Maternal Obesity Induces a Pro-Inflammatory Uterine Immune Response Associated with Altered Utero-Placental Development and Adverse Fetal Outcomes

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"*You must remember that caring for your prenatal health is vitally important. This is how you take care of your baby! To give your baby the best possible place to grow and develop during these critical months, you must take care of yourself"*.
Dr. William Sears, 2013

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Statement of Contribution

The thesis, "Maternal Obesity Induces a Pro-Inflammatory Uterine Immune Response Associated with Altered Utero-Placental Development and Adverse Fetal Outcomes" is composed of three research chapters, all of which were collaborative efforts mainly with Dr. Sandeep Raha's and Alison Holloway's research groups.

Chapter Two "Characterization of immune cells and cytokine localization in rat utero-placental unit during mid-late gestation ", was a collaborative effort. Dr. Sandeep Raha's and Alison Holloway's laboratories housed and mated the rats used for this study. Tissue collection was a joint effort between their laboratories and me. I conducted all other experiments in this study with the guidance of Dr. Andrée Gruslin and Dr. Julien Yockell-Lelièvre. In addition, Dr. Chandrakant Tayade helped in the preparation of the manuscript.

Chapter Three, "Maternal obesity is associated with a systemic and uterine pro-inflammatory state in the rat" was performed as a collaborative effort. The animal model used in this study was developed, housed and mated in Dr. Sandeep Raha's and Alison Holloway's laboratories. Tissue and sample collection was a joint effort between their laboratories and me. Quantification of MCP-1 was performed by Dr. Raha's laboratory and was published in the "Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity" manuscript (Hayes et al., 2014). I conducted all other experiments in this chapter with the guidance of Dr. Andrée Gruslin and Dr. Julien Yockell-Lelièvre.

Chapter Four, "Trophoblast Invasion and Blood Vessel Remodeling Are Altered in a Rat Model of Lifelong Maternal Obesity" was a collaborative effort. In this study the same obese rat model and tissue samples were used as in Chapter Three. Trophoblast invasion and vascular remodeling analysis was a joint effort between Dr. James Petrik, Emily Hayes and me. All statistical analysis of these results was conducted by Dr. Alison Holloway. MMP quantification was performed by Dr. Raha's research group. All data presented in this chapter with the exception of the morphology and invasion assay were published in the "Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity" manuscript in which Emily Hayes and I are co-first authors (Hayes et al., 2014). Manuscript preparation was a joint effort between Dr. Raha, Emily Hayes and me. Additional data includes the morphometric analysis of the rat utero-placental unit and human *in vitro* trophoblast invasion assay. I performed the morphometric analysis. Human trophoblast invasion assays utilized human serum samples from The Ottawa and Kingston Birth Cohort (Walker et al., 2011) and invasion assay was carried by me.

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Finally a special thank you to the Ottawa Hospital Research Institute for their financial support which has allowed me to complete my graduate studies.

Dedicated to the memory of:

Dr. Andrée Gruslin (1965-2014), Doctor, Scientist, Supervisor, Mentor and all around amazing person. I would like to dedicate this thesis to the loving memory of Dr. Andrée Gruslin. Her enthusiasm for life and science serves as an inspiration to all who have had the honour of working with her.

Abbreviations

Analysis of variance	ANOVA
Average for gestational age	AGA
Bovine serum albumin	BSA
Cluster of differentiation 68	CD68
Control Diet	CD
Decidua Basalis	Dec
Endovascular trophoblast	ENVT
Extravillous trophoblast	EVT
Fetal growth restriction	FGR
Free fatty acids	FFAs
Gestational age/day	GD
Gestational diabetes mellitus	GDM
High-fat Diet	HFD
Immunohistochemistry	IHC
Inflammatory bowel diseases	IBD
Interferon gamma	IFNγ
Interleukin 1 beta	IL-1β
Interleukin 10	IL-10
Interleukin 6	IL-6
Interstitial trophoblast	IST
Junctional zone	JZ
Labyrinth	Lab
Lipopolysaccharides	LPS
Matrix metalloproteinase	MMP
Monocyte chemoattractant protein-1	MCP-1
Myeloperoxidase	MPO
Phosphate buffered saline	PBS
Pre-eclampsia	PE
Smooth muscle actin	SMA
Tumor necrosis factor alpha	TNFα
Uterine/decidual natural killer	uNK/dNK

Abstract

Obese pregnant women have increased risk of a number of pregnancy complications, including poor maternal health, fetal growth restriction (FGR) and fetal demise. The success of pregnancy is dependent on precise regulation of the immune response within the utero-placental environment. Rats as a model for human related pregnancy complications are beginning to be widely used because of the similarities between these species in terms of trophoblast invasion and spiral artery remodeling. However our knowledge of immune cells and cytokine localization in the rat utero-placental tissue relating to these processes is limited. Therefore our first aim was to characterize the immune cell populations, such as uterine natural killer (uNK) cells, neutrophils and macrophages in the rat utero-placental unit at two crucial gestational ages relevant to trophoblast invasion and spiral artery remodeling, gestational day (GD) 15 and GD18. In addition, we characterized the cytokine distribution of TNFα, IFNγ and IL-10 in the utero-placental tissue at both above mentioned gestational ages. Our study has demonstrated co-localization of TNFα and IFNγ with uNK cells in the perivascular region of the spiral arteries in the rat mesometrial triangle. Neutrophils were localized at the maternal fetal interface and in the spiral artery lumen of the rat mesometrial triangle at both gestational ages. TNFα and IL-10 demonstrated a temporal change in the localization from GD15 to GD18 which coincides with the leading edge of trophoblast invasion into the mesometrial triangle. The results of the current study furthers our knowledge of the localization and temporal expression of uterine immune cells and relevant cytokines, and provides a base to research the function of these immune cells and cytokines during rat pregnancy as a model to study human pregnancy and complications related to immune functions.

Since obesity is associated with a peripheral and systemic pro-inflammatory state in humans, our second objective was to investigate whether maternal obesity could alter the utero-placental and systemic immune response in the rats. To characterize maternal obesity induced changes in uterine immune state we used pregnant rats fed a control diet (normal weight; CD) or a high fat diet (obese; HFD) at GD15 and GD18. We performed immunohistochemistry to localize TNFα and IL-10, and quantified the levels of TNFα, IL-1β and IL-10 in the uterine tissue by immunoassay. To assess the systemic immune state, circulating levels of pro-inflammatory cytokine MCP-1 were assessed by immunoassay. We demonstrated an increased concentration of the pro-inflammatory marker TNFα and a reduced anti-inflammatory IL-10-positive cell distribution in the rat mesometrial triangle in response to a HFD. In addition increased circulating MCP-1 was observed in the HFD-fed dams at both gestation ages. HFD induced obesity in our rat model leads to an increase in uterine and systemic pro-inflammatory markers. These markers have demonstrated the potential to alter utero-placental development.

Pregnancy complications such as FGR and fetal demise have been shown to be associated with impaired placental development as a result of altered trophoblast invasion and aberrant maternal spiral artery remodeling. Therefore, our third aim was to compare these parameters between the CD-fed rats and HFD-fed rats at GD15 and GD18. Early trophoblast invasion was increased by approximately 2-fold in HFD-fed dams with a concomitant increase in the expression of matrix metalloproteinase-9 protein, a mediator of tissue remodeling and invasion. By late gestation reduced trophoblast invasion was observed in HFD-fed dams. Furthermore, we also observed in late gestation significantly

higher levels of smooth muscle actin surrounding the uterine spiral arteries of HFD-fed dams, suggesting impaired spiral artery remodeling. We also determined the impact of human serum from obese mothers on trophoblast invasion. We compared the invasion of HTR-8/SVneo cells treated with pooled first-trimester serum from obese women with or without fetal growth restriction vs. cells treated with serum from normal-weight women with or without fetal growth restriction. First-trimester serum from obese pregnant women reduced invasion of the trophoblast cell line HTR8/SVneo compared to serum from normal-weight pregnant women. Taken together, the results of this study suggest that maternal obesity can negatively influence crucial utero-placental development processes resulting in the poor pregnancy outcomes and increased fetal demise.

To summarize, the HFD increased the pro-inflammatory marker TNFα which was associated with altered trophoblast invasion profiles and impaired vascular remodeling. These disturbances in utero-placental development were also associated with decreased birth weights (indication of FGR) and increased rates of stillbirths in our obese rat model.

In conclusion, we have made progress in defining the influence of maternal obesity (HFD) on utero-placental development. The importance of these studies is evident since FGR represents a leading cause of perinatal morbidity and mortality. Furthermore, FGR fetuses have an increased risk of becoming obese in their lifetime as a result of fetal programming, therefore resulting in the propagation of a transgenerational obesity cycle. Therefore by understanding the mechanisms by which maternal obesity influences utero-placental development leading to FGR, we may be able to impact short term morbidity and prevent the programming of obesity in future generations. In addition, characterization of maternal obesity's influence on utero-placental development will also help in the search for therapeutics or intervention strategies to help optimize fetal growth and improve pregnancy outcomes in obese women.

Resumé

Les femmes obèses courent un risque accru de subir plusieurs complications de grossesse, incluant des problèmes de santé maternelle, retard de croissance intra-utérin (RCIU) et décès fœtal. Le succès d'une grossesse dépend de la régulation précise de réponses immunitaires dans l'environnement utéro-placentaire. Les rats, en tant que modèle pour l'étude des complications reliées à la grossesse, sont de plus en plus utilisés grâce aux similarités aux humains en terme d'invasion trophoblastique et remodelage des artères spirales utérines. Par contre, les connaissances quant à l'emplacement des cellules immunitaires et cytokines dans l'unité utéro-placentaire chez le rat sont limitées. Pour cette raison, notre premier objectif fut de caractériser la population de cellules immunitaires comme, par exemple, les cellules tueuses naturelles utérines (uNK), les neutrophiles et les macrophages de l'unité utéro-placentaire à deux âges gestationnels cruciaux reliés à l'invasion trophoblastique et au remodelage des artères spirales, jour gestationnel (JG) 15 et JG 18. De plus, nous avons caractérisé la distribution des cytokines TNFα, IFNγ et IL-10 dans le tissu utéro-placentaire aux âges gestationnels mentionnés ci-dessus. Notre étude a démontré une co-localisation de TNFα et IFNγ avec les cellules uNK dans une distribution peri-vasculaire du triangle mésometrial chez le rat. Les neutrophiles étaient situés au niveau de l'interface maternel-fœtal et dans la lumière des artères spirales du triangle mésometrial chez le rat aux deux âges gestationnels. TNFα et IL-10 ont subit un changement temporel dans leur localisation entre le JG 15 et 18, ce qui coïncide avec l'origine d'invasion des trophoblastes dans le triangle mésometrial. Les résultats de cette étude approfondissent notre connaissance de la localisation et de l'expression temporelle de cellules immunitaires utérines et cytokines pertinentes, et fournissent une base pour l'étude de la fonction des cellules immunitaires et cytokines durant la grossesse murine en tant que modèle pour l'étude de la grossesse humaine et des complications reliées à la fonction immunitaire.

Puisque l'obésité est associée avec un état périphérique et systémique pro-inflammatoire chez les humains, notre deuxième objectif fut d'examiner si l'obésité maternelle pouvait modifier la réponse immunitaire utéro-placentaire chez le rat. Pour caractériser les changements de l'état immunitaire utérine induis par l'obésité maternelle, nous avons utilisé des rates en grossesse nourries par une diète contrôle (poids normal ; *control diet* [CD]) ou une diète riche en gras (obèse ; *high fat diet* [HFD]). Nous avons effectué des expériences d'immunohistochimie pour localiser TNFα et IL-10 et quantifier les niveaux de TNFα, IL-1β et IL-10 dans le tissu utérin par dosage immuno-enzymatique. Pour déterminer l'état immunitaire systémique, on a quantifié le niveau de la protéine chimio-attractive des monocytes-1 (MCP-1) en circulation sanguine par dosage immuno-enzymatique. Nous avons démontré une augmentation de la concentration du marqueur pro-inflammatoire TNFα et une réduction de la distribution de cellules positive au marqueur anti-inflammatoire IL-10 dans le triangle mésométrial murin suite à la diète riche en gras. De plus, une augmentation de MCP-1 en circulation a été démontrée suite à la diète riche en gras au deux jour gestationnels. L'obésité induite par la diète riche en gras dans le modèle murin a mené à une augmentation utérin et systémique de marqueur pro-inflammatoire. De plus, ces marqueurs pro-inflammatoire on le potentiel d'altérer le développement utéro-placentaire.

Les complications de grossesse telles que le RCIU et le décès fœtal sont associées au développement placentaire déficient dû à des modifications de l'invasion

trophoblastique et du remodelage des artères spirales utérines. Par conséquent, notre troisième objectif fut d'examiner ces paramètres chez des rates nourries par une diète contrôle (CD) ou une diète riche en gras (HFD) aux JG 15 et JG 18. L'invasion trophoblastique précoce fut approximativement deux fois plus élevée chez les rates nourries par HFD. Davantage, une augmentation concomitante dans l'expression de protéines metalloproteinase-9 matricielles (MMP-9), un médiateur d'invasion et remodelage, a été détectée. Vers la fin de la gestation, une diminution d'invasion trophoblastique fut observée chez les rates nourries par HFD. De plus, des niveaux considérablement plus hauts d'actine dans les muscles lisse entourant les artères spirales placentaires chez les rates nourries par HFD ce qui suggère un remodelage d'artères spirales incomplet. De plus, nous avons aussi déterminé l'impact du sérum humain sur l'invasion trophoblastique. Nous avons comparé l'invasion des cellules HTR-8/SVneo traitées avec du sérum du premier trimestre provenant de femmes obèses avec ou sans retard de croissance fœtale comparé à des cellules traitées avec du sérum provenant de femmes de poids normal avec ou sans retard de croissance fœtale. Le sérum du premier trimestre provenant de femmes enceintes obèses a causé une diminution de l'invasion de la lignée cellulaire trophoblastique HTR-8/SVneo comparé au sérum provenant de femmes enceintes de poids normal. Mis ensembles, ces résultats suggèrent que l'obésité maternelle peut avoir une influence négative sur des processus de développement utero-placentaire menant à des issues de grossesse défavorables et à l'augmentation du décès fœtal.

Pour résumer, la diète riche en gras a causé une augmentation du marqueur pro-inflammatoire TNFα ce qui fut associé à une modification de l'invasion trophoblastique et à une détérioration du remodelage vasculaire. Ces perturbations du développement utéro-placentaire furent aussi associées avec une diminution du poids à la naissance (un marqueur de RCIU) et une augmentation de mortinaissances dans notre modèle murin d'obésité.

En conclusion, nous avons fait du progrès dans la définition de l'influence de l'obésité maternelle sur le développement utéro-placentaire. L'importance de ces études est mise en évidence par le fait que le RCIU est une des causes principales de morbidité et mortalité périnatale. De plus, le RCIU est lié au développement de maladies relié à l'obésité chez l'adulte et peut avoir des effets trans-générationnels, ainsi affectant des générations futures. De ce fait, en déchiffrant le mécanisme par lequel l'obésité maternelle affecte le développement utéro-placentaire et mène au RCIU, nous pourrions influencer la morbidité à court-terme et prévenir la programmation de l'obésité chez les générations futures. Davantage, la caractérisation de l'influence de l'obésité maternelle sur le développement utéro-placentaire peut aussi servir au progrès dans la recherche pour des interventions thérapeutiques pour optimiser la croissance fœtale et améliorer les issues de grossesse chez les femmes obèses.

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Chapter 1

Introduction

Obesity is increasing in prevalence in industrialized countries. The Canadian Community Health Survey carried out by Statistics Canada in 1978/79 reported 15.7% of women were obese whereas by 2007-2009 24% of women were obese. This represents an astonishing 8.3% increase in obesity over 30 years. Obesity is defined as excess accumulation of fat mass in which negative effects on health are associated, including coronary heart disease, type 2 diabetes mellitus and pulmonary dysfunction (Arendas et al., 2008). Currently the most commonly used measurement to characterize an individual weight category is body mass index (BMI). BMI is calculated as such: body mass (kilograms) divided by the square of their height (meters). Normal BMI is between 18.5 and 24.9 kg/m^2, whereas, an obese individual has a BMI greater than 30.0 kg/m^2 (categorized by Health Canada in 2003, http://www.hc-sc.gc.ca/fn-an/nutrition/weights-poids/guide-ld-adult/bmi_chart-graph_imc-eng.php). With the increased prevalence of obese women in reproductive-age, an increase in obesity related reproductive complications are also observed. Indeed, obese women have higher risks of infertility or of developing complications during pregnancy such as gestational diabetes mellitus (GDM), pre-eclampsia (PE) (characterized by hypertension and end organ damage) (Arendas et al., 2008) (Figure 1.1). Fetal development is also affected by maternal obesity and fetal complications associated with maternal obesity include macrosomia, fetal growth restriction (FGR) and fetal death (Figure 1.1) (Radulescu et al., 2013). Although many obese women will deliver a large infant (macrosomia) a subset will deliver fetuses with growth restriction. FGR is mainly defined as a failure of the fetus to

reach their inherent growth potential mainly as a result of inadequate nutrient supply. These fetuses have an estimated weight below the 10^{th} percentile for their gestational age and have an abdominal circumference below the 2.5^{th} percentile (Peleg et al., 1998). FGR are also classified as symmetric or asymmetric. Symmetric growth restriction, classify fetuses in which the entire body is proportionately small while asymmetric growth restriction classify fetuses with disproportionate body composition as a result of being undernourished from placental insufficiency (Peleg et al., 1998). This disproportionate body composition occurs since energy restrictions force allocation of energy to vital organs such as the brain and heart while neglecting other organs such as liver, muscle and fat. This causes normal growth of the head but results in reduced abdominal size and scrawny limbs. The importance to studying FGR is evident since this group of fetuses/newborns appears to carry the most significant short and long term health complications. Short term complications include an increased risk of intrauterine fetal death, perinatal asphyxia, prematurity and stillbirth (de Bie et al., 2010). The long term consequences include poor neonatal health, increased risk of adult onset diseases such as metabolic syndrome and cardiovascular disease, systolic hypertension, obesity, insulin resistance, and diabetes type II (Boutsikou et al., 2010; Salam et al., 2014).

