together, these studies establish the basis for the possible role of TLR4 in the onset and progression of inflammation associated complications and progression as a result of metabolic stress in the form of obesity and diabetes.

Hypotheses

This thesis provides an overview of how maternal inflammatory stress, in the form of metabolic stress induced by GDM and MIA, affects placental health and fetal outcomes thus possibly influencing the developmental origin of health and disease. Our central hypothesis is that maternal inflammation alters placental physiology thus increasing the risk of adverse fetal outcomes. We apply this hypothesis to both human and mouse studies and in multiple scenarios looking at both metabolic stresses alone and in combination with the additional stress of maternal immune activation to assess the combined effect on placental and fetal health. The combination of these two common and often co-occurring stressors has never been interrogated.

Chapter 1 is an overview of the background information necessary for the understanding of where the field of maternal health is now regarding gestational diabetes and maternal immune activation. The now widely accepted viewpoint that the maternal environment during pregnancy can influence the health of the offspring, in both a negative and positive way, is also introduced. The work proposed and conducted in this thesis was developed under the framework of DOHaD to better understand how maternal immune stress during pregnancy affects not only maternal and placental health, but the long-term health of the offspring as well.

Chapter 2 tested the hypothesis that women with gestational diabetes exhibited altered placental physiology and placental macrophage phenotypes. We utilized a newly developed research tool at Vanderbilt University known as Pathlink which allowed us to search deidentified

patient medical records linked to archived formalin-fixed paraffin embedded (FFPE) placental tissue blocks obtained from the same patients. Comparison of placentae from women with GDM and healthy controls uncovered that GDM is associated with altered placental histology and altered placental macrophage phenotypes as evidenced by the higher level of expression of CD163 on macrophage-like cells of the chorion and decidua suggesting an increase in anti-inflammatory like macrophages. Increases in meconium-laden macrophages and greater iron stores within the placentae of women with GDM were noted and are consistent with reports that iron excess is associated with an increased risk for GDM. Overall, our results add to the growing amount of evidence that GDM has direct effects on placental structure.

In Chapter 3, we developed and characterized a rodent model of diet-induced gestational diabetes. This model was then utilized to test the combined effect of GDM and MIA via poly(I:C), both of which are inflammation-inducing psychiatric disorder risk factors, on markers of maternal inflammation and fetal brain gene expression patterns. Work presented in chapter 4 utilizes the same experimental premise as chapter 3 and assesses the placental gene expression patterns in the same mice after exposure to GDM, MIA, and the combination of the two stressors. For these studies, however, we were interested not only in how gene expression patterns change as a result of different sources of maternal inflammatory stress, but also the role of sexual dimorphism in influencing placental gene expression patterns.

Chapter 5 takes a more mechanistic approach investigating how metabolic stress alters placental macrophage IL-1β production. Dyslipidemia is often a hallmark of metabolic stress. Excess circulating FFAs can act as extracellular danger signals leading to the activation of the inflammasome culminating in the characteristic increased production of IL-1β seen in many

metabolic disorders including gestational diabetes. A summary of these findings as well as future directions for the field will be included in chapter 6.

CHAPTER 2

GESTATIONAL DIABETES MELLITUS IS ASSOCIATED WITH INCREASED CD163 EXPRESSION AND IRON STORAGE IN THE PLACENTA

Theresa L. Barke, Jeffery A. Goldstein, Alexandra C. Sundermann, Arun P. Reddy, Jodell E. Linder, Hernan Correa, Digna R. Velez-Edwards, David M. Aronoff

Introduction

GDM significantly increases the risk of pregnancy complications, including fetal macrosomia, neonatal hypoglycemia and hypocalcemia, preeclampsia, premature labor, and Cesarean delivery ("2. Classification and Diagnosis of Diabetes," 2017). It also increases the risk of postpartum complications in mother and child including late onset diabetes and cardiovascular disease ("2. Classification and Diagnosis of Diabetes," 2017). A major barrier to reducing the complications of GDM is a lack of clarity regarding how this disease changes placental physiology, morphology, and overall the pathophysiological mechanisms contributing to the impact of the disease.

Maternal hyperglycemia, as observed in GDM, alters placental morphometric characteristics, changes the structural integrity of syncytiotrophoblasts, and provokes major changes in placental gene expression related to chronic stress and inflammation (al-Okail & al-Attas, 1994; Calderon et al., 2007; Radaelli et al., 2003). Some evidence also suggests that within the placenta of women with GDM, the number of CD68+CD14+ cells, macrophages, increases compared to control groups (Yu et al., 2013). Elevated biomarkers of systemic

maternal inflammation have been described in women with GDM, including an increase in circulating levels of interleukin (IL)-6, IL-8, IL-18 and C-reactive protein (CRP) in conjunction with decreased levels of IL-10 (Kuzmicki et al., 2008). Multiple studies have shown that in conjunction with an increase in maternal circulating inflammatory mediators, GDM is also associated with pro-inflammatory changes within the placenta (Hara Cde et al., 2016; Kuzmicki et al., 2008; Li et al., 2013; Radaelli et al., 2003). In particular, macrophages, the primary phagocyte in the healthy placenta, appear to be impacted by this endocrine disorder (Mrizak et al., 2014; Sisino et al., 2013; Yu et al., 2013).

An identifying characteristic of placental macrophages is a high level of the anti-inflammatory marker CD163, which is involved in the clearance of hemoglobin-haptoglobin complexes and iron uptake (Svensson et al., 2011). The role of CD163 in iron recycling and handling is especially of interest in GDM as iron-containing proteins, iron availability, and levels of CD163 differ between women with GDM and non-diabetic pregnancies (Afkhami-Ardekani & Rashidi, 2009; Amiri et al., 2013; Bo et al., 2009; Chen et al., 2006; Helin et al., 2012; Rawal et al., 2017). Circulating levels of hemoglobin and iron are higher in GDM patients compared to controls (Afkhami-Ardekani & Rashidi, 2009). Elevated iron stores, as estimated by increased maternal serum hepcidin and ferritin levels, may be involved with the development of GDM (Amiri et al., 2013; Chen et al., 2006; Rawal et al., 2017). Two independent studies have shown that antenatal iron supplementation, leading to increased levels of maternal iron, increased the risk of GDM and was associated with glucose impairment and hypertension throughout gestation suggesting a link between imbalanced iron homeostasis and GDM (Bo et al., 2009; Helin et al., 2012).

Given the relationships among GDM, placental macrophage activation and iron homeostasis, we sought to conduct a retrospective, case-control, study of archived human placental tissue from women with or without GDM to better define placental pathology, placental iron stores, and both macrophage density and CD163 expression *in situ*.

Materials and Methods

Study Design

This study was reviewed and approved by the Vanderbilt University Institutional Review Board (protocol #150498). This study was a retrospective conceptualized and completed in collaboration with the Vanderbilt Institute for Clinical and Translational Research (VICTR) at Vanderbilt University Medical Center. Placental tissue specimens were identified through the Synthetic Derivative (SD), a queryable, real-time, deidentified electronic medical record (EMR)(Roden et al., 2008). The SD allowed us to identify patient medical records that were linked to archived formalin-fixed paraffin embedded (FFPE) placental tissue blocks obtained from the same patients. Placentae had been sent for histopathological review at the discretion of the attending obstetrician. The SD was queried to identify possible cases of women with GDM as well as healthy controls who also had placental FFPE blocks available. The databases were searched for key words including GDM, abnormal glucose, and International Statistical Classification of Diseases and Related Health Problems (ICD) 9 codes suggesting GDM including, Personal History of Gestational Diabetes (V12.21), Diabetes Mellitus Complicating Pregnancy Childbirth or the Puerperium (648.0), Gestational Diabetes, Antepartum (648.83). To identify unaffected controls, we required the absence of these key words and codes.

Participants

Our initial query identified 1,292 possible GDM cases and 554 possible control cases (**Figure 1**). The number of participants in this study was limited to 25 patients with GDM and 25 matched controls. The study design and sample distribution are outlined in Figure 1. From possible cases, we selected 25 cases, in which were able to confirm a diagnosis of GDM according to prespecified inclusion and exclusion criteria. Inclusion criteria included an abnormal oral glucose tolerance test (OGTT, defined below) and having access to a full FFPE placental cross-section, record of gestational age at birth, maternal age, and delivery method **(Supplemental Table 1)**.The presence of GDM was established if the woman had at least 2 values of the 100 g 3-hour OGTT above normal. Normal 100 g OGTT levels was defined as 95 mg/dl at fasting, 180 mg/dl at 1 hour, 155 mg/dl at 2 hours, and 140 mg/dl at 3 hours. Women were excluded from the study if they were diagnosed with preeclampsia, had previously diagnosed diabetes, or had tested positive for illicit drug use. We also excluded women who had active signs of infection during pregnancy or at the time of delivery based on placental pathology notes and noted fetal abnormalities during pregnancy or at delivery. Controls were then matched to cases on the basis of gestational age at delivery, maternal age, and method of delivery.

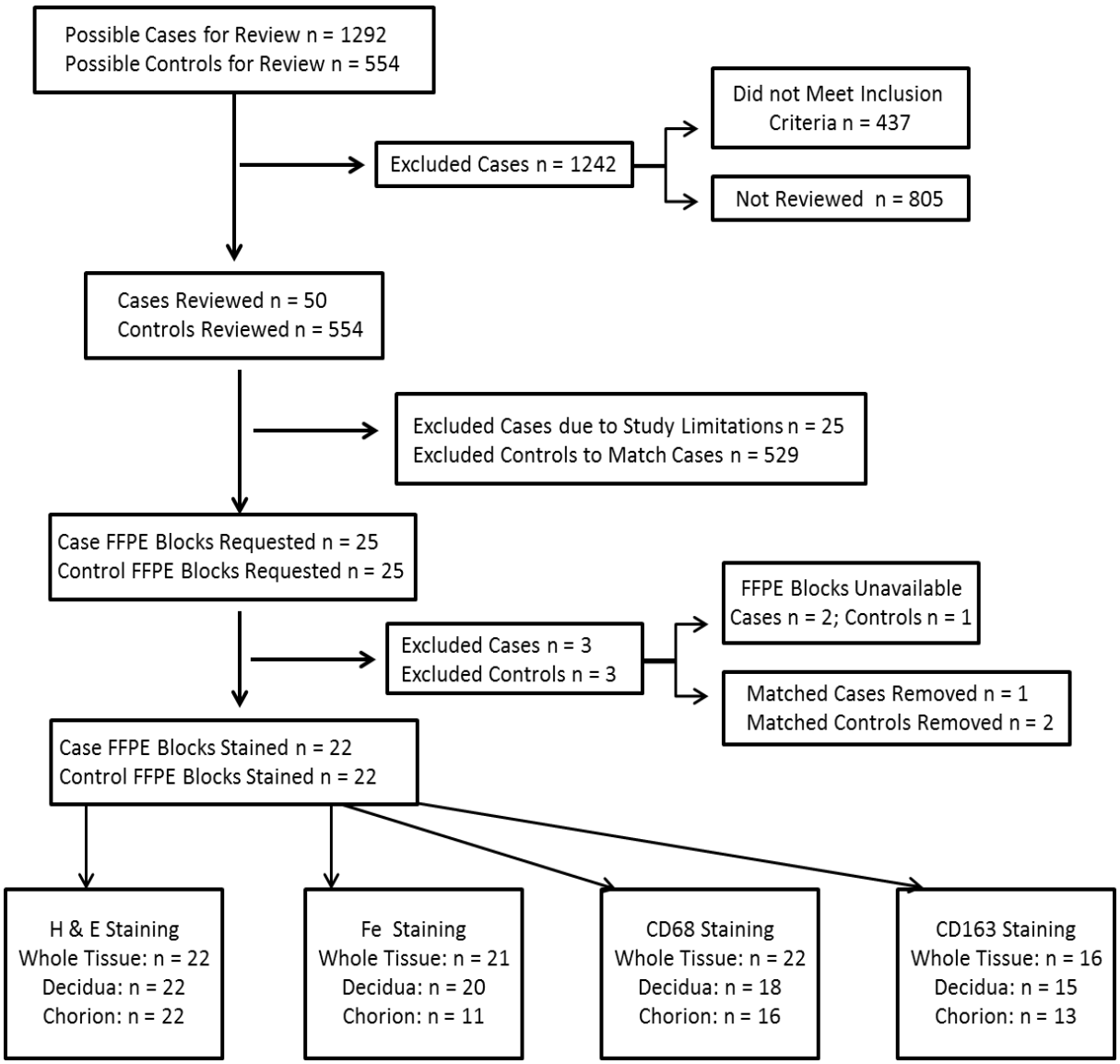


Figure 1 Study design and sample distribution Workflow diagram depicting study design and distribution of investigated population including the numbers of patients included and excluded from the study. Our goal for this pilot study was to include 25 cases and controls. Once we confirmed the first 25 cases, we excluded the remaining 830 possible cases from review.

Data Collection

Patient demographic information, vital statistics, and laboratory results including age, race/ethnicity, indication for delivery, body mass index (BMI), placental weight and volume, fasting glucose and 3-hr OGTT results, and maternal hematocrit were obtained through the SD. Not all patient records provided data on BMI or maternal hematocrit levels. We were able to collect data for calculating BMI for 27.2% of participants and maternal hematocrit for 86.3% of participants at time of delivery. Due to the nature of our study design, we could not recover all missing data points for these variables and have listed the missing number of data points where appropriate **(Table 1)**.

Characteristics of study participants by case status

	Case n=22		Control n=22		
Categorical Characteristics	n	%	n	%	p-value[1]
Race					0.45
White, non-Hispanic	12	55	12	55	
Black, non-Hispanic	4	18	3	14	
Hispanic	2	9	0	0	
Asian	2	9	5	23	
Other	1	5	0	0	
Unknown/Missing	1	5	2	9	
Method of delivery					
Cesarean section	14	64	14	64	1.00
Vaginal	8	36	8	36	
Continuous Variables	n	mean (S.D.)	n	mean (S.D.)	p-value
Maternal age at delivery (years)	22	29.3 (5.8)	22	27.0 (5.5)	0.19
Gestational age at delivery (weeks)	22	37.2 (1.8)	22	37.5 (2.1)	0.61
Pre-pregnancy BMI (kg/m^2)	7	25.5 (4.2)	2	23.5 (4.0)	0.58
1st Trimester BMI	8	26.8 (4.1)	4	27.6 (3.4)	0.78
Placental weight (g)	22	509.5 (184.0)	22	465.7 (102.5)	0.34
Placental volume (cm^3)	22	761.4 (326.2)	22	685.1 (243.7)	0.38
Glucose tolerance test, 50g	**21**	**157.4 (21.3)**	**11**	**110.5 (23.5)**	**<0.001**
Fasting glucose	22	90.0 (16.0)	0	—	
Oral glucose tolerance, 1 hour	22	190.7 (27.9)	0	—	
Oral glucose tolerance, 2 hours	22	186.8 (28.6)	0	—	
Oral glucose tolerance, 3 hours	22	140.6 (40.5)	0	—	
Maternal Hct	**20**	**35.3 (2.5)**	**18**	**33.0 (3.1)**	**0.02**
Maternal hemoglobin	10	12.1 (0.7)	10	11.3 (1.2)	0.12

Abbreviations: SD, standard deviation. Significant values are bolded.
[1]p-values derived from Pearson's chi-squared for categorical variables and from two-sample t test for continuous variables

Table 1 Characteristics of study participants by case status Cases and controls were matched by maternal age, gestational age, and indication for delivery. Maternal hematocrit (Hct) levels are from the day of delivery and were significantly higher in the case population compared to control with a p value of 0.02.

Immunohistochemistry and Pathological Analysis

A total of 50 FFPE blocks were requested from Pathlink, the Vanderbilt Tissue Biobank which accrues patient samples and is linked to a de-identified version of data extracted from an EMR system, in which all personal identifiers have been removed for analysis (Pulley, Clayton, Bernard, Roden, & Masys, 2010). FFPE blocks containing cross-sectional human placenta were sectioned and stained for analysis by the Translational Pathology Shared Resource (TPSR) at Vanderbilt University Medical Center. In some instances, full-thickness cross-sections containing the decidua, placental disc, and chorion, were unavailable. For these patients, pairs of blocks jointly forming a full-thickness cross section were obtained for analysis. Serial sections were prepared from the FFPE blocks and were stained with hematoxylin and eosin (H&E), Prussian blue, CD163 and CD68 at TPSR using standard protocols. Some requested samples were unavailable or in poor quality and thus were excluded from the study. While other samples, once cut from the FFPE blocks and stained, were missing sections of the decidua, chorion, or both. When this occurred, we excluded the corresponding tissue section in the matched sample. In total, 22 FFPE blocks from cases and 22 FFPE blocks from controls were available for analysis **(Figure 1)**.

All H&E-stained slides were reviewed by a pathologist blinded to GDM status and prior diagnostic findings using a custom data entry form. Amnion, villous disc, and decidual surface were systematically evaluated for multiple abnormalities including meconium laden macrophages, acute inflammation, infarcts, hydrops, villous maturity, and decidual and fetal vessel maturation. This allowed for uniformity and comparability in description. Histological findings were determined using standard clinical criteria. Representative images were captured under bright-field illumination with automated exposure.

Slide Imaging and Digital Quantification

Slides were digitally scanned at 20X magnification using a Leica SCN400 Slide Scanner by the Digital Histology Shared Resource (DHSR) at Vanderbilt University. Digital analysis and quantification was performed using Leica Biosystems Digital analysis software. For quantification of CD68, CD163, and iron within placental tissue, digital annotations were made segregating the chorion and decidua from the remainder of the placental disc (villous core). Cellular staining and iron staining were quantified for the entire placental tissue and individually for the chorionic and decidual annotated sections. The whole specimen encompasses all tissue on the slide including chorion and decidua if present as well as the villous core. CD68 and CD163 were quantified as the percentage of positively staining cells per total cells. Iron staining was quantified as the percentage of positively staining area of total tissue within the given region. When either the decidua or chorion was absent from a patient sample, the corresponding region was excluded from analysis for its matched control or case.

Statistical Analysis

Pearson's X^2 and two-sample t-tests were used to assess variation in categorical and continuous study participant characteristics, respectively. We used beta regression to predict the marginal effects of gestational diabetes for histological features that were measured as the proportion of cells with a given characteristic (proportion of cells to stain for CD68, CD163, and iron moderately, strongly, and overall). Beta regression accounts for a continuous outcome variable being bounded [0, 1], unlike linear regression. We used logistic regression to calculate the odds ratio (OR) of placental tissue from cases having abnormalities that were either present or absent as compared to controls. For histological characteristics with more than two levels, we

used polytomous regression to calculate ORs of the placental tissue from cases having the feature as compared to controls. We provide crude estimates and estimates adjusted for maternal hematocrit. In our supplemental analysis, we used linear regression to estimate the expected change in absorbance and intensity for CD68 and CD163 staining in cases compared to controls.

Results

Study Participant Characteristics

Study participant characteristics and data gathered from the SD are summarized in Table 1. The average maternal age for controls was 27.0 while cases had an average maternal age of 29.3 (S.D. for controls and cases were 5.5 and 5.8 respectively). Average gestational age at time of delivery for controls was 37.5 weeks and 37.2 for cases (S.D. for controls and cases were 2.1 and 1.8 respectively). The retrospective nature of this study limited our ability to collect BMI information on all subjects. The pre-pregnancy BMI and 1st trimester BMI information collected did not reveal significant difference between controls and cases. Placental weight and placental volume did not differ between cases and matched controls. The 50g OGTT was elevated in cases compared to controls, as expected. All control subjects had a normal 50g OGTT, therefore 3-hour OGTT was neither clinically indicated nor performed. Maternal hematocrit levels at delivery were significantly elevated in GDM women compared to controls at 35.3% versus 33% for controls (p=0.02). In light of hematocrit differences between cases and controls, and given the relationship between iron and hemoglobin, we adjusted analyses for differences in maternal hematocrit levels.

Differences in histological characteristics by gestational diabetes status

Variable	N	% of Cases	% of Controls	OR	95% CI	Adjusted OR[1]	95% CI
Amnion							
Abnormal	44	68.2	36.4	**3.75**	**1.08, 13.07**	**4.05**	**1.04, 15.83**
Reactive changes	44	50.0	36.4	1.75	0.52, 5.84	1.56	0.43, 5.69
Meconium-Laden Macrophages	44	36.4	4.6	**12.00**	**1.35, 106.80**	**12.51**	**1.26, 124.24**
Squamitization	44	13.6	4.6	3.32	0.32, 34.65	7.99	0.49, 130.65
Fetal Vessels							
Abnormal	44	22.7	13.6	1.86	0.39, 8.99	1.50	0.28, 8.08
Inflammation	44	4.6	0	–	–	–	–
Cushion Lesions	44	4.6	4.6	1.00	0.06, 17.07	–	–
Erythroblastosis	44	9.1	4.6	2.10	0.18, 25.01	1.91	0.13, 27.07
Villi							
Chorangiosis	44	4.6	4.6	1.00	0.06, 17.07	–	–
Hydrops	44	4.6	9.1	0.48	0.04, 5.67	0.48	0.03, 6.81
Chronic villitis	44	4.6	4.6	1.00	0.06, 17.07	–	–
Intervillositis	44	0.0	4.6	–	–	–	–
Abnormal size	44	77.3	45.5	**4.08**	**1.11, 15.02**	3.17	0.71, 14.10
Abnormal vessel	43	63.6	52.4	1.59	0.47, 5.39	1.03	0.24, 4.42
Hemosiderin-laden macrophages	44	18.2	22.7	0.76	0.17, 3.29	0.52	0.10, 2.70
Any Infarct	44	40.9	40.9	1.00	0.30, 3.33	1.25	0.28, 5.48
Other							
Calcifications	44	63.6	27.3	**4.67**	**1.30, 16.76**	3.70	0.86, 16.89
Fibrin Deposits	43	19.1	18.2	1.06	0.23, 4.92	0.88	0.14, 5.36
Maternal infarct	41	4.5	15.0	0.28	0.03, 2.98	0.13	0.01, 3.13
Villous Size[2]	44						
Normal		22.7	54.6	1.00	(referent)	1.00	(referent)
Immature		22.7	18.2	3.00	0.56, 16.07	3.24	0.48, 21.77
Accelerated maturation		40.9	18.2	**5.40**	**1.12, 26.04**	3.80	0.61, 23.54
Hypermature		13.6	9.1	3.60	0.45, 28.56	1.93	0.17, 21.66
Villous Vessels[2]	43						
Intermediate		36.4	47.6	1.00	(referent)	1.00	(referent)
Immature		27.3	9.5	3.54	0.59, 23.87	1.11	0.29, 4.21
External		36.4	42.9	5.74	0.45, 72.30	0.56	0.11, 2.80
Villous Infarct[2]	44						
None		59.1	59.1	1.00	(referent)	1.00	(referent)
Small (<4x field)		13.6	31.8	0.43	0.09, 2.03	3.00	0.51, 17.71
Large (>4x field)		26.3	9.1	0.44	0.06, 3.11	3.36	0.46, 24.51

Abbreviations: OR, odds ratio; CI, confidence interval. Significant values are bolded.
[1]Adjusted for maternal Hct
[2]Odds ratios for histological features for more than two levels were calculated using polytomous regression. All other odds ratios are from logistic regression models.

Table 2 Differences in histological characteristics by gestational diabetes status Histological characteristics were identified on H & E stained slides by a clinical pathologist blinded to gestational diabetes status.

GDM placentae exhibit an increase in abnormal histology

Meconium-laden macrophage levels within the amnion of GDM placentae were significantly higher at 36.4% compared to 4.6% of controls. Correspondingly, a greater percentage of GDM placentae showed amnion epithelial reactive changes suggestive of meconium exposure - 68.2% of GDM placentae versus 36.4% of controls **(Table 2, Figure 2)**.These differences remained statistically significant after adjustment for maternal hematocrit.

GDM placentae exhibited abnormal villous size compared to controls with the GDM placentae showing signs of accelerated maturation for gestational age. When adjusted for maternal hematocrit levels however, the difference was no longer significant. The presence of calcifications was significantly increased in GDM placentae in the crude, but not adjusted analysis **(Table 2)**.

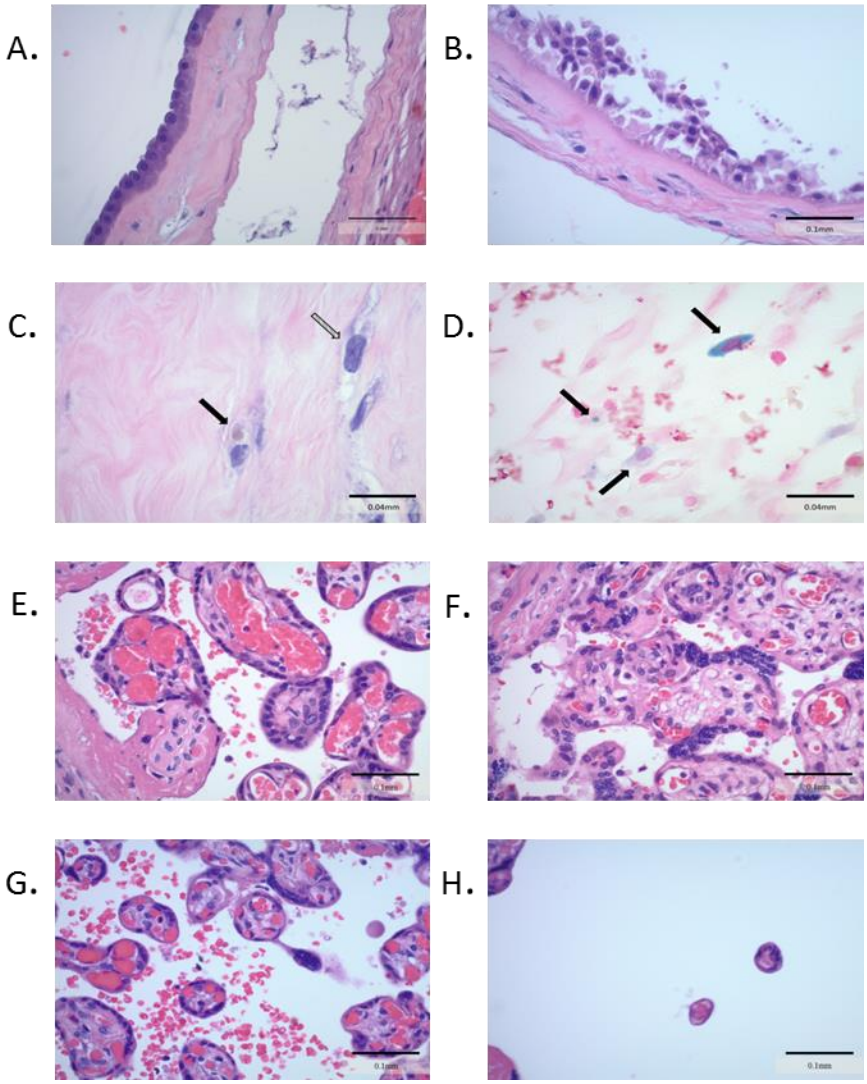


Figure 2 Histological characteristics of placenta from women with GDM and healthy controls. Histological characteristics were identified on H & E stained slides by a clinical pathologist blinded to gestational diabetes status. **A)** Image of normal amnion tissue from a control placenta (40x) **B)** Amnion displaying example of reactive changes suggestive of meconium in a placenta from a mother with GDM (40x) **C)** Black arrow indicating meconium-laden macrophage and stripped arrow indicates reactive macrophage from GDM placenta (100x) **D)** Prussian blue staining black arrows indicate hemosideran-laden macrophages in GDM placenta (100x) **E -H)** Representative H & E images showing differing levels of villous

maturation in placental villi (40x). Normal villi from control, large villi from GDM, accelerated maturation villi from control, and hypermature villi from control respectively.

CD163 and iron storage are increased in GDM placentae

To examine the impact of GDM on placental macrophages and iron stores, we stained full placental cross-sections for CD68, CD163, and iron. The chorion of GDM placentae contained a significantly higher number of moderately stained CD163 positive cells compared to controls **(Figure 3)**. The total number of positive cells as well as the total number of moderately staining CD163 positive cells was greater in the decidua of GDM placentae compared to controls. The higher expression of CD163 in the placentae of GDM mothers remained significant after adjustment for maternal hematocrit **(Table 3)**.

We examined the amount of iron storage within the placenta by Prussian blue staining. The difference in Prussian blue stained positive area was not significantly different in the chorion or decidual areas independently of the villous core **(Table 3)**. However we observed a significant increase in iron positive area within the entire tissue, indicating that the increase in iron staining was localized to the fetally-derived villous core. The difference remained significant after adjustment for the maternal hematocrit levels.

Table 3. Differences in CD68, CD163, and iron staining by gestational diabetes status

Variable	N	Mean % Case	Mean % Control	Change in %[1]	95% CI	Adjusted Change in %[2]	95% CI
CD68							
Whole Specimen							
Positive	44	8.59	8.84	-0.24	-2.90, 2.41	-0.27	-2.96, 2.41
Moderate	44	1.87	1.63	0.24	-0.38, 0.86	0.17	-0.10, 0.44
Strong	44	6.72	7.20	-0.48	-2.90, 1.93	-0.44	-2.94, 2.06
Chorion							
Positive	32	7.02	7.65	-0.63	-5.27, 4.01	-0.84	-5.34, 3.67
Moderate	32	1.89	1.42	0.47	-1.26, 0.32	0.37	-0.35, 1.10
Strong	32	5.13	6.23	-1.10	-5.35, 3.15	-1.09	-4.77, 2.60
Decidua							
Positive	36	16.98	16.50	0.48	-5.73, 6.68	1.27	-5.96, 8.49
Moderate	36	3.73	3.75	-0.02	-2.03, 1.99	0.73	-0.95, 2.42
Strong	36	13.93	12.50	1.44	-3.83, 6.70	1.67	-5.10, 8.43
CD163							
Whole Specimen							
Positive	38	20.2	18.0	2.23	-1.05, 5.51	3.29	-0.73, 7.31
Moderate	38	11.79	10.3	1.49	-0.53, 3.51	2.04	-0.44, 4.53
Strong	38	8.62	7.72	0.90	-1.05, 2.84	1.25	-0.91, 3.41
Chorion							
Positive	30	20.83	14.83	6.00	-0.37, 12.36	7.51	-1.97, 16.98
Moderate	30	15.96	11.25	**4.71**	**0.20, 9.22**	**5.51**	**0.04, 10.97**
Strong	30	4.86	3.57	1.29	-1.43, 4,01	2.03	-3.65, 7.71
Decidua							
Positive	36	24.83	18.31	**6.52**	**0.58, 12.48**	**9.29**	**1.96, 16.61**
Moderate	36	16.75	11.97	**4.77**	**0.59, 8.96**	**6.45**	**1.39, 11.51**
Strong	36	8.08	6.46	1.62	-0.79, 4.04	2.60	-0.75, 51.61
Iron							
Whole Specimen	42	0.82	0.59	**0.23**	**0.04, 0.42**	**0.24**	**0.02, 0.46**
Chorion	22	0.50	0.51	-0.02	-0.40, 0.36	0.00	-0.40, 0.40
Decidua	40	0.40	0.30	0.11	-0.12, 0.32	0.07	-0.26, 0.40

Abbreviations: CI, confidence interval. Significant values are bolded.
[1]Change in % represents the change in expect proportion of cells to have the histological characteristic when comparing cases to controls using beta-regression
[2]Change in % represents the change in expect proportion of cells to have the histological characteristic when comparing cases to controls adjusted for maternal hematocrit

Table 3 Differences in CD68, CD163, and iron staining by gestational diabetes status

Histological characteristics were identified on H & E stained slides by a clinical pathologist blinded to gestational diabetes status.