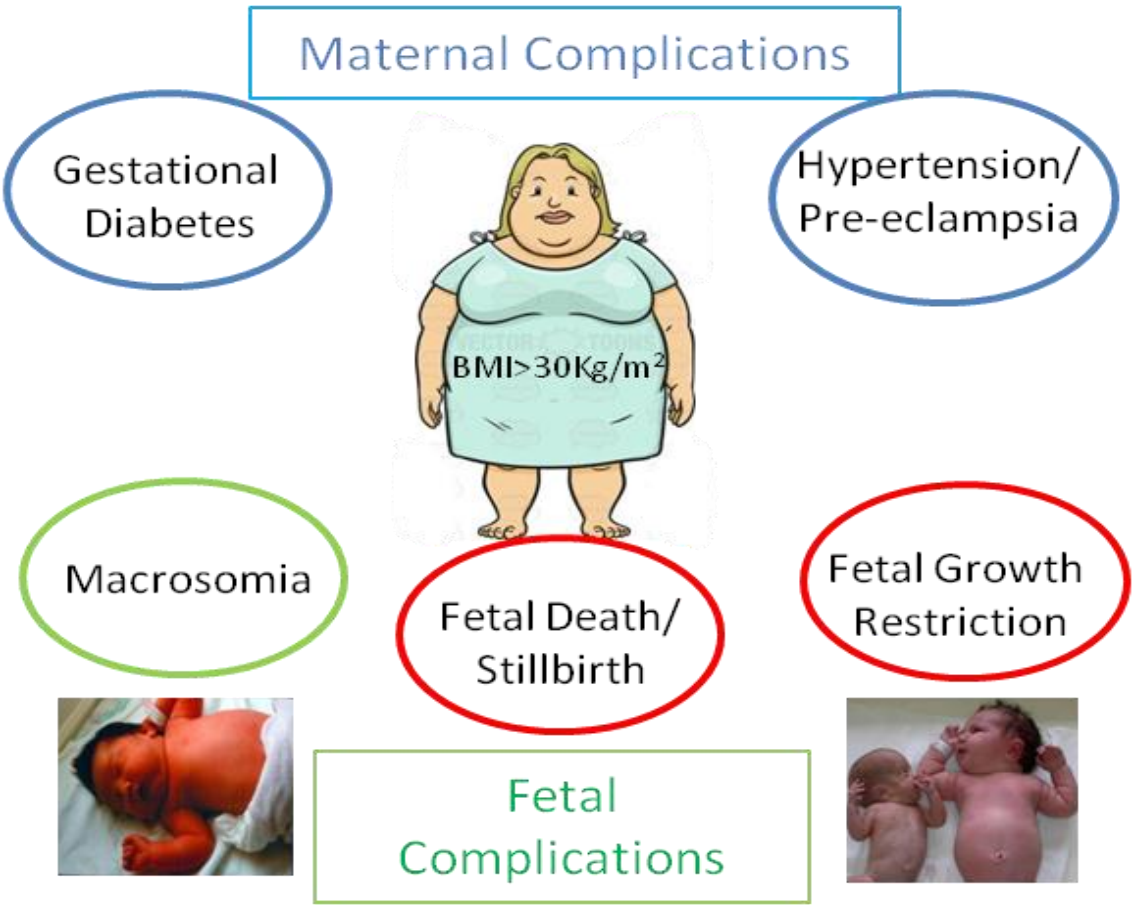

Figure 1.1 Maternal obesity associated pregnancy complications.

Background and Literature Review

In humans and in rodents, utero-placental development is characterized by establishment of the maternal-fetal interface to sustain fetal growth. Utero-placental development is crucial for adequate gas, nutrient and waste exchange between the mother and fetus. The maternal intrauterine immune response plays a vital role, in protecting the fetus against potential pathogens, in the tolerance of semi-allogeneic fetal derived trophoblast cells and in regulating utero-placental developmental processes (Rossant & Cross, 2001). To date, alterations in placental morphology, blood flow, oxygen delivery and nutrient transport have been characterized in pregnancies complicated by FGR (Higgins et al., 2011). However there has been little attention paid to the possible influence of maternal obesity on the uterine immune response and utero-placental development leading to adverse pregnancy outcomes such as FGR and stillbirths. Therefore we have focused our research on characterizing the influence of maternal obesity on the systemic and uterine immune state and on utero-placental development/morphology possibly leading to FGR. To achieve this we used a lifelong obesity rat model that mimics similar adverse pregnancy outcomes such as FGR and increased rates of stillbirths observed in obese women.

Human and Rat Pregnancy

Human pregnancy is defined by three distinct stages referred to as trimesters. In the first trimester, the following processes occur: fertilization, blastocyst formation, blastocyst implantation into the maternal endometrium, migration/invasion of trophoblast cells into the *decidua*, and early placental formation (Figure 1.2) (Benirschke et al., 2006). In the second and third trimesters an expansion and maturation of the placenta occurs to accommodate fetal demands/growth as well as to facilitate maintenance of pregnancy.

In humans implantation occurs approximately eight days after fertilization while in rats this occurs approximately on gestational day 4.5 (GD4.5) (Watson & Cross, 2005). One of the important steps during implantation is trophoblast cell invasion of the maternal endometrium (humans)/*decidua* (rats). In humans the endometrium is a thickened layer of stromal cells lining the uterus which forms a few days after ovulation and sheds if fertilization/implantation is unsuccessful (menstruation). In rats the formation of the *decidua* is referred to as decidualization and results in increased vascularisation of the uterine tissue. If fertilization is successful, the blastocyst will implant into the endometrium/*decidua*.

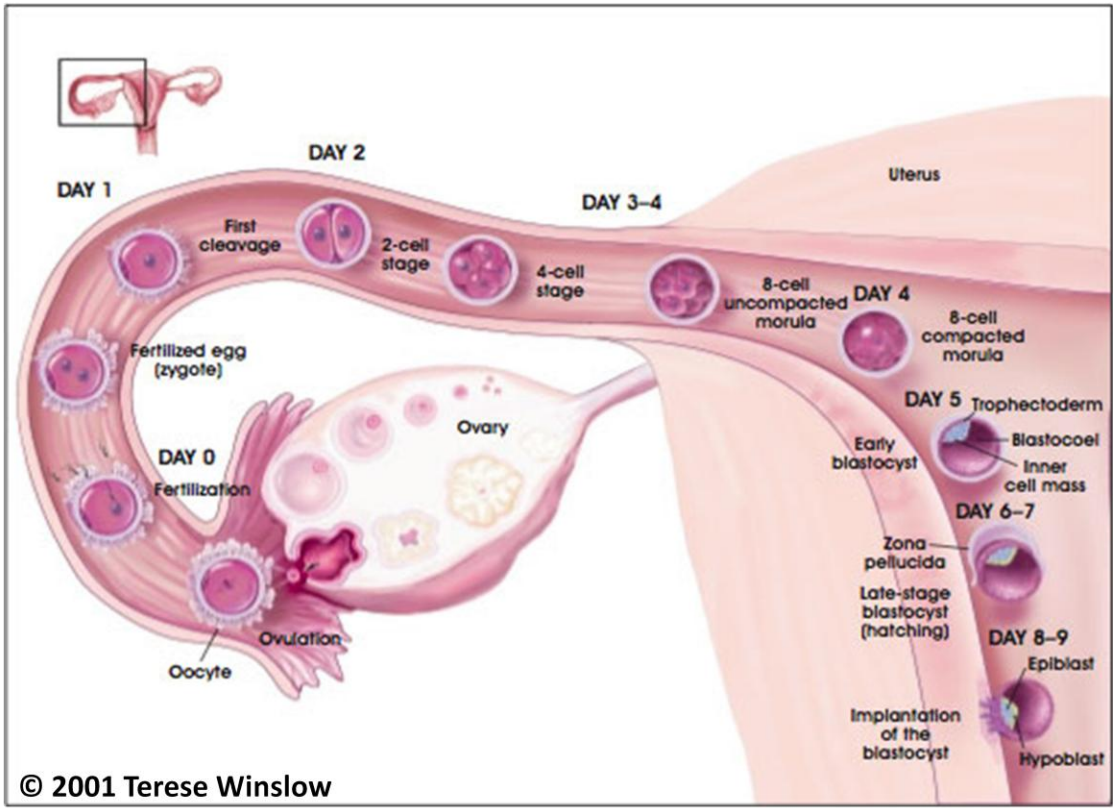

Figure 1.2 Development of the Preimplantation Blastocyst in Humans (http://stemcells.nih.gov/info/scireport/pages/appendixA.aspx)

In healthy pregnancies, the establishment of the placenta is a result of zygote-derived embryonic cells termed trophoblast cells which migrate and invade the maternal endometrium in order to form the proper maternal-fetal interface. Subsets of these trophoblast cells make up the placenta such as cytotrophoblast and syncytiotrophoblast whereas other lineages of trophoblast further invade into the uterine myometrium such as endovascular trophoblast (ENVT) and interstitial trophoblast (IST) (Benirschke et al., 2006). There are two main functions to this invasion. The first function is that trophoblast cells interact with the resident uterine immune population which includes natural killer (NK) cells, macrophages and T lymphocytes (Red-Horse et al., 2006). This interaction is believed to be responsible for the development of the decidual lymphatic vessels (Red-Horse et al., 2006). The development of a decidual lymphatic circulation is believed to play an important role in maintaining fluid balance during pregnancy and facilitates maternal immune cell trafficking (Red-Horse et al., 2006). The second function of invasive trophoblast cells is to participate in the degradation of vascular structural cells that control contraction of maternal uterine arteries. This degradation allows dilation of the vessels which results in increased blood supply to the placenta (Duc-Goiran et al., 1999). In rats trophoblast invasion of the *decidua* begins with the blastocyst trophoblast cells forming an ectoplacental cone which is well established by gestational day 8 (Figure 1.3) (Furukawa et al., 2011; Soares et al., 2012a; Watson & Cross, 2005). The ectoplacental cone contains giant trophoblast cells and glycogen cells at its periphery. These cells are responsible for decidual invasion. In humans, invasion is carried out by invading extravillous trophoblast cells which can reach up to the inner third of the maternal myometrium (Duc-Goiran et al., 1999).

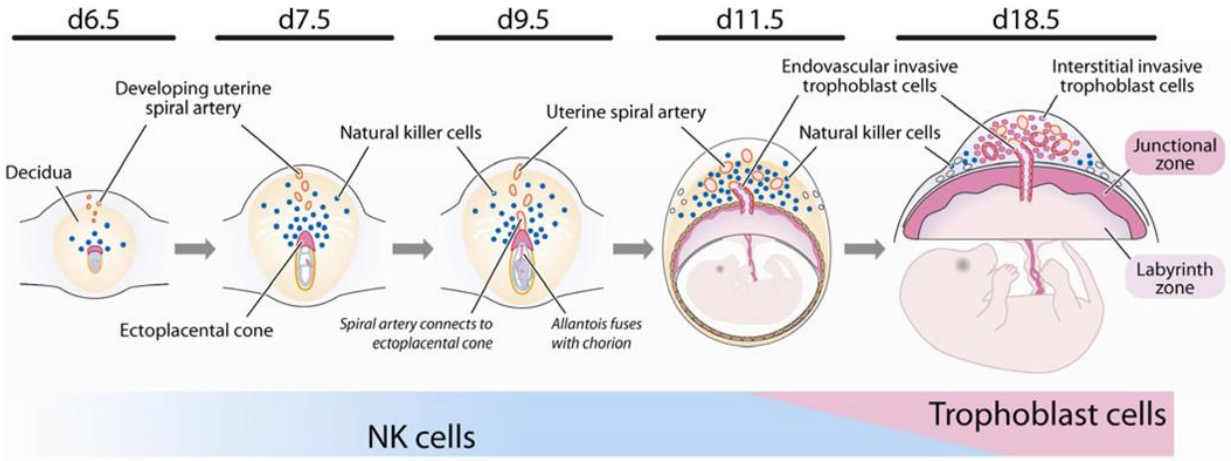

Figure 1.3 Schematic diagram of rat placentation (Soares et al., 2012a).

In humans the majority of the developmental processes in regards to the establishment of the placenta occur in the first trimester. In rats similar processes are observed however these occur at mid to late gestation (Pijnenborg & Vercruysse, 2006a). The development of the placenta and the interface of the placenta with uterine tissue can be visualized in cross sections as three distinct regions in humans and in rats (Tycko & Efstratiadis, 2002). These regions appear as early as 9.5 day post coitum in rats and 4 weeks after fertilization in humans and remain distinct until term. In rats/mice the first region, starting closest to the fetal side, is the chorioallantoic connective tissue which extends into the labyrinth zone (Figure 1.3 and 1.4) (Rossant & Cross, 2001; Soares, et al., 2012a). The placenta in both humans and rats is composed of differentiated and undifferentiated trophoblastic cells, mesenchymal cells and endothelial cells. In humans the placental structures resemble tree like protrusions (villi) which are perfused by maternal blood (Benirschke et al., 2006). In rats the placenta is structured in a much more intricate network of maternal blood sinuses and fetal blood channels and is referred to as the labyrinth. The fetal blood channel structure is the placental barrier separating maternal and fetal blood. In both species the placental barrier is the primary site of all nutrients/waste exchange (Georgiades et al., 2002). Interestingly, maternal and fetal circulations never come in direct contact with each other. This type of placental barrier in humans and rats is referred to as hemochorial. More specifically it is hemomonochorial (one layer of cells separating maternal and fetal circulations) in humans whereas in rats it is hemotrichorial (three cell layers separating maternal and fetal circulations) (Benirschke et al., 2006). The villi (human) are lined by differentiated multinucleated trophoblast cells called the syncytiotrophoblast facing the maternal circulation. Adjacent to the

syncytiotrophoblast cell layer are cytotrophoblast cells. These are stem-like cells and are the source of the syncytiotrophoblast cell layer as well as other trophoblast cell populations. Within the villi are the fetal connective tissues and the fetal vascular tissue. In the rat the hemotrichorial barrier is composed of three syncytiotrophoblast cells layers with the cytotrophoblasts facing maternal circulation. The main function of the hemochorial barrier is to facilitate exchanges between mother and fetus. With its large surface area it is ideal for exchange of gases, nutrients and waste.

The second region of the utero-placental structure is characterized as the basal plate in humans and the junctional zone (JZ) in mice/rats. In this region maternal blood channels are surrounded by zygote-derived cells. In rats, the JZ is composed of the trophospongium (also known as spongiotrophoblast) and junctional zone glycogen trophoblast cells. Passing through the JZ are the maternal blood vessels. The maternal blood flow enters by the maternal spiral arteries and returns through the junctional zone venous channels. In humans, the basal plate contains the cytotrophoblast cell columns, which are the source of endovascular trophoblast and interstitial trophoblast invasive cells. Similarly in humans, the maternal blood flow passes through the basal plate entering the placenta through the maternal spiral arteries and returns through the basal plate venous channels. One of the primary functions of the JZ/basal plate is to provide a source of undifferentiated invasive trophoblast cells. In rats, immediately following the JZ, are the giant trophoblast cells. In humans and rats the first and second regions combined make up the placenta. The third distinct cross sectional region in humans and rats is the maternal uterine tissue (Figure 1.4). In humans it is comprised of the endometrium and the myometrium and in rats it is composed of the *decidua*

basalis/mesometrial triangle in which the *decidua* is in direct contact with the JZ. There are many similarities between humans and rats in regards to uterine modifications in order to facilitate pregnancy. For example in both species the uterine tissue is primarily composed of maternal connective tissue, vascular structural cells and resident immune cells such as uterine natural killer cells, macrophages and other immune cells. These maternal derived immune cells are believed to regulate the trophoblast migration/invasion which occurs in late first trimester and early second trimester in humans and mid-late gestation in rats. The uterine tissue is also vascularised by maternal spiral arteries and veins in both humans and rats. In late first trimester in humans and in late gestation in rats extensive uterine spiral artery remodeling occurs (Caluwaerts et al., 2005; Pijnenborg, et al., 2006b). Arterial remodeling is essential to ensure sufficient dilatation of the maternal spiral arteries and to increase blood perfusion of the placenta. This allows optimal blood supply to facilitate nutrient/waste exchange between maternal and fetal circulations. As described above there are many similarities between rats and humans in terms of trophoblast invasion and spiral artery remodeling during pregnancy. Since my research was focused on the influence of maternal obesity on pregnancy induced uterine modifications, the rat was therefore an ideal model to study human related pregnancy complications.

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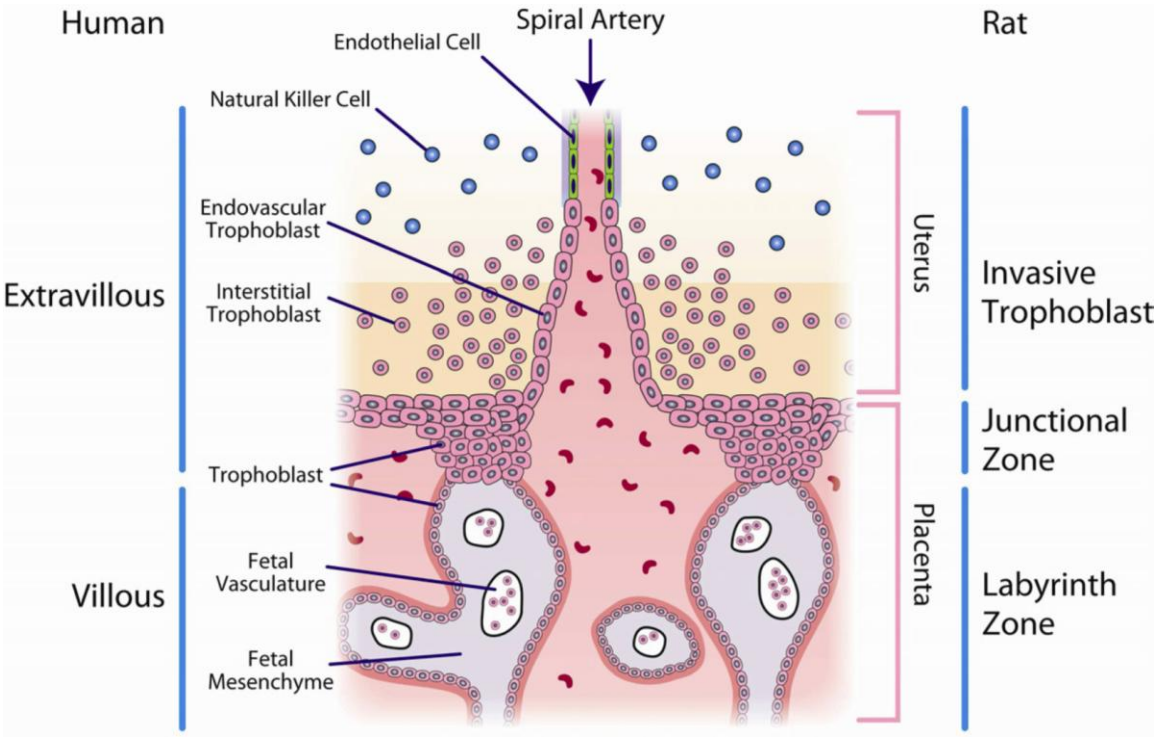

Figure 1.4 Comparative physiology diagram of the fully developed human and rat utero-placental units (Soares et al., 2012a).

Spiral artery remodeling

The blood supply to the placenta originates from the arcuate artery in the myometrium which branches into the radial arteries and finally into the spiral arteries as they enter into the endometrium (Whitley & Cartwright, 2010). As the vessels progress through the myometrium and into the endometrium the vessel diameters successively decrease and as the name suggests spiral arteries are coiled. The uterine spiral arteries are composed of a single layer of endothelial cells lining the lumen of the artery and a perivascular distribution of vascular smooth muscle cells (VSMCs), both of which are responsible for vessel integrity and vasomotor control (Pijnenborg et al., 2006b). These structural cells of the spiral arteries undergo remodeling which results in transformation of the spiral arteries from high resistance and low flow vessels to large dilated vessels capable of delivering increased blood supply to meet fetal demands. Uterine spiral artery remodeling consists of disorganization and clearance of the VSMC layer and clearance of the endothelial cells lining the walls of these arteries. Localized uterine immune cells and fetal derived trophoblast cells participate in these remodeling events. The main contributors to such events are the uterine/decidual natural killer cells (uNK) (Bulmer et al., 2012; Croy et al., 2012; Robson et al., 2012) and invasive extravillous trophoblast cells (EVTs) (Cross et al., 2002; Red-Horse et al., 2006) (Figure 1.5).

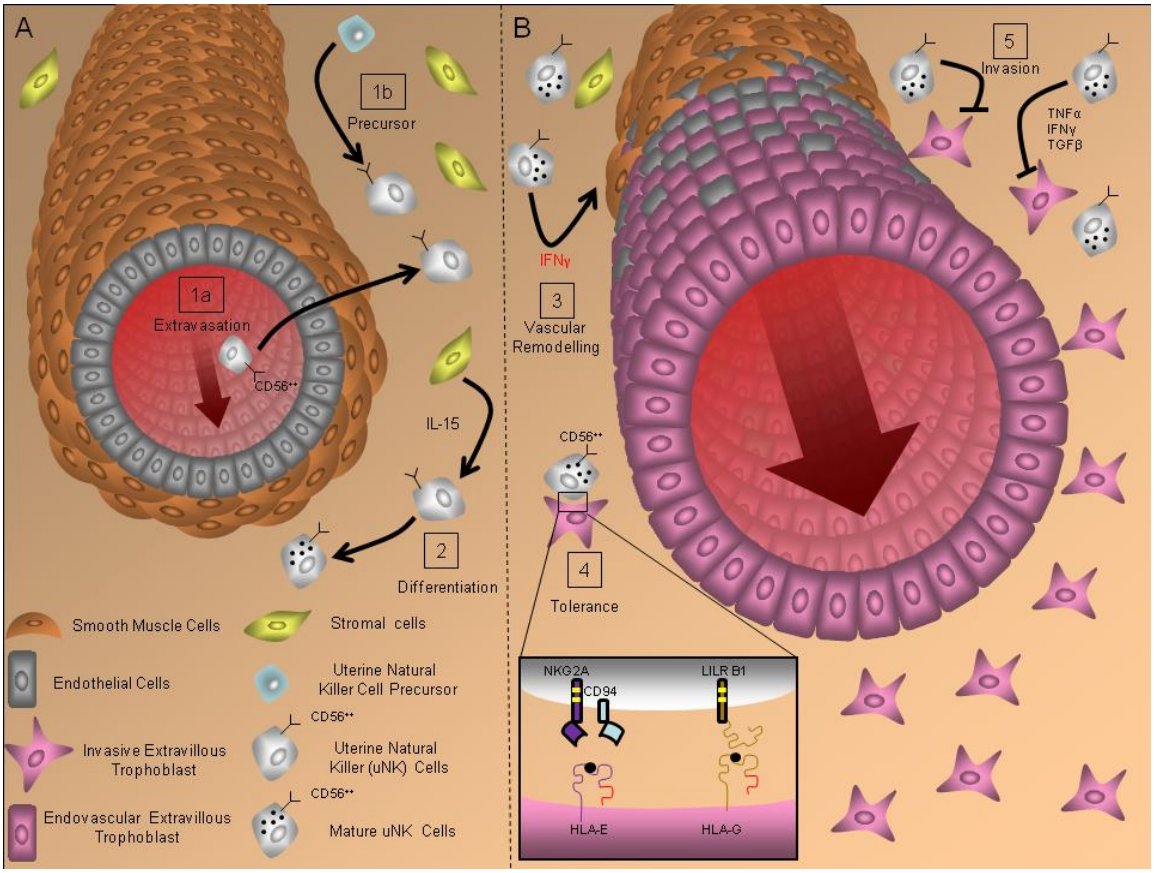

Figure 1.5 Schematic representation of human spiral artery remodeling in the *decidua basalis* and myometrium. (A) Establishment and maturation of uterine natural killer (uNK) cell population in the *decidua*/myometrium in early pregnancy: (1a) uNK cell extravasation and/or (1b) precursor NK cells (in mice) and (2) differentiation of uNK cells. (B) Spiral artery remodeling and trophoblast tolerance in the first trimester of pregnancy: (3) uNK induced spiral artery remodeling, (4) uNK interactions with extravillous trophoblasts (EVTs) perhaps for tolerance promotion and (5) uNK cell inhibition of EVT invasion. The large red intraluminal arrow indicates direction of blood flow. Small black arrows indicate molecules that trigger cell activation and the black 'line with the T termination' indicates inhibitory signaling (Tessier et al., 2014).

Uterine NK cells are the dominant immune cell population making up approximately 70% of total leukocytes ($CD45^+$ cells) within the uterine tissue (King et al., 1998). Their abundance in the *decidua*/mesometrium in early pregnancy suggests that vascular remodeling and placentation are in part regulated by the innate immune system. In humans, uNK cell populations are maintained during early to mid pregnancy (Bulmer & Lash, 2005). Activated uNK cells are mainly distributed in a perivascular pattern in humans and rodents. Their presence and accumulation coincide with the initiation and early stages of the arterial remodeling process (Croy et al., 2012; Smith et al., 2009). In humans, once vascular development nears completion, uNK cell number begins to decline (Bulmer & Lash, 2005; Delgado et al., 1996). This decline continues until low levels or no uNK cells are detected at term (Moffett-King, 2002). Similarly in rats as the end of gestation approaches a decline in the perivascular distribution of uNK is observed (Ain et al., 2003).

The current evidence show that initiation of the spiral artery remodeling process is carried out primarily by uNK cells, while the EVTs appear in the later stages of spiral artery remodeling and contribute to the completion of this process (Adamson et al., 2002; Charalambous et al., 2012; Craven et al., 1998). The exact function of uNK cells in arterial remodeling is not fully understood, however, they appear to be involved in the degradation/clearance of the maternal uterine spiral arterial smooth muscle cell layer and endothelial cells and in the regulation of trophoblast invasion (Figure 1.5). In mice interferon gamma (IFNγ) alone was shown to initiate arterial remodeling events (Ashkar et al., 2000). Since 90% of the IFNγ present in mouse *decidua basalis* is produced by uNK cells, this suggests that IFNγ production by uNK cells is a key regulator of uterine

arterial remodeling events. *In vitro* studies suggest that the remodeling of both VSMC and endothelial cells by uNK cells is an apoptotic process mediated by the Fas signalling pathway (Fraser et al., 2012). However others have demonstrated that this process does not involve the apoptosis of the VSMC but rather the disorganization of the VSMC layer, as well as rounding and migration of VSMCs away from the vessel lumen and toward the decidual stroma (Bulmer et al., 2012).

As initial stages of arterial remodeling occur, EVTs begin to migrate/invade replacing the degraded maternal smooth muscle cell layers and endothelial cells lining the spiral arterial walls (De Wolf, De Wolf-Peeters, Brosens, & Robertson, 1980) (Figure 1.6). This causes a loss of vasomotor control leaving dilated non-contractile tubes lined by trophoblast cells (Kaufmann et al., 2003), capable of delivering an increased blood volume to the placenta to meet fetal demands (Duc-Goiran et al., 1999).

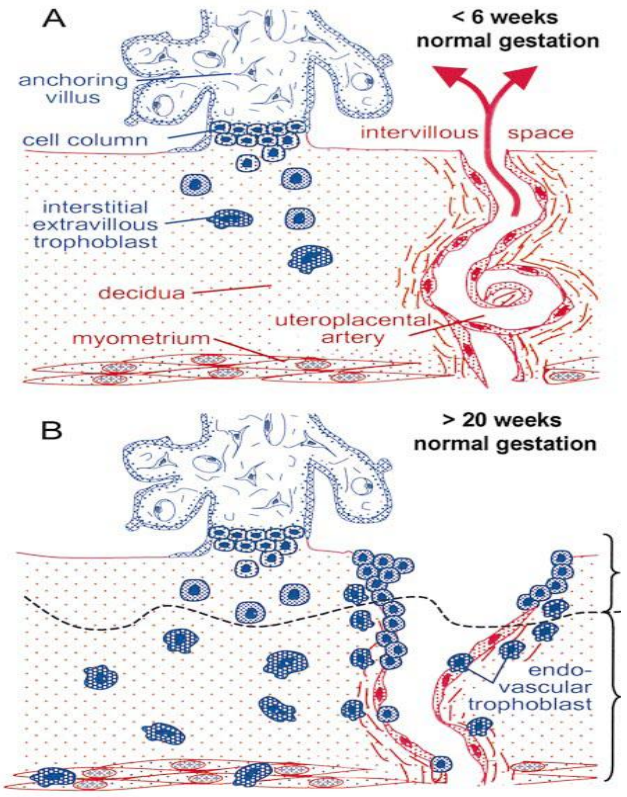

Figure 1.6 Schematic representation of interstitial and endovascular trophoblast invasion in human pregnancy before Week 6 of gestation (A), and after Week 20 of normal gestation (B). Blue: fetal tissues. Red: maternal tissues. Dotted line: zone of placental separation, where the basal plate (above, attached to the placenta) separates from the placental bed (remaining in the uterus after delivery) (figure modified from Kaufmann et al., 2003).

Invasive trophoblasts migrate/invade and reach the inner third of the uterine mesometrium via two distinct migratory pathways. The first is endovascular invasion via an intra-arterial luminal trajectory against the direction of blood flow and the second is by interstitial invasion in which EVTs migrate through the connective tissue of the *decidua*/endometrium to reach the vascular wall (Pijnenborg et al., 1980). This invasion is observed as early as eight weeks of gestation and contributes to the remodeling process by trophoblast colonization of maternal uterine spiral arteries (Pijnenborg et al., 1980). In the rat, glycogen cells (invasive trophoblast cells) are responsible for this invasion in the mesometrial triangle; the initiation of the interstitial trophoblast invasion occurs at gestational day 15 (GD15) while the peak of this invasion occurs at gestational day 18 (GD18) (Vercruysse et al., 2006). The contribution of EVTs in later stages of arterial remodeling is highly dependent on this ability to migrate/invade into the decidual stroma. The invasive properties of trophoblasts are a result of an epithelial to an endothelial phenotypic differentiation. The decidual cell population or extracellular components within the *decidua* appear to regulate this trophoblast invasion. In the absence of the *decidua*, due to uterine scar tissue or in sites of ectopic pregnancy where there are no uNK cells present, uncontrolled deep EVT invasion has been observed (Tantbirojn et al., 2008). This suggests that uNK cells within the uterine tissue are in part responsible for the regulation of trophoblast invasion. In support, *in vitro* studies have demonstrated that cytokines produced by uNK such as tumor necrosis factor alpha (TNFα) and IFNγ can inhibit EVT invasion (Lash et al., 2006).

Extravillous trophoblasts also have an additional role during arterial remodeling. They not only transiently replace the degraded endothelial cells lining the spiral arterial

wall but they also have the ability to degrade the extracellular matrix surrounding these vessels (Harris, 2011). In later stages of the remodeling process, EVTs also appear to influence migration/invasion of VSMCs. This was evident in spiral arteries from first trimester (8-10 weeks) placental bed biopsies which included *decidua*, myometrium and the presence of EVTs. In spiral arteries with associated EVTs, a more pronounced migration of VSMC away from the artery lumen was observed compared to arteries without the presence of EVTs (Bulmer et al., 2012).

Based on the clearance of the VSMC layers and the presence of invasive trophoblast, Smith et al., (2009), have elegantly demonstrated and summarized four distinct stages of spiral artery remodeling. The first stage consists of intact arteries/arterioles with a full perivascular VSMC layers, complete endothelial cell intramural coverage and absence of any EVTs. The second stage consists of initiation of VSMC layer disruption and misalignment with minimal presence of interstitial trophoblasts and the absence of endovascular trophoblasts. The third stage consists of major disorganization and clearance of the vessel structural cells (VSMCs and endothelial cells) with the presence of both perivascular interstitial and intramural endovascular invasive trophoblast. The fourth stage consists of a fully remodelled vessel with complete absence of the VSMC layer and complete replacement of the endothelial cells by endovascular invasive trophoblast embedded in fibrinoid deposits (Smith et al., 2009).

The regulation of spiral artery remodeling depends upon multiple factors, including the recruitment and activation of immune cells at the maternal-fetal interface. Evidence demonstrates that uNK cells play an important role in spiral artery remodeling

events and regulation of trophoblast invasion. Whether there are alterations to these events in obese pregnant women remains unexplored. Yet, in adipose tissue, vessel structure and tone are partly influenced by vasoactive inflammatory mediators such as cytokines (Zhang & Zhang, 2009). If such mediators are altered in obese pregnant women, they may influence the remodeling of the uterine spiral arteries (either directly or via a negative influence on trophoblast invasion), thus leading to FGR.

Maternal obesity associated pregnancy complications may be the result of a number of disturbances during utero-placental development. Maternal obesity may deregulate energy homeostasis, trophoblast migration/invasion, decidualization and/or inflammatory pathways (Hauguel-de Mouzon & Guerre-Millo, 2006). Moreover, temporal regulation of these processes may also be disturbed. However, the mechanisms by which these disturbances occur are currently unknown. In our studies, we focused on the effects of a life-long high fat diet leading to obesity before and during pregnancy on placentation as opposed to increased gestational weight gain. We focused on this group of individuals because of the astonishing increase of obese *pre-gravida* women.

Obesity and the immune response during pregnancy

Obesity is characterized by a chronic systemic low grade inflammatory state (Curat et al., 2006; Fantuzzi, 2005; Li et al., 2008). The main contribution to this inflammatory state is due to the increase in resident adipose tissue macrophages. These macrophages have been shown to be cluster of differentiation (CD) 68 positive and/or CD14 positive (Curat et al., 2006; Spencer et al., 2010; Wozniak et al., 2009). Furthermore the increases in adipose tissue macrophages have demonstrated to be proportionate to the individual's BMI. This increase in localized adipose tissue macrophages results in increased production and secretion of pro-inflammatory cytokines capable of reaching circulation leading to the chronic systemic low grade inflammatory state (reviewed by Wali et al., 2014). In non-pregnant obese individuals this low grade chronic inflammatory state negatively affects multiple organs including brain, pancreas, adipose tissue, and skeletal muscle (reviewed by Frias & Grove, 2012) and results in obesity associated diseases such as coronary heart disease, hypertension, and type 2 diabetes mellitus (Arendas et al., 2008). Interestingly a high fat diet induces the recruitment of these adipose tissue macrophages which in turn secretes the pro-inflammatory cytokine TNFα. It is therefore plausible that obese pregnant women will also display an increased inflammatory state which can negatively influence utero-placental development and ultimately fetal growth. During healthy (normal weight women) pregnancy TNFα serum levels significantly increase across gestation and demonstrate a similar low grade pro-inflammatory state near term as obese non-pregnant individuals (Christian & Porter, 2014). In obese pregnant women however, the levels of TNFα in maternal serum in early to mid pregnancy (14-20 weeks) were demonstrated to

be positively correlated with BMI (Gao et al., 2008). Interestingly, FGR pregnancy outcomes associated with placental insufficiencies, a common outcome in obese women (Radulescu et al., 2013), demonstrated elevated TNFα levels in serum near term compared to both FGR pregnancies without placental insufficiency and uncomplicated pregnancies (Bartha et al., 2003). Whether changes in circulating TNFα levels affect normal utero-placental development remains to be determined; however the uterine tissue is highly vascularised and therefore alteration in circulating TNFα and other pro-inflammatory cytokines could potentially affect the uterine immune response and utero–placental development. For example, *in vitro* studies have demonstrated that TNFα could inhibit HTR8/SVneo (human first trimester extravillous trophoblast cell line) migration/invasion (Renaud et al., 2007).

The anti-inflammatory cytokine, interleukin 10 (IL-10), has also demonstrated the potential to be involved in regulation of trophoblast physiology. IL-10 has been shown to be expressed by invasive trophoblasts and to positively regulate trophoblast migration/invasion (Renaud et al., 2007) by indirectly decreasing expression of the trophoblast invasion inhibitory cytokine TNFα (Rivera et al., 1998). In pre-eclampsia it is well documented that there is a decrease in trophoblast invasiveness of the spiral arterioles. This decrease coincides with a decrease in placental IL-10 levels as well as a decrease in circulating IL-10 levels (Keelan & Mitchell, 2007; Reister et al., 1999; Renaud et al., 2007). Taken together, these observations suggest that the balance between pro- and anti-inflammatory cytokines may be important in regulating trophoblast function. Therefore, an increase in pro-inflammatory state may result in decreased

trophoblastic migration/invasion and lead to utero-placental dysfunction. However, whether this balance is perturbed in obese pregnancy remains largely unexplored.