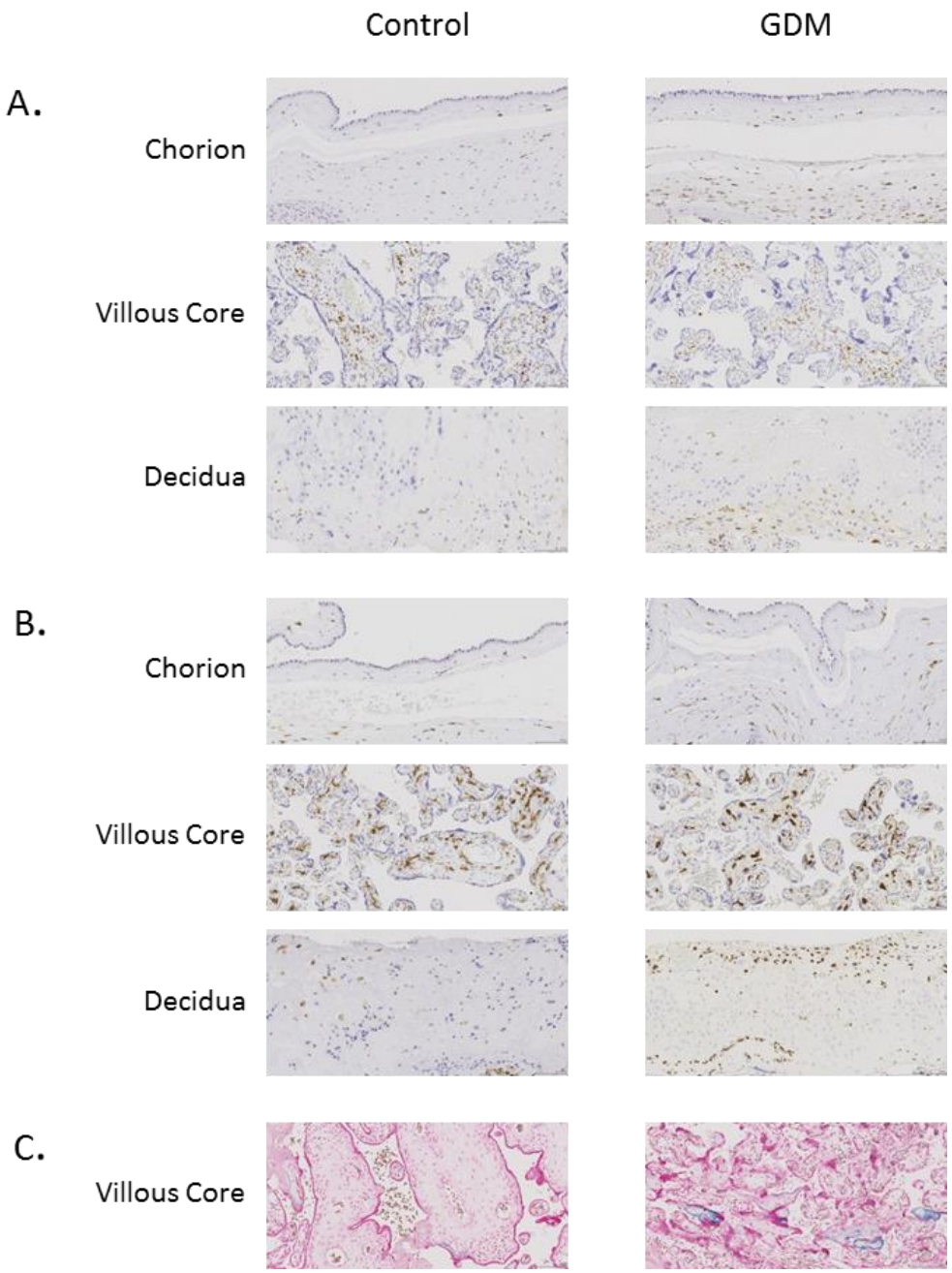


Figure 3 Differences in CD68, CD163, and iron staining by gestational diabetes status. FFPE blocks of placental specimens from women with GDM and control were stained for A) CD68 B) CD163 C) Prussian blue staining for iron. Slides were then digitally scanned and imaged for digital quantification purposes. Representative images were randomly captured at 20x.

Discussion

A growing body of evidence suggests and supports an association between GDM and altered histological and physiological characteristics within the placenta, an increase in maternal and placental inflammation, and an increase in maternal iron status (Afkhami-Ardekani & Rashidi, 2009; al-Okail & al-Attas, 1994; Amiri et al., 2013; Calderon et al., 2007; Chen et al., 2006; Kuzmicki et al., 2008; Mrizak et al., 2014; Pendeloski et al., 2015; Radaelli et al., 2003; Rawal et al., 2017; Rudge et al., 2011; Schober et al., 2014; Sisino et al., 2013; Yu et al., 2013). In this study we used a novel approach to retrieve archived placental tissue linked to de-identified patient records in order to investigate differences in placental histology, placental macrophage number, distribution, CD163 expression, and iron storage within the placenta in order to gain a better understanding of how GDM affects these placental characteristics. Indeed, we found an increase in abnormal placental histological characteristics, and increase in CD163 expression, and an increase in iron storage within the placenta of women with GDM. The findings presented here shed light on specific pathological and cellular changes that occur within the placenta as a result of GDM. These changes may mediate the pre and postnatal complications associated with GDM and confirm the need to look further into the role of iron and iron supplementation during pregnancy in women at risk for GDM.

GDM and Maternal Hematocrit

Our study identified an association between GDM and an increase in maternal hematocrit level, which is consistent with reports in pregnant and non-pregnant persons with diabetes. Associations between hematocrit levels and diseases of insulin resistance and metabolic syndrome have been reported, however, associations vary depending on study design and

population (Barbieri et al., 2001; Capoglu, Unuvar, Bektas, Yilmaz, & Kaya, 2002; Nebeck et al., 2012; G. Wannamethee & Shaper, 1994; S. G. Wannamethee, Perry, & Shaper, 1996). Lao reported women with GDM have higher hemoglobin, red blood cell count, and hematocrit levels in the third trimester, but not earlier in gestation (Lao & Ho, 2002). Another study reported no increase in hematocrit levels during pregnancy in cases of GDM; however, they described an increase in maternal hemoglobin levels throughout the duration of pregnancy and have a high hemoglobin and hematocrit level at baseline (Tan, Chai, Ling, & Omar, 2011). Elevated hematocrit levels have been reported to be an independent risk factor for the development of T3D and impaired glucose tolerance (Capoglu et al., 2002). Hyperinsulinemia and insulin resistance are features of metabolic syndrome, type 2 diabetes, and GDM. Hematocrit levels increase as a result of increased erythropoiesis, which might explain the increase observed in GDM patients. Previous studies have provided evidence for a relationship between hyperinsulinemia and an increase in erythropoiesis (Barbieri et al., 2001). Insulin resistance is associated with increased levels of insulin-like growth factor-1, which may lead to an increase in erythropoiesis, hence increasing hematocrit levels within GDM patients. This phenomenon has been demonstrated previously in rats (Kurtz et al., 1988). In healthy individuals, an increase in hematocrit levels would suggest superior oxygen delivery throughout the body. Alternatively, it is possible that GDM causes worse oxygen delivery to the placenta and the combination of elevated hematocrit levels and accelerated villous maturation that we report here are compensatory responses to an increased need for oxygen within the placenta. Future studies are needed to confirm our findings and assess these possible mechanisms.

Placental Histopathological Changes in GDM

The histopathological changes identified in the placentae of women with GDM were heterogeneous and varied in their severity. Our analysis discovered a significant increase in the presence of meconium-laden macrophages and epithelial changes presumed reactive to meconium within the amnion of GDM placentae. Meconium is fetal stool composed of bile acid, phospholipases, and other components. While the passage of meconium prior to 32 – 34 weeks is rare, 15 – 20 % of placentas are affected by the passage of meconium at delivery. The passage of meconium *in utero* may be indicative of fetal stress, typically hypoxic or ischemic stress, although not always present in cases of fetal stress. Although the exact implications of increased numbers of meconium-laden macrophages with the amnion are unknown, the uptake of meconium by alveolar macrophages has been shown to decrease macrophage phagocytosis and increase the release of TNF-α from macrophages (Craig, Lopez, Hoskin, & Markham, 2005; Lally, Mehall, Xue, & Thompson, 1999).

Villi arborize and mature throughout gestation, with more mature villi showing a smaller cross section and peripheral capillaries (Khong et al., 2016). These changes can be expected to maximize surface area and thus oxygen diffusion. Villous maturation varies in pathologic circumstances – immature villi have been repeatedly reported in GDM, while precocious maturity has been reported in conditions with impaired maternal-fetal circulation (Daskalakis et al., 2008; Khong et al., 2016; Rudge et al., 2011). In clinical practice, as in this study, villous maturation is based on a gestalt impression, rather than systematic measurement. Perhaps surprisingly, this gestalt impression has been shown to correlate with maturation-based gene expression changes (Leavey et al., 2017). It is unclear why our results differ from previously reported studies in GDM. There are several minor methodologic differences; however the most

likely cause is the use of a retrospective case: control design with matching by maternal and gestational age in the present study as compared to the prospective cohort-based designs in other studies. As noted in the results, the difference in villous maturation was no longer apparent when controlling for hematocrit. This suggests a unifying mechanism wherein GDM impairs oxygen delivery to the placenta, thus inducing the compensatory responses of accelerated villous maturation and increased red cell mass.

The presence of villous calcifications was elevated in GDM placentae compared to controls prior to adjustment for maternal hematocrit levels, with a fairly large effect size (unadjusted odds ratio of 4.67 (95% confidence intervals of 1.30-16.76, Table 2). Calcifications are indicative of an aging placenta (Jeacock, 1963). The clinical significance of such calcifications is not well defined although calcification is likely due to an increase in available calcium and phosphate within the placenta (Jeacock, 1963). Prominent in late term and post-term placentae, they can occur in the absence of other placental pathologies, particularly in peripheral areas. However, the mineralization process through which calcifications occur may be accelerated in some disorders of pregnancy. The early appearance of calcifications may represent accelerated placental maturation and or senescence brought about as a result of fetal stress and maternal complications such as maternal hypertensive disorders (Quinlan, Cruz, Buhi, & Martin, 1982). Quinlin and colleagues first noted that placentae from pregnancies complicated by diabetes develop calcifications earlier in gestation than non-complicated pregnancies and may represent early placental dysfunction (Quinlan et al., 1982). Although significance was lost after adjustment for maternal hematocrit, the effect size was 3.70 (95% confidence intervals of 0.86-16.89), suggesting that the small samples size might have played a role in the lack of significance. A difference in placental calcifications indicates that GDM may influence nutrient

availability within the placenta as well as leading to accelerated placental maturation and cellular senescence. The hypothesis that GDM may accelerate placental maturation is further strengthened by our reported finding that we observed an increase in villous maturation within the GDM cohort compared to control. The mechanism driving the accelerated maturation which could be linked to an increase in villous calcifications is yet to be defined.

Placental Macrophages and GDM

CD68 is a pan-macrophage marker found on monocytes and macrophages (Pulford, Sipos, Cordell, Stross, & Mason, 1990). We utilized this marker as way to localize and enumerate macrophages within the placenta. Our study identified no significant difference in the number or distribution of placental CD68+ cells between cases and controls. Another study which looked for CD68 expression levels within placental tissue of GDM and control patients found no significant difference in the levels of CD68 transcript levels (Abumaree et al., 2013). These findings contradict other reports where increases in the mRNA expression of CD68, in conjunction with increased expression levels of IL-6 and TNF- α, were observed in pregnancies complicated with GDM (Li et al., 2013). Mrizak et al also reported an increase in transcript levels of CD68 and CD14, both common human monocyte/macrophage markers, within placenta recovered from women with GDM (Schober et al., 2014). That study, however, only collected placentae from women diagnosed with GDM whose child was born with macrosomia. The presence of macrosomia as an inclusion criterion may have biased the results for more severe cases of GDM than we included. Other studies reported conflicting results regarding the level of CD68 mRNA in gene expression analysis studies. The discrepancies in results regarding the expression of CD68 mRNA or the enumeration of CD68+ stained cells via microscopy between

GDM and control placentae are not yet explained. The infiltration or expression of CD68 within placental tissue may depend on multiple factors, not just the presence or absence of GDM. The severity of insulin resistance, the amount of circulating inflammatory factors, and the degree to which glucose levels are controlled during pregnancy may all influence macrophage numbers and distribution within the placenta.

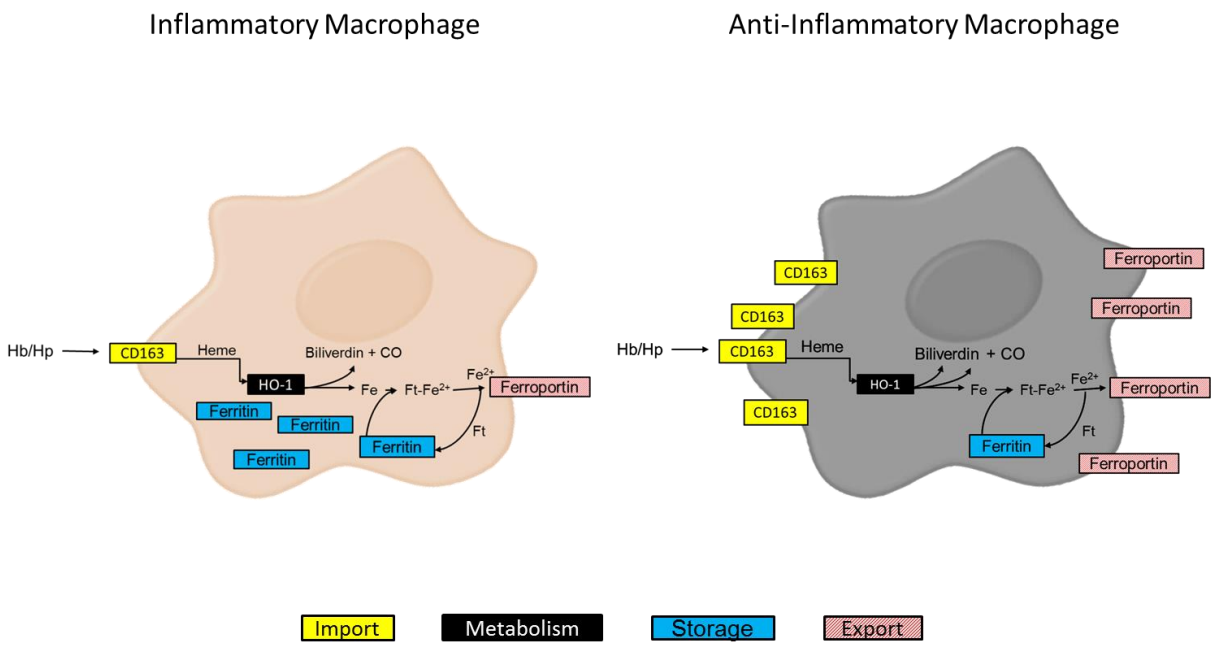


Figure 4 Macrophage Iron metabolism is coupled to inflammatory status The expression of iron regulatory proteins such as CD163, a hemoglobin scavenger receptor, ferritin, an iron storage protein, and ferroportin, an iron export protein, are affected by the inflammatory status of the macrophage. High levels of intracellular iron stores in the form of ferritin and low levels of CD163 and ferroportin expression are characteristic of M1 or pro-inflammatory macrophages. This phenotype allows for the sequestration of iron from the extracellular environment. M2 or anti-inflammatory macrophages store less intracellular iron and increase expression of CD163

and ferroportin permitting for increased uptake and degradation of hemoglobin/haptoglobin (Hb/Hp) complexes and the recirculation of iron to the extracellular environment.

A hemoglobin scavenger receptor that mediates the endocytosis of hemoglobin-haptoglobin complexes, CD163, is exclusively expressed on macrophages and is often utilized as a marker for placental macrophages (Kristiansen et al., 2001; Z. Tang et al., 2011). We identified an increase in CD163+ cells within the decidua of GDM placentae and an increase in the amount of moderately stained CD163+ cells within the chorion. Expression of CD163 has been associated with an anti-inflammatory (M2) macrophage phenotype (Abumaree et al., 2013; Z. Tang et al., 2013). Anti-inflammatory mediators such as IL-10 promote the expression of CD163 while pro-inflammatory mediators such as IFN-γ and TNF-α have been shown to decrease its expression (Buechler et al., 2000). CD163 also exists in soluble form, sCD163 (Etzerodt et al., 2010). Similar to TNF-α, the ectodomain of CD163 is cleaved from the surface of macrophages in an inflammation-driven fashion (Etzerodt et al., 2014). Levels of circulating sCD163 increase in mothers with GDM in conjunction with increases in circulating TNF-α and IL-6 (Bari et al., 2014). Placental tissue, as well as adipose tissue, from GDM mothers is a source of increased sCD163 circulating in maternal serum as described by placental and adipose tissue explant studies (Bari et al., 2014). GDM appears to not only increase the expression of CD163, but also increase levels of sCD163 in maternal serum. These findings are in agreement with previous findings of elevated sCD163 in GDM (Bari et al., 2014). The molecular mechanisms leading to the cleavage of CD163 from placental macrophages has yet to be elucidated in the case of GDM so future attention should be paid to discovering the mechanisms involved in this phenomenon.

Placental Iron Load Increases in GDM

Placental iron load for the tissue as a whole was significantly increased in GDM. However, close examination of both the decidua and chorion independently revealed no difference, indicating that the increase in iron load within the placenta is found primarily within the fetally-derived villous tissue. The opposite was found to be true for the presence of CD163. CD163 showed significance within the chorion and decidua individually, but not the tissue as a whole. The villous core, the majority of the placental specimen, is primarily fetally-derived tissue, bathed in maternal blood, and acts as the site of exchange between maternal and fetal circulation. As this area is saturated in maternal blood, it makes sense that the villous core stores iron at a higher level than within the chorion or decidua. Iron enters and exits macrophages in many different forms through many different pathways, including CD163 (Donovan et al., 2005; Soe-Lin et al., 2009). If macrophage polarization is impacted in cases of GDM, the relative expression of CD163 as well as iron storages within the macrophage may be altered in response. As reviewed by Cairo, *et al*., macrophage iron metabolism is influenced by the local inflammatory environment (Cairo et al., 2011). If GDM influences the inflammatory environment, CD163 expression and iron storage within the placenta may be effected as well. Multiple other cell types within the placenta are involved in iron homeostasis. Our findings of increased iron stores within GDM placentae correspond with previously reported data indicating that women with GDM have a significantly increased iron stores, as measured by hemoglobin, ferritin, and transferrin saturation (Afkhami-Ardekani & Rashidi, 2009). The physiological mechanism driving the increase in stored iron and more information regarding the cells in which the iron is stored needs further investigation and may shed light onto the apparent link between GDM, iron, and possible mechanisms driving adverse phenotypes.

Study Limitations

Our study has important limitations, which may reflect the retrospective nature of, and small number of subjects included in this study. Our pilot study was relatively small and designed to be inclusive of a large range of maternal ages and gestational ages while representing diverse ethnicities. This inclusivity may have introduced variability into our results. Future studies should be designed to account for variability due to gestational age, maternal age, and ethnicity. The previously archived placental tissues included in this study were sent to pathology at the discretion of the obstetrician present at delivery. While there are recommended indications for placental examination, they are inconsistently followed. This could introduce difficult to measure confounders, since normal-appearing placentas from normal pregnancies are rarely sent for pathology. This study was likely underpowered to confirm a previously reported association between increased placental weight and GDM (Taricco, Radaelli, Nobile de Santis, & Cetin, 2003). Although both placental weight and volume were higher in cases than in controls in our study, neither difference reached statistical significance. This may also reflect more aggressive treatment of GDM than in the time period when prior studies were conducted (Metzger et al., 2008). Prepregnancy obesity is a risk factor for GDM but we did not have prepregnancy height and weight measurements on most of the subjects in this study. Although we matched our GDM samples to our controls for variables such as maternal age and gestational age at birth, differences in other patient characteristics might have introduced variability. Differences in some of our measured parameters may be evident in some subgroups for which this pilot study is undersized. The nature of FFPE placental tissue and the age of some of the blocks themselves did not allow us to gather quantifiable information on all sections of the placenta due to quality and loss of tissue.

Conclusion

In conclusion, this study demonstrates that the presence of GDM influences multiple iron related parameters including an increase in maternal hematocrit levels, an increase in CD163 within the chorion and decidua, and an increase in iron stores within the fetally derived villous core. Our results also highlight GDM's influence on multiple placental physiological parameters such as meconium-laden macrophages, placental villous size and maturation, and an increase in the presence of villous calcifications. Taken together, the alterations exhibited by the placenta in cases of GDM may provide insight into the role of the placenta in the complications associated with this common metabolic disorder of pregnancy. However, future studies are needed to further elucidate the role of placental iron and inflammation in the development, progression, and postnatal complications associated with GDM.

CHAPTER 3

GESTATIONAL DIABETES MODIFIES MIA INDUCED GENE EXPRESSION IN THE FETAL BRAIN

Theresa Barke, Kelli M. Money, Krassimira A. Garbett, Karoly Mirnics, and David M. Aronoff

Introduction

Genetics alone cannot explain the development of many psychiatric disorders. Maternal exposure to a wide array of pro-inflammatory environmental factors can increase the risk of certain developmental and psychiatric disorders. It is not necessary that the inflammatory conditions be excessive or chronic in nature in order for the risk of psychiatric disorders to increase (Berk et al., 2013; Derry, Padin, Kuo, Hughes, & Kiecolt-Glaser, 2015). Multiple studies have revealed that *in utero* stressors have the potential to induce adverse consequences on brain development and influence behavior (Hamlyn, Duhig, McGrath, & Scott, 2013; Patterson, 2009; Rapoport, Giedd, & Gogtay, 2012). Most studies thus far have investigated the impact of a single environmental stressor and its influence on the development of psychiatric disorders. As a result of these individual studies, it has been shown that both maternal infection and gestational diabetes impact brain development and have been suggested to increase risk of developing psychiatric disorders later in life (Abell et al., 2015; Mandal et al., 2013; Urs Meyer, 2014; Patterson, 2011; Salbaum & Kappen, 2012; Torres-Espinola et al., 2015). Multiple studies have previously suggested that even low levels of chronic inflammation interact with environmental and genetic risk factors increasing the risk for psychiatric disorders (Maes et al., 2009; Michel,

Schmidt, & Mirnics, 2012; Patterson, 2009). Despite our current knowledge, it is currently unknown how maternal infection and gestational diabetes, two well established inflammation-inducing psychiatric disorder risk factors, interact with one another to influence fetal brain development. In order to address this, we characterized models of poly(I:C) induced MIA and high fat diet induced gestational diabetes then assessed the changes in fetal brain gene expression as a result of MIA, high fat diet induced GDM, and the combination of the two.

Materials and Methods

Animal procedures

All animal procedures were approved by the Vanderbilt Animal Care and Use Committee. Mice were housed under standard laboratory conditions and allowed *ad libitum* access to food and water. Female and male C57Bl/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Control female mice were given standard chow throughout the experiment (5LOD, Lab Diet, St. Louis, MO, USA). In order to induce GDM, females received 60% calories by fat diet (58Y1, Test Diet, St. Louis, MO, USA) from 4 weeks of age until 10 weeks of age and continued throughout pregnancy. At 10 weeks of age, mice were mated overnight, and the presence of a vaginal plug the following morning marked gestational day 0.5 (GD0.5). Pregnant females were left undisturbed except for cage changes and weight measurements.

High fat diet and control fed females were randomly assigned to the saline or poly(I:C) treatment groups. At GD12.5, 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 creating 4 treatment groups: control fed saline (SAL), high fat fed saline (HFS), control fed poly(I:C) injected (MIA), and high fat fed poly(I:C) injected (HFM). Pregnant mice were then sacrificed at one of two time points; either 3 hours after injection at GD12.5 or 4 days post injection at GD16.5. Experimental design is outlined in **Figure 5**. Maternal serum was collected from each pregnant female and fetal brains were collected from all viable pups. Maternal serum was utilized for cytokine, adipokine, and non-fasting insulin measurements. Cytokine and adipokine levels were measured with the assistance of the Vanderbilt Hormone core via antibody-conjugated bead-based multiplex assays. Brains were flash frozen until RNA isolation was performed, and tail snips were taken from each embryo for sex genotyping. Each group at each time point (4 groups, 2 time points) contained 9 pregnant females with the exception of GD12.5 MIA which only contained 8 females, making a total of 71 females.

Glucose tolerance testing and body composition analysis

A separate cohort of control fed and high fat fed females were bred and treated as described above for comparative glucose tolerance and body composition testing (n= 6 per group). At GD12.5, pregnant control fed and high fat fed females were fasted for 6 hours. Body composition was then measured via whole body NMR (Minispec Model mq7.5, Bruker Instruments) with the assistance of the Vanderbilt Mouse Metabolic Phenotyping Center. Blood samples utilized for glucose tolerance testing were obtained from tail snips. Fasting glucose and insulin samples were taken followed by intraperitoneal injection of 2g dextrose/kg body weight. Utilizing an Aviva Accu-Chek glucometer, blood glucose measurements were taken at 10, 20, 30, 45, 60, 75, 90, and 120 minutes post-injection. Blood samples were collected for insulin measurement at 10, 30, 60, 90, and 120 minutes post-injection and were processed by the

Vanderbilt hormone core. Dams were sacrificed after all testing was complete to confirm pregnancy and gestational age. In order to evaluate the effects of the high fat diet alone on glucose parameters and body composition, one 10-12 week old non-pregnant control group (n=8) was utilized as the control for non-pregnant high fat females age 10 weeks (n=7) and age 12 weeks (n=8).

Sex genotyping

Sex genotyping was performed on each embryo. Digested tail snips from each embryo were utilized for sex determination. Forward (5'-CGCTGCCAAATTCTTTGG - 3') and reverse (5'- TGAAGCTTTTGGCTTTGAG - 3') primers for *SmcXY* locus were utilized (Jacobs, Fogg, Emeson, & Stanwood, 2009). Thermocycler conditions started with 93°C for 2 minutes; followed by 30 cycles of 93°C for 30 seconds, 58°C for 45 seconds, and 72°C for 45 seconds; and ended with 72°C for 10 minutes. Female embryos resulted in an upper band at 330 bp while male embryos had both an upper (330 bp) and a lower (290 bp) band.

Antibody-conjugated bead-based analysis

Immediately after sacrifice, blood was collected directly from the heart via cardiac puncture. Approximately 0.5 to 1 mL of blood was collected from each pregnant dam and allowed to clot at room temperature for 30 minutes. The samples were spun at 8,000 rpm for 10 minutes at 4°C. Supernatant was transferred to a new sterile tube and spun again at 8,000 rpm for 10 minutes at 4°C in order to remove any residual red blood cells. The resulting supernatant, serum, was transferred to a new tube and stored at -80°C until antibody-conjugated bead-based analysis was performed.

Luminex analysis utilizes a multiplexed assay, using x-map technology via the MagPix system. Luminex analysis was performed by the Vanderbilt hormone core (http://hormone.mc.vanderbilt.edu), which is funded in part by NIH grants (DK059637 and DK020593). The Luminex multiplex panels (EMD Millipore, Darmstadt, Germany) used for analysis of the maternal serum were the mouse metabolic hormone panel (insulin and leptin – Cat# MMHMAG-44K), mouse adiponectin (Cat # MADPNMAG-70K-01), and mouse cytokine/chemokine panel 14 plex (eotaxin, IFNγ, IL1α, IL1β, IL4, IL6, IL10, IL12p40, IL13, IL17, KC, MCP1, RANTES, and TNFα- Cat# MCYTOMAG-70K).

RNA isolation and Nanostring gene expression analysis

The right hemisphere from each fetal brain, which included both the telencephalon and diencephalon, was utilized for RNA isolation. RNA was isolated according to the manufacturer's protocol in 1 mL Trizol. Resulting samples then underwent a Qiagen RNeasy clean-up, by following the protocol provided by the manufacturer. RNA concentration was measured using a Thermo Scientific Nanodrop 2000. Equal amounts of RNA (1250 ng) from each fetal brain were combined to create 1 pooled sample per litter. The resulting pooled samples underwent another Qiagen RNeasy clean-up step to remove any remaining contaminants. To measure final concentration and purity (260/280 values above 2 and 260/230 values above 1.5) the Thermo Scientific Nanodrop 2000 was utilized. The Agilent 2100 Bioanalyzer was employed to ensure RNA integrity, with all RNA integrity numbers falling between 9.5 and 10.

150 ng of purified RNA from each pooled sample was used for Nanostring nCounter analysis (http://www.nanostring.com/applications/technology). Two panels, the mouse inflammation V2 panel- 254 genes- and a custom inflammation and neurodevelopment panel

containing 4 genes, were utilized to measure gene expression values. Nanostring multiplexed gene expression technology utilizes a digital color-coded reporter tag to identity and count expression of genes. RNA samples were hybridized with an identifying reporter probe as well as capture probes at 65°C for 24 hours. The hybridized samples were then placed in the Nanostring nCounter prep station where the complexes were aligned and immobilized in the nCounter cartridge then read on the Nanostring nCounter digital analyzer. The resulting raw counts were normalized to positive and negative spike in's and the 5 included housekeeping genes (*Cltc, Gapdh, Gusb, Hprt,* and *Tubb5*). In order to reduce background noise, a baseline of 10 counts was set, and all counts below 10 were raised to 10 for analysis. Any results made statistically significant by this manipulation were excluded from the resulting analysis as being significant. Normalized counts were then $\log_2$ transformed for analysis. To investigate the effects of high fat diet or MIA alone, HFS and MIA were each compared to SAL. To investigate the effect of MIA in the context of high fat diet, HFM was compared to HFS. To uncover differences in the MIA induced changes in the context of high fat diet induced gestational diabetes compared to in the context of control diet, dMIA [each MIA $\log_2$(counts) – the average SAL $\log_2$(counts)] and dHFM [each HFM $\log_2$(counts) – the average HFS $\log_2$(counts)] was calculated and then compared.

Statistical Approaches

To investigate the effects of diet and treatment exposure on litter statistics, a two-way ANOVA with Bonferonni *post hoc* analysis was performed, with a p-value <0.05 considered significant. In all other instances, an unpaired student t-test with Welch's correction was used to compare values between two groups, with a p-value <0.05 considered significant. With regards

to the gene expression data, criteria for significance included having a >10% change (>0.138 ALR) in value and significance by an unpaired student t-test with Welch's correction which was not artificially created if the baseline was raised.

Results

High fat diet and poly(I:C) treatment affect dam weight, but not litter statistics

A total of 35 pregnant mice were used per GD12.5 and GD16.5 time point in our study; 9 pregnant dams per SAL, HFS, and HFM group and 8 pregnant dams in the MIA group. Poly(I:C) treatment has been shown to cause a transient increase in cytokine production, accompanied by anorexia, fever, and sickness behaviors that usually resolves within the first 24-48 hours. The poly(I:C) treatment in our study caused the dams to either lose weight or fail to gain weight compared to saline treated dams (SAL = 0.60 ± 0.33, MIA = -0.83 ± 0.34; GDM = 1.33 ± 0.25, GDM+MIA = -0.67 ± 0.31), $p<0.05$. Neither the high-fat diet nor the poly(I:C) treatment altered the average number of embryos per litter, the number of resorptions, embryo size, or the male: female ratio (**Supplemental Table 2**).

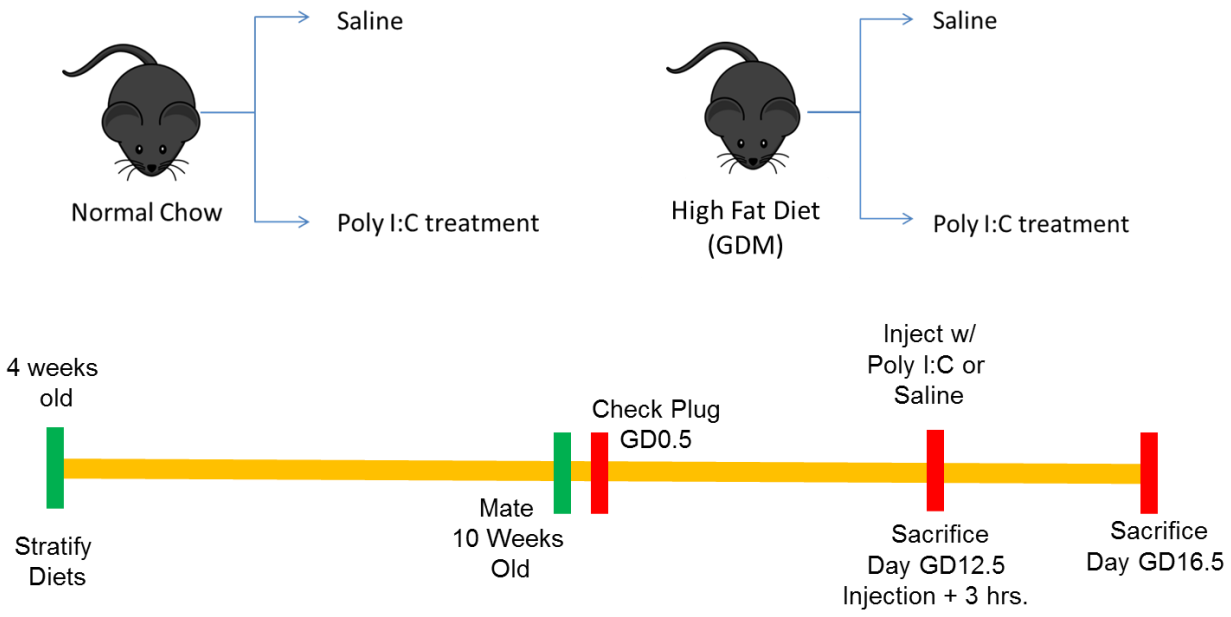


Figure 5. Diet and treatment experimental design. Mice were received at 4 weeks of age and randomly stratified into either the high-fat diet group or normal chow diet. Mice remained on their assigned diet for 6 weeks until they were mated at 10 weeks of age. 12 days after mating at GD12.5, for mice in which pregnancy was confirmed by the presence of a vaginal plug, the dams were randomly injected with either poly (I:C) or saline creating four distinct treatment groups.