Rat model of lifelong obesity

In our study, our goal was to characterize the influence of maternal obesity on the utero-placental immune state and on utero-placental development in hope of improving the success of pregnancy of these women. However, to achieve this we must first understand the mechanisms that are responsible for the pathologies related to obesity. Ethical and moral issues are always at the forefront in research on humans or human derived tissues. The use of animal models to study human disease is widely used because of the difficulties in obtaining human tissues, especially uterine tissue during early pregnancy. Therefore, we must first expand our knowledge through similar developing systems. The importance of developing rat models to study human pregnancy related complications lies in part in the similarities between the two species. Indeed processes of crucial importance to utero-placental development and successful pregnancy outcomes such as deep trophoblast invasion and extensive uterine vascular remodeling are common to both and, as such, lessons from rat can be partly extrapolated to the human (Soares et al., 2012a). By utilizing an obese rat model, we were able to initiate the characterization of maternal obesity's influence on uterine immune response and utero-placental development.

Our collaborators Drs. Raha and Holloway from McMaster University have developed a rat model of lifelong obesity that exhibits similar adverse pregnancy outcomes observed in human populations. Three week old female Sprague-Dawley rats are given either a control diet (CD) (17%) or a high fat diet (HFD), hyper-caloric diet (45% calories from fat) for 16 weeks starting after weaning, at which time they are mated with control diet Sprague-Dawley males. This HFD dietary intervention models the

effects of a poor diet during childhood, puberty and early adulthood and results in a significant increase in body weight (Figure 1.7). Similar to obese pregnant women, the HFD induced obese rat model demonstrated adverse pregnancy outcomes commonly observed in obese human pregnancies such as decreased birth weights (indicative of FGR) and increased rate of stillbirths (Radulescu et al., 2013). Moreover, the surviving offspring of the obese dams had reduced survival to post natal day 4 (PND4), an indicator of poor neonatal health (Figure 1.8). Taken together, the HFD-fed animal exhibits many of the features of pregnancy in obese humans and provides an excellent model to determine the underlying causes of placental pathology related to obesity.

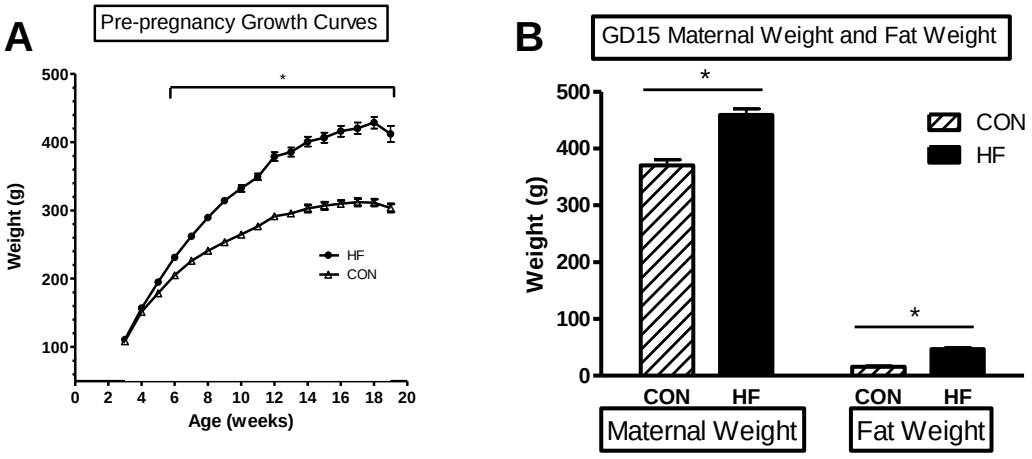

Figure 1.7 (A) Lifelong tracking of maternal body weight of CD and HFD rat model before the onset of pregnancy, n>28 dams per group. (B) Maternal weight and fat weight at gestational day 15, n>11 dams per group. Values represent mean +/- SEM; * p<0.05 (Hayes et al., 2012).

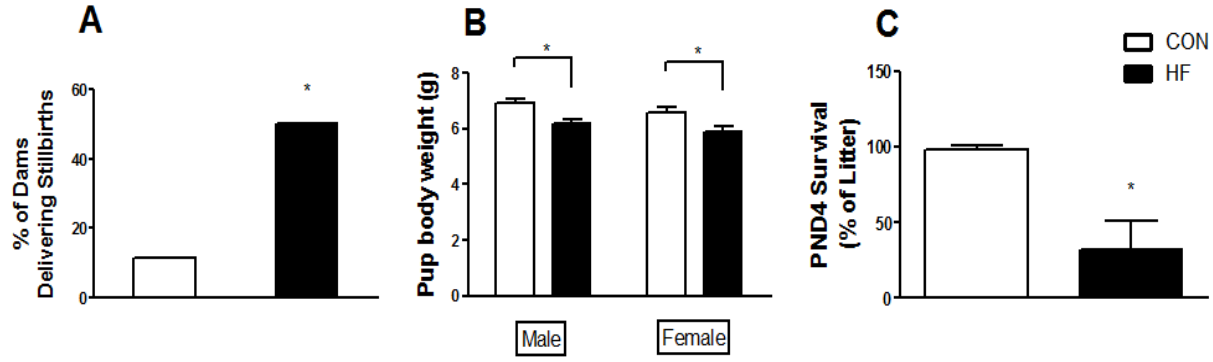

Figure 1.8 Pregnancy outcomes for offspring from CD and HFD rat model. (A) Rate of stillbirths. (B) Pup weight of males and females. (C) Post natal day 4 survival rates in percentage of litter. Values represent mean +/- SEM; * p<0.05; n>11 dams per group (Hayes et al., 2012).

Rationale:

We postulate that maternal obesity negatively influences fetal growth through an altered utero-placental development caused by deregulation of the uterine immune response. Obesity is associated with an increase in pro-inflammatory cytokine production which mediates a systemic low grade chronic inflammatory state (Fantuzzi, 2005). In addition, placental insufficiencies leading to FGR are associated with an increase in inflammatory response (Sitras et al., 2009). Independently these studies associate obesity with an increased inflammatory response and with poor utero-placental development; however no study to date have directly link maternal obesity with utero-placental inflammation, aberrant utero-placental development and adverse pregnancy outcomes. For these reasons, our goal was to characterize the influence of maternal obesity on the uterine inflammatory state and utero-placental development in order to elucidate the mechanisms leading to FGR. The importance of this information will help further our understanding of the influence of maternal obesity on utero-placental development (and therefore fetal growth and well being) and will potentially provide clues related to preventative and therapeutic approaches.

General Hypothesis:

Maternal obesity increases utero-placental production of pro-inflammatory cytokines, which mediates an imbalance in uterine immune response. Utero-placental inflammation inhibits trophoblast invasion leading to aberrant maternal vascular remodelling which results in decreased maternal blood supply and ultimately, FGR.

Objectives and Hypotheses:

Chapter 2: Characterization of immune cells and cytokine localization in rat utero-placental unit during mid-late gestation

Chapter 2, hypothesis

Since evidence in humans and rats have demonstrated the potential role of immune cells such as uNK cells, neutrophils, and macrophages in the utero-development we hypothesize that uNK cells, neutrophils, and macrophages as well as relevant cytokines TNFα, INFγ and IL-10 will be specifically localized in the rat mesometrial triangle.

Therefore, the objective of Chapter Two was to characterize the distribution of the immune cells, uNK cells, neutrophils and macrophages and the localization of the cytokines TNFα, INFγ and IL-10 in the healthy rat utero-placental tissue at GD15 and GD18.

Chapter 3: Maternal obesity is associated with a systemic and uterine pro-inflammatory state in the rat

Chapter 3, hypothesis

Since increased uterine tissue and systemic pro-inflammatory states are observed in obese pregnant women, our HFD induced obese rat model will also exhibit a uterine as well as systemic pro-inflammatory states compared to CD-fed rats.

Therefore, the objective of Chapter Three was to assess the differences in distribution of pro- and anti-inflammatory cytokines TNFα and IL-10 in the uterine tissue between CD- and HFD-fed rats at GD15 and GD18. In addition we assessed the levels of TNFα, interleukin 1 beta (IL-1β) and IL-10 at both gestational ages and compared between our study groups. Monocyte chemoattractant protein-1 (MCP-1) levels were also assessed in maternal serum for systemic analysis of the inflammatory state at both gestational ages and compared between our study groups.

Chapter 4: Trophoblast Invasion and Blood Vessel Remodeling Are Altered in a Rat Model of Lifelong Maternal Obesity

Chapter 4, hypotheses

The high fat diet/maternal obesity will negatively influence trophoblast invasion at both GD15 and GD18 and spiral artery remodeling at GD18 in the HFD-fed rats compared to CD-fed rats.

Therefore, the objectives of Chapter Four were to assess the differences in trophoblast invasion at GD15 and GD18 and to assess differences in vascular remodeling of the uterine spiral arteries at GD18 between HFD-fed rats and CD-fed rats. In addition we also assessed the influence of human first trimester serum from obese women on HTR8/SVneo (trophoblast cell line) invasion *in vitro* compared to first trimester serum from normal weight women.

Chapter 2

Characterization of immune cells and cytokine localization in the rat utero-placental unit during mid-late gestation

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Preface:

Chapter Two as a whole was accepted to the Journal of Reproductive Immunology on January 20th 2015. For details on author's contribution please refer to Statement of contribution on page VI. This chapter is a prelude to Chapters Three and Four. The characterization of immune cell distribution and cytokine localization during utero-placental development in normal weight rats was necessary to study the influence of maternal obesity on the uterine immune response (Chapter Three) and on utero-placental development (Chapter Four).

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Abstract:

The success of pregnancy is dependent on precise regulation of the immune response within the utero-placental environment. Rats are beginning to be widely used as a model for human related pregnancy complications. However our knowledge of immune cells and cytokine localization in the rat utero-placental tissue is limited. The current study aimed to localize the immune cell populations including uterine natural killer (uNK) cells, neutrophils and macrophages within the rat utero-placental unit at two crucial gestational ages, GD15.5* and GD18.5. In addition, we characterized the distribution of the cytokines TNFα, IFNγ and IL-10 in the utero-placental regions at both above mentioned gestational ages. Our study has demonstrated co-localization of TNFα and IFNγ with uNK cells in perivascular regions of the rat mesometrial triangle at both gestational ages. Neutrophils and IL-10 positive cells were localized at the maternal fetal interface and in the spiral artery lumen of the rat mesometrial triangle at both gestational ages. TNFα and IL-10 demonstrated a temporal change in the localization from GD15.5 to GD18.5 which coincides with the leading edge of trophoblast invasion into the mesometrial triangle. The current study furthers our knowledge of the localization of uterine immune cells and relevant cytokines and provides a base to research the function of these immune cells and cytokines during rat pregnancy as a model to study human immune related pregnancy complications.

Keywords: Rat pregnancy; uterine natural killer cells; neutrophils; tumor necrosis factor alpha; interferon gamma; interleukin 10

* Upon request of the Reviewers and Editor from the Journal of Reproductive Immunology the nomenclature referring to the first day post coitum (dpc) is represented as 0.5, however the remaining chapters' day 0 represents the first dpc.

2.1 Introduction

During pregnancy the uterine leukocyte population and associated cytokines are involved in multiple functions such as protection against pathogenic microorganisms, tolerance of the embryonic semiallogeneic cells and processes involved in regulation of trophoblast invasion and uterine spiral artery remodeling. Moreover, altered immune status caused by infection or other pro-inflammatory stimuli have the potential to lead to pregnancy complications such as preeclampsia, GDM and preterm delivery (Raghupathy, 2013; Vrachnis et al., 2010; Xu et al., 2014). To date, uterine immune cell populations and cytokine localization within the rat utero-placental regions have not been fully defined. This increases the difficulties in studying the immune responses at different gestational ages and/or different immune states and limits the interpretation of such studies. Innate immune cells such as uterine natural killer (uNK) cells and macrophages in humans are abundant within the uterine tissue and play an important role in regulating the immune response in the uterine environment (Hunt et al., 2000). Interestingly their highest levels occur in the first and second trimester (Williams et al., 2009). This coincides with major events in uterine development such as trophoblast invasion and maternal spiral artery remodeling (Pijnenborg et al., 1983). In the rat these events occur at the mid to late gestational time period, where GD15.5 marks the early phase of trophoblast invasion into the mesometrial triangle and uterine spiral artery remodeling and GD18.5 marks the peak of trophoblast invasion and spiral artery remodeling (Caluwaerts et al., 2005; Vercruysse et al., 2006). These uterine modifications result in dilatation of the spiral arteries and a sharp increase in blood flow to the placenta from GD15.5 to GD20.5 (Even et al., 1994) in order to meet increasing fetal demands. In mice

and in humans uterine leukocytes are involved in regulating these uterine modifications via the production of cytokines (Ashkar et al., 2000; Robson et al., 2012; Smith et al., 2009). Mice however have demonstrated differences in placentation events such as the depth of trophoblast invasion in to the uterine tissue when compared to humans. Rats on the other hand exhibit many similarities to humans in terms of deep trophoblast invasion and extensive spiral remodeling (Soares et al., 2012a). However, in rats, the uterine leukocyte and relevant cytokine localization and functions are not well delineated.

The distribution and function of uNK cells (also known as decidual natural killer cells or granulated metrial gland cells) have been partially defined in humans and in mice (Ain et al., 2003; Bulmer et al., 2012; Robson et al., 2012). These specialized immune cells have been shown to be major contributors to spiral artery remodeling in which degradation of the maternal uterine spiral artery endothelial cells and the clearance of the smooth muscle cell layer occurs (Bulmer et al., 2012; Fraser et al., 2012). In the rat, however, only a limited number of studies have demonstrated the distribution of uNK cells in the mesometrial triangle. Some of these studies focused on early to mid gestational time points (GD9.5 and GD13.5) (Chakraborty et al., 2011; Rosario et al., 2008) before the onset of trophoblast invasion (Vercruysse et al., 2006). Others studies have demonstrated an inverse relationship in the distribution of uNK and trophoblast cells during the mid to late gestational time periods. At GD14.5-15.5 initiation of trophoblast invasion into the *decidua* and large quantities of dispersed perforin-positive uNK cells are observed in the mesometrial triangle. In late gestation perforin positive granulated metrial gland cells were mainly localized in the perivascular regions of the mesometrial triangle

(Picut et al., 2009). At GD18.5 deep trophoblast invasion and significant reduction in uNK cells are observed (Ain et al., 2003).

Neutrophils localization in the rat utero-placental tissue is not well characterized. One study demonstrated their localized in the apex and lateral regions of the *decidua basalis* at gestation (GD18.5) (Peltier et al., 2003). Their localization and increased numbers following infection with *Mycoplasma pulmonis* suggest a potential role in host defense.

Macrophage characterization in the rat uterine tissue has proven to be challenging. The lack of specific markers and the existence of different macrophage subtypes have increased the complexity of this task. Macrophages in the rat utero-placental tissue were first demonstrated by van Oostveen et al., (1992) in which they described the presence of ED1 positive cells in the myometrium, endometrium, metrial gland, *decidua basalis* and labyrinth. CD68 (also known as ED1 in rat) a glycoprotein present in the cytoplasm of macrophages is currently being used to quantify the general macrophage population in multiple tissues in the rat including the uterine tissue. However in this study we question the use of CD68 as a marker for macrophage quantification.

Cytokines such as IFNγ, TNFα and IL-10, are secreted from uterine leukocytes and have demonstrated the potential to be important regulators of the uterine modifications during pregnancy (Ashkar et al., 2000; Huber et al., 2006; Renaud et al., 2007). IFNγ, in uterine tissues, is mainly produced by activated uNK cells (van der Meer et al., 2004). It has been demonstrated to be of central importance for spiral artery remodeling in mice (Ashkar et al., 2000). However in the rat, IFNγ distribution in the utero-placental tissue has not been well defined. TNFα is a pro-inflammatory cytokine

produced by numerous leukocytes such as macrophages, granulated metrial gland cells (uNK cells) and mast cells (Lea et al., 1998). TNFα has proven to be a good indicator of the inflammatory state within the uterine environment. In addition, *in vitro* studies have demonstrated that TNFα can inhibit trophoblast invasion (Otun et al., 2011; Renaud et al., 2007). IL-10 is known as an anti-inflammatory cytokine with the potential to regulate the expression of both IFNγ and TNFα. *In vitro* studies have demonstrated that IL-10 could enhance production of IFNγ in activated NK cells (Shibata et al., 1998) and reduce TNFα secretion from activated macrophages (Renaud et al., 2007).

The rat as a model for studying human related pregnancy complications is gaining popularity because of the multiple similarities which exist between the two species including similar trophoblast invasion profiles and maternal vascular remodeling events. However, localization of immune cells and cytokines in the rat utero-placental tissue relevant to these processes is not fully characterized. Therefore the objective of this study was to further characterize the localization of immune cells and associated cytokines relevant to trophoblast invasion and spiral artery remodeling in the rat utero-placental compartment during mid and late gestation time periods.

2.2 Materials and Methods

2.2.1 Animals

All animal procedures for this study were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol 07-07-40) in accordance with the guidelines of the Canadian Council of Animal Care. Female Sprague-Dawley rats, aged 21 days (84–100 g), were purchased from Charles River Laboratories (Wilmington, MA). Rats were maintained under controlled lighting (12 hr light – dark cycle) and temperature (22°C) with ad libitum access to food and water. Dams were fed a standard rat chow (17% kcal fat, 3.00kcal/g; Harlan Teklad, Madison, WI) for 16 weeks before being mated with age-matched Sprague-Dawley males fed the same diet. Copulation was confirmed by the presence of sperm in a vaginal flush; the day of copulation was designated gestational day 0.5. Ten animals underwent laparotomy at GD15.5 and another ten at GD18.5, exposing the uterine cavity with the fetuses. The second conceptus from each horn closest to the cervix was removed and fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical analysis.

2.2.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5μm paraffin sections mounted on Superfrost Plus slides (Fisher Scientific, ON, Canada). Following standard dewaxing and rehydration, antigen retrieval for CD68, myeloperoxidase (MPO), IL-10, IFNγ, TNFα and cytokeratin immunostaining was performed in a pressure cooker for 10 minutes in citrate buffer (10 mM Sodium citrate pH 6.0, 0.05% Tween 20). For perforin immunostaining no antigen retrieval was necessary. Endogenous peroxidase activity was

blocked with 3% (v/v) H_2O_2 in methanol followed by non-immune blocking using DAKO blocking solution (DAKO, Glostrup, Denmark). For leukocyte subtyping the following markers were used: mouse anti-rat CD68 for macrophages (2.5 µg/ml) (AbD Serotech, NC. U.S.A.), rabbit anti-rat perforin for activated NK cells (10 µg/ml) (Torrey Pines Biolabs Inc., NJ, U.S.A.) and rabbit anti-myeloperoxidase (MPO) for neutrophils (Abcam, Cambridge, MA, U.S.A.). For cytokine profiling antibodies used were: mouse anti-rat IFNγ (10 µg/ml) (R & D Systems, MN, U.S.A.), rabbit anti-rat IL-10 (2 µg/ml) (Genway Biotech Inc., CA, U.S.A.) and rabbit anti-rat TNFα (13.3 µg/ml) (Novus Biological, Littleton. CO, U.S.A.). Trophoblast immunohistological staining was performed using mouse anti-pan cytokeratin (1:300 dilution) (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies used were diluted in 1% bovine serum albumin (BSA)-phosphate buffered saline (PBS) buffer and incubated overnight at 4°C. PBS with 1% BSA and no primary antibody was used as a negative control. Thorough washes were performed in PBS prior to application of either anti-mouse or anti-rabbit DAKO EnVision™+ HRP labeled polymer secondary antibodies (DAKO, Glostrup, Denmark) corresponding to source of primary antibody. Positive antibody binding was detected with diaminobenzidine (DAB) (dark brown staining), and sections were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), dehydrated, and mounted. For comparison of cellular morphology and localization adjacent cross sections to the IFNγ immunohistochemistry slides were stained using hematoxylin and eosin (H&E) (standard protocol). All slides were scanned using brightfield microscopy with an Aperio Scanscope (Leica Biosystems, ON, Canada) and analyzed using Aperio Imagescope software (Leica Biosystems, ON, Canada). TNFα images in (Figure 2.1)

were analyzed using Image J software (developed by the National Institute of Health, U.S.A) to facilitate visual assessment of TNFα positive staining and to highlight TNFα distribution throughout the entire mesometrial triangle. Image J is a Java-based image processing program used for a variety of image analysis and processing tasks. In our case, it allowed for segmentation of areas of interest using specific color intensity thresholds (in this case DAB staining). Since the threshold parameters used across all images remain the same, Image J allows precise measurement and comparison of positively stained regions between multiple images. For example image processing was carried out by adjusting the color threshold using red as the color threshold and red/green/blue as color space (methods were modified from previously described analysis Walschus et al., 2011). Segmentation of areas of interest using specific color intensity, in this case DAB staining, thresholds were used and the selected areas were then highlighted in bright red to facilitate visual assessment. To focus on interstitial TNFα positive staining, images were further modified with the removal of all intraluminal spaces and inter tissue gaps. Identical image processing was used for immunostaining negative controls.

2.2.3 Blocking experiments

To test the specificity of the anti-IFNγ, anti-IL-10 and anti-TNFα in our immunohistochemical staining, primary antibodies were pre-incubated with corresponding rat recombinant protein at a ratio of 10:1 (recombinant concentration to antibody concentration) for 18 hrs at 4°C prior to application to cross sections. Source of recombinant rat proteins used in the primary blocking experiment are rIFNγ (R & D Systems, MN, U.S.A.), rIL-10 (Abnova, CA, U.S.A.) and rTNFα (Abnova, CA, U.S.A.)

2.3 Results

2.3.1 TNFα distribution and co-localization of TNFα and uNK cells in the rat utero-placental unit at GD15.5 and GD18.5

TNFα immunohistological staining revealed two distinct staining patterns in the mesometrial triangle tissue at both GD15.5 and GD18.5 (Figure 2.1.A and Figure 2.1.B). The first staining pattern observed was a diffuse interstitial distribution throughout the mesometrial triangle at both GD15.5 and GD18.5. At GD15.5 however a higher density of TNFα positive cells was observed at the base of the mesometrial triangle near or in the *decidua basalis* (Figure 2.1.A). By GD18.5 the higher density of TNFα positive cells was observed in the mid to upper portion of the mesometrial triangle (Figure 2.1.B). In early stages of trophoblast invasion (GD15.5) invasive trophoblasts are localized at the maternal fetal interface (*decidua*-junctional zone) (Figure 2.1.C) whereas by GD18.5, at the peak of trophoblast invasion, invasive trophoblasts are localized in the mid to upper portion of the mesometrial triangle (Figure 2.1.D). Interestingly, the higher density of TNFα positive cells observed at each gestational age coincided with the leading edge of the invasive trophoblast cells. The second TNFα staining pattern observed was in the perivascular regions of the spiral arteries. These TNFα positive cells demonstrated a distinct staining pattern primarily in granulated cells located in the vessel's perimeter. The distribution of TNFα positive cells in these areas demonstrated a similar staining pattern as perforin-positive uNK cells, suggesting an intracellular co-localization (Figure 2.2). The blocking experiments demonstrated the specificity of the anti-TNFα to recognize TNFα (Figure 2.2.E-G). Pre-incubation with recombinant rat TNFα completely abolished the capacity of the anti-TNFα to bind to the tissue (Figure 2.2.G).

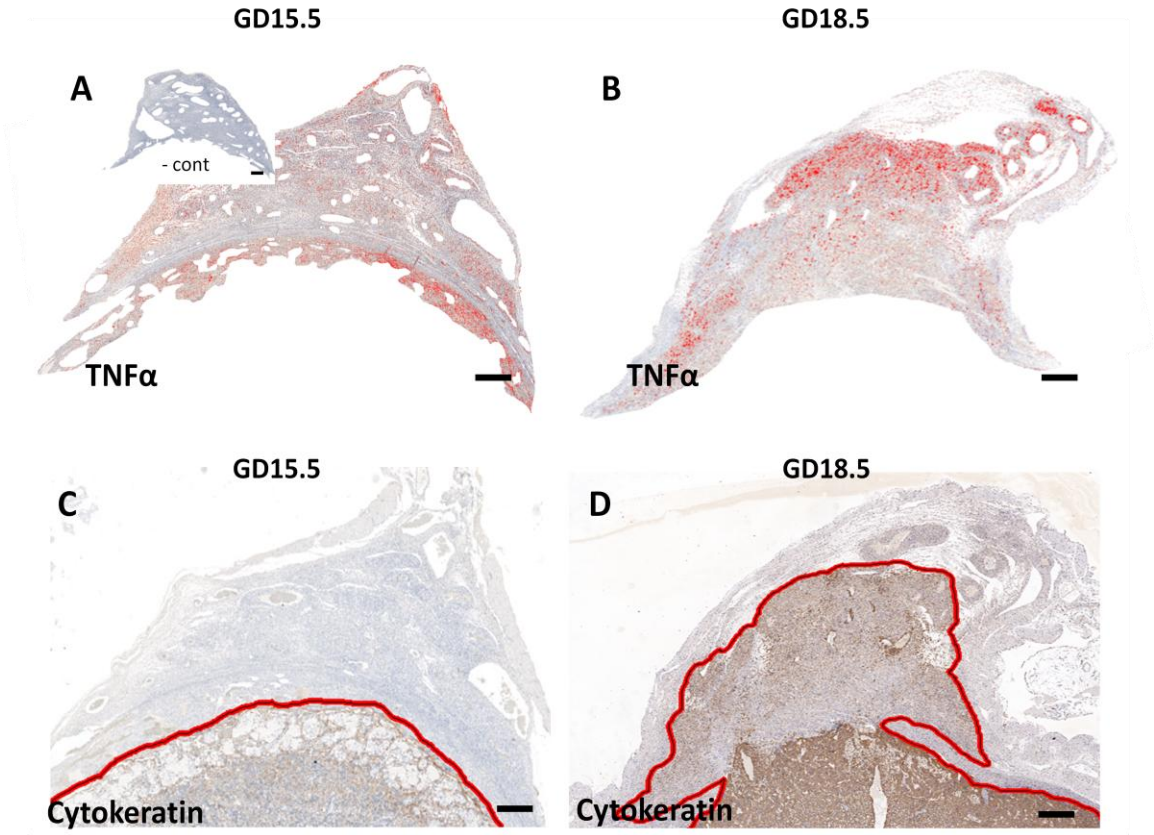


Figure 2.1 TNFα localization in the rat mesometrial triangle at GD15.5 and GD18.5. (A and C) Representative images of adjacent cross sections at GD15.5 and (B and D) are representative images of adjacent cross sections at GD18.5. (A-B) Immunohistochemistry using anti-TNFα primary antibody at GD15.5 and GD18.5 respectively. Images have been modified to facilitate visual assessment (see methods for details). Top left corner of image (A) demonstrated negative control (absence of anti-TNFα). (C-D) Representative images of the rat mesometrial triangle stained by immunohistochemistry using anti-cytokeratin (trophoblast marker) as primary antibody at GD15.5 and GD18.5 respectively. Red line delineates trophoblast invasion into the mesometrial triangle. Scale bars for negative control is 320 µm, (A and C) are 400µm and for (B and D) are 450µm.

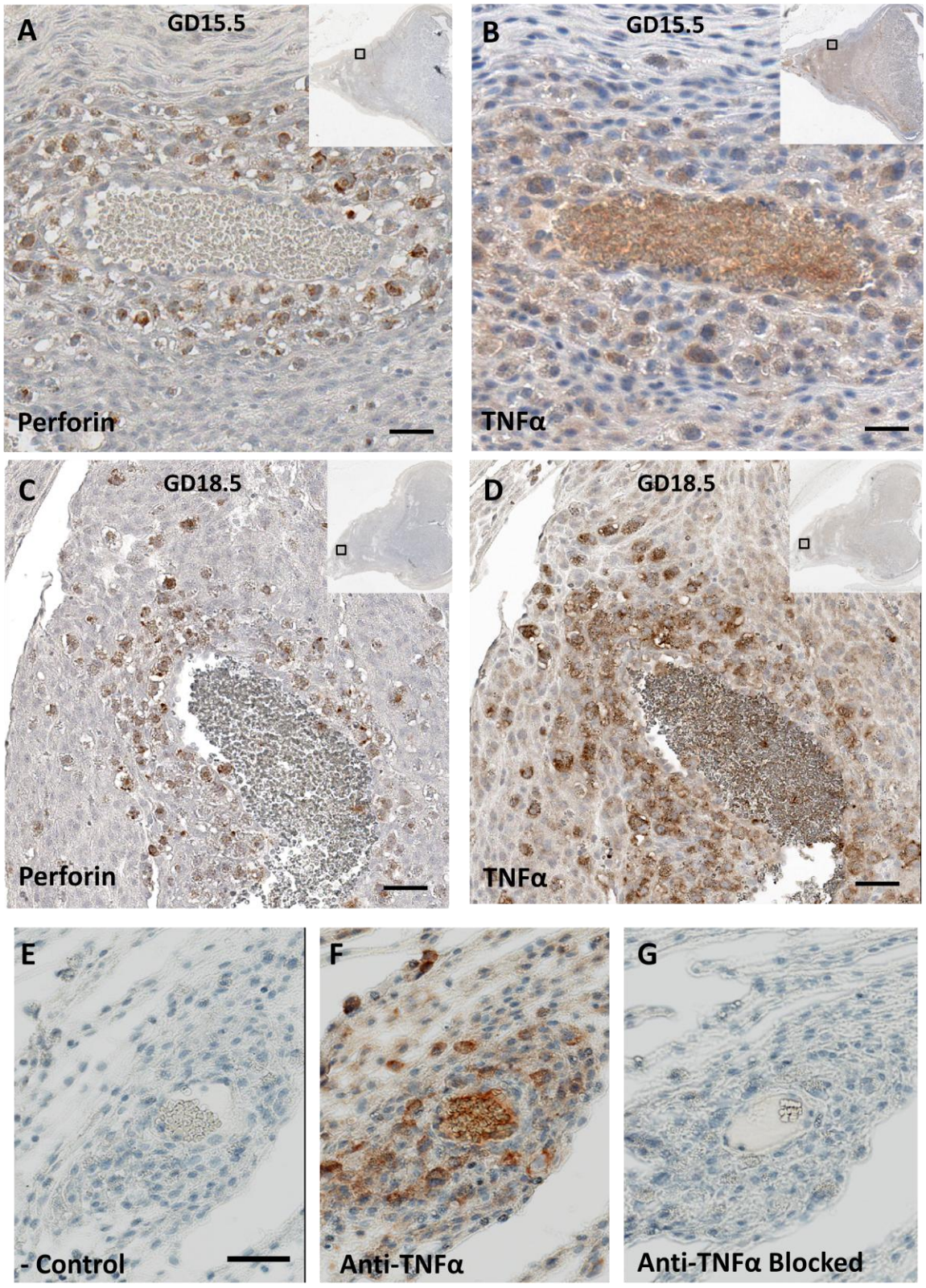

A
GD15.5
Perforin
B
GD15.5
TNFα
C
GD18.5
Perforin
D
GD18.5
TNFα
E
- Control
F
Anti-TNFα
G
Anti-TNFα Blocked

Figure 2.2 Co-localization of TNFα and perforin positive cells in the rat mesometrial triangle at GD15.5 and GD18.5. Immunohistochemistry of cross sections of the entire rat utero-placental units (images shown in top right corner of their respective panels (A-D)). Representative images of adjacent cross sections of a spiral artery (A-B) at GD15.5 and (C-D) at GD18.5. Immunohistochemistry (A and C) using anti-perforin and (B and D) using anti-TNFα. (E-G) GD15.5 adjacent cross sections of a uterine vessel from the anti-TNFα antibody blocking experiment. (E) Negative control (no primary antibody), (F) anti-TNFα primary antibody, and (G) anti-TNFα primary antibody pre-incubated with recombinant rat TNFα. Scale bars for (A-B) are 30μm, and for (C-G) are 50μm.

2.3.2 Co-localization of IFNγ and uNK cells in the rat utero-placental unit at GD15.5 and GD18.5

Histological assessment of perforin-positive uNK cells and IFNγ-positive cells in the mesometrial triangle at mid (GD15.5) and late (GD18.5) gestational time points revealed similar distribution, suggesting intracellular co-localization (Figure 2.3). The distribution of both perforin- and IFNγ-positive cells was observed in the perivascular regions of the spiral arteries at both GD15.5 and GD18.5. Our observed perforin-positive cell staining was consistent with uNK cell/granulated metrial gland cell staining previously described in the rat placenta (Head et al., 1994; Picut et al., 2009; Sladek et al., 1998).

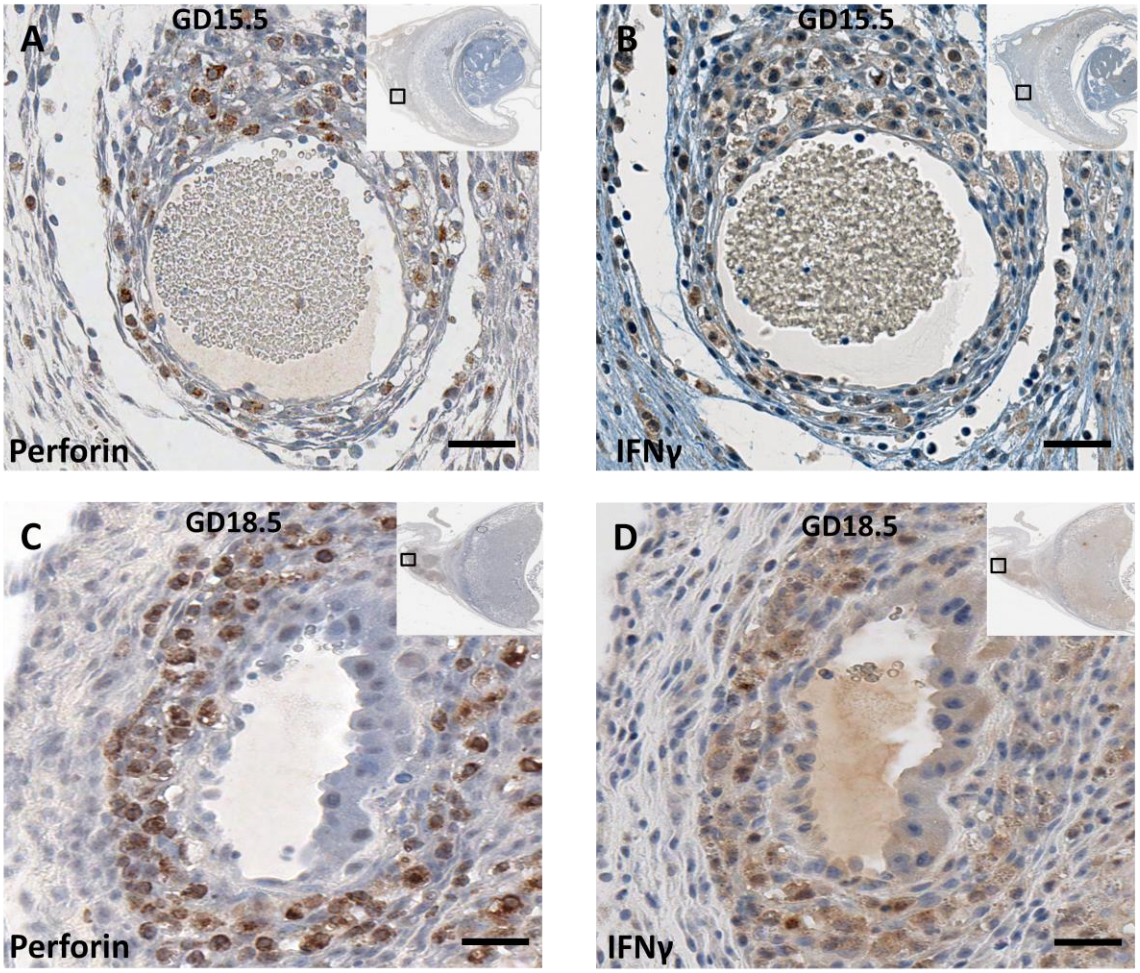


Figure 2.3 Co-localization of IFNγ and perforin positive cells in the rat mesometrial triangle at GD15.5 and GD18.5. (A-D) Immunohistochemistry of the entire rat utero-placental units (images shown in top right corner of their respective panel). Representative images of adjacent cross sections of a spiral artery (A-B) at GD15.5 and (C-D) at GD18.5. Immunohistochemistry (A and C) using anti-perforin and (B and D) using anti-IFNγ. Scale bars for (A-B) are 40µm and for (C-D) 30µm.