High fat diet leads to GDM mid-gestation

Although the high fat diet dams were on a 60% by calorie fat diet for 6 weeks prior to mating and throughout pregnancy, these mice were not obese compared the control diet fed mice. Weight differences between the high-fat diet group and the control fed group were not significantly different at GD0.5, however gained significance, across the entire cohort, at GD9.5 (control diet = 24.6 g ± 0.3, high fat diet = 25.8 g ± 0.4, $p<0.05$) and GD12.5 time points (**Figure 6A**). To demonstrate that the high-fat diet successfully produced a gestationally diabetic

phenotype, glucose tolerance testing was conducted on mice fed normal or high-fat diets. Compared to control diet fed dams, the high-fat fed dams had increased body weight and percent fat mass at GD12.5 (**Figure 6 A/B**). In addition, the high fat diet dams had both impaired fasting glucose and increased fasting insulin after a 6 hour fast (**Figure 6 C/D**). When challenged with 2 g/kg dextrose after the 6 hour fast, high fat diet fed dams displayed significant glucose intolerance and exaggerated glucose-induced insulin secretion that remained high even at 2 hours post-injection (**Figure 6 E/F/G**).

It was important to confirm whether or not the diabetic phenotype is truly gestation-dependent. In order to test this, two separate cohorts of high-fat fed dams, age matched at GD0.5 and GD12.5, were used to compare to our pregnant cohorts for selected assessments. Confirming that the diabetic phenotype is gestation dependent, high-fat fed dams at GD0.5 showed no significant differences in body weight, composition, fasting glucose, or fasting insulin (**Supplemental Figure 1**). High-fat fed dam at GD0.5, when challenged with 2 g/kg dextrose, did not show significant differences in blood glucose levels at any time point throughout the test (**Supplemental Figure 1**). This data suggests that the high-fat fed dams did not have a diabetic phenotype at conception. To test whether or not the diabetic phenotype is a result of being on the high-fat diet for the duration of the study, GD12.5 age-matched non-pregnant high-fat fed dams were assessed. The non-pregnant high-fat and control diet fed dams age-matched to the GD12.5 cohort did not demonstrate a diabetic phenotype although they did show trends towards an impaired glucose tolerance and displayed an increase in weight gain (**Supplemental Figure 2**). It is likely that over time the high-fat diet would lead to glucose intolerance, however, the pregnancy induced increase in glucose production and insulin insensitivity is the likely the cause of the diabetic phenotype at an earlier time point (Abell et al., 2015; Gallou-Kabani et al., 2007).

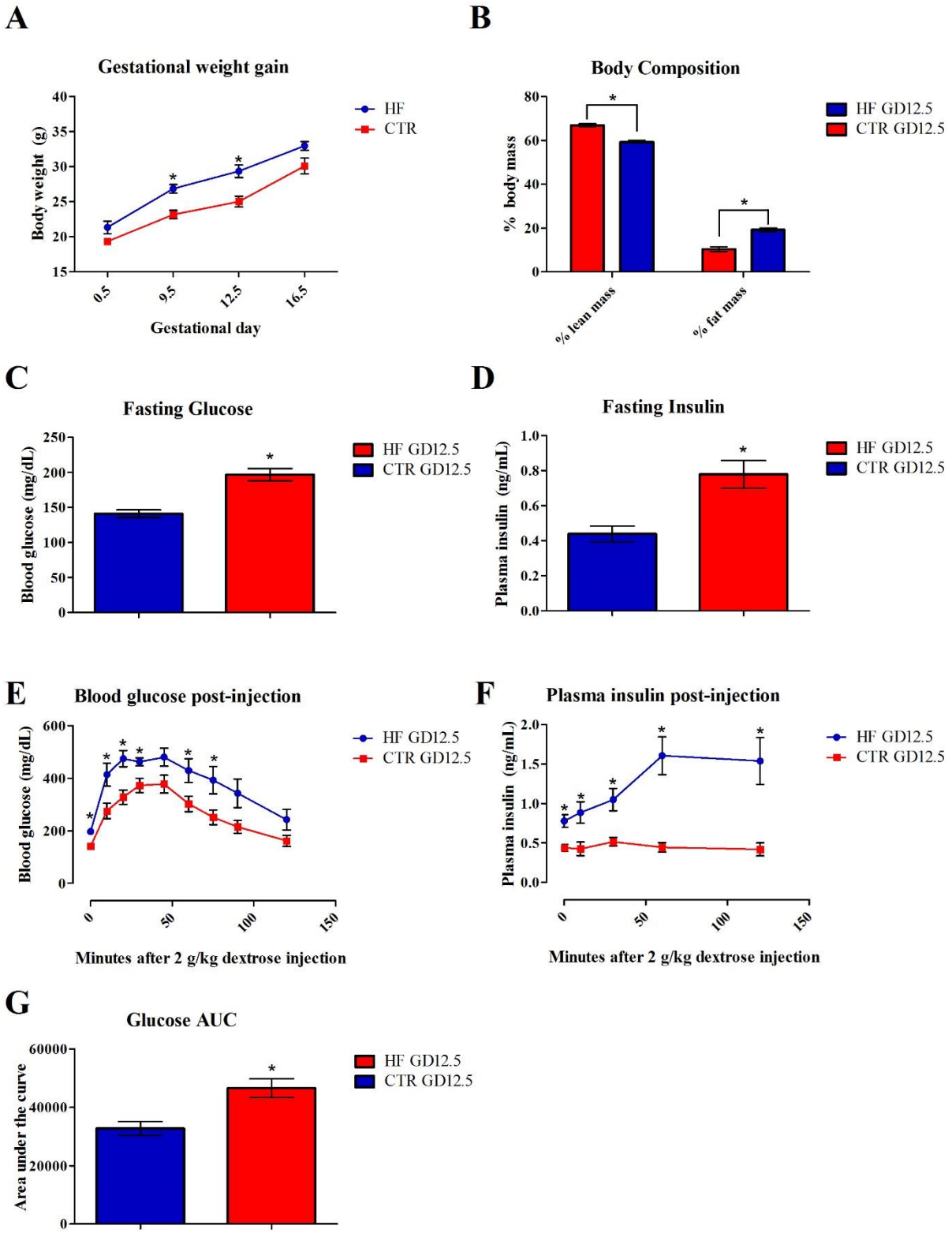


Figure 6. High fat diet produces a diabetic phenotype midgestation. Body composition, 6

hour fasting glucose and insulin, and glucose tolerance measurements were performed on a cohort of high fat(HF) and control(CTR) fed GD12.5 pregnant dams (n=6 per group). (**A)** High fat fed dams (HF) showed significant increases in body weight at GD9.5 and GD12.5. (**B**) Whole body NMR at GD12.5 showed significant changes in % body mass in high fat dams with increased % fat mass and decreased % lean body mass. (**C-D**) 6 hour fasting blood glucose and serum insulin were both significantly increased in high fat dams. (**E-G**) After a 6 hour fast, dams were injected intraperitoneally with 2 g dextrose/kg body weight. High fat fed dams demonstrated a higher peak and prolonged increase in blood glucose with significant differences from control dams observable at 0 10, 20, 30, 60, and 75 minutes post-injection, which is supported by a significantly increased area under the curve. A significant heightened insulin response that was maintained up until the 2 hour end point was also observed in high fat dams. Error bars represent SEM. Significance of $p<0.05$ is indicated by * and determined by unpaired student t-test with Welch's correction in all assays, n=6 dams per group.

MIA produces an acute increase in maternal cytokines and chemokines

In order to assess the immune response within pregnant dams as a result of poly(I:C) as well as differences between normal and GDM mice, a panel of common cytokines and chemokines were measured in maternal serum at the time of sacrifice either 3 hours post poly(I:C) or saline exposure at GD12.5 or 4 days post poly(I:C) or saline exposure at GD16.5. 3 hours after either poly(I:C) or saline injection, MIA-exposed dams in both the control and GDM groups demonstrated increases in Eotaxin, IL-1β, IL-6, IL-10, IL-12p40, IL-17, KC, TNF-α, MCP1 (CCL2), and RANTES (CCL5) (**Figure 7**). A subset of the cytokines and chemokines interrogated either exceed the range of the assay, in the case of MCP1 and IL-6, or fell below the

level of detection. In these cases, the analyte was analyzed at either the lowest or the highest detectable value. Not surprisingly, by GD16.5, almost no serum factor changes with MIA were observed, suggesting that the single poly(I:C) injection effects were acute and had largely subsided (**Supplemental Figure 3**).

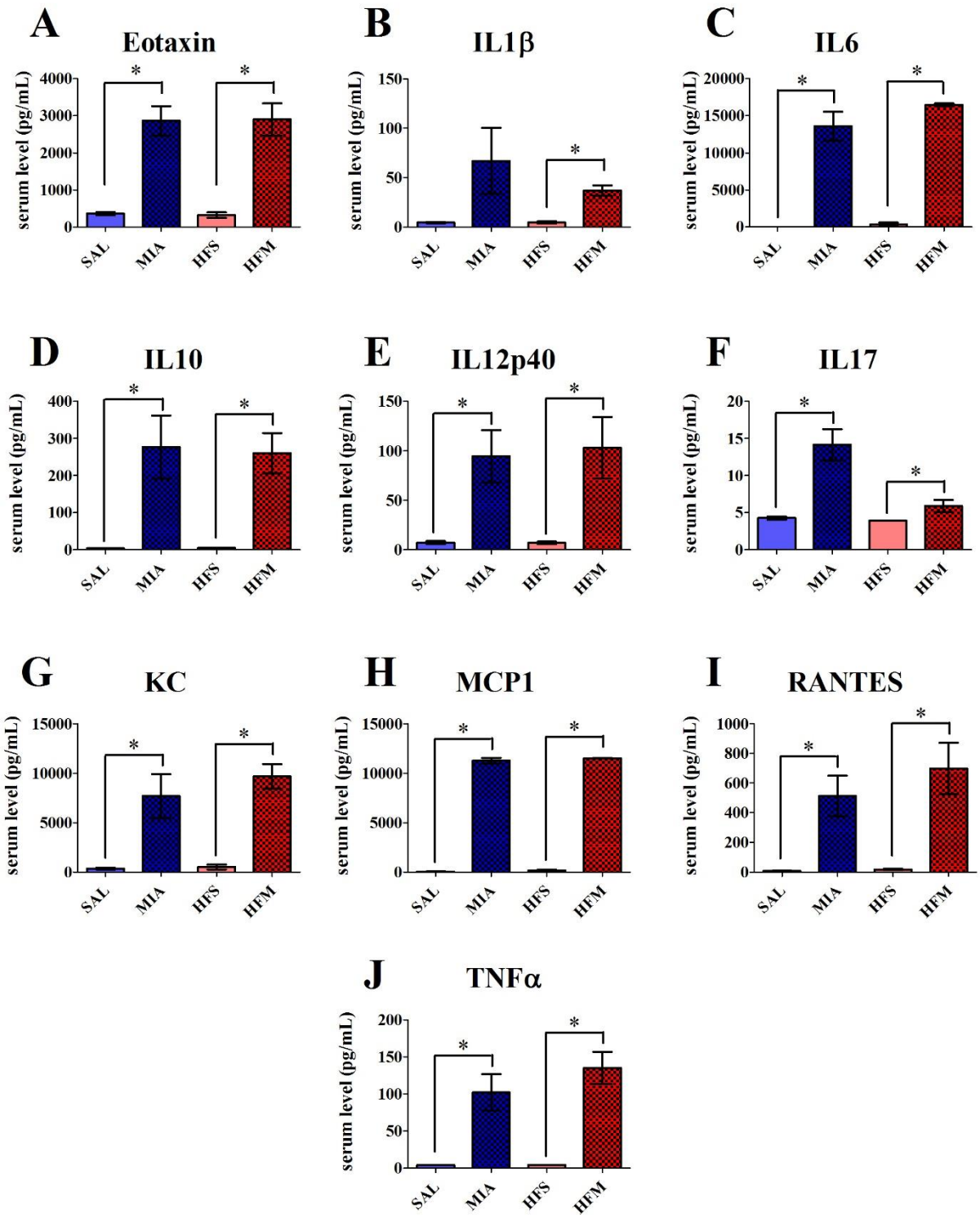


Figure 7. MIA increases maternal serum levels of chemokines and cytokines. Maternal serum collected 3 hours post injection on GD12.5 was analyzed for chemokine and cytokine

levels via antibody-conjugated bead-based multiplex assay. (**A-J**) Significant increases were observed with MIA in both the context of control diet and high fat diet in all shown but IL1β, which demonstrated a significant increase with MIA exposure only in the context of high fat diet induced gestational diabetes. Error bars represent SEM. Significance of $p<0.05$ is indicated by * and determined by unpaired student t-test with Welch's correction in all assays.

Maternal metabolic hormones are altered by both HFS and MIA

Maternal serum levels of metabolic hormones were found to be altered by both high-fat saline, diet-induced gestational diabetes, and MIA at GD12.5. HFS mice were found to have decreased levels of circulating adiponectin. These levels were not found to be further altered by the addition of MIA in the context of high-fat diet induced gestational diabetes (**Figure 8A**). Non-fasting serum insulin levels trended toward an increase with HFS alone, but the combination of HFS and MIA exposure produced a significant increase in serum insulin levels not seen with MIA exposure alone (**Figure 8B**). Interestingly, the immune-activating, satiety-inducing hormone leptin was significantly increased by HFS and increased even further by the combination of high-fat diet induced gestational diabetes and MIA exposure (**Figure 8C**). At GD16.5, reduced adiponectin and increased leptin levels were still observable in the high-fat diet induced gestational diabetes. Not previously seen at GD12.5, at GD16.5 we observed a significant increase in adiponectin levels induced by MIA in the context of gestational diabetes (**Supplemental Figure 4**). Although the effect of gestational diabetes on metabolic hormones remains consistent between GD12.5 to GD16.5, the combined effect of inflammatory and metabolic stress appears to evolve as the effect of MIA moves from the acute phase to the post-acute phase. Taken together, the maternal serum metabolic hormone levels likely promote a pro-

inflammatory phenotype in diet-induced gestational diabetes, which is intensified in combination with MIA. Thus, the combined stressors of GDM and MIA induce a pro-inflammatory profile as shown through the levels of cytokines, chemokines, and metabolic hormones.

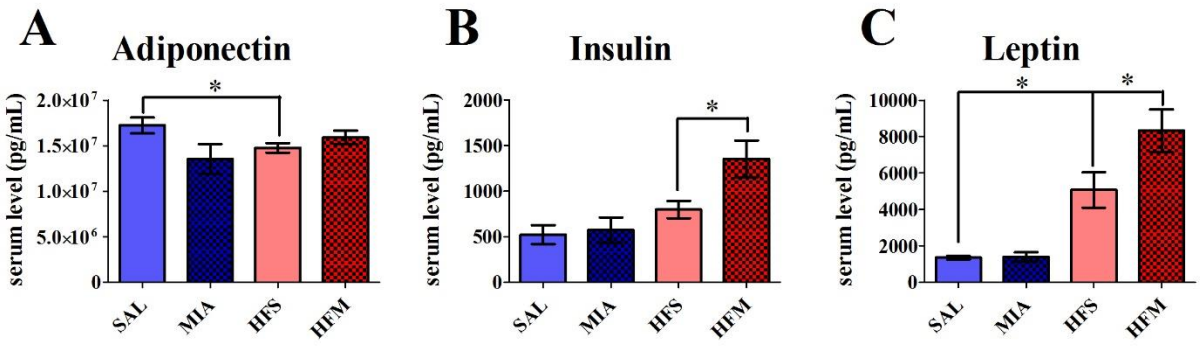


Figure 8. High fat diet induced gestational diabetes alters non-fasting levels of metabolic hormones. Maternal serum collected 3 hours post injection on GD12.5 was analyzed for non-fasting adipokine and insulin levels via antibody-conjugated bead-based multiplex assay. (**A**) The anti-inflammatory metabolic hormone adiponectin is significantly decreased by high fat diet induced gestational diabetes, with a trend towards a decrease with MIA. (**B**) Non-fasting serum insulin levels are not quite significantly increased with high fat diet induced gestational diabetes alone, but MIA in the context of high fat diet does produce a significant increase. (**C**) The pro-inflammatory satiety hormone leptin is significantly increased by high fat diet induced gestational diabetes. Leptin levels are also significantly increased by MIA in the context of high fat diet induced gestational diabetes. Error bars represent SEM. Significance of $p<0.05$ is indicated by * and determined by unpaired Student t-test with Welch's correction in all assays. Serum was collected from dams whose embryos were utilized for Nanostring gene expression analysis (SAL n=9, MIA n=8, HFS n=9, HFM n=9).

Inflammation and development related gene expression in the embryonic brain is disrupted by HFS and MIA

Both high fat diet-induced GDM (HFS) and MIA resulted in altered gene expression in the fetal brain at GD12.5, with brains being collected 3 hours after poly(I:C) treatment. We evaluated numerous genes with a predominant inflammation related function along with a small number of genes related to neurodevelopment as well as the major neuronal glucose transporters. When comparing gene expression changes significantly different between SAL and MIA, HFS, or HFS+MIA in a supervised hierarchical clustering analysis, each diet x treatment group aggregated together, demonstrating group-specific gene expression profiles **(Figure 9**, **Supplemental table 3**).

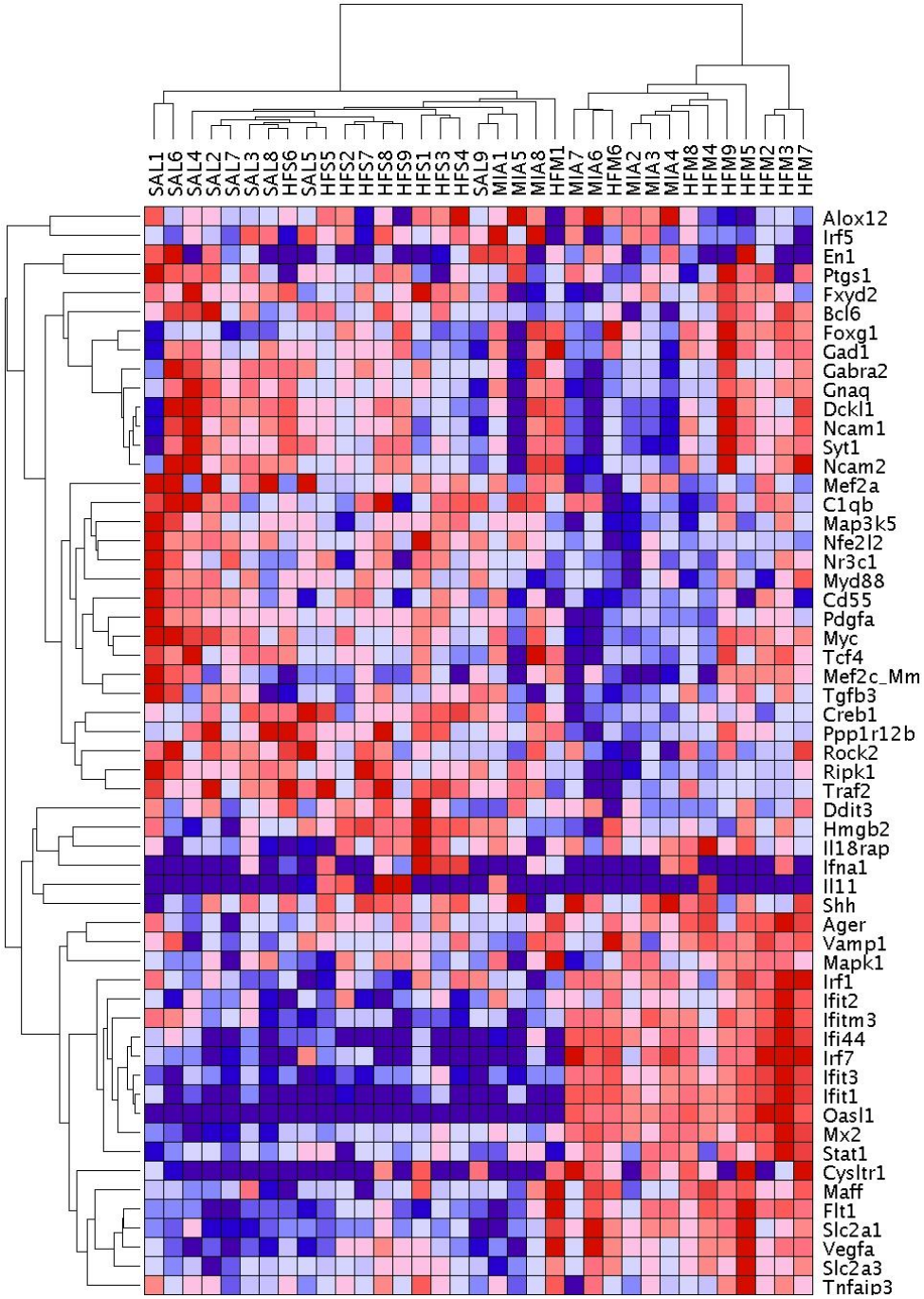


Figure 9. Both HFS and MIA alter gene expression at GD12.5. Genes that demonstrated a significant change in expression in either HFM, MIA, or HFS when compared to SAL (57 genes) were utilized for supervised hierarchical clustering analysis. Each diet x treatment group aggregates together, demonstrating distinct gene expression changes in each group. Significance

was determined by unpaired student t-test with Welch's correction and $p<0.05$. Values are in **Supplemental table 3.**

High-fat diet alone displayed significant differences in gene expression when compared to SAL. Neurodevelopmental genes involved in patterning (*En1*, *En2*) and cholinergic signaling (*Chat)* were repressed (Chiang et al., 1996; Sadler, Liu, & Augustine, 1995). A number of inflammation-related genes were also induced (e.g. *Cls, Ccl8, Ifit3, Mx2*) while others were repressed (e.g. *Ccl21a, Ifi44, Tgfb2, Tgfb3*), with these genes functioning in IFN/antiviral response, growth, cell cycle regulation, and apoptosis (Liu, Sanchez, & Cheng, 2011; McKinsey, Zhang, & Olson, 2002; H. Yanai et al., 2009). Moreover, GDM increased *Vegfa* expression, which is associated with a hypoxic environment (**Figure 10, Supplemental Table 3**) (Paschen, Gissel, Linden, Althausen, & Doutheil, 1998; Pearce, Butler, Abrassart, & Williams, 2011).

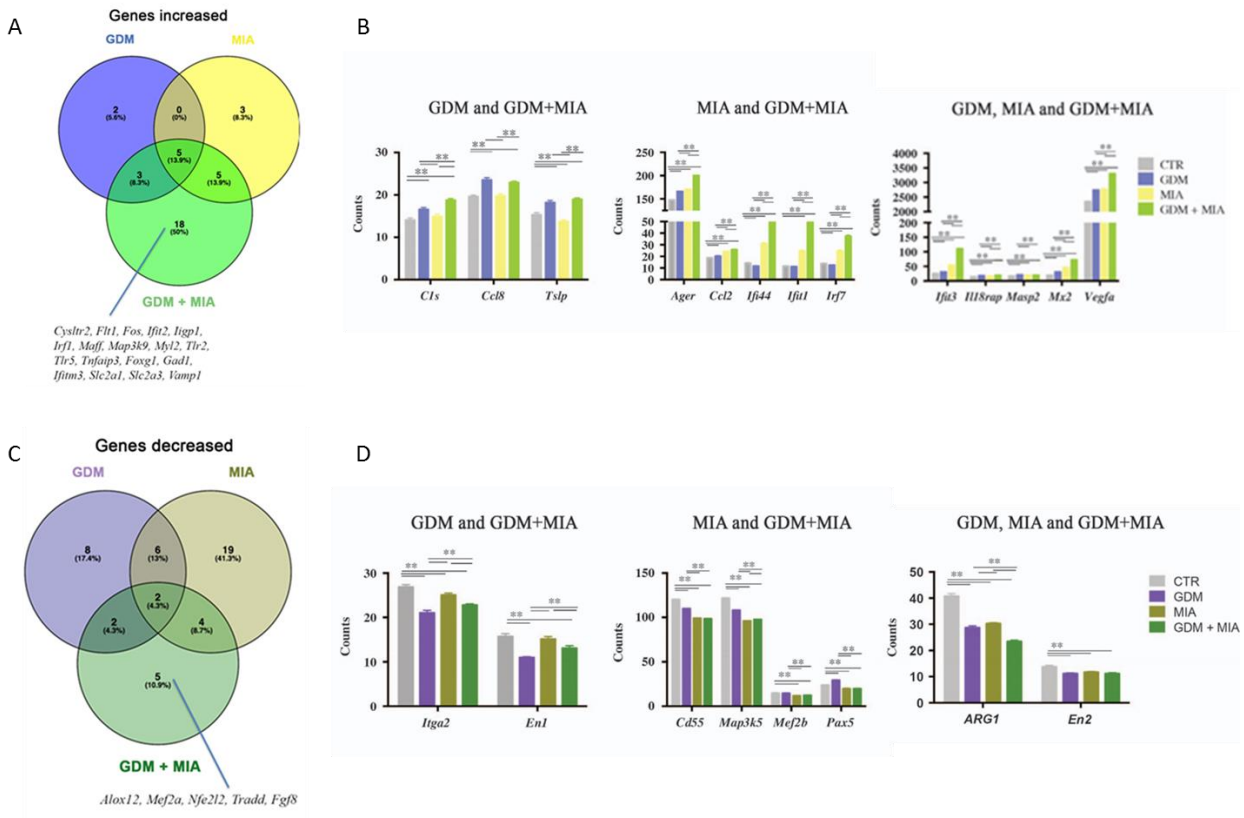


Figure 10. **Gestational diabetes mellitus (GDM) and maternal immune activation (MIA) alter expression of neurodevelopmental and inflammatory genes at GD12.5.** Venn diagrams denote the number of genes increased (**A**) or decreased (**C**) in diet induced gestational diabetes (GDM), MIA, or GDM + MIA when compared to control mice. The graphics represent gene counts increased (**B**) or decreased (**D**) in GDM and GDM + MIA, MIA and GDM + MIA or GDM, MIA, and GDM + MIA as indicated. (**A and C**) Significant changes in gene expression were determined by multiple t-tests, and genes with $p<0.05$, false discovery rate (FDR) <0.01 were selected. (**B and D**) Tukey's multiple comparisons two-way analysis of variance (ANOVA) was used to determine significance (**$p<0.05$) among the groups.

Diet-induced gestational diabetes potentiates MIA induced gene expression changes

Three hours following injection, poly(I:C) treatment in the context of high-fat diet induced GDM produced marked induction of multiple inflammation and neurodevelopment genes (**Figure 11**). The combined treatment of HFS and MIA both induced (*Foxg1, Gad1, Mbp, Vamp1*) and repressed (*En1, En2, Fgf8, Pax5*) subsets of neurodevelopment genes involved in patterning, migration, and oligodendrocyte development (Danesin & Houart, 2012; Marin, 2013; Ohtsuka et al., 2013; Ozgen et al., 2014; Smith et al., 2011; Toma, Kumamoto, & Hanashima, 2014; Walshe & Mason, 2003). A number of inflammation related genes were induced by the combination of the metabolic and inflammatory stress of MIA and diet-induced GDM and were largely involved in the antiviral/IFN response (*Ifi44, Ifit1, Ifit2, Ifit3, Ifitm3, Irf1, Irf7, Maff, Mx2, Oasl1*) and the generalized innate immune response (*Ager, Ccl21a, Csf1, Il1r1*), however, other genes known for their role in cell growth (*Tgfb2*) and apoptosis (*Mef2d*) were also induced (Barton & Medzhitov, 2003; Liu et al., 2011; McKinsey et al., 2002; X. Zhou et al., 2013). Contrastingly, the combination of diet-induced GDM and MIA (HFS+MIA) also led to the repression of a number of genes associated with the antiviral/IFN response (*Irf3, Irf5*), apoptosis/cell cycle regulation (*Ddit3, Hmgb2, Ripk1, Tradd, Traf2*), and cell growth (*Pdgfa*) (Demoulin & Essaghir, 2014; Liu et al., 2011; Spellman et al., 1998). Also repressed in the combined metabolic and inflammatory stress condition of HFS+MIA were genes critical for intracellular signaling pathways associated with both pro- and anti-inflammatory processes such as *Creb1, Jun,* and *Nfe2l2* (Bryan, Olayanju, Goldring, & Park, 2013; Janeway & Medzhitov, 2002). Furthermore, both the constitutively expressed GLUT1 and high affinity neuronal specific GLUT3 genes (*Slc2a1, Slc2a3*) as well as the hypoxia-associated genes *Flt1* and *Vegfa* were induced in HFS+MIA compared to HFS alone. Despite the fact that several inflammation-related

genes were found to be repressed, many more genes responsible for encoding apoptosis-regulating proteins and other innate immune response proteins were induced when MIA and diet-induced GDM were combined. This data suggests that the combination of the two stressors further disrupts the complex metabolic and inflammatory environment of the developing fetal brain. This disruption is made evident by both unique and differentially altered transcript changes induced by MIA and/or diet-induced GDM **(Figure 10).** This interesting phenomenon is enhanced in changes in both dopamine neuronal differentiation pathways as well as multiple inflammation related pathways (**Figure 12**).

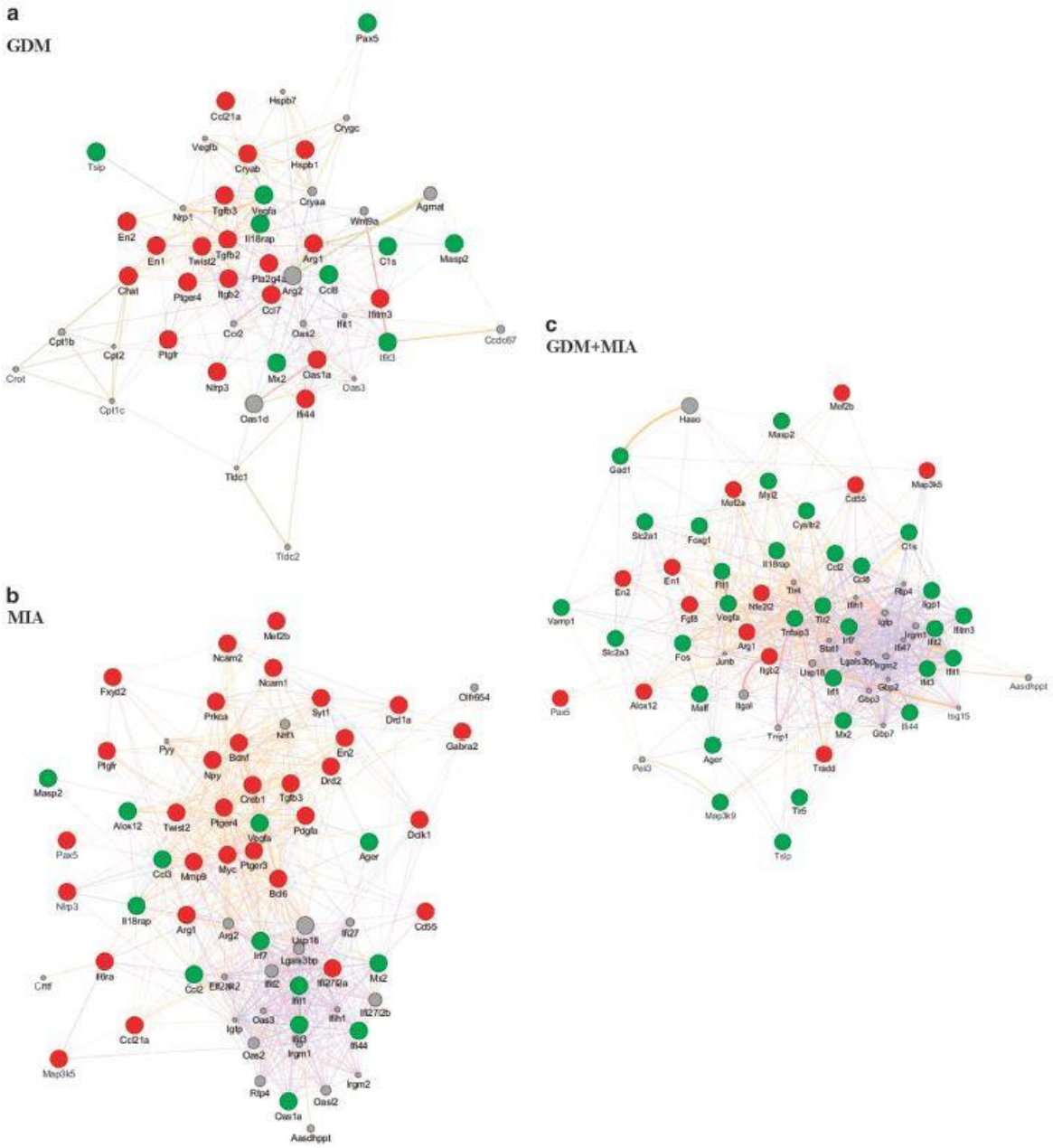


Figure 11. Maternal immune activation (MIA), gestational diabetes mellitus (GDM) and GDM+MIA produce unique transcriptional changes in the developing fetal brain. Genes significantly altered in either GDM (a), MIA (b), or GDM+MIA (c) compared with control were utilized for functional gene network diagrams. Predicted functional gene networks were generated with Genemania's algorithm available within Cytoscape. For each condition in

comparison with control, genes significantly induced are shown in green, whereas genes significantly repressed are shown in red. Gray nodes represent additional genes strongly associated with the predicted network, with node size proportional to strength of association of gene within the network. Lines demonstrate functional association with line thickness indicating association strength. Significant genes were determined by one-way analysis of variance (ANOVA) with post hoc Bonferonni correction (P<0.05, false discovery rate (FDR) <0.01). (P<0.05, false discovery rate (FDR) <0.01).

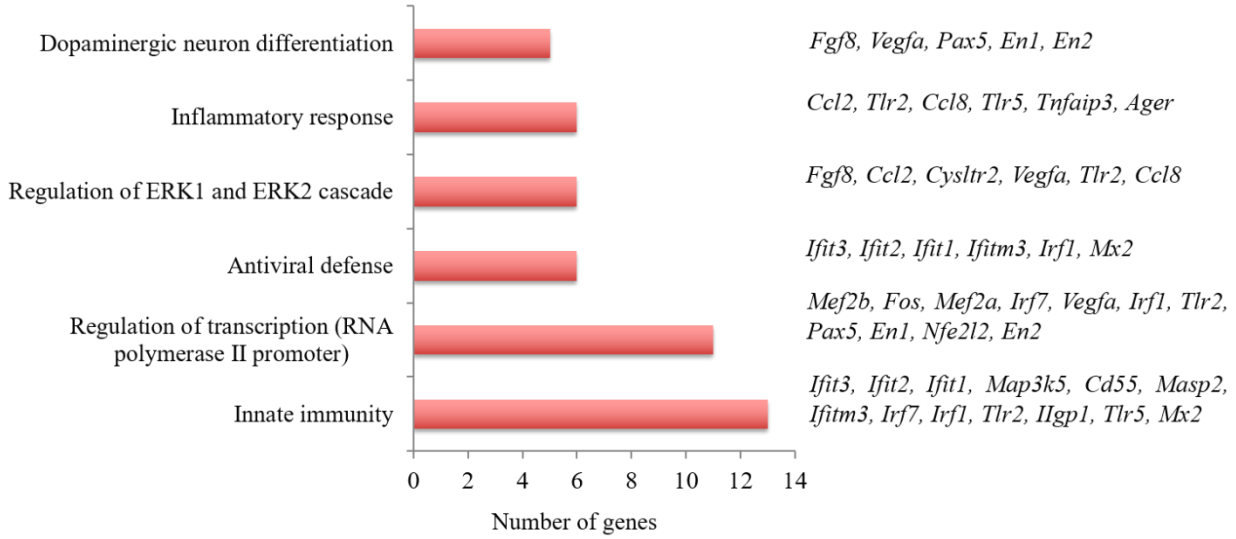


Figure 12. Differentially expressed transcripts in diet-induced GDM and maternal immune activation (GDM+MIA) condition at GD12.5. Gene ontology (GO) was performed using genes significantly altered in GDM+MIA when compared to control mice fed a normal diet and treated with saline. Clustering was performed using DAVID bioinformatics database analysis (p<0.05).

At GD16.5, smaller and fewer changes in relative gene expression were detected with MIA and GDM+MIA. This decreased level of change was as expected since the acute immune response induced by poly(I:C) exposure passes after 24 – 48 hours (**Supplemental Figure 5, Supplemental Table 5**). In contrast to the combined stress of MIA and diet-induced GDM, the effect produced by GDM alone is still occurring at GD16.5, we observed a similar number of gene expression changes produced by GDM alone at GD16.5 as was observed at GD12.5. The changes in fetal brain gene expression patterns that remain at GD16.5 include the induction of neurodevelopment genes (*En2, Pax5*) and both the repression (*Ccr1, Cd163, Itgb2*) and induction (*Hras1, Ptger3*) of inflammation genes. This data supports the hypothesis that GDM has continued effects on the fetal brain. All be it subtle, this evolving phenotype reflects ongoing changes in both neurodevelopment and the immune response in the brain of the developing fetus.

Discussion

There is abundant epidemiological evidence supporting an association between either GDM or infection-associated MIA and neurocognitive disabilities in offspring, including disorders such as autism and schizophrenia (Atladóttir et al., 2010; Brown et al., 2004; Krakowiak et al., 2012; Xiang et al., 2015). Despite these associations, the extent to which these two stressors interact with one another has not yet been closely assessed. The clinical relevance of the potential interactions between GDM and MIA are growing in developed countries such as the United States, where the incidence of GDM is increasing (DeSisto et al., 2014), and where infections continue to pose a persistent threat to pregnant mothers. In low- or middle-income countries this possible interaction may be even more important due to increasingly high rates of

infectious diseases and the recent and ever increasing shift from undernutrition to excess adiposity (J.A. Goldstein, S.A. Norris, & D.M. Aronoff, 2017).

The work discussed in this chapter presents new evidence for a significant interaction between GDM and MIA in the developing fetal brain as well as their impact on maternal inflammation. We modeled two commonly occurring environmental stressors that can and often times occur during gestation, are known to induce inflammation, and have been associated with increased risk for psychiatric disorders later in life- GDM, as modeled in this study by the high fat diet induction of gestational diabetes (HFS) mice, and maternal infection, as modeled by MIA (Atladóttir et al., 2010; Brown et al., 2004; Gardener et al., 2009; Xiang et al., 2015; Yamashita et al., 2003). The combination of these two models demonstrates that 1) individually, both GDM and MIA produce a pro-inflammatory maternal state and alter inflammation-associated and neurodevelopmental embryonic gene expression, 2) the combination of GDM and MIA intensifies the maternal inflammatory state and produces a novel transcriptional phenotype, and 3) these novel transcriptional changes are associated with pathways implicated in psychiatric disorders such as the dopamine neuron differentiation and innate immune response pathways. These findings provide insight into how multiple environmental stressors interact with one another and the impact they may have on increasing the risk for developing psychiatric disorders later in life.

Consistent with previous reports (Liang et al., 2010), we found that administration of a high fat diet for 6 weeks prior to mating and throughout pregnancy successfully induced a GDM phenotype in mice. Although elevated maternal body mass index (BMI) is not necessary for the development of GDM, it is a strong predictor of the disorder (Whiteman et al., 2015). After 8 weeks of consuming a high fat diet, mice showed a significant increase in weight independent of

pregnancy. Despite significant weight gain, non-pregnant mice did not show the same level of glucose intolerance and insulin insensitivity/resistance present in their pregnant counterparts, confirming that these phenomena were gestationally-dependent.

The two environmental stressors we chose to study, GDM and MIA, are both known to induce a state of maternal inflammation. It is known that prolonged inflammation has the potential to produce a "primed" state, thus altering and potentially exacerbating the bodies response to any additional inflammatory stimuli that may occur (Abell et al., 2015; Leboyer, Oliveira, Tamouza, & Groc, 2016). Environmental stressors such as midgestation maternal infection, perinatal hypoxia, or early childhood trauma, are considered co-factors and are known to trigger this "primed" state (Maes et al., 2009; Millan et al., 2016).

The maternal serum data discussed in this chapter supports a pro-inflammatory maternal state in the setting of MIA, in both the presence and absence of GDM. These results also confirm previous models of MIA in mice, where analogous transient increases in cytokine and chemokine levels were observed during the acute-phase response to poly(I:C) (Arrode-Bruses & Bruses, 2012; Khan et al., 2014; Mandal et al., 2013). Most studies have reported very subtle or no increases in cytokines or chemokines in maternal serum between 1 – 8 days post injection, which is expected given the transitory maternal response to poly(I:C) (Arrode-Bruses & Bruses, 2012; Mandal et al., 2013; U. Meyer, Engler, Weber, Schedlowski, & Feldon, 2008). Our results further support the body of evidence that poly(I:C) administration at GD12.5 produces an acute, but strong increase in multiple cytokines and chemokines, including IL-6, that is no longer present 4 days post injection at GD16.5 in maternal serum. We speculate that this increase in pro-inflammatory cytokines within the maternal environment may be the driving force behind the changes in gene expression within the fetal brain.

In addition to markers of inflammation, we investigated the extent to which either GDM, MIA, or the combination of the two induced changes in metabolic hormone levels in the maternal serum. Increased serum leptin and insulin levels, as well as leptin and insulin insensitivity have been previously reported in adult mice fed a high fat diet (Gallou-Kabani et al., 2007; Van Heek et al., 1997). In our studies however, non-fasting serum insulin levels were only significantly affected at GD12.5 and only with the combination of GDM and MIA. As the acute inflammatory period of the poly(I:C) induced MIA passed the levels were no longer significantly different at GD16.5; suggesting that MIA had a greater impact on circulating, non-fasting insulin levels then GDM. The significant increase in pro-inflammatory leptin levels in GDM was further increased when MIA was introduced at GD12.5. This increase dissipated by GD16.5, with only the combination of GDM+MIA still producing significant increases in leptin at this time point (Gallou-Kabani et al., 2007). Although the effects of GDM on metabolic hormones appeared to remain consistent between the GD12.5 and GD16.5 time points, the interaction between MIA and GDM appeared to evolve to a mostly normalized state. The observed alterations in insulin and leptin levels likely contributed to the pro-inflammatory phenotype seen in GDM, which was further exacerbated when combined with MIA. In summation, the combined effect of GDM and MIA resulted in a pro-inflammatory profile of cytokines, chemokines, and metabolic hormones in dams.

The hypoxic response caused by both GDM and MIA was interesting. Most likely resulting as a consequence of the antiviral response mounted in response to poly(I:C), signs of hypoxia were detected in the MIA-exposed fetal brain. As for the increase in hypoxic markers in the GDM-exposed fetal brain, it is known that oxygen demand can increase with the heightened metabolic demand associated with maternal hyperglycemia (Bastian et al., 2015; Cerf et al.,

2010). VEGF is known to increase under conditions of hypoxia and has been shown to mediate the vascular remodeling that occurs with chronic hypoxia, which is likely the cause of the *Vegfa* and *Flt1* induction observed within the fetal brains of our GDM and MIA models (Nilsson, Shibuya, & Wennstrom, 2004; Osada-Oka, Ikeda, Imaoka, Akiba, & Sato, 2008; Pearce et al., 2011). This change aligns with microvascular and cerebral blood volume alterations that are theorized to increase risk for psychiatric disorders observed in patients with schizophrenia (Meier et al., 2013; Talati, Rane, Skinner, Gore, & Heckers, 2015).

We also set out to determine if GDM and MIA interact with one another in order to produce a novel phenotype. We found that MIA differentially affected inflammation and neurodevelopment associated gene expression when in combination with GDM, suggesting that GDM and MIA interacted with one another leading to an exacerbated transcriptional response within the fetal brain. The genes differentially regulated in the the comined treatment of GDM and MIA are multifunctional neurodevelopmental genes and include crucial patterning genes that are part of the neuronal fate differentiation of dopaminergic neurons found within the developing midbrain (Stoykova & Gruss, 1994). Partial or complete disruption of these factors has been linked to failed development and differentiation of dopaminergic neurons as well as midbrain degeneration with models that reach adulthood (Schwarz, Alvarez-Bolado, Urbanek, Busslinger, & Gruss, 1997; Simon, Bhatt, Gherbassi, Sgado, & Alberi, 2003; Sonnier et al., 2007). Our work suggests that dopaminergic disruption as a consequence of MIA, which is strongly tied to psychiatric dysfunction, can be exacerbated in combination with additional environmental factors, such as GDM. Although the most prominent response was observed in the MIA group at GD12.5, both the induction and repression of neurodevelopment and inflammation associated genes was observed in all treatment groups including GDM, MIA, and GDM+MIA. When the

effects of MIA were observed in the context of GDM, the fetal brain gene expression response at both time points was unique than that observed for the treatments individually, suggesting an interaction between these two inflammation-associated risk factors. It is clear that the effects of MIA on fetal brain gene expression are modified by GDM, indicating that high fat diet induced gestational diabetes, when it is accompanied by maternal infection, induces a novel phenotype worth further investigation.

In summary, we have shown that the combined inflammatory interactions of GDM and MIA influence transcriptional responses within the developing mouse brain. It is important to note that neither GDM or MIA, either in alone or in combination, are specific to a particular outcome later in life, but they have been shown to predispose some to a variety of brain disorders, with diagnosis-specificity being genetic context dependent (Horvath & Mirnics, 2014). The effects of the co-occurrence of GDM and an infection during pregnancy are most likely depend on the genetic makeup of the mother, the developing fetus, or both. Ultimately, our data suggests that children born to mothers with GDM that have been exposed to mid-gestation infections/immune activation have an increased vulnerability to psychiatric disorders later in life. These findings should be further investigated and studied in a large-scale epidemiological study.

CHAPTER 4

SEX MODIFIES PLACENTAL GENE EXPRESSION IN RESPONSE TO METABOLIC AND INFLAMMATORY STRESS

Theresa L. Barke, Kelli M. Money, Liping Du, Ana Serezani, Maureen Gannon, Karoly Mirnics, David M. Aronoff

Introduction

Disruptions to normal immune homeostasis within the fetal-placental unit can disrupt neurodevelopment and predispose to psychiatric disorders (Van Lieshout & Voruganti, 2008). Metabolic stress (*e.g.*, gestational diabetes mellitus (GDM) and obesity) and infections are common during pregnancy, both of which impact not only fetal development, but the lifelong health of offspring as well. Among the most prevalent maternal stressors are obesity (Brenseke, Prater, Bahamonde, & Gutierrez, 2013), gestational diabetes mellitus (GDM) (Breyer, Bagdassarian, Myers, & Breyer, 2001; Tomar et al., 2015; J. Yan & Yang, 2014)) and infection (Howerton & Bale, 2012; Monk, Georgieff, & Osterholm, 2013; Rupérez et al., 2017). How these comorbidities impact fetal development and long-term health outcomes for offspring remains an open question. Another interesting unanswered question includes the observation that some DOHaD-associated health outcomes exhibit sexual dimorphism, meaning that male and female offspring are affected differently (Jeffrey S. Gilbert & Mark J. Nijland, 2008).

The placenta, a critical organ important for numerous physiological phenomenon, is also an important biological conduit that could mediate the non-genomic transmission of risk for NCDs across generations (Konkel, 2016; Peng et al., 2017). To advance our understanding of the extent to which metabolic and inflammatory stressors impact placental immune activation, we utilized a pregnant mouse model of GDM combined with an acute inflammatory MIA stress (Money et al., 2017). GDM was modeled using a high fat diet in female C57/BL6 mice prior to and during pregnancy (Money et al., 2017). 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 (L. Shi, Fatemi, Sidwell, & Patterson, 2003; L. Shi, Tu, & Patterson, 2005). Placental gene expression for 248 genes involved in innate and adaptive immunity were evaluated because immune tolerance is important for fetal development (Trowsdale & Betz, 2006) and both metabolic and infectious stressors have been shown to alter the inflammatory state of the placenta (Altmae et al., 2017; E. L. Johnson & Chakraborty, 2016; Pantham, Aye, & Powell, 2015). This study design allowed us to test the tripartite hypothesis that (1) either metabolic stress or MIA alone can induce changes in inflammatory gene expression within the placenta, (2) these stressors can interact to influence gene expression when both are occurring in the same gestation and (3) the effects of these perturbations on gene expression is different depending on fetal/placental sex.

Materials and Methods

Animal procedures

Animal procedures were approved by the Vanderbilt Animal Care and Use Committee and conducted according to our previously detailed protocol (Money et al., 2017). Briefly, female and male C57Bl/6J 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, females 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 (Money et al., 2017). At 10 weeks of age, mice were mated, and the presence of a vaginal plug marked gestational day 0.5 (GD0.5) **(Figure 13A)**. 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) (MIA) as reported (Money et al., 2017), 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) (Money et al., 2017). GD12.5 was chosen as a mid-gestation timepoint in mice, 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 hours after injection at GD12.5. Sex genotyping was performed for each embryo using a previously published protocol (Jacobs et al., 2009). 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.

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 separated and both 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, 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 (Money et al., 2017). All pups were measured to have crown/rump lengths between 8 mm to 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.

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 untilized to measure

RNA concentration and purity (260/280 values above 2 and 260/230 values above 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 (150ng) 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 **(Supplemental Table 6)**.

Nanostring® gene expression analysis and data visualization

The 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 and placenta gender groups, 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).

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 (Mi et al., 2017). All genes upregulated or down regulated within a particular 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 is color-coded so that each pathway is represented by the same color in all figures and are 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 are differentially expressed between male and female that contains 3 or less implicated genes.

Results

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, three hours 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 initially by a principal coordinate analysis (PCA) plot, in which each data point represents an individual placental tissue. The closer data points are to each other, the more closely related are the transcriptional responses. Transcriptional responses varied most dramatically between mice exposed to MIA versus saline, regardless of the presence of diet-induced GDM (**Figure 13B**). 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 as the other experimental group, suggesting a more chaotic transcriptional response compared to the other experimental conditions (**Figure 13B**). The potential impact of placental sex on transcriptional profiles was also observed from the plot, particularly among mice not exposed to poly(I:C).

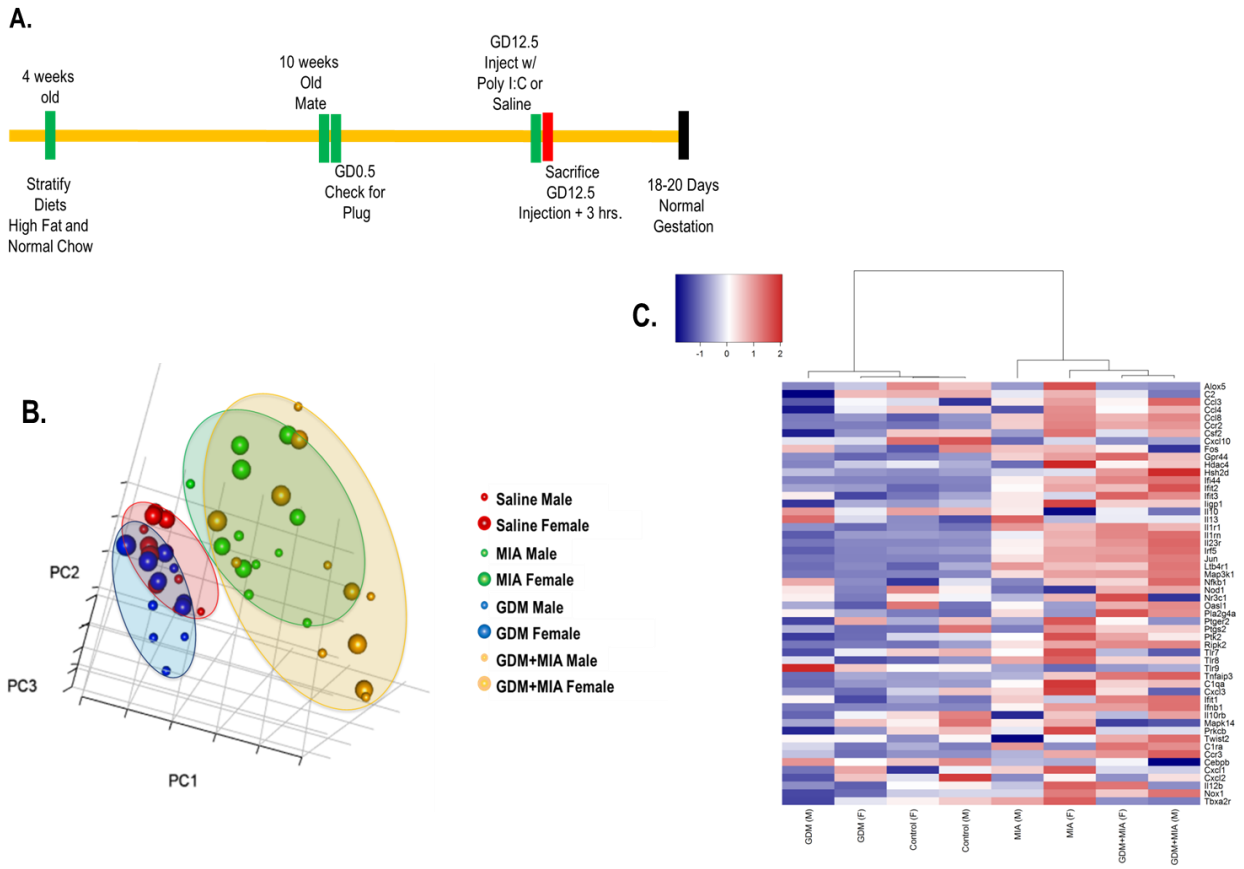


Figure 13. Visual comparison of gene expression profiles for male and female placentae exposed *in utero* to gestational diabetes mellitus (GDM), maternal immune activation (MIA) or both GDM and MIA. Control animals received normal chow and saline injections. (A). Experimental timeline highlighting the 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. Heat map was generated under R version 3.5.1.

From the regression analysis to test the interaction between sex of placenta and treatment, we selected a subset of genes that were statistically significantly perturbed by sex in response to GDM, MIA or both GDM+MIA when compared with control (normal diet, saline-injected) animals, and generated a heat map with hierarchical clustering on group using the average level of gene expression for each group (**Figure 13C**). 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) (**Figure 13C**). 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.

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**. The sex specific differences in gene expression patterns in normal diet saline treated mice indicate that even in the absence of any external or environmental stressors, the sex of the fetus influences gene expression patterns in the placenta.

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 (**Figure 14A**). 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 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 (**Figure 14B**). Female placentae saw unique differences within the Insulin/IGF/MAPK pathway (**Figure 14C**).

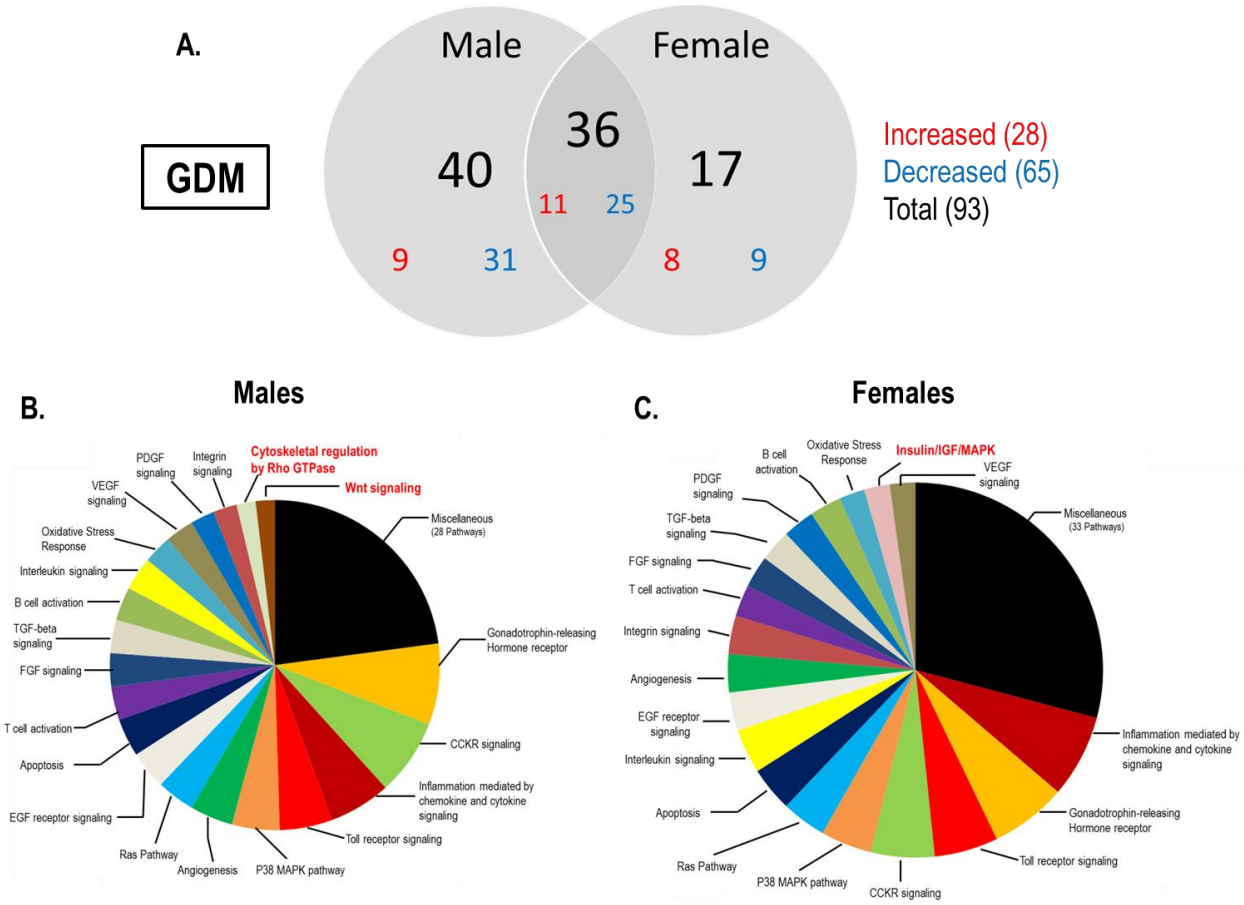


Figure 14. Sex-associated changes in gene expression in the placenta induced by diet-induced gestational diabetes mellitus (GDM) compared to the saline treated, normal-diet controls. (A.) Number of genes whose expression levels were statistically-significantly impacted by GDM compared to control ($p<0.05$). 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.

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 response differently to MIA exposure. Expression levels of 114 genes (of 248; 46%) showed statistically significant changes with MIA exposure (**Figure 15A**). Of these 114 genes, 52 were similarly impacted by MIA in male and female placental tissues (39 induced and 13 suppressed), 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-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 (**Figure 15B and C**).

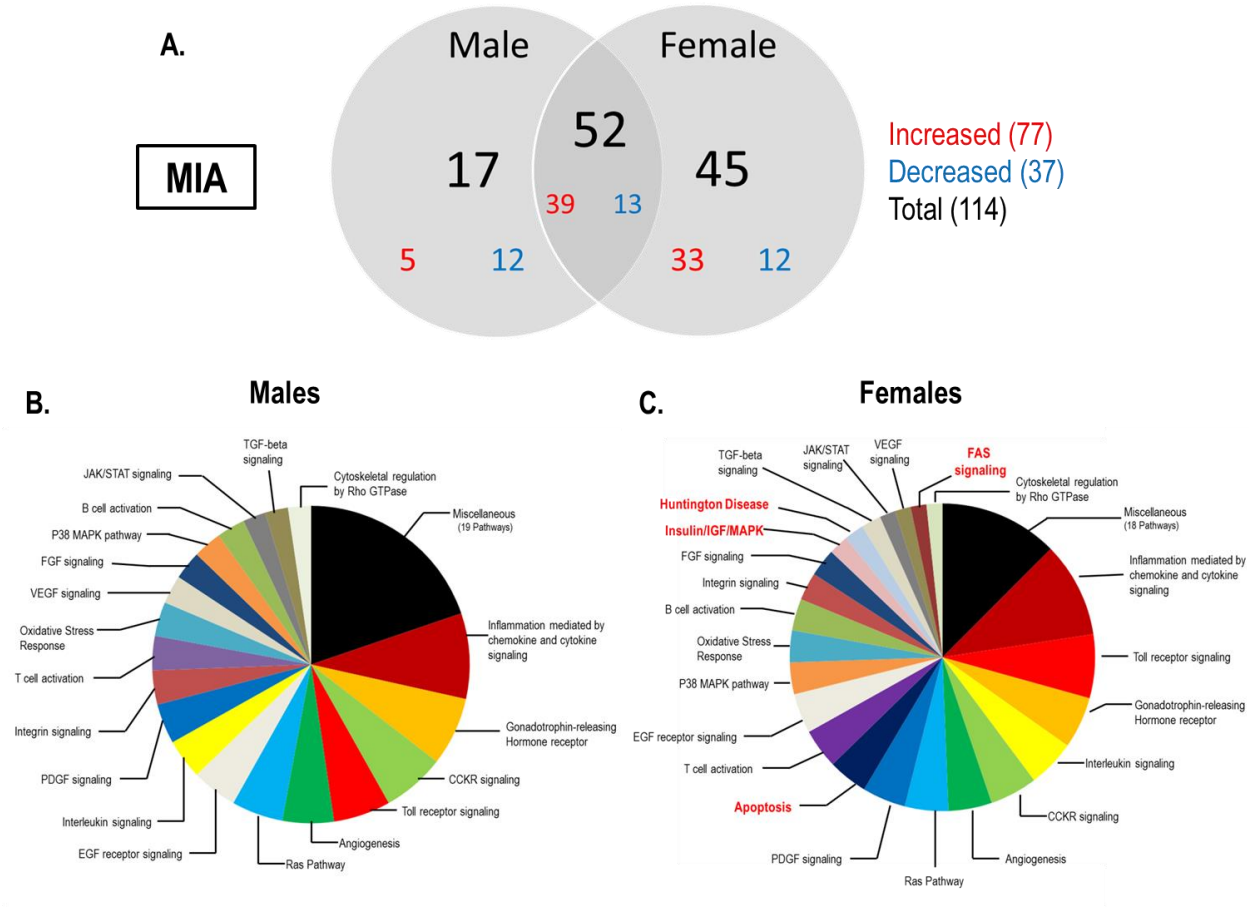


Figure 15. Sex-associated changes in gene expression in the placenta induced by maternal immune activation (MIA) compared to the saline treated, normal-diet controls. (A.) Number of genes whose expression levels were statistically-significantly impacted by MIA compared to control ($p<0.05$). Large black 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.

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

The impact of sex was next evaluated 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 93 genes (of 248; 37.5%) showed statistically significantly alteration after GDM and MIA exposure (**Figure 16A**; **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 (**Figure 16B and C**).