2.3.3 IFNγ positive nucleated fetal blood cells at GD15.5

Immunostaining for IFNγ of the entire utero-placental unit also revealed positive signal from the majority of fetal nucleated blood cells within the vessel of the umbilical cord cross section and within the fetal capillaries of the labyrinth at GD15.5 (Figure 2.4.B and D). Almost all the fetal blood cells at GD15.5 are still nucleated within the vessel of the umbilical cord cross section and within the fetal capillaries of the labyrinth as demonstrated with the H&E staining (Figure 2.4.A and C). In the umbilical cord cross section at GD18.5 we observed that the majority of the red blood cells have become mature erythrocytes (lack of nucleus) demonstrated by H&E staining (Figure 2.4.E.). Interestingly, mature erythrocytes within the umbilical cord cross section did not stain positive for IFNγ (Figure 2.4.F). We did however observe a fraction of blood cells that were still nucleated in the umbilical cord cross section in the fetal capillaries of the labyrinth which retained their IFNγ positivity (image not shown). The anti-IFNγ blocking experiments demonstrated the specificity of the anti-IFNγ to recognize IFNγ (Figure 2.4.G-I). The pre-incubation with the recombinant rat IFNγ completely abolished the capacity of the anti-IFNγ to bind to the tissue (Figure 2.4.I).

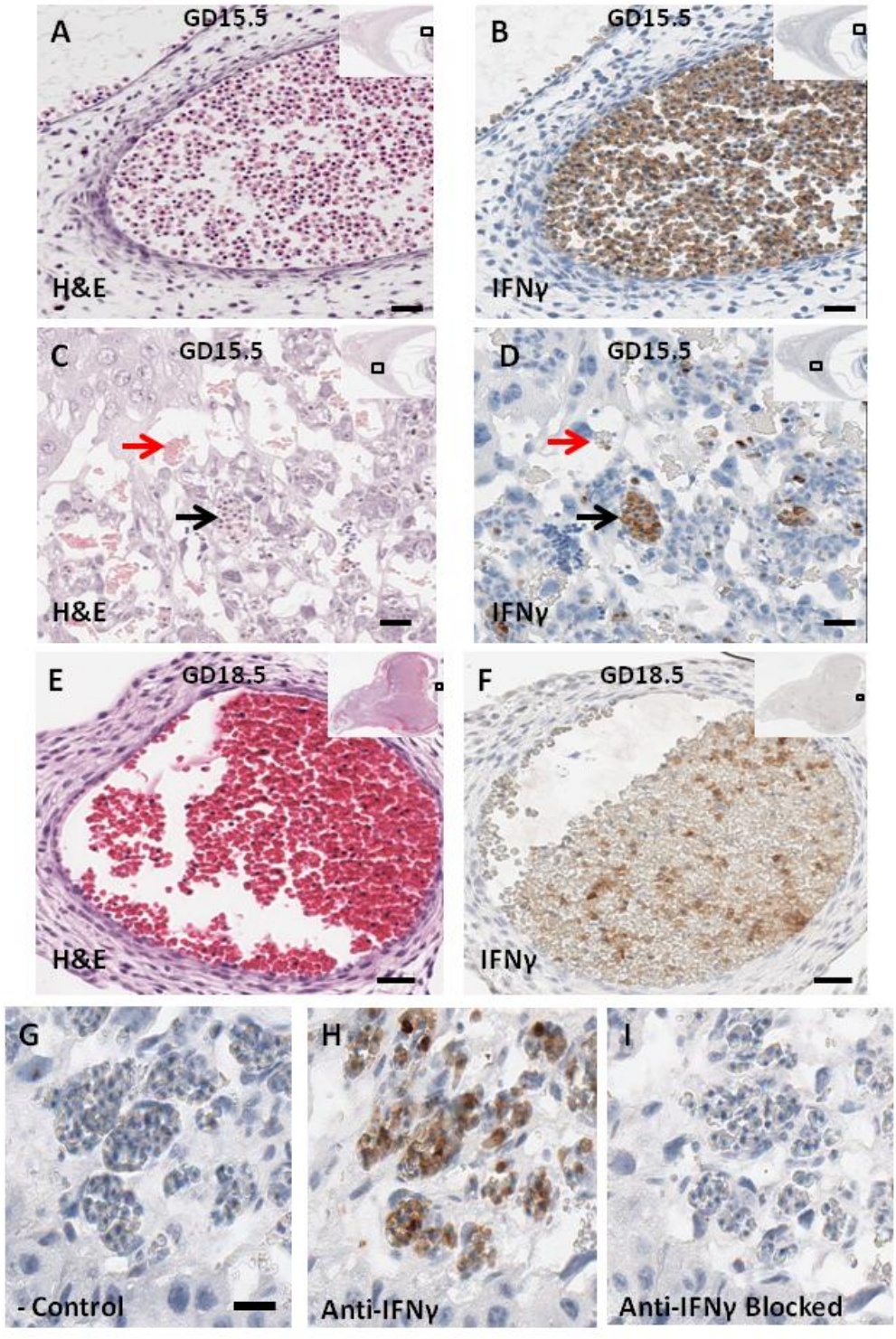

A
GD15.5
H&E
B
GD15.5
IFNγ
C
GD15.5
H&E
D
GD15.5
IFNγ
E
GD18.5
H&E
F
GD18.5
IFNγ
G
- Control
H
Anti-IFNγ
I
Anti-IFNγ Blocked

Figure 2.4 IFNγ positive cells in rat fetal circulation at GD15.5 and GD18.5. Representative images of the entire utero-placental unit are shown in the top right corner of their respective panels (A-F). Representative images (A, C, and E) of H&E staining and (B, D and E) immunohistochemistry using anti-IFNγ as primary antibody. (A-B) Adjacent cross sections of the umbilical cord and (C-D) adjacent cross sections of the labyrinth at GD15.5. Red arrows indicate maternal blood sinuses and black arrows indicate fetal capillaries. (E-F) Adjacent cross sections of the umbilical cord at GD18.5. (G-I) GD15.5 adjacent cross sections of the rat labyrinth from blocking experiment using anti-IFNγ as primary antibody. (G) Negative control (no primary antibody), (H) anti-IFNγ as primary antibody and (I) anti-IFNγ as primary antibody which was pre-incubated with recombinant rat IFNγ. Scale bars for (A-F) are 30μm, (G-I) are 20μm.

2.3.4 Co-localization of neutrophils and IL-10 positive cells and IL-10 distribution in the utero-placental unit at GD15.5 and GD18.5

MPO (marker for neutrophils) and IL-10 positive cells at GD15.5 and GD18.5 localized in distinct regions within the utero-placental unit; MPO and IL-10 immunostaining had a similar pattern of localization suggesting that neutrophils are IL-10 positive (Figure 2.5). At GD15.5 the greatest concentration of MPO and IL-10 positive cells was observed in the *decidua basalis*. These cells are located at the maternal fetal interface (*decidua*-junctional zone) in proximity of the invasive trophoblast (Figure 2.5.A-B). In addition these cells are found throughout the entire cross section of the *decidua basalis* with the greatest concentration near the apex of the placenta. By GD18.5 the MPO and IL-10 positive cells were still present in the decidual layer, however they appear less abundant compared to GD15.5 (Figure 2.5.E-F). The second area of co-localization of MPO and IL-10 positive cells was in the intraluminal space of the spiral arteries, at both GD15.5 and GD18.5 (Figure 2.5.C-D and G-H). These cells were distributed predominately in close proximity with vessel endothelial cells. Higher magnification also revealed that IL-10 positive cells not only co-localize with MPO positive cells but also had the distinctive neutrophilic characteristic of displaying either the multi-lobular nuclei or band form nuclei. In late gestation (GD18.5) we also observed interstitial IL-10 positive cells in the mesometrial triangle (Figure 2.6). These cells were primarily observed in perivascular regions of the spiral arteries and at the leading edge of invasive interstitial trophoblast (Figure 2.6). Very few interstitial IL-10 positive cells were observed in the center of the mesometrial triangle at this gestational age (Figure 2.6.A-3). Anti-IL-10 blocking experiments demonstrated the specificity of the anti-IL-10

to recognize IL-10 (Figure 2.5.I-K). The pre-incubation with the recombinant rat IL-10 completely abolished the capacity of the anti-IL-10 to bind IL-10 positive decidual and intraluminal cells described above.

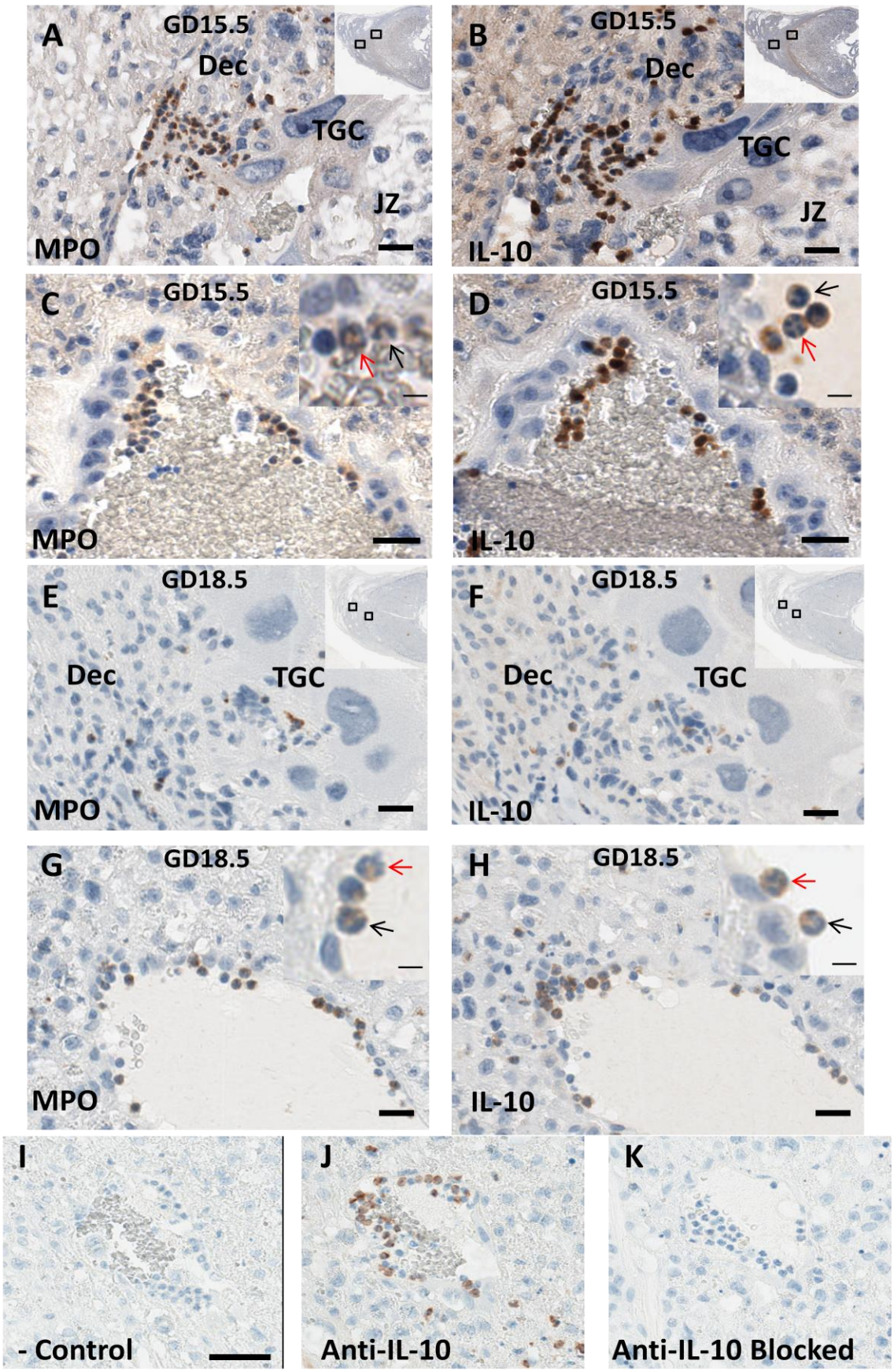

A
GD15.5
Dec
TGC
JZ
MPO
B
GD15.5
Dec
TGC
JZ
IL-10
C
GD15.5
MPO
D
GD15.5
IL-10
E
GD18.5
Dec
TGC
MPO
F
GD18.5
Dec
TGC
IL-10
G
GD18.5
MPO
H
GD18.5
IL-10
I
- Control
J
Anti-IL-10
K
Anti-IL-10 Blocked

Figure 2.5 Neutrophils and IL-10 positive cells co-localize at the maternal fetal interface and in the intraluminal space of the spiral arteries of the rat utero-placental unit at GD15.5 and GD18.5. Immunohistochemistry (A, C, E and G) using anti-MPO as primary antibody (neutrophil marker) and (B, D, F and H) using anti IL-10 as primary antibody. (A-B) Top right images represent adjacent cross sections of the entire utero-placental unit at GD15.5. Right boxed areas in the utero-placental unit (A-B) represent magnified images of the maternal fetal interface respectively and left boxed areas in the utero-placental unit (C-D) represent magnified images of a spiral artery lumen respectively. (C-D) Top right images represent a higher magnified image of MPO (C) and IL-10 (D) positive cells. (E-F) Top right images represent adjacent cross sections the entire utero-placental unit at GD18.5. Right boxed areas of the utero-placental unit (E-F) represent magnified images of the maternal fetal interface respectively and left boxed areas utero-placental unit (G-H) represent magnified images of a spiral artery lumen respectively. (G-H) Top right images represent a higher magnified image of MPO (G) and IL-10 (H) positive cells. Red arrows indicate an multi-lobular nucleated cell and black arrow indicate a horseshoe nucleated cell (Dec: *decidua basalis*, JZ: junctional zone, TGC: trophoblast giant cells). (I-K) GD15.5 adjacent cross sections of a uterine vessel from anti-IL-10 antibody blocking experiment. (I) Negative control (no primary antibody), (J) anti-IL-10 as primary antibody, and (K) anti-IL-10 as primary antibody pre-incubated with recombinant rat IL-10. Scale bars for (A-D) are 20µm, for (I-K) are 40µm and for (C-D and G-H) top right panels are 5µm.

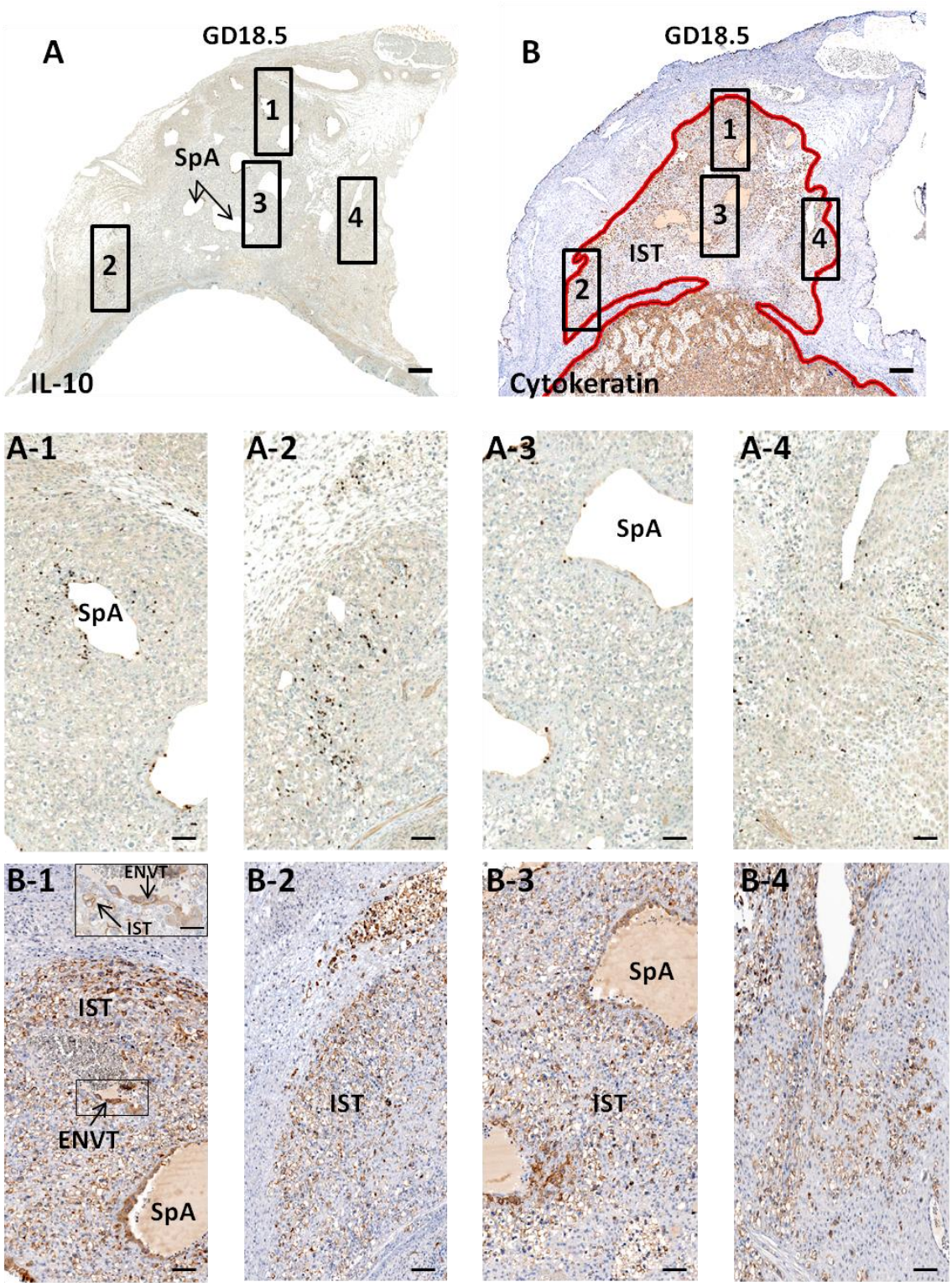

A
GD18.5
1
SpA
3
4
2
IL-10
B
GD18.5
1
3
4
IST
2
Cytokeratin
A-1
SpA
A-2
A-3
SpA
A-4
B-1
ENVT
IST
IST
ENVT
SpA
B-2
IST
B-3
SpA
IST
B-4

Figure 2.6 Interstitial IL-10 positive cells are localized to the leading edge of invasive interstitial trophoblast at GD18.5. (A-B) Representative images of adjacent cross sections of the mesometrial triangle at GD18.5. Immunohistochemistry (A) using anti-IL-10 as primary antibody and (B) using anti-cytokeratin (trophoblast marker) as primary antibody. Red line indicates the leading edge interstitial trophoblast invasion into the mesometrial triangle. (A-1 to A-4) Magnified images of the boxed area shown in (A). (B-1 to B-4) Magnified images of the boxed area shown in (B). (B-2) Thumbnail represents boxed area and demonstrates interstitial and endovascular trophoblast cells in respect to the spiral artery structure. Positive staining is shown as dark brown staining. SpA: spiral artery, IST: Interstitial trophoblast and ENVT: endovascular trophoblast. Scale bars for (A-B) are 400µm, (A-1 to B-4) are 65µm and (B-1) thumbnail is 25µm.