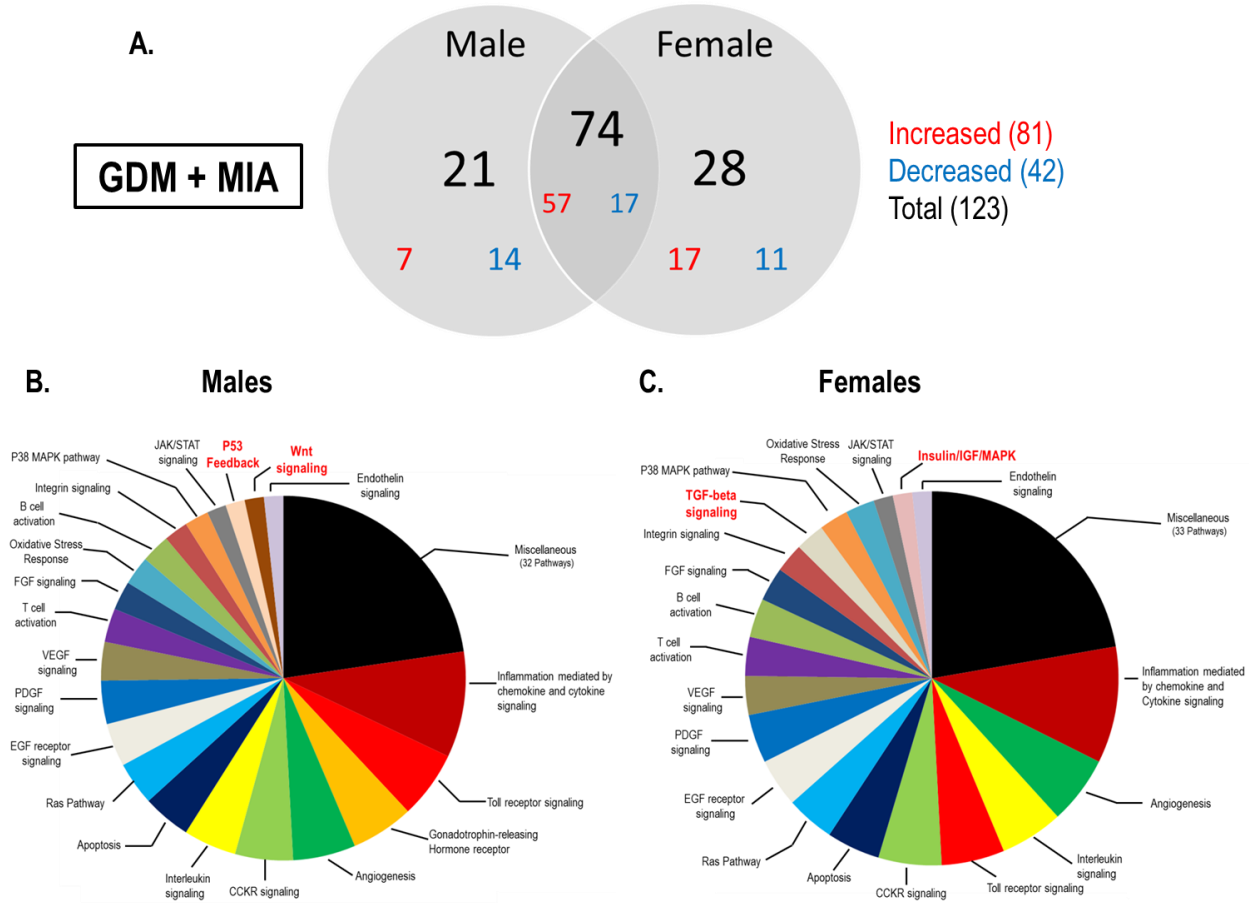


Figure 16. Sex-associated changes in gene expression in the placenta induced by the combination of maternal immune activation and diet-induced gestational diabetes (GDM + MIA) compared to the saline treated, normal-diet controls. (A.) Number of genes whose expression levels were statistically-significantly 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.

Discussion

Investigating the impact of maternal antenatal stress on the placenta provides an important opportunity to understand fetal origins of disease in offspring. Both antenatal metabolic stress and infection pose tremendous risks to fetal health and development, risks that might be transmitted through actions within the placenta. However, compared to other organs, the placenta is a relatively understudied tissue. The present study newly demonstrates two fundamental observations relevant to understanding how stressors impact placental immunobiology. First, both metabolic stress and immune activation, or the combination of the two, perturbs immune gene expression in the placenta. Second, such effects exhibit sexual dimorphism.

Because much of the placenta is fetally-derived, particularly the metabolically highly-active trophoblast, its sex is largely fetal (Rosenfeld, 2015). This is reflected in the fact that male, rather than female, placentae can be more responsive to changes in the maternal environment, a circumstance referred to as sexual dimorphism (Bale, 2016; Clifton, 2005; Mueller & Bale, 2008; Rosenfeld, 2015).

The premise of DOHaD has largely been accepted. A wealth of data supports the association between maternal stressors during pregnancy on health outcomes in offspring and has been linked to various causal mechanisms including epigenetic changes and damage to fetal

tissue (Gabory et al., 2012; D. W. Kim, Young, Grattan, & Jasoni, 2014; Reynolds, Vickers, Harrison, Segovia, & Gray, 2015). 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 (J. A. Goldstein et al., 2017). Our group has recently demonstrated the unique impact of combined maternal stressors (GDM and MIA) on the fetal brain, supporting the importance of more realistic *in utero* investigations (Money et al., 2017). Equally important is that studies of these factors incorporate sex as a biological variable. Sex differences in the fetal origins of disease are frequently identified (Abuznait, Qosa, Busnena, El Sayed, & Kaddoumi, 2013; J. S. Gilbert & M. J. Nijland, 2008). Increasingly, the placenta is being viewed as a key mediator of DOHaD-related sexual dimorphism (Andres et al., 2015; Gabory, Roseboom, Moore, Moore, & Junien, 2013).

At baseline we found that the transcription of some immune genes differed significantly between male and female placentae (**Supplemental table 7**). While only 13 genes were significantly differentially expressed between male and female tissues, it was interesting that several chemokines (*Ccl4, Ccl24, Cxcl2*), pathogen recognition receptors (*Tlr5, Tlr6, Tlr8*) and the prostaglandin-generating, inducible cyclooxygenase-2 (*Ptgs2*) were among these. The male-specific increased expression of *Ptgs2* is interesting given that prostaglandins are critically important in labor and sexual dimorphism has been observed in the incidence of preterm labor (Verburg et al., 2016). Also, cyclooxygenase inhibitors were shown to have sex-specific effects in mollifying 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 (Bronson & Bale, 2014). Similar to our data, but in humans, Sood and colleagues (Sood, Zehnder, Druzin, & Brown, 2006) 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, genes expressed at higher levels in female placentae included immunoregulatory genes such as *JAK1*, *IL2RB*, *Clusterin*, *LTBP*, *CXCL1*, and *IL1RL1* (Sood et al., 2006). A more recent study of late first trimester placentae from humans demonstrated sex differences in the transcriptome that included a set of 18 autosomal genes (Gonzalez et al., 2018).

The TLR3 dsRNA ligand poly(I:C) is commonly used to model viral infection (Reisinger et al., 2015), most notably to define the impact of MIA on fetal brain development and neurocognitive function in offspring (Money et al., 2017) (Reisinger et al., 2015). Notably, antenatal poly(I:C) exposure has been shown to differentially alter behaviors in male vs. female mouse offspring (Xuan & Hampson, 2014). To our knowledge, this is the first study that has examined changes in a large number of inflammatory genes within the placenta provoked by poly(I:C) in a sex-specific manner. The impact of this stress on placental inflammation has been studied before (E. Y. Hsiao & P. H. Patterson, 2011) but in a more limited way. Hsiao and Patterson conducted a study in C57BL/6 mice similar to ours, finding that inflammatory gene expression was markedly induced 3 hours following MIA induction (E. Y. Hsiao & P. H. Patterson, 2011). However that study examined a small number of prespecified inflammatory genes and did not examine sex-specific differences in placental 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. Similar to GDM, 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 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, while males contained higher levels of growth hormones (Geary, Pringle, Rodeck, Kingdom, & Hindmarsh, 2003). 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 (Clifton et al., 2010). The role for the IGF axis within the 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 (J. E. Hsu & Jones, 2005; Pantham et al., 2015). Obesity's impact on placental inflammation appears to affect male and female tissues differently (Leon-Garcia et al., 2016) and mice fed a high fat diet have been found to have divergent patterns of gene expression in male vs. female placentae (Gabory et al., 2012). 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 of the genes found to be differentially affected based on sex revealed two pathways more highly changed in males compared to females; the cytoskeletal regulation of Rho GTPase and the Wnt signaling pathways. The female placentae showed a higher degree of change in the Insulin/IGF/MAPK signaling pathway. Studies have interrogated the IGF axis as it relates to fetal development and outcomes in maternal asthma, however not in the context of GDM (Clifton, 2010). It is potentially important that the prior studies concluded that the IGF axis was regulated in a sex-dependent manner (Clifton et al., 2010).

A fascinating aspect of our study was the combined GDM and MIA stressors. A total of 123 genes within the placenta were significantly changed 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 results from the GDM and MIA conditions alone. In addition to the insulin/IGF/MAPK pathway, the TGF-□ signaling pathway was more significantly changed in female placentae. TGF-□ has been implicated in supporting maternal-fetal immune tolerance (Alijotas-Reig, Llurba, & Gris, 2014) so disturbing its expression could have important consequences. Similar to 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 (Sharp et al., 2014). It will be important for future studies to establish how sex influences these pathways.

Limitations of our study are important. Gene expression was assessed using a preselected set of 248 immune genes, which introduces bias and limits conclusions about many other functions of the placenta, such as nutrient transport or metabolism. Our GDM model was generated with a high fat diet, which itself could produce inflammation in the absence of diabetes (D. Zhou & Pan, 2015). We used this model because mice consistently exhibited enhanced glucose intolerance and hyperinsulinemia following a glucose challenge only while pregnant (Money et al., 2017). 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 (Malassine, Frendo, & Evain-Brion, 2003). 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 to define. Despite these limitations, our work sheds new light on the convergence of stressors at the maternal-fetal interface that deserves ongoing attention.

In summary, our 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 in offspring. We strongly support that future studies continue to model multiple stressors and pay heed to sex-related effects.

CHAPTER 5

METABOLIC STRESS AND IL-1β PRODUCTION BY THE PLACENTAL MACROPHAGE

Theresa L. Barke, Lisa M. Rogers, David M. Aronoff

Introduction

IL-1β has long been known to exert metabolic effects, most notably being implicated in the progression of insulin resistance and obesity. The consequences of IL-1β signaling is context, time, and tissue dependent. For example, in the absence of acute inflammatory stimuli, IL-1β signaling is essential for maintaining adipose tissue homeostasis and normal body weight (Garcia et al., 2006; Matsuki, Horai, Sudo, & Iwakura, 2003; Somm et al., 2005). In the context of chronic inflammation, however, as in obesity and diabetes, IL-1β signaling largly corresponds with a pro-inflammatory phenotype and an increase in pathogenic inflammation (McGillicuddy et al., 2011; Miura et al., 2010; Nov et al., 2013; Stienstra et al., 2010). IL-1β plays such an important role in the pathogenesis of metabolic disease that its use as a drug target is actively being studied with promising results. Pharmaceutical intervention blocking IL-1 signaling in mouse models of T2D and atherosclerosis was effective and improved glycemia, β-cell function, and reduced inflammation releaving the T2D phenotype (Bhaskar et al., 2011; Ehses et al., 2009).

Differential effects of IL-1β can be seen impacting metabolic signaling in multiple different organs and cells types. It has been reported that IL-1β is a potent inhibitor of insulin

signaling in adipocytes as well as interfering with adipocyte differentiation (Lagathu et al., 2006; Stienstra et al., 2010). In models of non-alcholic fatty liver disease, IL-1 signaling induces lipogenesis leading to the detrimental accumulation of tiglycerides in the liver (Negrin et al., 2014). Important in the context of diabetes and obesity, macrophage inflammasome activation in obese individuals within the pancrease leads to increased levels of IL-1β subsequently inducing β-cell dysfunction and death (Eguchi et al., 2012; Steer, Scarim, Chambers, & Corbett, 2006; Thomas, Darwiche, Corbett, & Kay, 2002). The tissue specific effects of IL-1 in the context of obesity and diabetes make it clear that not all tissues and their responses are created equal. While limited studies to date have interrogated the role of metabolic stress on the placental macrophage IL-1β pathway, unanswered questions still remain. It is important to study tissue and organ specific phenotypes in a clinically and contextually relevant manner. The studies put forth in this chapter were designed to shed light on the interraction between placental macrophage IL-1β expression and metabolic stress in the form of glucose and free-fatty acids (FFA).

Type-2 diabetes was the first disease shown to involve the NLRP3 inflammasome in its pathogenesis and subsequent progression (R. Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010). Obesity has been well establihed as a major risk factor for the development of T2D and insulin resistance explaining why T2D and GDM are often found in conjunction with maternal obesity (K. E. Martin et al., 2015; Whiteman et al., 2015). The loss of metabolic homeostasis in obese individuals results in an increase in the levels of glucose and fatty acids (FFA) in circulation and within organs (Donath & Shoelson, 2011). Although less research has focused on GDM compared with T2D and obesity, GDM has also been characterized by low-grade inflammation endotoxemia and elevated circulating FFA (Catalano, 2002; Lappas, 2011; Winzer, 2004; Wolf, 2004). Multiple studies have demonstrated that these byproducts of diabesity constitue metabolic

danger signals with the ability to alter the activation status of the NLRP3 inflammasome (L'Homme et al., 2013; Vandanmagsar et al., 2011; Wen et al., 2011; Y. Yan et al., 2013). If these findings are correct, then it can be hypothesized that FFAs have the ability to offer both signals necessary for inflammasome activation by both priming and activating the inflammasome in the setting of diabetes and obesity.

In humans, oleate (C18:1), palmitate (C16:0), and stearate (C10:0) make up roughly 80% of the circulating FFA at a ratio of 1.6:1.0:0.5 (Hagenfeldt, Wahren, Pernow, & Raf, 1972). As a consequence of diabetes and obesity, dyslipidemia increases the levels of these FFA in circulation making them available to act as ligands for inflammasome activation. Although the findings regarding how FFA may induce inflammasome activation are often times conflicting, the evidence that FFA play a role in IL-1β production and inflammasome activation is overwhelming. In a human monocyte cell line, THP-1 cells, palmitate treatment increased caspase activity in parallel with an increase in IL-1β release eventually leading to cell death (Pillon et al., 2016). Again in THP-1 cells, another group demonstrated that in the presence of high glucose, palmitate and stearate increased TLR2 and TLR4 expression, increased NF-κB activity, and increased the release of IL-1β and MCP-1 in a dose and time dependent manner. Futhermore, when they reported that the silencing of TLR2 and TLR4 significantly reduced NF-κB activity and IL-1β and MCP-1 secretion in palmitate treated cells in the presence of high glucose (Dasu & Jialal, 2011). It has since been reported that palmitate directly activates TLR2 inducing inflammasome-mediated IL-1β production in human monocytes (Snodgrass, Huang, Choi, Rutledge, & Hwang, 2013). These studies, as well as many others, demonstrate the ability for palmitate and potentially other FFA to induce IL-1β production in a TLR-dependent

inflammasome mediated fashion. These studies, however compelling, do not recapitulate the placental environment.

How these extracellular danger signals in the form of FFA activate the inflammasome culminating in the characteristic increased production of IL-1β seen in metabolic disorders is a difficult question to answer. Again, tissue specificity is key. Evidence suggests that FFA and glucose may initiate the pro-inflammatory release of IL-1β in an inflammasome-dependent pathway through the activation of TLRs. Although relatively widely accepted to occur in many tissues, evidence from the placenta is lacking. TLR4 has been reported to play a role in the pathogenesis and inflammation associated with obesity and T2D through its interaction with FFA (F. Kim et al., 2007; Lu et al., 2015). Placental trophoblast cells produce increased levels of multiple pro-inflammatoy mediators in response to saturated fatty acids in a TLR4 dependent manner (Yang et al., 2015). TLR expression is altered in the diabetic state as evidenced by a study that determined that women with GDM have increased TLR4 expression levels on peripheral blood monocytes (Xie et al., 2014). TLR2 and TLR4 are the only TLRs whose expression is at high levels within the placenta as well as on placental macrophages throughout gestation (Abrahams, 2008) promting us to question the potential role of TLR activation in placental inflammation through the production of IL-1β as a consequence of FFA exposure. Taken together, the evidence introduced here, as well as evidence presented in the following chapter give precedence for our studies investigating the role of FFA- induced IL-1β production by placental macrophges.

Materials and Methods

Animal procedures

Animal procedures were approved by the Vanderbilt Animal Care and Use Committee and conducted according to our previously detailed protocol (Money et al., 2017). Briefly, female and male C57Bl/6J 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, females 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 (Money et al., 2017). At 10 weeks of age, mice were mated, and the presence of a vaginal plug marked gestational day 0.5 (GD0.5) **(Figure 13A)**. 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) (MIA) as reported (Money et al., 2017), creating 4 experimental groups: control fed saline, high fat fed saline, control fed poly(I:C), and high fat fed poly(I:C) (Money et al., 2017). GD12.5 was chosen as a mid-gestation timepoint in mice, 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 hours after injection at GD12.5. 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.

Antibody-conjugated bead-based analysis

Immediately after sacrifice, amniotic fluid was removed from each intact amniotic sac and pooled together resulting in one representative amniotic fluid sample per pregnant dam. Approximately 0.5 to 1 mL of amniotic fluid was collected from each pregnant dam and kept at -80°C until submitted for antibody-conjugated bead-based analysis.

Luminex analysis utilizes a multiplexed assay, using x-map technology via the MagPix system. The reactants are attached to the surfaces of tiny fluorescent microspheres. Each set of microspheres carries a unique biological reagent distinguishable by internal dye ratios. Identification of an analyte is based upon specific fluorescent emission spectra of the bead associated with the analyte. Two LEDs with high speed digital signal processors and computer algorithms distinguish which analyte is being carried on each microsphere while quantifying the reaction based on fluorescent reporters signals. This allows for analysis of multiple analytes from a single aliquot of sample. Luminex analysis was performed by the Vanderbilt hormone core (http://hormone.mc.vanderbilt.edu), which is funded in part by NIH grants (DK059637 and DK020593). The Luminex multiplex panels (EMD Millipore, Darmstadt, Germany) used for analysis of the maternal serum were mouse cytokine/chemokine panel 14 plex (eotaxin, IFNγ, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-13, IL-17, KC, MCP-1, RANTES, and TNF-α Cat# MCYTOMAG-70K).

Cell isolation and stimulation

Primary placental macrophages (PMs) were isolated from healthy donors undergoing term scheduled C-sections using a previously published protocol (Z. Tang et al., 2011). Briefly, placental tissue was enzymatically digested then subjected to various rounds of stepwise

filtrations followed by Percoll gradient to separate possible cells of interest. The resulting cells underwent CD14+ positive selection via magnetic sorting. In all cell experiments, freshly isolated cells were allowed to rest overnight in RPMI-1640 with 1% antibiotic and 10% FBS media then pretreated with a glucose free RPMI-1640 (#11879 Invitrogen) media for 1 hour prior to experimental exposure. Experimental exposures included a metabolic cocktail (MetaC) comprised of 30 mM glucose, 0.4 mM palmitate, and 10 nM human insulin for 4 or 24hr. Euglycemic conditions were 5mM glucose and acted as the control. PMs were also stimulated with 0.4 mM palmitate, 10 nM insulin, or 30 mM glucose (hyperglycemic condition) alone. For inhibition of caspase-1, PMs were exposed for 1hr prior to experimental conditions in glucose-free RPMI to an irreversible caspase-1 inhibitor zYVAD-FMK (Millipore #218746) at a concentration of 10uM.

ELISA

Freshly isolated PMs were plated at a density of 3.5e6 PMs in 6 well dishes and allowed to rest overnight in RPMI with 1% antibiotic and 10% FBS. Prior to experimental treatment, cells were pretreated with a glucose free RPMI media for 1 hour. Experimental exposures included MetaC, 30 mM glucose, 0.4 mM palmitate, 10 nM human insulin, or 0.4 mM Oleate for either 4 or 24hrs. Supernatants were collected for ELISA analysis after 4 hours or 24 hours of exposure to the treatment conditions. IL-1β was measured using the Duo-set ELISA from R&D and caspase-1 was measured using Human Caspase-1/ICE Quantikine ELISA kit from R&D.

Immunofluorescence for NLRP3-ASC localization

Freshly isolated PMs were plated at a density of 300,000 cells per chamber slide and allowed to rest overnight in RPMI with 1% antibiotic and 10% FBS. Prior to experimental treatment, cells were pretreated for 1 hour in glucose-free RPMI. Experimental exposures included a metabolic cocktail that simulates a state of diabesity which is comprised of 30 mM glucose, 10 nM human insulin, and 0.4 mM palmitate (MetaC), 5 mM glucose and 0.4 mM palmitate for 4 hrs. Immunofluorescence to interrogate NLRP3-ASC complex formation was completed using the Duolink In Situ PLA Kit from Sigma following manufacturer's instructions, using Anti-ASC pAB (AL177) at 1:200 and anti-NLRP3 mAB (Cryo-2) at 1:200 (AdipoGen).

Results and Future Directions

Numerous studies have indicated that gestational diabetes and metabolic stress impact placental macrophage (PM) number, polarization, and expression patterns to adopt a more pro-inflammatory phenotype (Altmae et al., 2017; Barke et al., 2018; Mrizak et al., 2014; Pantham et al., 2015; Yu et al., 2013). Macrophages regulate inflammation in the context of many systems, tissues, and disease states and have the ability to control both local and systemic inflammation. While conducting our experiments outlined in chapter 3 and chapter 4 we discovered that within the amniotic fluid of mice fed a high-fat diet (exhibiting a phenotype consistant with GDM), there was a significant increase in the levels of IL-1β **(Figure 17).** The increase in IL-1β was present only in the context of GDM and GDM+MIA, but not MIA alone. This increased level of IL-1β was also not observed in the maternal serum. Li, *et al*. described an increase in IL-1β levels in placental tissues harvested from mice fed a high-fat diet exhibiting signs consistent with GDM compared to healthy control mice (Li et al., 2013). In our studies we did not see an

increase in the levels of IL-1β in the maternal serum, but we did see an increase in the amniotic fluid in the GDM and GDM+MIA mice. Taken together with the data that placentae from GDM mice produce higher levels of IL-1β, we hypothesized that the increased levels of IL-1β originate from the placenta and more specifically macrophages as they are the predominant immune cell in the placenta throughout gestation and have been shown to have increased numbers within the placenta and produce high levels of IL-1β in the context of GDM (Sisino et al., 2013; Yu et al., 2013).

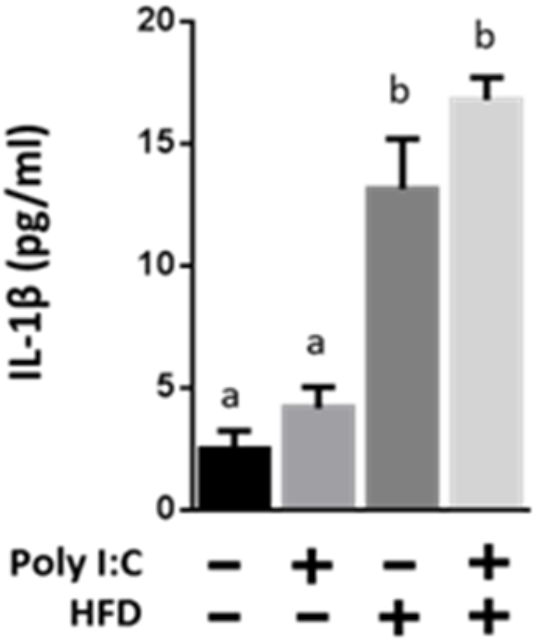


Figure 17. GDM and MIA alter cytokine, chemokine and metabolic hormone profiles within amniotic fluid at GD12.5 Amniotic fluid was collected via membrane puncture for all pups and pooled together for each individual dam. Amniotic fluid collected 3 hours post injection with either saline or 20mg/kg Poly I:C potassium salt on GD12.5 was analyzed for chemokine and cytokine levels via conjugated bead array multiplex assay. Significance indicated by letters with similar letters dictating no significance as determined by one-way ANOVA with

Tukey's multiple comparisons post test in all assays. (n=9 except for Poly I:C treated non-HFD where n=8).

Given the association between dyslipidemia and diabetes we interrogated the idea that FFAs can induce the increased expression and release of IL-1β from placental macrophages. At the cellular level, FFAs act as messengers with the ability to modulate a wide array of signaling transduction pathways (H. Shi et al., 2006). As thouroughly introduced earlier in this document, ample evidence suggests that FFAs, in the form of palmitate and stearate, have the ability to induce the production and subsequent release of IL-1β from multiple different cells and tissues. The ability of primary placental macrophages to produce increased levels of IL-1β in a FFA-dependent manner has not been reported to date. Not only did we want to see if FFAs alone were enough to stimulate the cells to produce more IL-1β we also sought to induce a more GDM like environment with the use of a metabolic cocktail that more closely mimics the high glucose, high insulin, high FFA environment of the GDM patient. Media containing high levels of glucose, insulin, and palmitate, termed the metabolic cocktail (MetaC), was previously developed and utilized to induce a metabolically activated phenotype in adipose tissue macrophages (Kratz et al., 2014). When we treated freshly isolated PMs with MetaC we saw a significant increase in the amount of IL-1β secreated from the cells into the supernantants **(Figure 18A).** Similar to treatment with MetaC, palmitate alone increased the release of IL-1β from the cells. This increase was not seen when PMs were treated with insulin or glucose alone **(Figure 18A)**.

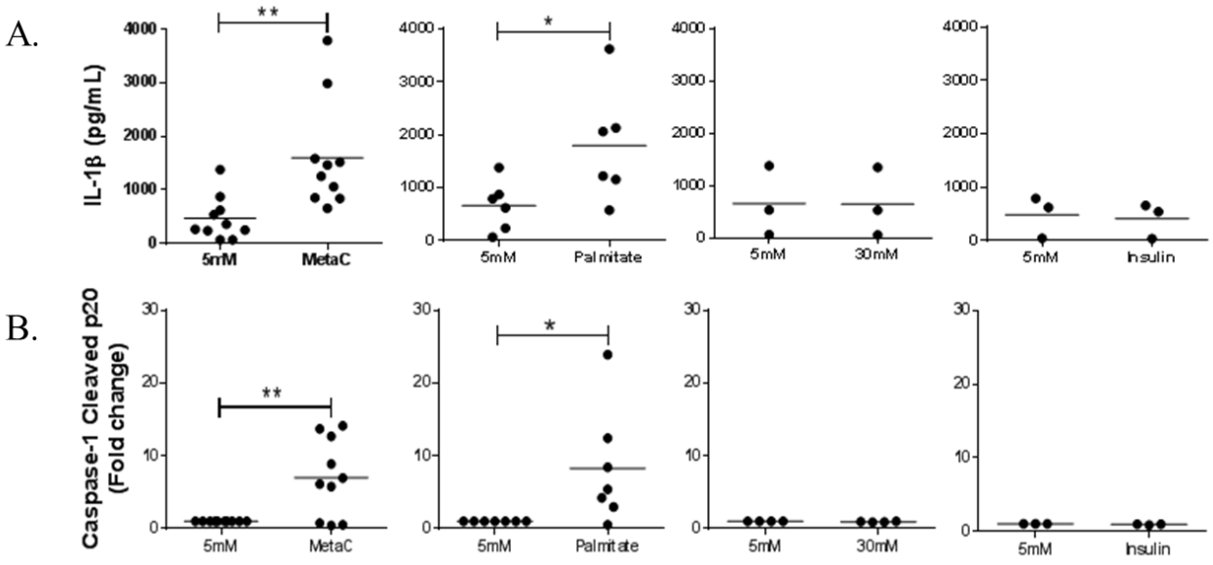


Figure 18. Metabolic stress increases IL-1β and cleaved caspase-1 (p20) in placental macrophages. PMs were treated for 24 hours with 5 mM glucose, MetaC, palmitate, 30 mM glucose, or insulin alone. A) Levels of extracellular IL-1β from cell culture supernatants as measured by ELISA B) Levels of extracellular caspase-1 from supernatants as measured by ELISA

Cleavage of pro- IL-1β into its active secreted form requires caspase-1 (Wilson et al., 1994). In a similar pattern to IL-1β, we saw an increase in the amount of extracellular cleaved caspase-1 in the MetaC and palmitate treated PMs **(Figure 18B)**. This increase was not seen after treatment with high glucose levels or insulin alone suggesting that the increase is as a result of palmitate activation of the PMs.

IL-1β processing requires active caspase-1. Caspase-1 activation in turn requires assembly of the NLRP3 inflammasome. Activation of the NLRP3 infammasome, in this case through FFA activation, induces oligomerization of NLRP3 leading to the recruitment of ASC, which in turn, forms large structures leading to the recruitment of pro-caspase-1. Autocatalytic

cleavage of pro-caspase-1 into the p10 and p20 subunits allows for the formation of active caspase-1 as the two subunits form hetero-tetramers which are then able to convert pro-IL-1β into their bioactive secreted forms (C. A. Dinarello, 2009; Wilson et al., 1994). To interrogate if MetaC and its components lead to inflammasome assembly in PMs we performed immunoflourescence to visualize inflammasome assembly. In this assay, if ASC is in complex with NLRP3 it will flouresce red. PM stimulation with MetaC and palmitate show evidence of inflammasome formation as represented by the red specs within the cells. Quantification of the number of specs per cell indicates that both MetaC and palmitate induced inflammasome formation in PMs **(Figure 19)**.

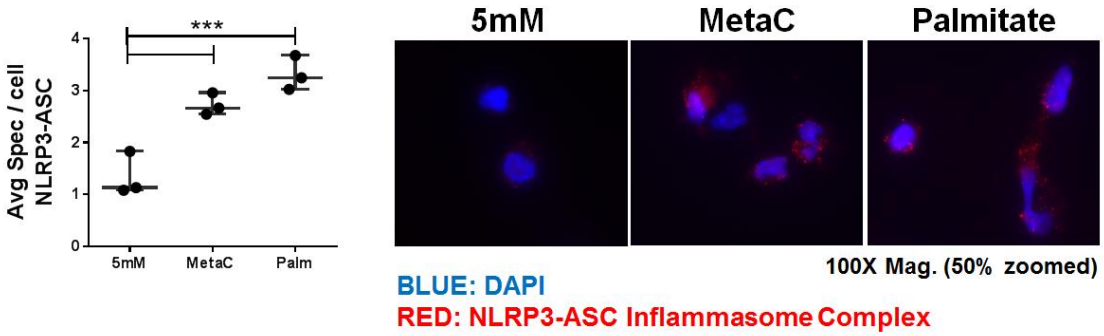


Figure 19. Metabolic stress activates the NLRP3-ASC inflammasome in placental macrophages. PMs were treated for 4h with 5mM glucose, MetaC, or palmitate alone and ASC-NLRP3 complex formation was quantified as indicated by specs per cell.

Thus far, our studies have concluded that palmitate alone is capable of inducing IL-1β production and NLRP3 inflammasome assembly as evidenced by the formation of the NLRP3-ASC complex in PMs. High levels of glucose and insulin are not, on their own, enough to

stimulate a similar response in IL-1β production. Concluding that in instances of metabolic stress such as diabetes, the increased levels of FFA could be the driver of IL-1β production in PMs. As for the receptor or receptors that mediate this interaction there are a multitude of possibilies. Many studies have shown that TLR2 and TLR4 may be possible receptors for FFAs and thus play a role in the production of IL-1β in PMs (J. Y. Lee & Hwang, 2006; Schaeffler et al., 2009; H. Shi et al., 2006). Given the fact that both TLR2 and TLR4 are rather ubiquitously expressed throughout the placenta, and on PMs, during all three trimesters these receptors are likely candidates. A recent study, however, has reported that TLR4 is not a receptor for saturated fatty acids although it does play a role in lipid-induced sterile inflammation through the action of altering macrophage metabolic programming (Lancaster et al., 2018). Even though evidence now shows that palmitate does not directly bind to and activate TLR4 in the classical sense, their findings confirming that palmitate does indeed induce a TLR4-dependent primig that alters cellular metabolism and gene expression gives precedence to the importance of this pathway and the need to further study this interaction in the context of PMs and inflammasome-dependent IL-1β production.

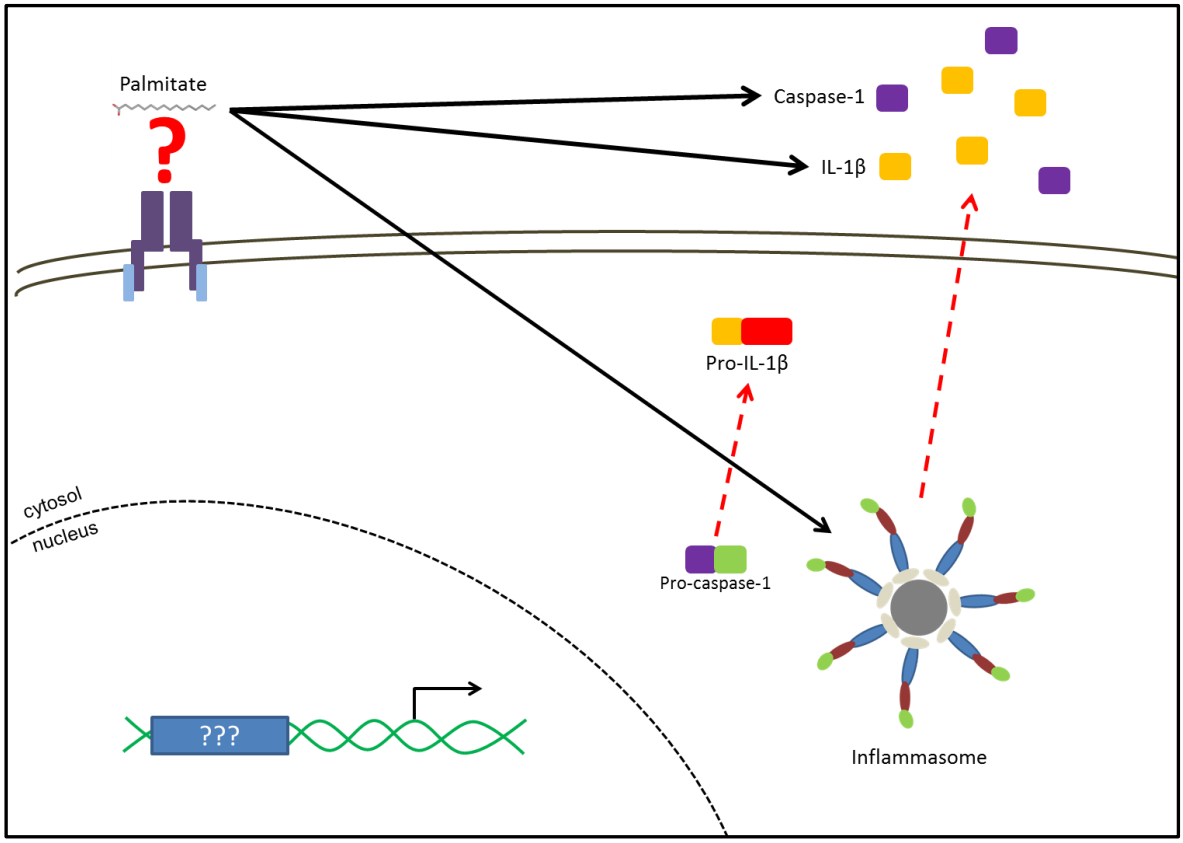


Figure 20. Hypothetical model of palmitate-induced IL-1β production in human placental macrophages This model outlines what the studies outlined in this chapter have confirmed as indicated by solid black arrows. Unanswered questions that require further investigation are indicated by the dashed red arrows.

Although preliminary studies investigating the role of palmitate in initiating the release of IL-1β from primary PMs have been completed, much work remains ongoing and many more questions need to be addressed **(Figure 20)**. The most important questions to answere next are represented in figure 22 by the red dotted arrows. First we need to address the question if indeed plamitate is inducing the release of IL-1β in an inflammasome/caspapse-1 dependent manner. In order to test this we would block caspase-1 activity through the use of zYVAD-FMK, an irreversible caspase-1 inhibitor, and repeat the above experiments looking at IL-1β release

through ELISA and western blots. If IL-1β release is due to the activity of caspase-1, we would expect to see a decrease in the amount of extracellular IL-1β in supernatants and an increase in intracellular pro-IL-1β through western blot. Secondly, we will test whether the palmitate-dependent induction of IL-1β release is dependent upon TLR4 in PMs. Similar sets of experiments as outlined previously using rs-LPS, a potent antagonist of TLR4, would be completed to test the cleavage and subsequent release of IL-1β through ELISA and western blots. The NLRP3/ASC complex assembly would also be interrogated after PM stimulation with palmitate in the presence of rs-LPS to test if TLR4 is necessary for palmitate-induced inflammasome assembly. Although preliminary in nature, these studies are essential to the understanding of the machanism by which IL-1β increases under metabolic stress.

Taken together these studies will lay the ground work for future studies investigating the role of metabolic stress induced production of IL-1β in the placenta by PMs. This work is important to the understanding of how maternal inflammation and metabolic stress in the form of dyslipidemia can impact the fetus through placental mechanisms and could possibly be leveraged to decrease fetal responses to adverse maternal stimuli.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

The prenatal environment is widely recognized to play a vital role in the development of non-communicable diseases (NCD) later in life. The idea that many NDCs have their origins in prenatal life is the basis of the DOHaD paradigm. Exposure to prenatal insults such as metabolic stress, maternal immune activation, and infections have been associated with an increased risk for the development of neurocognitive disorders, such as autism and schizophrenia, as well as the increased risk of developing metabolic diseases such as coronary heart disease and T2D (Buka et al., 2001; Eriksson, Sandboge, Salonen, Kajantie, & Osmond, 2014; Gardener et al., 2009). The mechanisms through which this prenatal programming occurs likely involves complex interactions between the maternal immune environment, the placenta, and the fetus and can be influenced by factors such as fetal sex and developmental stage during gestation (Bale, 2011; Dunn, Morgan, & Bale, 2011; Mao et al., 2010). Better understanding the mechanisms that drive the increased risk of NCDs in response to maternal stress is vital to developing new and effective intervention techniques.

A major barrier to reducing the risks and complications associated with GDM is a lack of clarity regarding how this disease changes placental physiology, morphology, and the overall pathophysiological mechanisms within the placenta contributed to this disease. The placenta acts not only as a barrier between the mother and the developing fetus, but as a regulatory conduit through which all communication between mother and fetus occurs.

Given the relationship among GDM, placental macrophage activation and iron homeostasis, chapter 2 summarized a retrospective, case-control, study of archived human placental tissue from women with or without GDM to better define placental pathology, placental iron stores, and both macrophage density and CD163 expression *in situ*. We tested the hypothesis that women with gestational diabetes exhibited altered placental physiology and placental macrophage phenotypes.

It had been reported previously that macrophage numbers were increased in placentae from women with GDM and that this increase in macrophage number was accompanied by an increase in pro-inflammatory cytokines (Yu et al., 2013). Their assessment did not factor in macrophage localization throughout the different placental regions. Our comparison of placentae from women with GDM and healthy controls did not find a significant difference in the number or localization of CD68+ cells in the placenta. We did however; find a significant difference in the expression of CD163 on macrophages within the chorion and decidual regions of the placenta indicating either a change in macrophage polarization phenotype, iron metabolic potential, or both. In conjunction with the increase in CD163+ cells, we reported a significant increase in the amount of iron in the placenta. This iron appeared to be localized to macrophages, but was also detectable within other cells in smaller amounts. High maternal iron has been linked to glucose metabolism disorders such as GMD and T2D (Afkhami-Ardekani & Rashidi, 2009; Barbieri et al., 2001; Choi et al., 2003; Rawal et al., 2017). As iron can be highly toxic to cells in high amounts, this increase in stored iron within the placenta may be one mechanism by which placental macrophages sequester and recycle iron in an attempt to reduce the transfer of iron to the fetus. Overall, our results add to the growing amount of evidence that GDM has direct effects on placental structure and macrophage phenotypes.

In chapter 3 and 4, we proposed that GDM modifies MIA-induced gene expression changes within the fetal brain and placenta. To interrogate this hypothesis, we studied the combined effect of two commonly occurring insults during pregnancy, GDM and maternal infection. We based these hypotheses on the following findings. First, both MIA and GDM increase inflammation (Abell et al., 2015; Deverman & Patterson, 2009). Secondly, both GDM and MIA have their peak influence on programming midgestation (Buka et al., 2001; Xiang et al., 2015). Third, both have been shown to alter cytokine levels that can cross the placenta (Elaine Y. Hsiao & Paul H. Patterson, 2011; Hsiao & Patterson, 2012; Li et al., 2013). And lastly, both GDM and MIA have been linked to increased occurrence of autism and schizophrenia (Atladóttir et al., 2010; Mary Cannon et al., 2002; B. K. Lee et al., 2015; Van Lieshout & Voruganti, 2008; Xiang et al., 2015). We chose to induce GDM through the administration of a high fat diet (Pasek & Gannon, 2013). In our model, the presence of impaired glucose tolerance, fasting glucose, and fasting insulin are consistent with a gestationally diabetic phenotype. To model maternal infection and induce MIA, we utilized the viral mimetic poly(I:C).

To gain insight into the maternal inflammatory status of these models, we measured maternal serum cytokine, chemokine, adipokine, and insulin levels 3 hours after MIA exposure at GD12.5 and four days after exposure at GD16.5. At GD12.5 the MIA-induced cytokine/chemokine profile was similar to what was observed in GDM, suggesting that GDM does not modify the cytokine/chemokine response to MIA. Although no changes reached significance with GDM alone, we did observe significantly increased leptin and decreased adiponectin levels. This metabolic profile is expected with a diabetic phenotype (Fasshauer, Blüher, & Stumvoll, 2014; Gallou-Kabani et al., 2007; Kautzky-Willer et al., 2001; Van Heek et

al., 1997; Vitoratos et al., 2001). Taken together, the maternal serum data supports that 1) MIA produces a transiently heightened inflammatory state 2) that GDM induces adipokine changes consistent with a diabetic phenotype 3) that MIA and GDM interact to produce an even higher increase in pro-inflammatory leptin levels. This demonstrates that our models of MIA and GDM are pro-inflammatory as well as provides novel insight into the interaction at the maternal level of MIA and GDM.

We utilized these models to gain insight into gene expression changes within the fetal brain in order to better understand how MIA and GDM could impact neurodevelopmental pathways. Overall our results demonstrate that 1) GDM alters expression of IFN response, growth and cell cycle regulation, apoptosis, and fetal brain neuronal patterning genes in the brain 2) MIA alters expression of genes both alone (antiviral/IFN response, lipid peroxidation, cell cycle regulation/apoptosis, cell growth, inflammation-associated intracellular signaling, hypoxia, and neuronal migration) and in the context of GDM (antiviral/IFN response, innate immune response, cell cycle regulation/apoptosis/growth, inflammation-associated intracellular signaling, neuronal patterning/migration/myelination, hypoxia, and glucose transport), 3) GDM modifies MIA gene expression in the fetal brain with a stronger induction of inflammation associated genes, and 4) the majority of gene expression changes are only present during the acute immune response at GD12.5.

As mentioned earlier, fetal sex can also be a driver of NCD risk during development (Al-Qaraghouli & Fang, 2017). To our knowledge, this study is the first of its kind to investigate the combined stressors of GDM and MIA from the standpoint of sexual dimorphism and placental gene expression. The data gathered will lay the groundwork for future studies to elucidate the mechanistic basis for sex-dependent risk for NCDs. At baseline we found some genes to be

differentially expressed between male and female placentae. Interestingly several chemokines, pathogen recognition receptors, and the prostaglandin-generating, inducible cyclooxygenase-2 (*Ptgs2*) were among these. The male-specific increased expression of *Ptgs2* is interesting given that prostaglandins are critically important in labor and sexual dimorphism has been observed in the incidence of preterm labor (Verburg et al., 2016). Also, cyclooxygenase inhibitors were shown to have sex-specific effects in mollifying 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 (Bronson & Bale, 2014). Taken together, the role of cyclooxygenase should be reviewed more in depth for its sex-specific role in fetal outcomes independent of maternal inflammation.

GDM induced a significant change in the expression of 37.5% of the genes interrogated. Of the genes that decreased in expression, 62% displayed sexual dimorphism and of those with increased expression 61% displayed differential expression in a sex-dependent manner. Our data is consistent with others who have reported that obesity impacts placental inflammation in a sex-dependent manner (Leon-Garcia et al., 2016) and that mice fed a high fat diet have been found to have divergent patterns of gene expression in male vs. female placentae (Gabory et al., 2012). Males had upregulated expression of the cytoskeletal regulation of Rho GTPase and the Wnt signaling pathways while female placentae showed a higher degree of change in the Insulin/IGF/MAPK signaling pathway. Prior studies have concluded that the IGF axis was regulated in a sex-dependent manner (Clifton et al., 2010). Making this pathway one that deserves further interpretation in the context of maternal inflammation and placental function.

Mice exposed to MIA revealed significant changes in gene expression in 46% of the total genes interrogated. Of the genes with decreased expression levels, 65% displayed sexual dimorphism while only 49% of those with increased expression differed based upon sex. The pathways with the most perturbations were related to TLR signaling, interleukin signaling, and cytokine and chemokine signaling in both male and female placentae. Similar to GDM, 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 not interrogated in the context of GDM, 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 (Clifton et al., 2010). The role for the IGF axis within the placenta has not been well established although its possible importance in the sex-dependent response to inflammatory insults should be investigated further.

The most fascinating aspect of our study was the combination of GDM and MIA. The combined inflammatory stress of GDM and MIA reveals changes in 38% of the genes in our panel. Of the genes decreased in expression, 60% were regulated in a sex-dependent manner while only 30% of those genes increasing in expression displayed sexual dimorphism. Again, the insulin/IGF/MAPK pathway was more significantly changed in female placentae compared to male, stressing its possible importance in the placental response to maternal inflammatory stress. Although a more comprehensive study on the placental transcriptome is necessary to make any conclusions regarding the role of sexual dimorphism in the context of maternal inflammatory stress, these studies have laid the groundwork for studies to come. In summary, this work suggests that the common antenatal stressors of GDM and MIA impact immune gene expression

within the placenta and that when combined they appear to interact changing placental gene expression patterns in a sex-dependent manner.

The underlying mechanism driving the increased risk of NDCs in the context of maternal inflammation is unknown, although multiple studies have described how metabolic stress in the form of dyslipidemia can influence inflammatory mediators (Robbins, Wen, & Ting, 2014). In chapter 5 we began to mechanistically test some of the observations described in earlier chapters. The increases in IL-1β seen in the amniotic fluid of GDM mice, but not in the maternal serum, lead us to hypothesize that the placenta may be the source of this pro-inflammatory cytokine. The role of the IL-1 family of cytokines has been well established in T2D (Banerjee & Saxena, 2012) and studies have identified its dysregulation in cases of GDM (Lappas, 2014; S. Yanai et al., 2016). We established that primary PMs secrete increased levels of IL-1β in response to stimulation with palmitate, but not in response to levels of glucose and insulin physiologically relevant in diabetic states. This increase in IL-1β was accompanied by an increase in caspase-1 release. Pro-caspase-1 is autocatalytically cleaved into its active form upon activation and assembly of the NLRP3 inflammasome (Wilson et al., 1994). We were also able to show that inflammasome assembly was occurring in PMs as a consequence of palmitate exposure by NLRP3-ASC complex formation. Although much works needs to be done in order to fully understand the mechanisms through which palmitate is stimulating increased levels of IL-1β production by PMs we were able to establish that PMs are indeed able to produce IL-1β in response to palmitate and that this increase is accompanied by inflammasome assembly and increased levels of caspase-1.

Altogether, this body of work concludes that maternal stress, either in the form of metabolic stress induced by GDM or immune stress as induced by maternal infection, affects

multiple aspects of development and maternal and fetal health in some cases in a sexual dimorphic manner. Defining the phenomenon that occur as a result of maternal inflammatory stress within the placenta and fetal brain will allow for more focused and insightful future questions regarding the underlying mechanisms to be interrogated. Ultimately these studies will help lead to a better understanding how maternal stressors influence the developmental origins of health and disease.

Future directions

Behavioral studies of GDM and MIA mice

First and foremost, I believe that one of the most important studies that should be completed to round out the work presented in chapters 3 and 4 are behavioral studies of the offspring born to mothers who were exposed to our models of GDM, MIA, and the combination of the two. The research conducted by our collaborators and others defined the neurocognitive phenotypes in the offspring of MIA mice and pioneered our understanding of the topic leading us to form the hypotheses presented here. I feel like it is essential to move beyond just gene expression studies and look at real world phenotypes that are characteristic of autism and schizophrenia in mice. These behavioral studies would truly allow for us to see if the interactions between the two maternal stressors of GDM and MIA culminate in differential or synergistic behavioral phenotypes in the offspring. Once the baseline phenotypes were evaluated, we could expand our knowledge by repeating the entire set of experiments using inhibitors of or antibodies against particular mediators of interest such as IL-6 and IL-1β in order to gain mechanistic insight into the phenotypes of interest.

Use of live flu virus in conjunction with GDM

Although widely used as a viral mimic, poly(I:C) is not a live virus. As mentioned in previous sections there are many advantages to using poly(I:C) over live viruses, however, live viruses may initiate phenotypes and interact in ways that you would not see given the use of a viral mimic. I would propose to repeat the experimental design outlined and utilized in chapters 3 and 4 using live Influenza virus in the context of GDM. The use of live virus may initiate perturbations not otherwise seen and interact with cells in ways independent of its contact with the immune system. In conjunction with the use of live Influenza virus, I would also propose to use other viruses known to cause complications either during pregnancy or to the offspring as a result of maternal infection such as Cytomegalovirus. These experiments would allow us to identify virus specific outcomes in gene expression, maternal and fetal inflammation, and offspring behavioral studies.

In conjunction with the use of live virus, I would propose to expand the gene expression panel to a more global and non-biased RNAseq platform. This way we could better monitor global changes in fetal brain and placental development as a consequence of infection and GDM.

Explore sexual-dimorphism in neurobehavioral comorbidities in MIA and GDM

Our study design outlined in chapter 3 did not make it possible for us to explore the role of sexual-dimorphism in fetal brain gene expression patterns. As evidence mounts suggesting that fetal and placental sex play a large part in conferring the risk of NCDs to the offspring as a result of maternal inflammatory insults I think it will be of the utmost importance to include fetal and placental sex as a variable in data analysis.

The effect of paternal inflammatory status on fetal outcomes

The developing fetus, and thus the placenta, is genetically influenced by both the mother and the father equally. The vast majority of studies that interrogate the DOHaD paradigm do so from the stand point of the mother and how the maternal environment influences fetal outcomes. Although limited in number, studies have shown that paternally derived factors also have the ability to influence pregnancy related outcomes and offspring health (Ding, Lambert, Aronoff, Osteen, & Bruner-Tran, 2018; Ding, Mokshagundam, Rinaudo, Osteen, & Bruner-Tran, 2018). It would be interesting to expose the paternal germ line to inflammatory insults such as MIA, live viral infections, and or GDM prior to mating in order to assess how the heightened inflammatory status of the father affects his progeny. Assessment of fetal brain gene expression patterns and placental gene expression patterns when compared to our data from maternal stimulation would allow for a direct comparison looking at the contribution of both parents to changes in fetal outcomes.

The impact of GDM and MIA on the maternal, fetal gut, and placental microbiome

Until recently the placenta was considered a sterile pathogen-free zone. Recent evidence has suggested otherwise. In fact, the placenta contains a distinct microbial signature (Aagaard et al., 2014). Furthermore, it has been reported that these distinct placental microbes begin to colonize the fetal gut shortly before birth (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016). These fascinating findings lead me to my most exciting future direction which explores the impact of maternal inflammatory stress in the form of viral infection and GDM on the fetal gut and placental microbiome in a sex-dependent manner. This future direction even ties in our mechanistic studies presented in chapter 5 as it is known that inflammasomes are important regulators of the gut microbiome and gut homeostasis (Henao-Mejia et al., 2012). These sets of

experiments, although ambitious, would lead to a great deal of insight into the DOHaD paradigm while incorporating multiple different realms of biomedical research.

SUPPLEMENTAL INFORMATION

Supplemental Table 1. Gestational diabetic case demographics

25 placental samples from women diagnosed with GDM were chosen for this study. Displayed below is the case patient information gathered from the synthetic derivative electronic medical record. These cases were matched to controls based upon the information provided in this table.

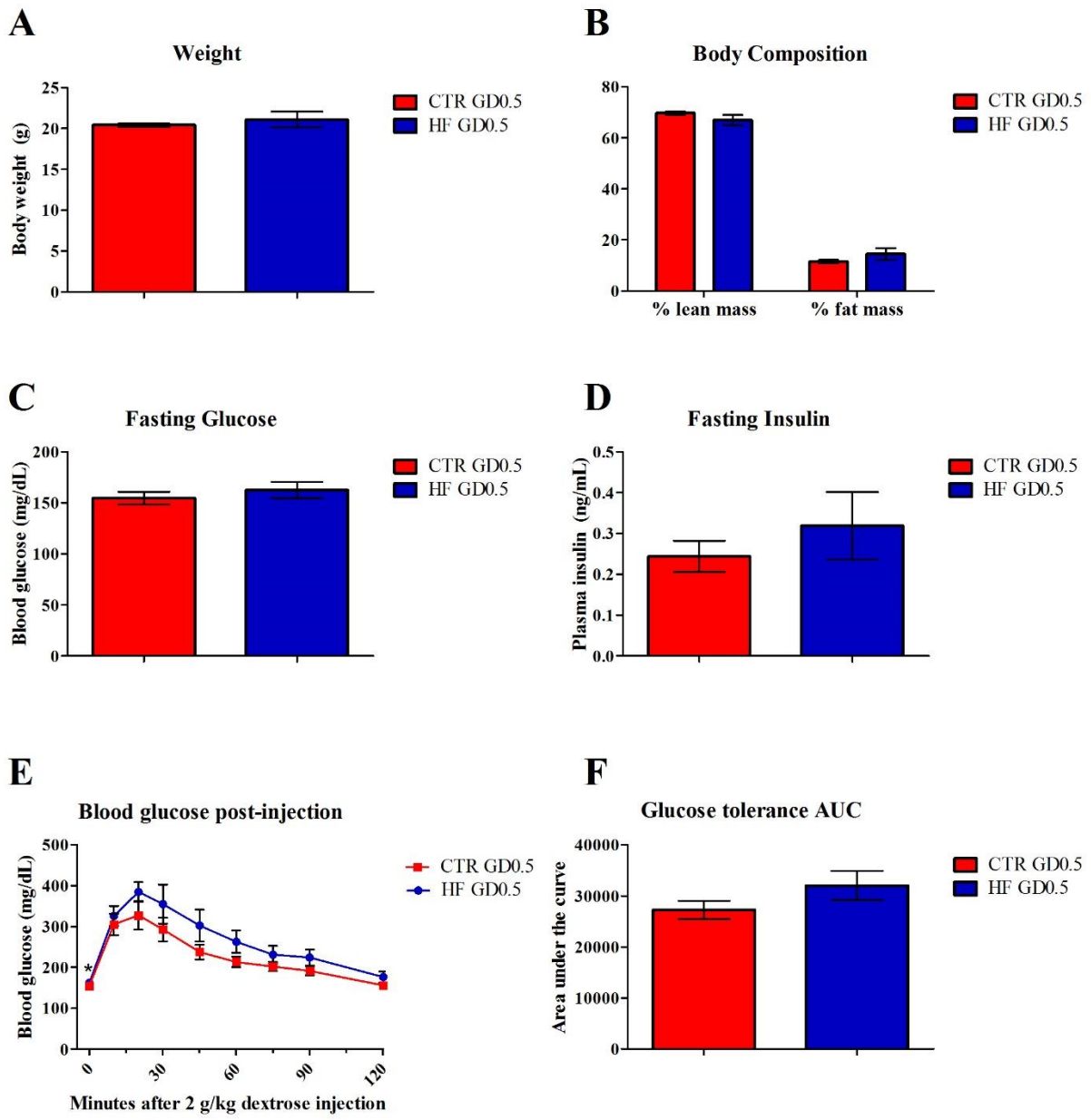
maternal age	weeks gestation	Race/Ethnicity	delivery method
39	37 2/7	Caucasian	cesarean
25	38 2/7	Caucasian	cesarean
19	38 5/7	African American	cesarean
28	36	Caucasian	cesarean
21	35 5/7	African American	cesarean
19	38 1/7	African American	vaginal
26	33 6/7	Caucasian	vaginal
34	39	Caucasian	cesarean
28	39	Caucasian	cesarean
28	35 3/7	Caucasian	vaginal
37	36 3/7	Caucasian	vaginal
38	39	Asian-pacific	Vaginal
33	36	Caucasian	Vaginal
31	38	Caucasian	Vaginal
36	33	African American	Vaginal
31	39	Asian-pacific	cesarean
32	37	Unknown	cesarean
23	36.2	Caucasian	cesarean
28	36	Caucasian	vaginal
27	34	Caucasian	cesarean
29	39	African American	cesarean
31	34 1/7	other non-Hispanic	vaginal
32	39	Caucasian	cesarean

41	39	Hispanic	cesarean
25	39	African American	cesarean

Supplemental Table 2. Litter statistics

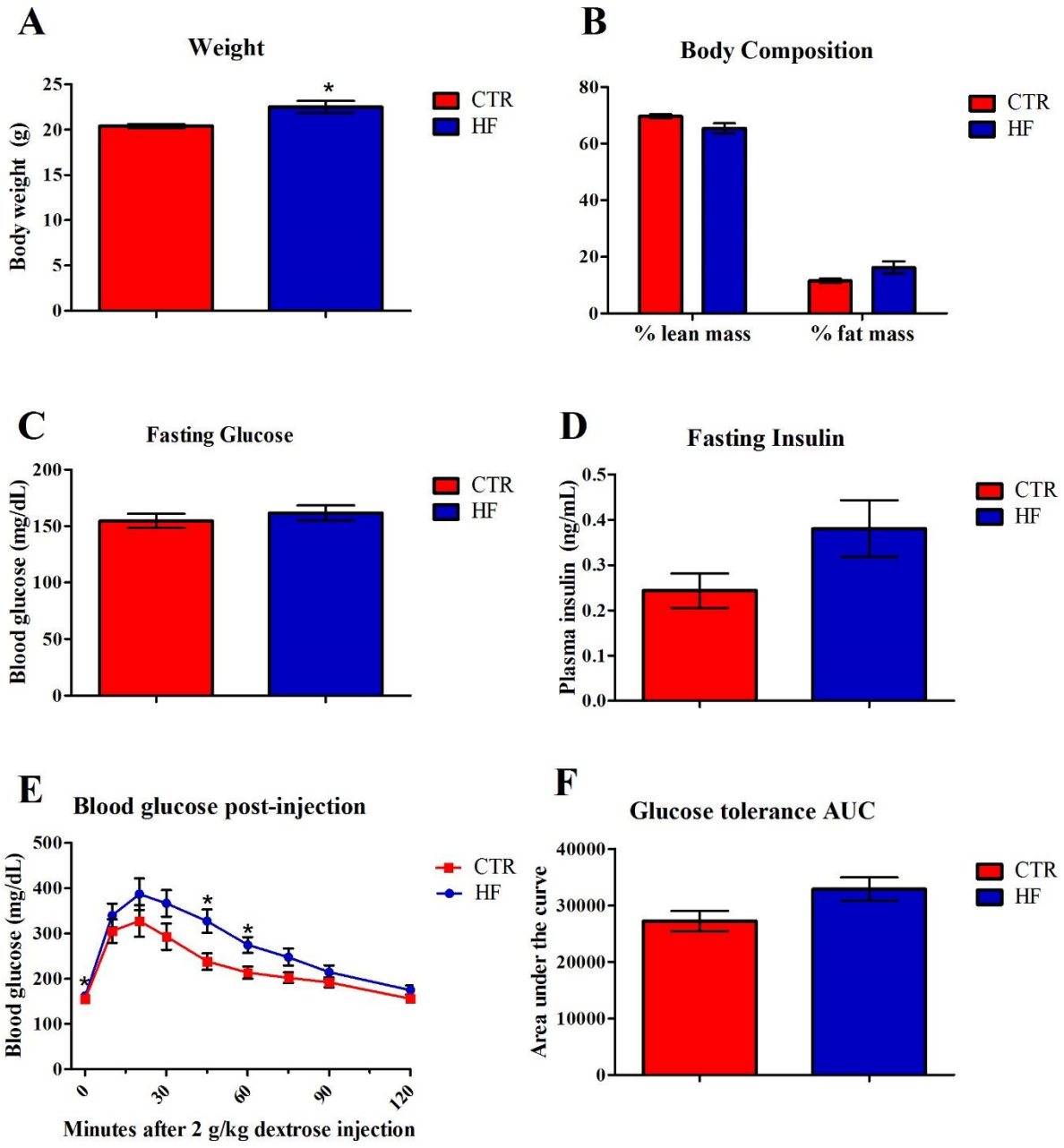
35 GD12.5 pregnant dams and 36 GD16.5 pregnant dams were sacrificed, and the embryos collected. Average litter size, average number of resorptions and mal-developed pups (runts, severe hemorrhage or hypoxia) per litter, average ratio of male:female embryos, and average embryo crown-rump length are listed with SEM in the table below.

Diet	**Exposure**	**GD age**	**Number of litters**	**litter size**	**resorptions**	**mal-developed embryos**	**male : female ratio**	**Crown – rump length**
CTR	SAL	12.5	9	7.0 ± 0.4	1.3 ± 0.7	0.2 ± 0.2	0.6 ± 0.1	8.6 ± 0.1
CTR	MIA	12.5	8	7.8 ± 1.0	1.6 ± 0.6	0.3 ± 0.3	0.6 ± 0.1	8.3 ± 0.1
HF	SAL	12.5	9	8.0 ± 0.5	1.1 ± 0.6	0.2 ± 0.2	0.5 ± 0.1	8.4 ± 0.1
HF	MIA	12.5	9	7.4 ± 0.4	2.0 ± 0.8	0.1 ± 0.1	0.5 ± 0.0	8.4 ± 0.1
CTR	SAL	16.5	9	6.8 ± 0.8	1.8 ± 0.8	0.3 ± 0.2	0.5 ± 0.1	15.5 ± 0.2
CTR	MIA	16.5	9	7.3 ± 0.4	1.4 ± 0.4	0.1 ± 0.1	0.6 ± 0.1	14.8 ± 0.1
HF	SAL	16.5	9	7.8 ± 0.6	0.9 ± 0.3	0.1 ± 0.1	0.7 ± 0.1	15.1 ± 0.2
HF	MIA	16.5	9	6.7 ± 0.7	2.4 ± 0.6	0.2 ± 0.2	0.6 ± 0.1	14.9 ± 0.2

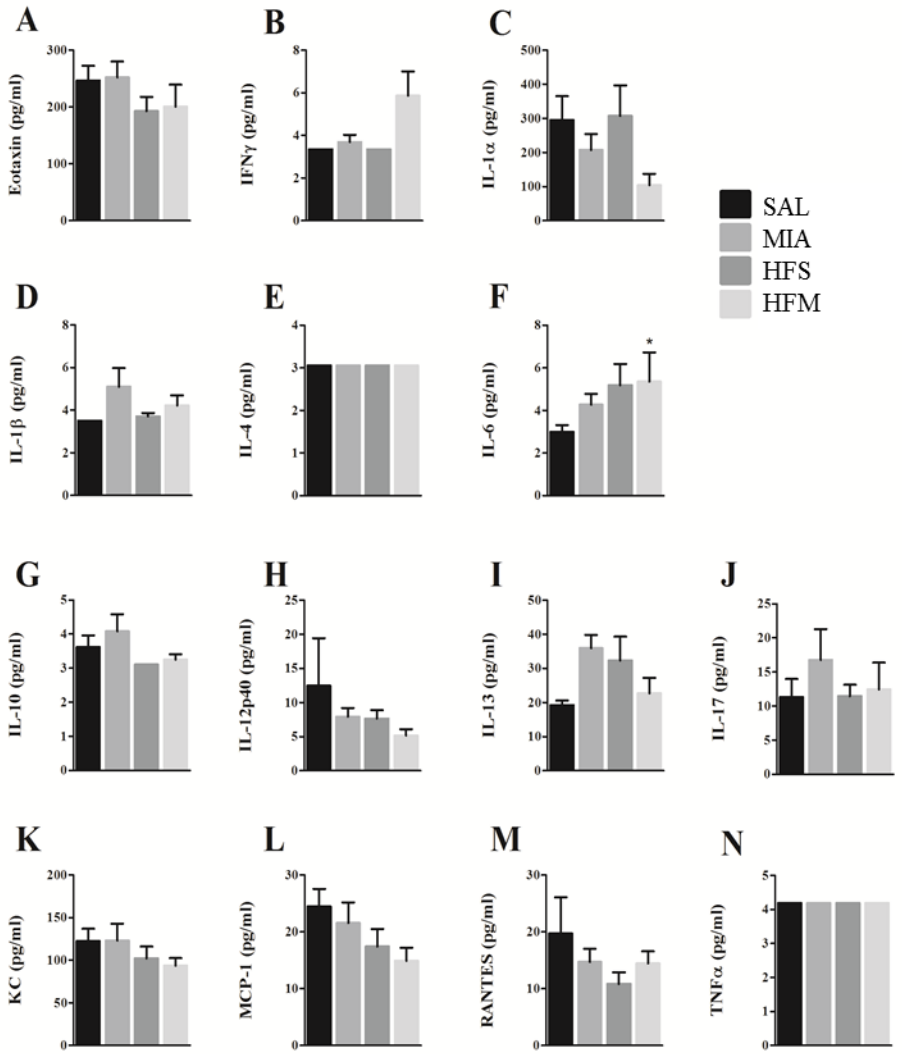


Supplemental Figure 1. High fat diet does not produce a diabetic phenotype at GD0.5. Body composition, 6 hour fasting glucose and insulin, and glucose tolerance measurements were performed on a cohort of high fat and control fed GD0.5 pregnant dams (n=8 per group). (**A)** High fat fed dams did not show significant increases in body weight. (**B**) Whole body NMR also did not show significant changes in % body mass. (**C-D**) Neither 6 hour fasting blood glucose or serum insulin were significantly altered in high fat dams. (**E-F**) After a 6 hour fast, dams were injected intraperitoneally with 2 g dextrose/kg body weight. No significant difference in blood

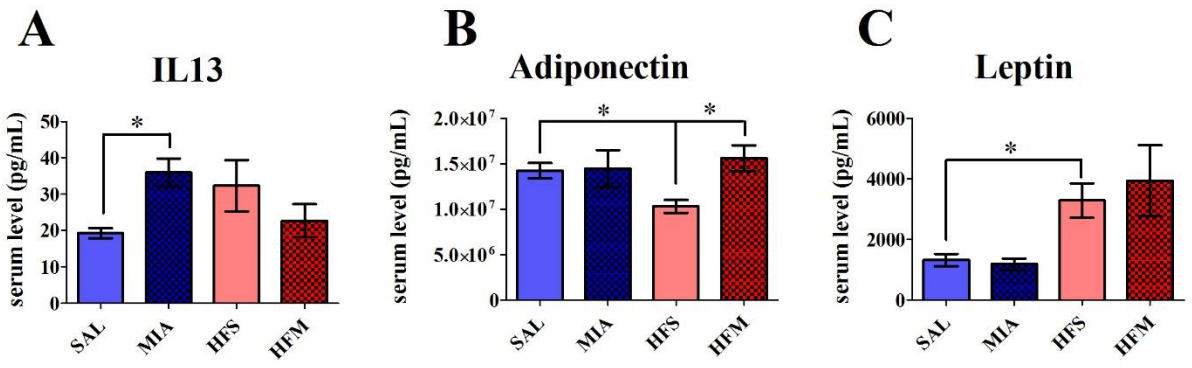
glucose between control and high fat dams was observable at 0 10, 20, 30, 60, and 75 minutes post-injection. Error bars represent SEM. Significance of $p<0.05$ is indicated by * and determined by unpaired student t-test with Welch's correction in all assays, n=6 dams per group.