2.3.5 CD68 positive macrophages are localized in three distinct areas of the mesometrial triangle at both GD15.5 and GD18.5

Immunohistochemistry of the entire utero-placental unit using anti-CD68 revealed a large number of CD68 positive cells within three regions in the mesometrial triangle. The first and most distinct staining occurs in the outer perimeter of the spiral artery lumen just beyond the perivascular granulated metrial gland cell layer (Figure 2.7.A-1). The second area of staining was in the upper portion of the mesometrial triangle bordering the myometrium (Figure 2.7.A-2). The CD68 positive cells in this area were clustered and in large numbers. The third area of staining in the utero-placental unit occurred near the base of the mesometrial triangle in the *decidua basalis* (Figure 2.7.A-3). Large clusters of decidual cells in this region were CD68 positive. In addition the CD68 positive *decidua* cell cluster was observed throughout the entire length of the *decidua basalis* cross section (Figure 2.7.A). In all three regions, CD68 positive cellular staining was confined to the cytoplasmic granules in a pattern consistent with previously characterized CD68 staining (Damoiseaux et al., 1994). Similar CD68 positive cell distribution was observed at GD18.5 (images not shown). The positive control (Figure 2.7.B), demonstrated specific CD68 positive cell staining consistent with previously characterized lamina propria CD68 positive macrophages of the rat small intestine (Honkanen et al., 2005).

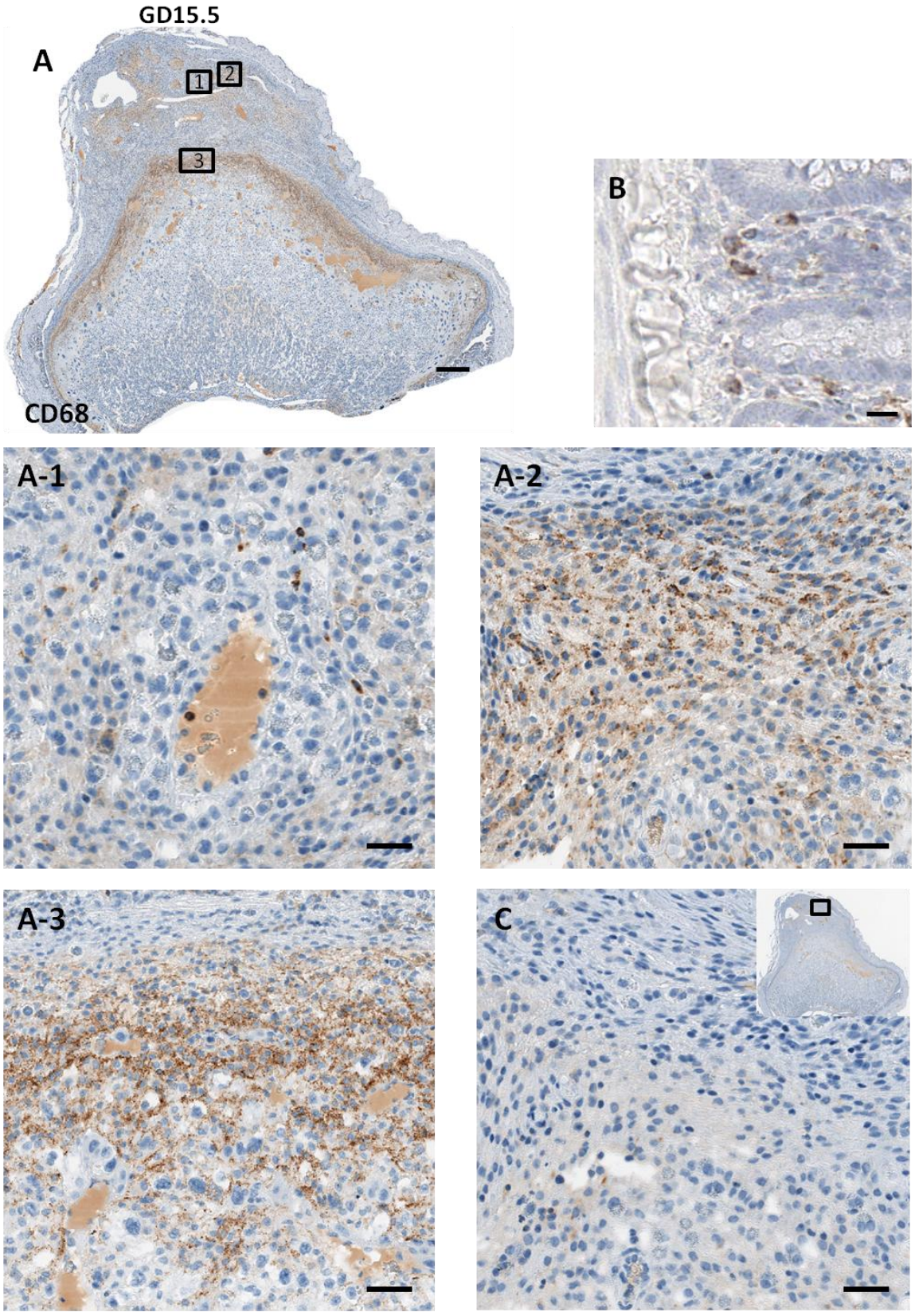

GD15.5
A
1
2
3
CD68
B
A-1
A-2
A-3
C

Figure 2.7 CD68 positive cell distribution in the rat utero-placental unit at GD15.5. (A-B) Representative images of immunohistochemistry using anti-CD68 as primary antibody in the entire utero-placental unit and rat small intestine cross section respectively. (A-1 to A-3) Magnified images of boxed region indicated in image (A). (A-1) maternal vessel (A-2) upper region of the mesometrial triangle and (A-3) *decidua basalis*. (C) Negative control (with no primary antibody), an adjacent cross section to (A) (image shown in top right corner). The magnified image shown in (C) corresponds to the adjacent cross section shown in (A-2). Scale bars for (A) is 400µm, for (B) is 15µm, for (A-1 and A-2 and C) are 30µm and for (A-3) is 50µm.

2.4 Discussion

The strength of using rat models to study human pregnancy related complications such as pre-eclampsia, intrauterine fetal growth restriction and pre-term labour lies in part in the similarities between the two species. Indeed processes of crucial importance to utero-placental development and successful pregnancy outcomes such as deep trophoblast invasion and extensive uterine vascular remodeling are common to both and as such lessons from rat can be partly extrapolated to the human (Soares et al., 2012b). Since the uterine leukocyte populations in humans have demonstrated a potential role in regulating trophoblast invasion and spiral artery remodeling, it is important to characterize them in the rat utero-placental environment. Therefore the primary objective of this study was to characterize the distribution of uNK cells, neutrophils and macrophages as well as associated cytokines such as IFNγ, TNFα and IL-10 in the rat utero-placental unit at two distinct gestational ages relevant to trophoblast invasion and spiral artery remodeling. To achieve this goal we used immunohistochemistry which is a well defined technique for cellular and protein localization while visually maintaining tissue morphology; however it does present some limitations. Firstly quantification of cytokine expression levels is not possible using this technique. Secondly our co-localization findings were performed using adjacent cross sections and therefore only represented partial match of cells. We emphasize partial match since a significant number of cells are not represented on both adjacent cross sections; therefore our results only demonstrate a fraction of cells that are represented on both cross sections. Because of these limitations further studies using immunofluorescence microscopy and quantification techniques would provide further characterization of these immune cells

and relevant cytokines. This study furthers our knowledge of immune cell and cytokine localization in the rat utero-placental unit. The benefit of this knowledge is that it provides evidence of their potential involvement in the regulation of trophoblast invasion and vascular remodeling events. In addition, the characterization of their distribution within the utero-placental tissue can be used in the designing of region specific quantification studies.

TNFα positive staining was observed throughout the mesometrial triangle. Additionally a gestational specific higher density of TNFα positive cells was observed in isolated regions of the mesometrial triangle. Interestingly, these areas of higher density of TNFα positive cells specific to each gestation day coincided with a proximal localization with the gestational distinct leading edge invasive trophoblast. TNFα has been shown to inhibit human trophoblast invasion *in vitro* (Huber et al., 2006). However, whether TNFα is capable of regulating interstitial trophoblast invasion *in vivo* remains to be elucidated. TNFα was also shown to be localized in the perivascular regions of the spiral arteries and to be co-localized with perforin positive uNK cells. Similarly, human uNK cells and mouse granulated metrial gland cells have been previously characterized to express TNFα (Jokhi et al., 1994; Otun et al., 2011; Parr et al., 1995). Yelavarthi et al., (1991) demonstrated TNFα mRNA expression peaks at GD15.5 in the rat uterine tissue. They also describe granulated metrial gland cells (uNK cells) near the vessels to be faintly positive for TNFα mRNA which is consistent with our findings of perivascular distribution. Our results at the mid and late gestation in healthy rat pregnancy, the intensity of TNFα staining and the co-localization of TNFα and uNK cells indicate that uNK cells may be an important source of TNFα in the mesometrial triangle. In addition,

the region specific localization at both studied gestational time points suggests a potential role of TNFα in regulating trophoblast invasion and spiral artery remodeling in the rat. Therefore deregulation of TNFα expression levels may result in adverse utero-placental development. In support, a lipopolysaccharide (LPS) induced inflammatory state (elevated levels of TNFα) in the rat uterine tissue was associated with decreased extravillous trophoblast invasion depth and aberrant spiral artery remodeling (Cotechini et al., 2014). Whether TNFα is directly responsible for these pathologies remains to be determined.

Rat uNK distribution and IFNγ localization are not well defined in the mesometrial triangle. In our study we demonstrate that indeed rat uNK cells and IFNγ positive cells co-localize. In addition, the majority of these cells co-localized in the perivascular regions of the spiral arteries. This co-localization is consistent with findings of IFNγ producing uNK cells in mice and humans (Croy & Kiso, 1993; Jokhi et al., 1994; Platt & Hunt, 1998; Saito et al., 1993). Moreover, multiple studies in humans and mice have demonstrated that IFNγ is essential in vascular remodeling (Ashkar et al., 2000; Robson et al., 2012). In contrast, by GD18.5 we still observed an abundance of uNK cells and IFNγ positive cells even though most of the remodeling process has occurred. This suggests that IFNγ producing uNK cells may serve other functions in late stages of gestation.

Fetal nucleated red blood cells are observed in healthy human first trimester feto-placental circulation prior to the third month of pregnancy (Benirschke et al., 2006). In the rat, Vadgama et al., (1987) described at GD16.5 that fetal red blood cells resemble the normoblastic stage in the human red blood cell development. In normal progression of

differentiation, erythroblasts shed their nucleus. We demonstrate that in the rat umbilical cord and placental fetal capillaries at mid gestation (GD15.5) the majority of red blood cells are still nucleated. However by GD18.5 the majority of these cells have lost their nucleus. The reduction in nucleated red blood cells in late gestation in the rat is consistent with the absence of nucleated red blood cells in healthy human fetal circulation near term. Interestingly, we report for the first time that the majority of nucleated blood cells at GD15.5 were IFNγ positive, in which a portion of these IFNγ positive blood cells resemble nucleated red blood cells (similar to the normoblastic stage/erythroblast) observed in human erythropoeitic development. Interestingly, at GD18.5 the maturation of the red blood cells (loss of their nucleus) observed by H&E coincided with the loss of IFNγ positivity, supporting our findings that a large portion of the IFNγ positive cells are potentially red blood cells. IFNγ positive nucleated red blood cells have been previously described in erythroids isolated from human embryonic liver (Sennikov et al., 2002). IFNγ has also been shown to regulate differentiation and proliferation during erythropoiesis (Sennikov et al., 2004). Therefore the presence of IFNγ in nucleated red blood cells of the rat at early to mid gestation may have similar functions. At GD18.5 we still observed a small portion of IFNγ positive blood cells within the umbilical vessel. Whether these are nucleated red blood cells that have not yet matured or circulating IFNγ positive leukocytes remains to be determined.

Neutrophils in the uterine tissue are best associated with infection leading to aberrant placental and fetal development (Peltier et al., 2003). A prior study using neutrophilic scoring from H&E stained cross sections at mid to late gestation assessed the neutrophil population in the rat. This revealed neutrophils to be localized at the apex of

the placenta in the *decidua basalis* and lateral regions of the *decidua* near the chorionic plate, with the majority of the neutrophils in the lateral regions. Furthermore, Peltier and Brown (2005) described an increased number of neutrophils in the *decidua capsularis* after infection resulting in chorioamnionitis. An abundance of neutrophils in the antimesometrial *decidua* (*decidua parietalis*) has also been described (Correia-da-Silva et al., 2004). In our studies we have demonstrated that MPO positive neutrophils displayed similar cellular morphology and distribution as IL-10 positive cells which suggest cellular co-localization. Our results also demonstrate that at GD15.5 a high density of myeloperoxidase (MPO) positive neutrophils and IL-10 positive cells are localized throughout the entire cross sectional length of the *decidua basalis*. This localization coincides with the site of early trophoblast invasion. By GD18.5 we observed fewer IL-10 positive cells in the *decidua* and that interstitial IL-10 positive cells were localized at the leading apical and lateral edge of interstitial invasive trophoblast. This close proximity to the trophoblast invasion front observed at both gestational age suggests a potential role of IL-10 in regulation of trophoblast invasion. IL-10 has been described as an anti-inflammatory cytokine, reviewed by Prud'homme, (2004). Furthermore *in vitro* studies have demonstrated that IL-10 could reduce pro-inflammatory TNFα secretion from LPS induced activated macrophages (Renaud et al., 2007). In the same study the secretion of TNFα from activated macrophages inhibited trophoblast invasion *in vitro*. Interestingly, IL-10 was shown to indirectly promote trophoblast invasion by attenuating the secretion of TNFα from these activated macrophages (Renaud et al., 2007). MPO and IL-10 positive cells were also co-localized in the perivascular regions and in the intraluminal space of the spiral arteries. Taken together our studies demonstrate a

gestation and region specific localization of MPO and IL-10 positive cells in proximity to invasive trophoblast and maternal spiral arteries nevertheless whether these cells play a role in regulation of trophoblast invasion and spiral artery remodeling remains to be determined.

The characterization of CD68 positive cells as macrophages in the rat uterine and placenta tissue has been established over the last two decades (Bosquiazzo et al., 2005; Cotechini et al., 2014; Hamilton et al., 2012; van Oostveen et al., 1992). However no study has ever shown the entire cross section of the rat utero-placental unit stained with anti-CD68. As described in our results CD68 positive cells were distributed in three distinct regions within the uterine tissue. The first region was the outer perimeter of the perivascular region which contains very few intensely stained CD68 positive cells surrounding the granulated cell ring of the spiral arteries. The second region was in the upper portion (near the apex) of the mesometrial triangle and the third was the *decidua basalis*. The second and third regions demonstrated large clusters of CD68 positive cells. In all three areas of interest we observed positive cytoplasmic granule staining consistent with the previously characterized CD68 positive staining (Dijkstra et al., 1985). The decidual CD68 positive cell clusters were observed in the entire length of the *decidua basalis* cross section. This was previously characterized with ED1 (CD68) positive staining in the *decidua basalis*, where the immunohistological CD68 staining was described as a diffuse brown layer containing only a few macrophages from GD15.5 to GD21.5 (van Oostveen et al., 1992). The distribution and abundance of CD68 positive cell clusters in the *decidua* and the upper portion of the mesometrial triangle places the use of CD68 as a reliable macrophage marker into question. The specificity of the ED1

mouse monoclonal antibody to recognize CD68 present in uterine macrophages is well established. Damoiseaux et al., (1994) elegantly demonstrated the specificity of the ED1 (CD68) monoclonal antibody for macrophages in different macrophage cell source isolates. In addition, to eliminate the possibility that the ED1 clone antibody was non-specific in the uterine tissue we used an alternate rabbit polyclonal anti-CD68. This alternative antibody produced similar patterns of staining as we previously observed (images not shown). The concern raised in this study is not the specificity of the CD68 (ED1) antibody or other CD68 antibodies to recognize CD68 positive macrophages but the possibility that cell types other than macrophages present in the rat uterine tissue may express CD68 or a glycoprotein similar to CD68. In support, the cell composition of the *decidua* has been described as highly immunogenic and to be composed of granulated metrial gland cells containing many glycoproteins and glycogen (Stewart & Peel, 1982). Therefore CD68 positive cells in the *decidua* may be misinterpreted as uterine macrophages. Therefore caution must be applied when using the CD68 as a marker for quantifying the macrophage population in the *decidua basalis* and in the extremities of the mesometrial triangle since overestimation of the actual macrophage population may occur.

As we further our knowledge of the immune cell population and corresponding cytokine localization in the rat uterine tissue, we will be better equipped at determining the function of these cytokines in regulating trophoblast invasion and spiral artery remodeling events. In addition, this knowledge will be beneficial in understanding pregnancy related pathologies in the rat pregnancy and potentially model some of the pathologies of human pregnancy complications.