Supplemental Figure 2. 8 weeks of high fat diet produces increased body weight and a trend towards altered glucose tolerance. Body composition, 6 hour fasting glucose and insulin, and glucose tolerance measurements were performed on a cohort of high fat and control fed non-pregnant dams (n=8 per group). The purpose of this experiment was to determine if the GD12.5 high fat dams were glucose intolerant due to pregnancy + 8 weeks of high fat diet or if 8 weeks of high fat diet alone was sufficient to induce glucose intolerance. (**A-B**) High fat dams had a significantly increased body weight but not altered body composition. (**C-D**) Fasting glucose and insulin after a 6 hour fast were NOT impaired in HF dams, unlike that seen in the pregnant cohort. (**E-F**) When challenged with 2 g/kg dextrose after a 6 hour fast, HF dams show significant differences at two time points (45 and 60 minutes post-injection), and there is a strong trend although not significant of a significantly increased area under the curve (best measure of glucose intolerance). Error bars represent SEM. Significance of $p<0.05$ indicated by * and determined by unpaired student t-test, n=8 for HF and CTR.



Supplemental Figure 3. HFD and MIA do not alter cytokine or chemokine profiles within maternal serum at GD16.5 Maternal serum collected via cardiac puncture 4 days post injection with either saline or 20mg/kg Poly I:C potassium salt on GD12.5 was analyzed for chemokine and cytokine levels via conjugated bead array multiplex assay. Error bars represent SEM. Significance indicated by letters with similar letters dictating no significance as determined by one-way ANOVA with Tukey's multiple comparisons post-test in all assays. (n=9 except for Poly I:C treated non-HFD where n=8).



Supplemental Figure 4. High fat diet induced gestational diabetes and MIA continue to alter serum factor levels at GD16.5. Maternal serum collected at GD16.5- 4 days after poly(I:C) exposure- was analyzed for chemokine, cytokine, and metabolic hormone levels via Luminex multiplex assay. Only those serum factors that demonstrated a significant difference in at least one comparison (SAL vs. MIA, SAL vs. HFS, or HFS vs. HFM) are shown. (**A**) The only cytokine/chemokine significantly increased by MIA at GD16.5 was IL13, which actually was not significantly different at GD12.5. This was only observed in the control context and not in the context of high fat diet induced gestational diabetes. (**B**) The anti-inflammatory metabolic hormone adiponectin remained significantly decreased by high fat diet induced gestational diabetes at GD16.5, but was also increased by MIA in the context of high fat diet induced gestational diabetes, bringing the HFM group to a similar level as MIA and SAL. (**C**) The pro-inflammatory satiety hormone leptin also remained significantly increased by high fat diet induced gestational diabetes, but the further increase by MIA only in the context of high fat diet induced gestational diabetes was no longer observable. Error bars represent SEM. Significance of $p<0.05$ is indicated by * and determined by unpaired student t-test with Welch's correction in all assays. Serum was collected at GD16.5, four days after poly(I:C) exposure, from dams whose embryos were utilized for Nanostring gene expression analysis (SAL n=9, MIA n=9, HFS n=9, HFM n=9).

Supplemental Table 3. Both high fat diet and MIA alter gene expression at GD12.5.

Genes listed demonstrated a significant change in expression in either HFM, MIA, or HFS when compared to SAL (57 genes). The $\log_2$(counts) values, which are listed with SEM, for each group was utilized for a supervised hierarchical clustering analysis shown in **Figure 7**. Significance was determined by unpaired student t-test with Welch's correction and $p<0.05$ (significant values are shaded).

	SAL	**HFS**			**MIA**			**HFM**		
Gene	**$\log_2$ (counts)**	**$\log_2$ (counts)**	**ALR (HFS-SAL)**	**p-value**	**$\log_2$ (counts)**	**ALR (MIA-SAL)**	**p-value**	**$\log_2$ (counts)**	**ALR (HFM-SAL)**	**p-value**
Ager	7.21 ± 0.11	7.38 ± 0.07	0.17	*0.182*	7.41 ± 0.04	0.21	*0.088*	7.64 ± 0.07	0.44	*0.003*
Alox12	4.37 ± 0.08	4.43 ± 0.21	0.06	*0.778*	4.89 ± 0.12	0.52	*0.003*	3.96 ± 0.18	-0.42	*0.045, ns*
Bcl6	4.89 ± 0.13	4.73 ± 0.09	-0.16	*0.302*	4.22 ± 0.12	-0.67	*0.001*	4.81 ± 0.11	-0.08	*0.637*
C1qb	9.05 ± 0.04	8.99 ± 0.05	-0.06	*0.303*	8.97 ± 0.05	-0.08	*0.242*	8.91 ± 0.05	-0.14	*0.030*
Cd55	6.90 ± 0.12	6.78 ± 0.06	-0.13	*0.342*	6.63 ± 0.05	-0.27	*0.049*	6.62 ± 0.07	-0.29	*0.051*
Creb1	7.64 ± 0.06	7.66 ± 0.06	0.02	*0.793*	7.34 ± 0.09	-0.30	*0.014*	7.45 ± 0.05	-0.19	*0.025*
Cysltr1	3.45 ± 0.09	3.50 ± 0.13	0.06	*0.718*	3.73 ± 0.18	0.28	*0.188*	3.90 ± 0.17	0.45	*0.030*
Dckl1	12.19 ± 0.09	12.11 ± 0.05	-0.08	*0.45*	11.87 ± 0.10	-0.32	*0.024*	12.19 ± 0.07	0.00	*0.994*
Ddit3	9.69 ± 0.03	9.84 ± 0.05	0.14	*0.017*	9.71 ± 0.04	0.02	*0.631*	9.69 ± 0.05	0.00	*0.988*
En1	3.97 ±	3.46 ±	-0.51	*0.049*	3.92 ±	-0.05	*0.855*	3.71 ±	-0.27	*0.360*

	0.23	0.06			0.21			0.19		
Flt1	8.84 ± 0.02	8.92 ± 0.03	0.08	*0.051*	9.04 ± 0.07	0.20	*0.025*	9.24 ± 0.05	0.41	*0.000*
Foxg1	10.75 ± 0.03	10.85 ± 0.04	0.11	*0.052*	10.83 ± 0.08	0.09	*0.311*	11.07 ± 0.05	0.33	*0.000*
Fxyd2	7.09 ± 0.06	7.15 ± 0.06	0.06	*0.509*	6.83 ± 0.10	-0.26	*0.048*	7.07 ± 0.06	-0.03	*0.730*
Gabra2	9.42 ± 0.09	9.30 ± 0.04	-0.11	*0.243*	9.01 ± 0.13	-0.41	*0.018*	9.26 ± 0.07	-0.16	*0.162*
Gad1	9.74 ± 0.09	9.77 ± 0.06	0.02	*0.837*	9.64 ± 0.10	-0.11	*0.396*	10.07 ± 0.06	0.33	*0.006*
Gnaq	12.67 ± 0.04	12.62 ± 0.02	-0.05	*0.295*	12.52 ± 0.04	-0.15	*0.028*	12.65 ± 0.03	-0.02	*0.722*
Hmgb2	9.92 ± 0.05	10.11 ± 0.04	0.19	*0.009*	9.88 ± 0.05	-0.03	*0.636*	9.97 ± 0.04	0.05	*0.464*
Ifi44	3.82 ± 0.19	3.54 ± 0.12	-0.28	*0.197*	4.94 ± 0.35	1.12	*0.017*	5.70 ± 0.40	1.88	*0.001*
Ifit1	3.54 ± 0.12	3.47 ± 0.09	-0.07	*0.627*	4.61 ± 0.34	1.07	*0.017*	5.63 ± 0.41	2.09	*0.001*
Ifit2	8.86 ± 0.04	8.81 ± 0.05	-0.04	*0.483*	8.95 ± 0.04	0.09	*0.074*	9.18 ± 0.09	0.32	*0.004*
Ifit3	4.66 ± 0.14	4.93 ± 0.16	0.27	*0.197*	5.77 ± 0.32	1.11	*0.010*	6.79 ± 0.38	2.13	*0.000*
Ifitm3	8.41 ± 0.10	8.15 ± 0.05	-0.26	*0.027*	8.54 ± 0.13	0.13	*0.410*	8.79 ± 0.12	0.38	*0.017*
Ifna1	3.36 ± 0.04	3.86 ± 0.20	0.51	*0.026*	3.44 ± 0.08	0.09	*0.350*	3.54 ± 0.13	0.18	*0.191*
Il11	3.32 ± 0.00	3.56 ± 0.10	0.23	*0.043*	3.35 ± 0.03	0.03	*0.374*	3.39 ± 0.07	0.06	*0.358*
Il18rap	3.79 ±	4.15 ±	0.36	*0.102*	4.02 ±	0.23	*0.161*	4.30 ±	0.51	*0.002*

	0.10	0.19			0.12			0.11		
Irf1	7.01 ± 0.08	7.01 ± 0.07	0.00	*0.973*	7.14 ± 0.09	0.13	*0.270*	7.28 ± 0.10	0.27	*0.040*
Irf5	4.86 ± 0.08	4.82 ± 0.07	-0.05	*0.637*	4.92 ± 0.11	0.06	*0.685*	4.64 ± 0.04	-0.22	*0.019*
Irf7	3.78 ± 0.16	3.64 ± 0.13	-0.14	*0.478*	4.62 ± 0.36	0.84	*0.060*	5.22 ± 0.35	1.45	*0.002*
Maff	6.21 ± 0.07	6.21 ± 0.11	0.00	*0.985*	6.35 ± 0.10	0.14	*0.258*	6.69 ± 0.07	0.48	*0.000*
Map3k5	6.93 ± 0.13	6.76 ± 0.07	-0.17	*0.238*	6.58 ± 0.08	-0.34	*0.033*	6.61 ± 0.08	-0.32	*0.040*
Mapk1	11.22 ± 0.04	11.26 ± 0.04	0.04	*0.416*	11.26 ± 0.05	0.04	*0.514*	11.37 ± 0.04	0.15	*0.009*
Mef2a	9.38 ± 0.07	9.19 ± 0.03	-0.19	*0.027*	9.16 ± 0.07	-0.23	*0.031*	9.12 ± 0.05	-0.26	*0.007*
Mef2c_Mm	8.94 ± 0.09	8.76 ± 0.04	-0.18	*0.074*	8.71 ± 0.03	-0.23	*0.026*	8.87 ± 0.05	-0.06	*0.500*
Mx2	4.29 ± 0.17	4.93 ± 0.09	0.64	*0.004*	5.54 ± 0.21	1.25	*0.000*	6.18 ± 0.21	1.88	*0.000*
Myc	9.85 ± 0.07	9.72 ± 0.03	-0.13	*0.081*	9.53 ± 0.09	-0.32	*0.010*	9.72 ± 0.05	-0.13	*0.140*
Myd88	7.52 ± 0.06	7.40 ± 0.03	-0.12	*0.097*	7.32 ± 0.04	-0.20	*0.016*	7.34 ± 0.06	-0.19	*0.043*
Ncam1	13.75 ± 0.08	13.71 ± 0.04	-0.05	*0.603*	13.49 ± 0.07	-0.26	*0.020*	13.82 ± 0.05	0.07	*0.483*
Ncam2	9.41 ± 0.08	9.37 ± 0.02	-0.04	*0.646*	9.17 ± 0.09	-0.24	*0.048*	9.46 ± 0.06	0.06	*0.560*
Nfe2l2	10.52 ± 0.04	10.44 ± 0.06	-0.08	*0.238*	10.34 ± 0.04	-0.18	*0.006*	10.26 ± 0.04	-0.26	*0.000*
Nr3c1	8.80 ±	8.70 ±	-0.10	*0.261*	8.63 ±	-0.17	*0.047*	8.65 ±	-0.15	*0.073*

	0.07	0.06			0.03			0.04		
Oasl1	3.32 ± 0.00	3.32 ± 0.00	0.00	*1.000*	4.39 ± 0.34	1.07	*0.016*	5.30 ± 0.36	1.97	*0.000*
Pdgfa	9.60 ± 0.09	9.50 ± 0.03	-0.09	*0.299*	9.27 ± 0.07	-0.33	*0.010*	9.37 ± 0.05	-0.23	*0.026*
Ppp1r12b	9.07 ± 0.05	9.08 ± 0.04	0.01	*0.877*	8.88 ± 0.05	-0.19	*0.016*	9.01 ± 0.03	-0.06	*0.255*
Ptgs1	6.28 ± 0.06	6.07 ± 0.07	-0.21	*0.031*	6.16 ± 0.07	-0.11	*0.199*	6.15 ± 0.09	-0.13	*0.236*
Ripk1	8.28 ± 0.04	8.25 ± 0.05	-0.03	*0.648*	8.07 ± 0.06	-0.21	*0.014*	8.06 ± 0.04	-0.22	*0.001*
Rock2	9.88 ± 0.04	9.81 ± 0.03	-0.07	*0.180*	9.73 ± 0.05	-0.15	*0.026*	9.78 ± 0.04	-0.10	*0.077*
Shh	10.71 ± 0.07	10.90 ± 0.03	0.19	*0.024*	10.88 ± 0.09	0.17	*0.143*	10.83 ± 0.05	0.12	*0.180*
Slc2a1	11.23 ± 0.02	11.28 ± 0.01	0.05	*0.040, ns*	11.37 ± 0.06	0.14	*0.046*	11.46 ± 0.05	0.23	*0.001*
Slc2a3	9.61 ± 0.04	9.73 ± 0.02	0.12	*0.021, ns*	9.70 ± 0.06	0.10	*0.201*	9.90 ± 0.07	0.29	*0.003*
Stat1	9.31 ± 0.03	9.34 ± 0.03	0.03	*0.457*	9.43 ± 0.05	0.12	*0.046, ns*	9.50 ± 0.07	0.19	*0.025*
Syt1	9.55 ± 0.09	9.53 ± 0.06	-0.02	*0.864*	9.26 ± 0.09	-0.29	*0.025*	9.61 ± 0.06	0.07	*0.517*
Tcf4	13.84 ± 0.04	13.75 ± 0.03	-0.09	*0.095*	13.65 ± 0.07	-0.19	*0.040*	13.80 ± 0.04	-0.04	*0.519*
Tgfb3	8.24 ± 0.17	7.95 ± 0.06	-0.29	*0.119*	7.80 ± 0.08	-0.45	*0.028*	8.07 ± 0.09	-0.18	*0.349*
Tnfaip3	7.33 ± 0.06	7.46 ± 0.05	0.13	*0.107*	7.29 ± 0.09	-0.04	*0.733*	7.58 ± 0.09	0.25	*0.036*
Traf2	9.62 ±	9.67 ±	0.05	*0.326*	9.46 ±	-0.15	*0.040*	9.44 ±	-0.18	*0.008*

	0.03	0.04			0.06			0.05		
Vamp1	7.78 ± 0.08	7.89 ± 0.03	0.11	*0.233*	7.81 ± 0.08	0.03	*0.765*	8.09 ± 0.03	0.31	*0.004*
Vegfa	11.20 ± 0.03	11.42 ± 0.04	0.22	*0.000*	11.44 ± 0.10	0.24	*0.033*	11.69 ± 0.07	0.49	*0.000*

Supplemental Table 4. GDM and MIA alter gene expression at GD12.5.

Genes listed demonstrated a significant change in expression in HFS, MIA, or HFS+MIA when compared to S. The difference of $\log_2$ (counts) between control and each treatment/diet group was utilized for Venn diagram in **Figure 10**. Significance was determined by multiple t-tests with FDR<0.01 and p<0.05.

Genes induced in GD12.5 high-fat diet induced GDM				
	Difference	Fold Change	FDR	p-value
C1s	2.51	1.18	*	7.33E-09
Ccl8	3.91	1.20	*	6.43E-12
Hspb1	14.79	1.20	*	9.51E-21
Ifit3	5.20	1.21	*	1.62E-14
Il18rap	3.94	1.29	*	1.72E-12
Masp2	4.26	1.26	*	2.13E-09
Mx2	10.89	1.56	*	2.46E-20
Tslp	2.89	1.19	*	2.27E-09
Pax5	5.45	1.23	*	1.22E-15
Vegfa	394.12	1.17	*	0.00E+00

Genes repressed in GD12.5 high-fat diet induced GDM				
	Difference	Fold Change	FDR	p-value
Arg1	-12.04	0.70	*	2.42E-15

Ccl21a	-42.76	0.84	*	9.51E-28
Ccl7	-2.73	0.82	*	5.66E-10
Ifi44	-2.52	0.82	*	1.90E-09
Itgb2	-5.38	0.80	*	1.16E-12
Nlrp3	-2.60	0.81	*	2.45E-13
Oas1a	-3.56	0.84	*	7.15E-10
Pla2g4a	-16.20	0.85	*	8.64E-24
Ptger4	-3.37	0.78	*	4.14E-11
Ptgfr	-9.61	0.77	*	1.37E-16
Tgfb2	-121.72	0.84	*	1.81E-38
Tgfb3	-55.39	0.82	*	5.81E-32
Twist2	-23.33	0.75	*	6.96E-18
Chat	-2.11	0.85	*	4.39E-10
Cryab	-6.48	0.75	*	1.35E-11
En1	-4.70	0.70	*	5.39E-13
En2	-2.63	0.81	*	2.06E-09
Ifitm3	-55.76	0.84	*	1.52E-35

Genes induced in GD12.5 MIA				
	Difference	Fold Change	FDR	p-value
Ager	22.62	1.15	*	4.95E-27
Alox12	9.06	1.44	*	6.42E-20
Ccl2	5.58	1.30	*	3.38E-13
Ccl3	3.89	1.15	*	8.25E-13
Ifi44	16.63	2.18	*	3.06E-17
Ifit1	12.84	2.11	*	3.47E-16
Ifit3	29.20	2.15	*	8.91E-22
Il18rap	2.38	1.17	*	1.22E-10
Irf7	10.85	1.79	*	1.44E-14
Masp2	2.68	1.16	*	1.08E-05

Mx2	27.07	2.38	*	8.62E-23
Oas1a	6.65	1.29	*	4.41E-13
Vegfa	423.91	1.18	*	0.00E+00

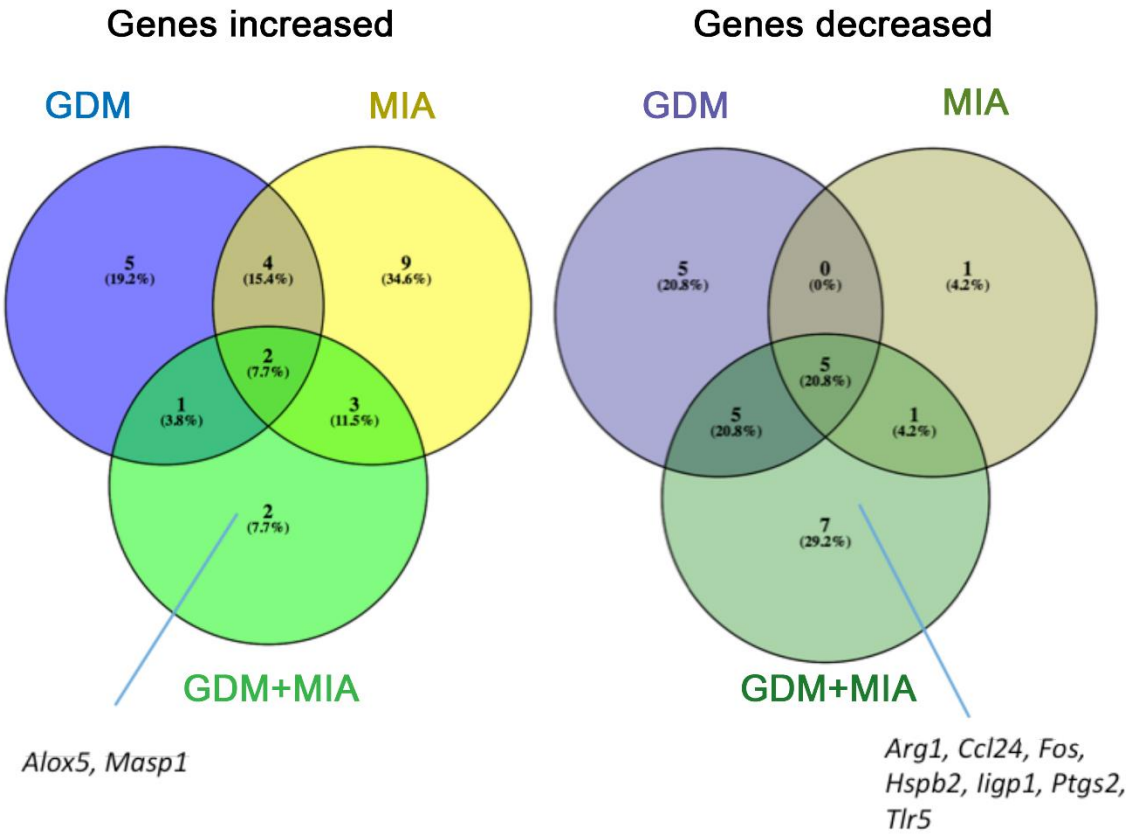
Genes repressed in GD12.5 MIA				
	Difference	Fold Change	FDR	p-value
Arg1	-10.47	0.74	*	2.66E-14
Bcl6	-11.03	0.63	*	9.79E-20
Ccl21a	-60.27	0.77	*	7.00E-28
Cd55	-20.79	0.83	*	1.21E-25
Creb1	-37.13	0.81	*	5.81E-31
Fxyd2	-22.69	0.83	*	4.33E-27
Ifi27l2a	-6.04	0.77	*	2.94E-12
Il6ra	-3.39	0.76	*	5.24E-12
Map3k5	-25.65	0.79	*	3.62E-26
Mef2b	-2.85	0.80	*	6.19E-09
Mmp9	-31.25	0.76	*	1.95E-24
Myc	-183.11	0.80	*	0.00E+00
Nlrp3	-2.07	0.85	*	5.55E-10
Pdgfa	-156.80	0.80	*	0.00E+00
Prkca	-73.22	0.84	*	5.61E-34
Ptger3	-13.07	0.79	*	1.91E-17
Ptger4	-2.49	0.83	*	1.59E-07
Ptgfr	-11.97	0.71	*	3.76E-17
Tgfb3	-80.95	0.73	*	3.37E-32
Twist2	-15.91	0.83	*	3.74E-13
Bdnf	-8.10	0.82	*	2.49E-16
Dckl1	-925.97	0.80	*	0.00E+00
Drd1a	-13.08	0.66	*	1.09E-16
Drd2	-13.28	0.80	*	6.89E-22

En2	-2.09	0.85	*	2.20E-07
Gabra2	-168.67	0.75	*	1.89E-38
Ncam1	-2295.92	0.83	*	0.00E+00
Ncam2	-102.90	0.85	*	3.46E-37
Npy	-112.55	0.80	*	4.26E-35
Pax5	-3.85	0.84	*	1.83E-11
Syt1	-135.50	0.82	*	1.33E-38

Genes induced in GD12.5 GDM + MIA				
	Difference	Fold Change	FDR	p-value
Ager	52.37	1.35	*	4.54E-34
C1s	4.71	1.33	*	1.16E-13
Ccl2	7.23	1.39	*	5.01E-16
Ccl8	3.32	1.17	*	5.39E-13
Cysltr2	5.62	1.19	*	2.59E-15
Flt1	148.59	1.32	*	0.00E+00
Fos	5.07	1.16	*	2.71E-14
Ifi44	37.87	3.68	*	3.95E-23
Ifit1	37.87	4.26	*	2.26E-23
Ifit2	115.49	1.25	*	0.00E+00
Ifit3	85.49	4.38	*	1.97E-29
Iigp1	3.43	1.30	*	3.06E-09
Il18rap	5.84	1.42	*	1.01E-17
Irf1	26.56	1.21	*	2.62E-29
Irf7	23.66	2.72	*	7.09E-21
Maff	28.93	1.39	*	2.46E-31
Map3k9	19.09	1.21	*	2.09E-25
Masp2	3.12	1.19	*	2.35E-07
Mx2	52.75	3.69	*	6.72E-29
Myl2	3.70	1.32	*	2.73E-07

Tlr2	4.92	1.23	*	3.51E-12
Tlr5	3.60	1.30	*	2.44E-11
Tnfaip3	30.35	1.19	*	5.03E-31
Tslp	3.61	1.24	*	1.41E-11
Foxg1	437.41	1.25	*	0.00E+00
Gad1	220.45	1.26	*	0.00E+00
Ifitm3	102.30	1.30	*	1.62E-37
Slc2a1	415.71	1.17	*	0.00E+00
Slc2a3	175.35	1.22	*	0.00E+00
Vamp1	52.83	1.24	*	1.66E-36
Vegfa	953.36	1.41	*	0.00E+00

Genes repressed in GD12.5 GDM + MIA				
	Difference	Fold Change	FDR	p-value
Alox12	-5.17	0.75	*	4.61E-15
Arg1	-17.33	0.57	*	2.06E-18
Cd55	-21.52	0.82	*	3.81E-27
Itgb2	-4.06	0.85	*	6.48E-13
Map3k5	-24.12	0.80	*	1.45E-27
Mef2a	-111.62	0.83	*	0.00E+00
Mef2b	-2.32	0.84	*	1.29E-07
Nfe2l2	-244.85	0.83	*	0.00E+00
Tradd	-8.93	0.84	*	5.59E-20
En1	-2.64	0.83	*	6.64E-08
En2	-2.60	0.81	*	4.53E-09
Fgf8	-23.17	0.84	*	8.28E-27
Pax5	-3.92	0.83	*	5.72E-12



Supplemental Figure 5. GDM and MIA exposure still show altered gene expression patterns at GD16.5. Venn diagrams representing the number of genes induced or repressed in GDM, MIA or GDM+MIA when compared to control mice fed a normal diet and treated with saline. Significant changes in gene expression were determined by multiple t tests, and genes with $p<0.05$, FDR<0.01 were selected.

Supplemental Table 5. GDM andMIA exposure continues to show altered gene expression patterns at GD16.5. Genes found to be significantly different between SAL and GDM, MIA, and GDM+MIA are shown. Significance was determined by multiple t-tests with FDR<0.01 and p<0.05. Although less prominent than at GD12.5, each condition continues to show significant differences in both inflammation and neurodevelopment gene expression. This demonstrates that MIA continues to alter gene expression patterns days after the immune stimulus has passed, and GDM continues to have an effect late gestation.

Genes induced at GD16.5 in diet-induced GDM				
	Difference	Fold Change	FDR	p-value
C8b	12.10	1.50	*	9.83E-14
Ccl22	4.99	1.20	*	4.23E-13
Ccl8	2.39	1.20	*	2.82E-06
Cd55	77.91	1.16	*	1.21E-34
Hras1	23.34	1.18	*	1.12E-33
Il1a	2.89	1.16	*	1.10E-06
Maff	26.20	1.18	*	4.80E-28
Max	400.42	1.16	*	0.00E+00
Mef2b	3.84	1.22	*	1.33E-06
Ptger3	25.02	1.15	*	4.38E-31
En2	6.27	1.53	*	4.07E-09
Pax5	13.62	1.96	*	5.60E-15

Genes repressed at GD16.5 in diet-induced GDM				
	Difference	Fold Change	FDR	p-value
Ccl17	-8.92	0.58	*	4.15E-13
Ccl7	-4.66	0.82	*	5.97E-07
Ccr1	-5.29	0.75	*	4.58E-15
Cd163	-10.69	0.79	*	5.05E-24
Cfb	-4.20	0.75	*	2.34E-10
Csf1	-32.71	0.79	*	1.54E-24
Ifi27l2a	-8.81	0.83	*	1.37E-17
Ifit1	-10.15	0.67	*	7.58E-14
Irf7	-5.23	0.75	*	4.36E-12
Itgb2	-12.64	0.68	*	4.91E-21
Lta	-2.09	0.84	*	2.11E-08
Oasl1	-2.95	0.77	*	1.89E-10
Tlr7	-3.07	0.82	*	5.02E-10
Tlr9	-4.30	0.81	*	1.39E-14
Fgf8	-4.81	0.75	*	4.69E-13

Genes induced at GD16.5 in MIA				
	Difference	Fold Change	FDR	p-value
C1ra	2.75	1.21	*	3.40E-08
C2	6.13	1.16	*	9.01E-15
C8b	6.20	1.26	*	8.60E-10
Ccl17	5.84	1.28	*	2.17E-08
Ccl22	6.71	1.27	*	2.59E-14
Ccl7	21.32	1.82	*	6.42E-17
Ccl8	4.24	1.36	*	7.80E-09
Ccr2	8.82	1.47	*	2.38E-13
Fasl	4.69	1.26	*	7.74E-11
Ifi44	13.62	1.46	*	9.50E-19
Il1a	5.84	1.32	*	1.33E-12
Irf7	8.14	1.38	*	4.99E-15
Ltb	3.36	1.16	*	2.59E-09
Mef2b	16.73	1.94	*	4.37E-15
Myl2	12.86	1.24	*	6.67E-18
Nlrp3	3.57	1.16	*	1.53E-07
Tlr2	6.50	1.26	*	1.13E-13
Pax5	5.40	1.38	*	5.25E-11

Genes repressed at GD16.5 in MIA				
	Difference	Fold Change	FDR	p-value
Cd163	-8.69	0.83	*	1.44E-19
Cfb	-3.53	0.79	*	2.06E-09
Csf1	-29.35	0.82	*	6.66E-24
Gngt1	-2.29	0.82	*	1.39E-07
Itgb2	-6.00	0.85	*	1.81E-19
Oasl1	-2.31	0.82	*	1.10E-08
Chat	-8.93	0.77	*	2.56E-19

Genes induced at GD16.5 in diet-induced GDM+MIA				
	Difference	Fold Change	FDR	p-value
Alox5	3.08	1.23	*	2.66E-11
C2	7.25	1.18	*	2.53E-16
Ccl22	4.44	1.18	*	3.90E-12
Masp1	504.87	1.17	*	0.00E+00
Nlrp3	3.86	1.18	*	9.03E-08
Tlr2	12.50	1.50	*	4.11E-19
En2	3.00	1.25	*	1.78E-08
Pax5	11.68	1.82	*	1.44E-15

Genes repressed at GD16.5 in diet-induced GDM+MIA				
	Difference	Fold Change	FDR	p-value
Arg1	-5.75	0.74	*	6.34E-13
Ccl17	-10.47	0.51	*	1.07E-14
Ccl24	-6.69	0.85	*	6.18E-19
Ccr1	-4.97	0.77	*	4.46E-15
Cd163	-12.32	0.76	*	1.39E-21
Cfb	-5.07	0.70	*	1.73E-11
Csf1	-24.08	0.85	*	1.69E-22
Fos	-11.48	0.84	*	5.13E-23
Hspb2	-2.68	0.83	*	6.09E-11
Ifit1	-8.10	0.74	*	4.38E-11
Iigp1	-5.23	0.83	*	1.26E-11
Irf7	-4.19	0.80	*	1.81E-09
Itgb2	-11.18	0.71	*	3.50E-20
Oasl1	-2.73	0.79	*	6.52E-10
Ptgs2	-2.07	0.84	*	1.20E-06
Tlr5	-2.65	0.82	*	1.39E-08
Tlr9	-4.48	0.80	*	2.78E-13
Chat	-9.62	0.76	*	7.39E-19

Supplemental Table 6. Genes that are included in the Nanostring V2 Mouse Inflammation panel utilized in the placental gene expression analysis

Gene Names Included in the Nanostring V2 Mouse Inflammation Panel						
C1s	**Ccl20**	**Cxcr4**	**Il10rb**	**Ltb4r1**	**Nfatc3**	**Rock2**
C4a	**Ccl22**	**Cysltr1**	**Il11**	**Ltb4r2**	**Nfe2l2**	**Rps6ka5**
C7	**Ccl24**	**Cysltr2**	**Il12a**	**Ly96**	**Nfkb1**	**Shc1**
Ccl19	**Ccl3**	**Daxx**	**Il12b**	**Maff**	**Nlrp3**	**Smad7**
Ccl21a	**Ccl4**	**Ddit3**	**Il13**	**Mafg**	**Nod1**	**Stat1**
Ccl8	**Ccl5**	**Elk1**	**Il15**	**Mafk**	**Nod2**	**Stat2**
Chi3l3	**Ccl7**	**Fasl**	**Il17a**	**Map2k1**	**Nos2**	**Stat3**
Defa-rs1	**Ccr1**	**Flt1**	**Il18**	**Map2k4**	**Nox1**	**Tbxa2r**
Ifit3	**Ccr2**	**Fos**	**Il18rap**	**Map2k6**	**Nr3c1**	**Tcf4**
Ifna1	**Ccr3**	**Fxyd2**	**Il1b**	**Map3k1**	**Oas1a**	**Tgfb1**
Il1a	**Ccr4**	**Gnaq**	**Il1r1**	**Map3k5**	**Oas2**	**Tgfb2**

Il22	**Ccr7**	**Gnas**	**Il1rap**	**Map3k7**	**Oasl1**	**Tgfb3**
Rhoa	**Cd163**	**Gnb1**	**Il1rn**	**Map3k9**	**Pdgfa**	**Tgfbr1**
Ager	**Cd4**	**Gngt1**	**Il2**	**Mapk1**	**Pik3c2g**	**Tlr1**
Alox12	**Cd40**	**Gpr44**	**Il21**	**Mapk14**	**Pla2g4a**	**Tlr2**
Alox15	**Cd40lg**	**Grb2**	**Il22ra2**	**Mapk3**	**Plcb1**	**Tlr3**
Alox5	**Cd55**	**H2-Ea-ps**	**Il23a**	**Mapk8**	**Ppp1r12b**	**Tlr4**
Areg	**Cd86**	**H2-Eb1**	**Il23r**	**Mapkapk2**	**Prkca**	**Tlr5**
Arg1	**Cdc42**	**Hc**	**Il3**	**Mapkapk5**	**Prkcb**	**Tlr6**
Atf2	**Cebpb**	**Hdac4**	**Il4**	**Masp1**	**Ptger1**	**Tlr7**
Bcl2l1	**Cfb**	**Hif1a**	**Il5**	**Masp2**	**Ptger2**	**Tlr8**
Bcl6	**Cfd**	**Hmgb1**	**Il6**	**Max**	**Ptger3**	**Tlr9**
Birc2	**Cfl1**	**Hmgb2**	**Il6ra**	**Mbl2**	**Ptger4**	**Tnf**
C1qa	**Creb1**	**Hmgn1**	**Il7**	**Mef2a**	**Ptgfr**	**Tnfaip3**
C1qb	**Crp**	**Hras1**	**Il9**	**Mef2b**	**Ptgir**	**Tnfsf14**
C1ra	**Csf1**	**Hsh2d**	**Irf1**	**Mef2c_Mm**	**Ptgs1**	**Tollip**
C2	**Csf2**	**Hspb1**	**Irf3**	**Mef2d**	**Ptgs2**	**Tradd**
C3	**Csf3**	**Hspb2**	**Irf5**	**Mknk1**	**Ptk2**	**Traf2**
C3ar1	**Cxcl1**	**Ifi27l2a**	**Irf7**	**Mmp3**	**Rac1**	**Trem2**
C6	**Cxcl10**	**Ifi44**	**Itgb2**	**Mmp9**	**Raf1**	**Tslp**
C8a	**Cxcl2**	**Ifit1**	**Jun**	**Mrc1**	**Rapgef2**	**Twist2**
C8b	**Cxcl3**	**Ifit2**	**Keap1**	**Mx1**	**Rela**	**Tyrobp**
C9	**Cxcl5**	**Ifnb1**	**Kng1**	**Mx2**	**Relb**	
Ccl11	**Cxcl9**	**Ifng**	**Limk1**	**Myc**	**Retnla**	
Ccl17	**Cxcr1**	**Iigp1**	**Lta**	**Myd88**	**Ripk1**	
Ccl2	**Cxcr2**	**Il10**	**Ltb**	**Myl2**	**Ripk2**	

Supplemental Table 7. Pathways represented in PANTER analysis based upon the genes in the Nanostring V2 inflammation panel. All genes that were assessed for gene expression changes in the Nanostring V2 inflammation panel were put into the PANTER pathway analysis software. The pathways represented by the V2 panel and the number of genes in each of the pathways is noted in the below table.

Annotated Pathways in V2 Mouse Inflammation Panel	**Number of Genes in Pathway**
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	**52**
Gonadotropin-releasing hormone receptor pathway (P06664)	**42**
CCKR signaling map (P06959)	**33**
Angiogenesis (P00005)	**27**
Toll receptor signaling pathway (P00054)	**27**
Apoptosis signaling pathway (P00006)	**25**
Interleukin signaling pathway (P00036)	**24**
Ras Pathway (P04393)	**22**
EGF receptor signaling pathway (P00018)	**21**
PDGF signaling pathway (P00047)	**20**
p38 MAPK pathway (P05918)	**18**
VEGF signaling pathway (P00056)	**17**
Integrin signalling pathway (P00034)	**17**
FGF signaling pathway (P00021)	**17**
T cell activation (P00053)	**16**
Oxidative stress response (P00046)	**15**
TGF-beta signaling pathway (P00052)	**14**
B cell activation (P00010)	**14**
Endothelin signaling pathway (P00019)	**12**
Wnt signaling pathway (P00057)	**10**
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	**10**
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)	**9**
PI3 kinase pathway (P00048)	**8**
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	**7**

(P00027)	
Alzheimer disease-amyloid secretase pathway (P00003)	**6**
Interferon-gamma signaling pathway (P00035)	**6**
FAS signaling pathway (P00020)	**6**
5HT2 type receptor mediated signaling pathway (P04374)	**6**
Parkinson disease (P00049)	**5**
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	**5**
p53 pathway feedback loops 2 (P04398)	**5**
Thyrotropin-releasing hormone receptor signaling pathway (P04394)	**5**
Oxytocin receptor mediated signaling pathway (P04391)	**5**
Huntington disease (P00029)	**5**
Histamine H1 receptor mediated signaling pathway (P04385)	**5**
Cytoskeletal regulation by Rho GTPase (P00016)	**5**
Axon guidance mediated by netrin (P00009)	**4**
JAK/STAT signaling pathway (P00038)	**4**
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	**4**
Axon guidance mediated by Slit/Robo (P00008)	**3**
p53 pathway (P00059)	**3**
Enkephalin release (P05913)	**3**
Cortocotropin releasing factor receptor signaling pathway (P04380)	**3**
Axon guidance mediated by semaphorins (P00007)	**2**
Alpha adrenergic receptor signaling pathway (P00002)	**2**
Plasminogen activating cascade (P00050)	**2**
Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)	**2**
Metabotropic glutamate receptor group I pathway (P00041)	**2**
Metabotropic glutamate receptor group II pathway (P00040)	**2**
Endogenous cannabinoid signaling (P05730)	**2**
Hypoxia response via HIF activation (P00030)	**2**
Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction (P00028)	**2**
Nicotine pharmacodynamics pathway (P06587)	**2**
Dopamine receptor mediated signaling pathway (P05912)	**2**
Histamine H2 receptor mediated signaling pathway (P04386)	**2**
Beta3 adrenergic receptor signaling pathway (P04379)	**2**
Beta2 adrenergic receptor signaling pathway (P04378)	**2**

Beta1 adrenergic receptor signaling pathway (P04377)	**2**
5HT4 type receptor mediated signaling pathway (P04376)	**2**
Toll pathway-drosophila (P06217)	**1**
DPP signaling pathway (P06213)	**1**
BMP/activin signaling pathway-drosophila (P06211)	**1**
Activin beta signaling pathway (P06210)	**1**
Alzheimer disease-presenilin pathway (P00004)	**1**
Transcription regulation by bZIP transcription factor (P00055)	**1**
GABA-B receptor II signaling (P05731)	**1**
Metabotropic glutamate receptor group III pathway (P00039)	**1**
Insulin/IGF pathway-protein kinase B signaling cascade (P00033)	**1**
2-arachidonoylglycerol biosynthesis (P05726)	**1**
Opioid proopiomelanocortin pathway (P05917)	**1**
Opioid prodynorphin pathway (P05916)	**1**
Opioid proenkephalin pathway (P05915)	**1**
Blood coagulation (P00011)	**1**
5HT1 type receptor mediated signaling pathway (P04373)	**1**

Supplemental Table 8. Placental immune genes differentially regulated by sex in the normal diet, saline control group

Gene Name	p value	Effect Estimate
C1ra	0.00742	-4.81
Ccl24	0.02055	1.96
Ccl4	0.01453	7.14
Cfl1	0.00539	2095.12
Cxcl2	0.02904	8.43
Ddit3	0.01489	368.37
Ifna1	0.0308	11.13
Jun	0.00645	257.26
Ptgs2	0.0433	151.79
Rac1	0.03921	703.26
Tlr5	0.04321	-44.74

Tlr6	0.0489	8.53
Tlr8	0.00525	6.99

Supplemental Table 9. Inflammatory genes significantly down-regulated in GDM male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox5	0.001926946	-42.54733333
Arg1	0.004757934	-29.95466667
Bcl2l1	0.00911303	-294.4806667
Birc2	0.022424927	-86.65766667
C1qa	5.42307E-06	-67.33933333
C1qb	7.78421E-06	-87.36233333
C2	0.034250549	-287.2493333
Ccl17	0.045018675	-13.04566667
Ccl5	0.024391854	-14.35166667
Ccl8	0.001327809	-67.039
Ccr2	0.001593047	-55.074
Ccr7	0.000917256	-5.360666667
Cd55	0.000180472	-2299.446667
Cdc42	8.19668E-07	-3787.151333
Chi3l3	0.002931513	-38.79766667
Fasl	0.000834616	-36.11966667
Fos	0.032260602	-737.5546667
Gngt1	0.022292491	-16.52533333
H2_Eb1	0.000448036	-60.784
Hc	0.000424382	-43.80033333
Ifi27l2a	0.000334374	-271.203

Ifit1	0.038105124	-186.4636667
Ifit2	0.003321288	-422.8946667
Ifit3	0.042938592	-237.8013333
Iigp1	0.003083693	-127.727
Il18	0.001186363	-12.35166667
Il1rap	0.032796542	-98.54566667
Il1rn	0.003423275	-8.426
Il6ra	0.000600867	-940.8706667
Irf1	0.00522281	-151.1853333
Irf5	0.014295347	-16.592
Ltb4r1	3.75591E-06	-15.95766667
Ly96	0.000508472	-206.2116667
Map3k7	0.014557956	-337.1053333
Mapk1	0.013142938	-378.2956667
Masp1	0.00409261	-1236.857
Mef2a	0.000318597	-179.538
Mx1	0.001411058	-242.3123333
Oas2	0.000716271	-61.958
Pla2g4a	0.04766698	-94.13366667
Prkcb	0.01605893	-14.98533333
Ptger2	0.00012342	-15.66466667
Rac1	0.001496487	-1468.581
Retnla	2.43648E-07	-18.823
Ripk1	0.037597673	-51.07433333
Rock2	0.001292389	-222.8103333
Stat3	0.017098294	-624.4743333
Tgfb1	0.000300543	-696.5766667
Tgfb2	0.026228814	-480.876
Tlr4	0.002427201	-452.5416667
Tlr8	1.75227E-05	-10.84533333
Tnfaip3	0.002507121	-76.42766667
Tollip	0.013209681	-542.8513333

Trem2	0.000159557	-868.547
Twist2	0.001434843	-49.602
Tyrobp	0.000131002	-826.4123333

Supplemental Table 10. Inflammatory genes significantly up-regulated in GDM male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Cxcl2	0.047137709	11.61766667
Grb2	0.035753225	436.8726667
Hdac4	0.000646108	485.1873333
Hspb1	1.66271E-06	8406.071
Il1r1	0.000860141	510.4433333
Irf3	0.025725625	17.51666667
Limk1	0.01536897	588.6323333
Mafg	0.000571474	323.146
Mafk	0.026932154	257.3866667
Map2k6	0.009178745	209.9556667
Map3k1	0.002014505	103.2533333
Mapk14	0.000391098	433.9283333
Mef2b	0.008633252	9.527666667
Mef2d	0.041015838	262.925
Myc	0.003347919	432.0903333
Myd88	0.030464185	236.6826667
Nfatc3	0.000848957	220.507
Nos2	1.07767E-06	432.735
Nr3c1	0.000140557	178.3166667
Tgfb3	0.004105075	768.624

Supplemental Table 11. Inflammatory genes significantly down-regulated in GDM female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox5	0.002657516	-42.90666667
Birc2	0.001678911	-103.39
Ccl8	0.020947974	-40.58
Ccr2	0.005000052	-49.24666667
Cd40	0.002869934	-33.01166667
Cd55	0.0086493	-1381.636667
Cdc42	1.69164E-07	-3718.875
Chi3l3	0.001457773	-37.78
Fasl	0.011263235	-27.7
Gngt1	0.000238865	-21.525
Hc	0.000408656	-38.05333333
Il10rb	0.006717435	-277.9683333
Il12b	0.019578984	-3.125
Il13	0.035996966	-1.623333333
Il18	0.003353307	-17.66333333
Il1rap	0.005261877	-59.72833333
Il6ra	0.000753243	-658.4133333
Irf1	0.01914598	-102.8866667
Itgb2	0.02514249	-108.9133333
Ly96	7.30122E-06	-211.9716667
Map2k4	0.009317877	-459.2166667
Map3k7	2.02865E-05	-486.6683333
Mapk1	6.31208E-05	-357.5
Mef2a	8.72661E-05	-145.555
Ppp1r12b	0.04974014	-15.77166667
Rac1	2.00025E-05	-1502.036667
Rela	0.014490209	-113.105
Rps6ka5	0.047803932	-25.605

Stat3	0.024819031	-455.045
Tgfb1	0.001524355	-627.045
Tnfaip3	0.016694721	-51.63
Tollip	0.000831075	-542.7566667
Trem2	6.08834E-05	-816.0333333
Tyrobp	8.93488E-05	-670.7366667

Supplemental Table 12. Inflammatory genes significantly up-regulated in GDM female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Ccl24	0.032027172	2.115
Ccl3	0.024972918	17.865
Csf3	0.003082224	6.7
Cxcl2	0.004442088	17.14166667
Daxx	0.019088553	61.27666667
Gnas	0.017920168	1096.776667
Grb2	0.012391565	422.545
Hspb1	4.82945E-14	7136.168333
Limk1	0.002211185	954.4483333
Maff	0.001052794	606.1133333
Mafg	0.048580393	123.6216667
Mafk	0.036375918	164.645
Mapk14	0.018899014	106.0633333
Myc	0.026384777	398.9766667
Myd88	0.013242547	156.0516667
Nfatc3	0.032867819	88.93833333
Nos2	1.32928E-05	299.8416667
Tlr7	0.026354793	10.08666667
Traf2	0.041488015	50.87333333

Supplemental Table 13. Inflammatory genes significantly down-regulated in MIA male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox5	0.028290457	-38.305
C8a	0.037954604	-2.596666667
Ccr2	8.32652E-10	-85.41666667
Cd55	0.030422733	-1474.191667
Cdc42	0.000218025	-2717.34
Cfl1	0.002627437	-2944.211667
Csf2	0.046893204	-0.476666667
Cxcr1	0.040973271	-18.895
Cxcr4	0.001597391	-59.555
H2_Eb1	0.003301646	-48.40666667
Hc	0.010176615	-32.42166667
Hmgb1	0.00271586	-296.3283333
Keap1	0.004597594	-74.84833333
Ly96	0.004404948	-132.26
Mapk14	0.018386112	-195.635
Masp1	0.027012034	-910.0916667
Mef2a	0.007008629	-145.815
Pla2g4a	0.005003258	-113.5766667
Ppp1r12b	0.025255939	-13.00833333
Ptgs2	0.003698711	-310.4966667
Rac1	0.019066249	-1034.328333
Retnla	0.043272012	-10.215
Tgfb1	0.009808497	-459.92
Tlr8	0.007260952	-9.355
Tollip	0.022370573	-416.54

Supplemental Table 14. Inflammatory genes significantly up-regulated in MIA male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Bcl6	4.92493E-05	48.58166667
C1ra	0.026043722	6.55
Ccl4	0.000222395	47.79
Ccl5	3.23584E-05	137.82
Cebpb	0.000136427	1197.366667
Cxcl10	2.78515E-13	1869.075
Cxcl9	6.06393E-05	87.38
Daxx	9.92234E-10	1291.538333
Grb2	0.015151275	617.8966667
Hdac4	0.001243608	498.1866667
Hsh2d	1.294E-10	79.58333333
Ifi44	0.000867071	258.1933333
Ifit1	4.68501E-12	1104.11
Ifit2	3.06416E-05	739.365
Ifit3	4.32565E-15	2338.716667
Iigp1	1.93422E-20	1277.725
Il15	7.86613E-05	26.085
Il18rap	0.002163094	5.238333333
Il1r1	0.015684705	591.06
Il6	0.000384512	26.64166667
Irf1	1.39681E-13	590.73
Irf7	1.48353E-07	889.6433333
Jun	0.001181313	765.3483333
Limk1	0.00079421	1077.361667
Maff	0.000899847	523.2583333
Mafk	0.000723943	366.1666667
Map2k1	0.001468616	1773.581667
Map3k1	6.80898E-09	283.4466667

Mx1	0.007468486	230.75
Mx2	9.37471E-20	1072.621667
Myc	3.21305E-05	574.11
Myd88	1.828E-08	1282.4
Nod1	0.032530351	37.17833333
Nod2	8.57939E-10	124.4866667
Nos2	4.78772E-05	524.1266667
Nr3c1	0.0032139	241.0616667
Oas1a	2.36618E-05	2625.05
Oasl1	3.03153E-09	441.2716667
Ptk2	0.025180371	469.545
Stat1	9.2138E-09	2480.65
Stat2	8.56089E-09	2823.29
Stat3	8.97837E-06	1434.323333
Tlr3	5.9249E-06	97.89333333
Tlr5	0.037040533	60.605

Supplemental Table 15. Inflammatory genes significantly down-regulated in MIA female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Ccr2	4.51236E-05	-50.69833333
Cdc42	4.16829E-05	-2518.83
Cfl1	0.001907284	-2405.546667
Cxcr1	0.026250746	-19.37333333
Cxcr4	1.28666E-09	-105.0666667
Gpr44	5.37534E-05	-195.49
Hmgb1	0.000113252	-316.885
Hmgb2	0.040267232	-6.491666667
Hmgn1	0.006506691	-1159.956667
Il10rb	0.001170154	-358.7816667

Keap1	0.03801625	-71.37666667
Ly96	0.000453134	-123.9683333
Map2k4	0.015424441	-289.9033333
Map3k7	1.34679E-06	-396.7666667
Mapk14	3.25408E-05	-285.7333333
Mapk8	0.001910214	-157.0166667
Mef2a	0.001033832	-128.03
Mknk1	0.029765683	-38.68666667
Ppp1r12b	0.000588804	-17.47
Ptgfr	4.09749E-06	-42.45333333
Rac1	9.16008E-06	-1154.17
Rps6ka5	0.010006455	-25.52666667
Tgfbr1	0.000179131	-402.1816667
Tollip	7.45155E-06	-468.9783333
Tradd	0.026831407	-31.47

Supplemental Table 16. Inflammatory genes significantly up-regulated in MIA female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox12	0.003629169	21.16666667
Bcl6	0.000687227	64.91166667
C1ra	0.031070011	4.181666667
Ccl11	0.045540276	3.43
Ccl19	0.01878264	9.558333333
Ccl2	0.004767201	211.14
Ccl21a	0.014500722	10.62166667
Ccl3	0.006851076	88.76
Ccl4	4.56563E-06	79.74666667
Ccl5	0.000280704	199.8266667
Ccl7	0.015602657	38.92833333

Cd86	0.028374925	11.57666667
Cebpb	4.50835E-08	1828.663333
Csf1	2.69127E-06	578.1533333
Csf3	0.007866434	60.34166667
Cxcl1	0.03566611	143.4666667
Cxcl10	8.16294E-08	2797.3
Cxcl2	0.000320823	85.195
Cxcl9	0.000284436	91.69166667
Daxx	1.71012E-13	1066.87
Defa_rs1	0.009160059	6.001666667
Fos	5.09553E-05	1651.5
Grb2	0.01408914	407.4666667
Hif1a	0.026364669	984.345
Hsh2d	3.49472E-08	58.605
Ifi44	7.27311E-11	554.315
Ifit1	1.08477E-06	1725.543333
Ifit2	3.77589E-06	1447.66
Ifit3	1.77799E-11	3384.315
Ifnb1	0.035851348	5.513333333
Iigp1	2.90761E-11	1857.776667
Il10	0.038565405	0.38
Il15	0.002444093	26.215
Il18rap	0.015146386	7.27
Il1a	9.63652E-07	30.25333333
Il1rn	0.002115208	8.963333333
Il23a	0.001772555	71.49666667
Il23r	0.000578064	2.325
Il6	0.001860776	58.6
Irf1	1.12114E-05	952.8633333
Irf5	0.001296574	25.09666667
Irf7	2.06557E-07	882.9216667
Jun	1.59424E-08	1433.095

Limk1	0.000462344	1533.311667
Lta	0.004037424	2.671666667
Ltb4r1	0.006243247	9.195
Maff	0.000171343	900.065
Mafk	0.01019094	202.5233333
Map2k1	0.000355357	872.8083333
Map3k1	4.27795E-07	455.6716667
Mmp9	0.002644901	14.48
Mx1	5.79395E-06	481.7316667
Mx2	1.22494E-12	1181.58
Myc	0.000656724	428.0033333
Myd88	4.29751E-08	1026.875
Nfkb1	0.009908969	254.0583333
Nlrp3	0.032609536	6.735
Nod1	1.77147E-05	116.9733333
Nod2	3.87012E-13	128.31
Nos2	0.007556955	292.5333333
Oas1a	6.83764E-08	2171.593333
Oas2	0.002815711	94.85833333
Oasl1	7.27486E-09	771.2
Relb	0.019019242	198.8083333
Ripk2	0.005675255	46.44
Stat1	5.06974E-09	2517.636667
Stat2	8.65277E-09	2206.95
Stat3	2.75973E-05	1638.223333
Tlr2	0.000398621	32.04
Tlr3	7.8327E-06	84.40833333
Tlr7	0.000143085	13.16166667
Tnfaip3	0.008358732	110.4916667
Twist2	0.029954362	34.705

Supplemental Table 17. Inflammatory genes significantly down-regulated in GDM + MIA male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox5	0.009382917	-40.7
C1qa	0.02583044	-44.125
C8a	0.043156103	-2.525
Ccr2	4.96434E-06	-69.925
Ccr3	0.019303864	-4.325
Cd4	0.006978826	-2.748333333
Chi3l3	0.000233442	-45.94833333
Cxcr1	7.47959E-06	-34.23666667
Cxcr4	0.001819166	-55.275
Ddit3	0.008834223	-472.5216667
Elk1	0.018923354	-11.32
Gngt1	0.015863006	-18.86666667
Gpr44	0.035287198	-123.4816667
Il12b	0.011832861	-3.893333333
Il6ra	0.009179431	-781.9233333
Keap1	0.034227363	-77.96833333
Ltb4r1	0.000474657	-15.15833333
Ltb4r2	0.031927157	-4.535
Ly96	0.000442878	-186.6933333
Mapk14	0.009402756	-180.24
Nox1	0.001977326	-3.24
Pik3c2g	0.000755689	-1.78
Ppp1r12b	0.005513036	-16.04333333
Prkcb	0.003412472	-19.85666667
Ptgir	0.048415925	-5.196666667
Retnla	0.000373759	-15.80333333
Tbxa2r	6.05595E-06	-10.74833333
Tollip	0.000477353	-668.345

Tradd	0.004108641	-45.54333333
Trem2	0.038806055	-616.9483333
Tyrobp	0.018122399	-619.5733333

Supplemental Table 18. Inflammatory genes significantly up-regulated in GDM + MIA male placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Bcl6	3.63744E-05	54.5
C1ra	0.000238432	10.855
Ccl2	3.09502E-06	548.4266667
Ccl3	5.05631E-06	72.43333333
Ccl4	1.22171E-05	66.69333333
Ccl5	1.87953E-05	338.96
Ccl7	0.004807987	89.98166667
Cd86	0.049983817	11.22833333
Cebpb	2.08105E-06	1347.821667
Csf1	0.004412482	869.8666667
Csf3	4.20971E-08	74.21833333
Cxcl1	1.21847E-09	589.1683333
Cxcl10	1.42746E-09	3830.006667
Cxcl2	2.92157E-09	109
Cxcl3	0.017252275	21.08
Cxcl9	1.17372E-08	156.9933333
Cysltr1	0.013820893	18.50333333
Daxx	9.94678E-11	1556.9
Grb2	0.00363946	946.8583333
Hif1a	0.001636509	4285.185
Hsh2d	4.95459E-09	68.22166667
Hspb1	2.8863E-06	6599.301667

Ifi44	0.001850172	800.0316667
Ifit1	5.6215E-05	2462.636667
Ifit2	0.001212532	1793.168333
Ifit3	9.47698E-06	4089.02
Ifnb1	0.006433125	5.723333333
Iigp1	4.75649E-06	2180.101667
Il15	0.008892757	35.51833333
Il1r1	0.00260618	873.5316667
Il23a	0.006320893	73.015
Il6	0.0004182	71.05166667
Irf1	2.63843E-06	1176.798333
Irf7	8.93603E-07	986.505
Jun	8.39928E-09	1402.135
Limk1	0.048149749	651.13
Map2k1	2.88349E-06	2529.4
Map3k1	0.000289534	219.0866667
Mapkapk2	0.005794269	424.4483333
Mx1	0.024350915	352.35
Mx2	4.26271E-10	1248.198333
Myc	1.03332E-05	925.3633333
Myd88	3.01594E-07	1823.355
Nfkb1	0.001913947	424.7266667
Nod2	2.83017E-08	141.8533333
Nos2	0.00022415	421.7916667
Nr3c1	2.19463E-07	366.1683333
Oas1a	4.268E-06	2743.151667
Oasl1	0.000146218	948.275
Plcb1	0.016734861	12.81
Ptk2	0.025906364	488.845
Rela	0.00412249	179.6783333
Relb	1.34072E-05	529.145
Ripk1	0.029750362	80.19166667

Ripk2	1.38362E-08	65.975
Stat1	4.6516E-07	3307.063333
Stat2	1.69532E-08	2403.615
Stat3	8.23455E-08	1521.023333
Tcf4	0.021048757	689.32
Tgfb3	0.021186903	890.565
Tlr2	0.000177751	29.75
Tlr3	9.53174E-05	71.23833333
Tnf	5.53161E-08	58.045
Tnfaip3	0.014218011	109.585

Supplemental Table 19. Inflammatory genes significantly down-regulated in GDM + MIA female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Alox5	0.003532975	-53.17666667
Ccl17	0.017581283	-11.52333333
Ccr2	8.06551E-06	-69.265
Cd163	0.027758806	-3.298333333
Cd55	0.005511445	-1650.815
Chi3l3	0.000173896	-45.40666667
Cxcr1	0.001137625	-26
Cxcr4	2.67261E-07	-83.26
Gngt1	0.001902236	-22.365
Hc	0.003995914	-40.765
Hmgb1	0.045130659	-146.8016667
Il6ra	0.001627881	-766.1016667
Itgb2	0.000939113	-180.1716667
Ly96	6.95627E-07	-209.065
Mapk14	0.002091425	-197.2833333

Mapk3	1.76091E-06	-274.6433333
Ppp1r12b	0.008442819	-18.77
Prkcb	0.003312925	-12.29666667
Ptgfr	0.005900359	-36.83166667
Ptgir	0.020011913	-4.73
Retnla	0.003555347	-19.145
Tgfb1	0.025148466	-482.03
Tlr4	0.042272554	-308.44
Tollip	1.77951E-06	-619.595
Tradd	0.00267707	-60.4
Trem2	0.000234666	-868.6833333
Twist2	0.020610161	-25.87833333
Tyrobp	0.00159687	-750.8666667

Supplemental Table 20. Inflammatory genes significantly up-regulated in GDM +MIA female placentae compared to control (normal diet, saline-injected mice)

Gene Name	p value	Effect Estimate
Bcl6	0.000296618	79.42833333
Ccl11	0.003942223	0.768333333
Ccl19	2.49961E-05	11.61666667
Ccl2	1.47849E-05	422.4566667
Ccl20	6.60833E-05	10.46333333
Ccl21a	0.033885906	7.008333333
Ccl3	1.71476E-14	72.79666667
Ccl4	1.57615E-08	67.32333333
Ccl5	4.11082E-06	249.965
Ccl7	0.029596889	56.15333333
Ccr1	0.03131221	13.65166667
Cd86	3.18647E-05	19.62833333

Cebpb	3.09099E-08	2308.996667
Cfl1	0.00761819	4554.248333
Csf1	3.78088E-07	1026.656667
Csf3	0.001952187	63.56333333
Cxcl1	3.24085E-08	402.095
Cxcl10	3.14418E-09	3609.576667
Cxcl2	5.42194E-07	75.845
Cxcl3	0.00564542	29.68
Cxcl5	0.005009756	3.613333333
Cxcl9	4.30051E-07	166.4733333
Cxcr2	0.040361123	356.345
Daxx	3.419E-10	1616.273333
Fos	5.8073E-05	817.76
Gnas	0.026555136	1781.951667
Grb2	0.004143636	818.1983333
Hif1a	0.001850287	4110.808333
Hsh2d	1.29079E-08	77.145
Hspb1	1.22542E-06	6696.811667
Ifi44	6.61454E-05	688.625
Ifit1	8.13286E-07	2070.381667
Ifit2	5.47057E-05	1478.818333
Ifit3	9.51437E-08	3666.286667
Ifnb1	0.01834975	5.208333333
Iigp1	1.12885E-07	1918.485
Il15	0.002451438	28.93333333
Il1a	1.3243E-06	35.76333333
Il1r1	6.27158E-05	1030.191667
Il23a	0.012122437	44.78333333
Il6	1.69472E-05	56.21166667
Irf1	2.2915E-06	1047.678333
Irf7	8.73794E-10	1192.223333
Jun	9.67415E-07	1619.001667

Limk1	0.007448809	1141.67
Maff	0.000192269	580.3583333
Mafk	0.005232445	304.35
Map2k1	5.39551E-09	2474.553333
Map2k6	0.012109299	363.8366667
Map3k1	1.87912E-06	276.655
Mknk1	0.013391738	47.48
Mx1	0.000230181	406.7433333
Mx2	4.83749E-13	1173.906667
Myc	7.11576E-06	803.48
Myd88	5.40278E-08	1897.143333
Nfkb1	0.000691407	403.2116667
Nod2	1.23466E-09	127.3633333
Nos2	0.000102455	511.6316667
Nr3c1	0.004874238	288.035
Oas1a	5.86408E-07	2834.136667
Oasl1	4.08791E-06	769.0283333
Ptk2	0.010638704	453.0783333
Relb	8.05649E-06	424.87
Ripk2	0.001923797	52.2
Stat1	4.88281E-08	3156.975
Stat2	2.11636E-09	2645.03
Stat3	9.24011E-09	1509.233333
Tcf4	0.019953409	521.7816667
Tgfb3	0.011948836	1221.581667
Tlr2	1.65059E-07	38.30333333
Tlr3	0.000361793	95.76833333
Tnf	3.20358E-06	46.49666667
Traf2	0.004237746	84.115
Tslp	0.0122762	21.62833333

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Barke, T. L., Money, K. M., Du, L., Serezani, A., Gannon, M., Mirnics, K., & Aronoff, D. M.(2019). Sex modifies placental gene expression in response to metabolic and inflammatory stress. 78:1-9. *Placenta.* Doi: 10.1016/j.placenta.2019.02.008.

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