Acknowledgments

We would like to thank Emily K. Hayes for all the animal care and processing of the utero-placental tissue and Christopher Patrick and Gen-Sheng Wang from the Dr. Fraser Scott lab for providing the rat gut cross sections (used as controls) and for their technical expertise.

Chapter 3

Maternal obesity is associated with a systemic and uterine pro-inflammatory state in the rat

Preface:

Chapter Three is a composition of unpublished and published data. For details on the authors' contribution please refer to Statement of contribution on page VI. The unpublished data includes sections 3.3.1 and 3.3.2 of the results. The unpublished data assesses the uterine immune state with the distribution analysis of TNFα and IL-10 in the rat mesometrial triangle and the quantitative analysis of TNFα, IL1β and IL-10 concentrations in the uterine tissue of the HFD-fed animals compared to CD-fed animals. The published data are shown in section 3.3.3 of the results in which we assessed the rat systemic immune state with the quantitative analysis of the levels of MCP-1 in maternal serum of the HFD-fed animals compared to CD-diet fed animals. These data have been published in the journal Reproductive Sciences.

Hayes, E. K., Tessier, D. R., Percival, M. E., Holloway, A. C., Petrik, J. J., Gruslin, A., & Raha, S. (2014). Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity. *Reproductive Sciences (Thousand Oaks, Calif.), 21*(5), 648-657. doi:10.1177/1933719113508815

The remaining data from this manuscript, examining the influence of obesity/HFD on utero-placental development are presented in Chapter Four.

3.1 Introduction

Aberrant utero-placental development is believed to be an important contributor to adverse maternal and fetal outcomes (Ball et al., 2006; Khong et al., 1986). However the causes for poor maternal and fetal health are not well defined. Altered maternal immune response associated with maternal obesity has emerged as a potential cause for such outcomes.

In humans, obesity is in part characterized by a low-grade chronic systemic inflammatory state (Curat et al., 2006; Fantuzzi, 2005; Friis et al., 2013; Li et al., 2008). This systemic inflammation is a result of increased levels of circulating pro-inflammatory cytokines secreted by excess adipose tissue. During the course of normal weight pregnancy increased pro-and anti-inflammatory cytokines have been reported (Christian & Porter, 2014; Winkler et al., 2002). However evidence suggests that a pro-inflammatory state increases as pregnancy progresses (Christian & Porter, 2014; Rusterholz et al., 2007). In combination, obesity and pregnancy have demonstrated even higher levels of pro-inflammatory cytokines (Christian & Porter, 2014; Friis et al., 2013). Furthermore, obese pregnant women have been observed to exhibit increased levels of circulating pro-inflammatory MCP-1 in the first trimester and second trimester (Friis et al., 2013; Madan et al., 2009). Obese pregnant women with an elevated systemic pro-inflammatory state have also demonstrated increased expression of pro-inflammatory cytokines in the uterine environment near term (Challier et al., 2008; Roberts et al., 2011). Multiple studies in humans have quantified the inflammatory state of the utero-placental tissue in humans near term however the inflammatory state associated to obesity in the first and second trimesters when the majority of utero-placental

development occurs is not well defined. In the rat, obesity associated systemic or utero-placental pro-inflammatory markers also remain largely unexplored. Interestingly, TNFα, IL-1β and IL-10, have demonstrated the potential to impact normal utero-placental development. More specifically, all three of these cytokines have been shown to modulate trophoblast invasion *in vitro* (Prutsch et al., 2012; Renaud et al., 2007). Of these cytokines, TNFα has demonstrated the capacity to inhibit migration/invasion of the human extravillous trophoblast cell line, HTR-8/SVneo (Huber et al., 2006; Renaud et al., 2007). In addition, placental macrophages from obese women have demonstrated significantly greater expression of TNFα mRNA near term (Challier et al., 2008). Moreover, TNFα is elevated in FGR pregnancies with placental insufficiency (Bartha et al., 2003). Maternal obesity is also associated with significantly elevated IL-1β mRNA expression in the placenta (Roberts et al., 2011). However, in contrast to TNFα, IL-1 β was shown to promote trophoblast migration (Prutsch et al., 2012), in part, through an up-regulation of matrix metalloproteinase (MMP) 2 and 9 (MMP-2, MMP-9) and urokinase type plasminogen activator (Karmakar & Das, 2002). The anti-inflammatory cytokine IL-10 has been shown to decrease the expression of both pro-inflammatory cytokines TNFα and IL-1β (Rivera et al., 1998) and can induce extravillous trophoblast invasion by indirect inhibition of TNFα secretion (Renaud et al., 2007). Collectively, these observations suggest that obesity induced disruption of cytokine expression patterns may have deleterious effects on trophoblast function and ultimately fetal development. Therefore, specific pro/anti-inflammatory cytokines in circulation and in the rat utero-placental tissue during the onset of trophoblast invasion (GD15) and peak of trophoblast

invasion (GD18) (Vercruysse et al., 2006) in the context of maternal obesity needs to be characterized and is the focus of this chapter.

3.2 Materials and Methods

3.2.1 Animals

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care (animal utilization protocol # 070740). These studies were conducted using a rat model of life-long maternal HFD, which has been previously described (Hayes et al., 2012). This model results in a significant increase in body weight prior to and during pregnancy, and the offspring of HFD-fed dams have decreased birth weights and an increased incidence of stillbirths (Hayes et al., 2012). In addition, the offspring also have reduced survival rates, an indicator of poor neonatal health.

Sprague-Dawley rats, aged 21 days (84-100g), were purchased from Charles River Laboratories (Wilmington, MA). Rats were maintained under controlled lighting (12-hr light – dark cycle) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned to receive either standard rat chow CD; 16% kcal fat, 3.82 kcal/g; Harlan Teklad, Madison, WI) or a purified HFD (45% kcal fat, 4.70 kcal/g; Research Diets, New Brunswick, NJ). Twenty dams were assigned to the CD group and 30 to the HFD group (due to a diet-induced reduction in fertility). Dams were maintained on their respective diets for 16 weeks before being mated with age-matched Sprague-Dawley males that were fed CD. Copulation was confirmed by visualization of sperm in a vaginal flush. The presence of sperm indicated day 0 of pregnancy (GD0). HFD- and CD-fed dams Blood sampling was performed by cardiac puncture prior to euthanasia. Utero-placental tissue was isolated by laparotomy at either GD15 (early trophoblast invasion) or GD18 (the peak of trophoblast invasion and completion of spiral

artery remodeling events) (Vercruysse et al., 2006). Two entire conceptuses from the second position closest to the cervix, in each uterine horn, were removed and preserved in 10% neutral buffered formalin for immunohistological analysis. The remaining placentas were removed from the uterus, and the underlying mesometrial triangles were excised, snap frozen in liquid nitrogen and stored at -80^{o}C for quantitative analysis.

3.2.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed on 5µm paraffin cross sections of the central area of the utero-placental unit (including placenta, *decidua* and mesometrial triangle) that were mounted on Superfrost Plus slides (Fisher Scientific, ON, Canada). Following standard dewaxing and rehydration, antigen retrieval was performed in a pressure cooker in citrate buffer (10 mM sodium citrate pH 6.0, 0.05% Tween-20). Nonspecific staining was blocked with 3% (v/v) H_2O_2 in PBS followed by non-immune blocking using DAKO blocking solution (DAKO, ON, Canada). Rabbit anti-rat TNFα (13.3 µg/mL) (Novus Biological ON, Canada) and rabbit anti-rat IL-10 (2 µg/mL) (Genway Biotech Inc., CA, U.S.A.) were used for IHC. Primary antibodies were diluted in 1% BSA-PBS buffer and incubated overnight at 4°C. PBS buffer containing 1% BSA was used as a negative control. Thorough washes were performed in PBS prior to application of anti-rabbit DAKO EnVision+ HRP-labeled polymer secondary antibodies (DAKO, ON, Canada). Positive antibody binding was detected with 3,3'-diaminobenzidine and sections were counterstained with Harris Hematoxylin (Sigma-Aldrich, ON, Canada), dehydrated, and mounted. One cross section of the central area of the utero-placental unit per animal, six animals (n=6) per diet group at each time point

(i.e., GD15 and GD18) were scanned at 20x magnification using the brightfield Scanscope and analyzed using Imagescope software (Aperio, Leica Biosystems, ON, Canada).

3.2.3 Semi-quantitative analysis of cytokine distribution

TNFα and IL-10 IHC images were analyzed using Image J software to assess positive staining within the entire mesometrial triangle and to facilitate visual assessment of specific regions of staining. (ImageJ, Version 1.37, NIH, Bethesda, MD, USA). Image analysis was performed from previously established methods (van der Avoort et al., 2007; Walschus et al., 2011) and modified for our tissue and cytokine distribution. In brief, images were analyzed by adjusting the color threshold, using red as the color threshold and red/green/blue as color space. The color threshold values were batch-dependent; CD-fed rats and HFD-fed rats were always matched within a batch and were analyzed using the same color threshold values. Normalization of background staining was performed by subtracting values from the batch-specific negative control. To focus on interstitial TNFα immune positive staining, TNFα images were modified by Adobe® Photoshop® (Adobe Systems Incorporated, U.S.A) to remove all intra-luminal spaces and inter-tissue gaps within the mesometrial triangle. In addition, these modifications were also necessary to accurately assess the actual mesometrial triangle total tissue surface area. Due to the diffuse localization of TNFα positive staining, the percentage of the surface area of the mesometrial triangle covered by TNFα was evaluated. Perivascular TNFα-positive staining was analyzed as the percentage of surface area of the perivascular regions covered by TNFα. The perivascular region corresponds to the interstitial tissue of

the mesometrial triangle areas within a 50μm perimeter of a maternal blood vessel. IL-10 demonstrated cell-specific staining; therefore, quantification was carried out by calculating the IL-10-positive cell counts divided by the surface area (mm^2) of the mesometrial triangle. All immunohistological analysis was performed by an investigator blinded to study groups.

3.2.4 Cytokine quantitative analysis

Quantitative analysis of TNFα, IL-1 β and IL-10 in uterine tissue was performed by immunoassay. Briefly, rat maternal decidual/mesometrial tissue was collected from HFD- and CD-fed rats at both GD15 and GD18. 100mg decidual/mesometrial tissue was thawed and washed in cold 1x PBS (9.1 mM dibasic sodium phosphate, 1.7mM monobasic sodium phosphate, 150mM NaCl, pH 7.4) to eliminate any residual serum. Washed tissue was transferred to 1mL lysis buffer (50mM Hepes, 150mM NaCl, 1mM Ethylenediaminetetraacetic acid, 10mM NaPPi, 1.5mM $MgCl_2$, 100mM NaF, 10% glycerol, 1% Triton X) containing an anti-protease cocktail (1mM phenylmethylsulfonyl fluoride, 10μg/mL aprotinin and 1mM sodium orthovanadate) and diced into minuscule pieces (Qiu et al., 2004). Homogenization of tissue was performed using Zirconia beads and Qiagen TissueLyser II® (Qiagen, Valencia, CA). Total protein was recovered from the supernatant after centrifugation at 10,000g for 10 minutes. DC Protein assay (BioRad, Hercules, CA) was used for total protein quantification. Quantification of cytokines of interest was performed using a specific TNFα, IL-1β and IL-10 custom Milliplex® Map Rat Cytokine Magnetic Bead Panel Immunology Multiplex Assay (EMD Millipore Billerica, MA, USA) according to manufacturer's specifications.

3.2.5 Monocyte Chemoattractant Protein 1 enzyme linked immunosorbent assay

Total MCP-1 in maternal serum, from CD and HFD-fed dams at GD15 and GD18 was quantified using a rat MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Serum samples were diluted 1:25 for the assay.

3.2.6 Statistical analysis

Statistical analysis was performed in SigmaPlot 11.0 (Systat Software Inc. San Jose, CA). TNFα and IL-10 distribution as well as TNFα, IL-1β, IL-10 and MCP-1 quantification were analyzed using a two-way analysis of variance (ANOVA) with diet and age as the independent variables. Bonferroni's post hoc testing was performed if a significant interaction effect of these variables was observed. Experiments with p values of <0.05 were considered to be statistically significant.

3.3 Results

3.3.1 TNFα and IL-10 distribution in the mesometrial triangle of CD and HFD fed rats at GD15 and GD18

Immunolocalization of TNFα staining in both diet groups at GD15 and GD18 demonstrated a diffuse distribution of TNFα throughout the mesometrial triangle and stronger staining in the perivascular regions of the uterine vessels (Figure 3.1.A-D). Semi-quantitative analysis revealed no differences in the total surface area covered by TNFα in the mesometrial triangle compared between the diet groups or between gestational ages (Figure 3.1.E). In addition, no differences were observed in the surface area covered by TNFα in the perivascular regions between diet and age groups (Figure 3.1.F).

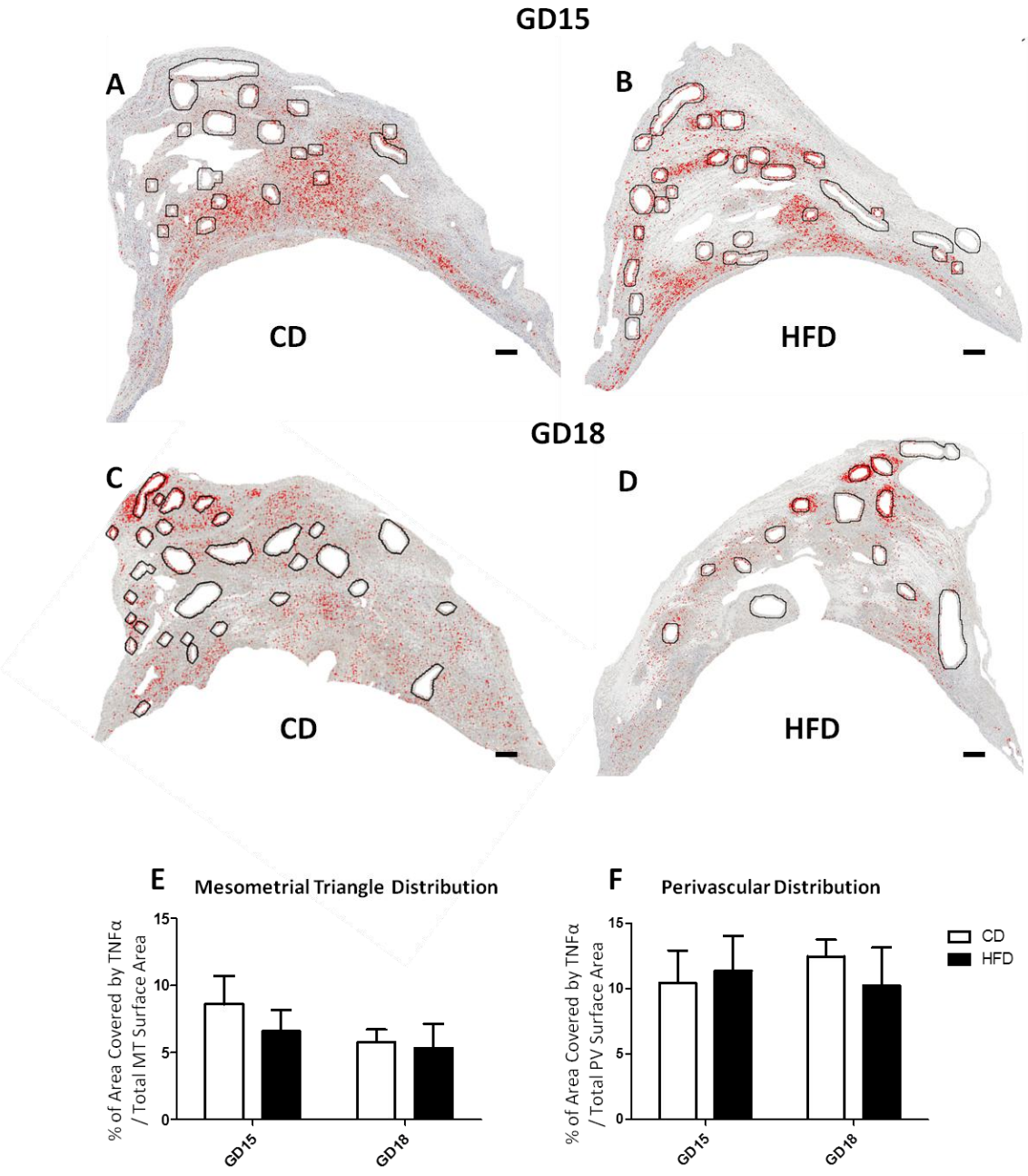

GD15
A
CD
B
HFD
GD18
C
CD
D
HFD
E
Mesometrial Triangle Distribution
% of Area Covered by TNFα / Total MT Surface Area
15
10
5
0
GD15
GD18
F
Perivascular Distribution
% of Area Covered by TNFα / Total PV Surface Area
15
10
5
0
GD15
GD18
CD
HFD

Figure 3.1 TNFα distribution in the mesometrial triangle of CD- and HFD-fed rats at GD15 and GD18. (A-B) Representative images of the rat mesometrial triangle at GD15 from CD and HFD respectively. (C-D) Representative images of the rat mesometrial triangle at GD18 from CD and HFD respectively. (A-D) IHC using anti-TNFα as primary antibody. Images have been modified to exclude intra luminal spaces and gaps in tissue in order to focus on TNFα positive staining of the interstitial space. For better visualization of TNFα localization Image J imaging software was used to enhance TNFα positive staining by adjusting color threshold (see methods for details). Scale bar for (A and B) = 250μm and for (C and D) = 400μm. (E-F) Graphical representation of the percentage of the surface area covered by TNFα positive staining in the mesometrial triangle (MT) and percentage of the surface area covered by TNFα positive staining in the perivascular (PV) regions (delineated in black) respectively. All data represent the mean +\- SEM (standard error of the mean). A total of six animals per group and one mesometrial triangle cross section per animal was analyzed (n=6). There was no main effect of either diet or gestational age or any interaction effect as determined by two-way ANOVA.

IL-10 immunostaining revealed similar localization patterns between the diet groups and gestational age groups. IL-10 positive cells were observed in the intraluminal space of the uterine vessels, in the mesometrial interstitial space and in the decidual tissue (Figure 3.2.A-H). As described in Chapter 2 a shift in IL-10 positive cells localization occurs from GD15 to GD18 in the CD-fed rats. At GD15 we observe a higher density of these cells in the decidua (Figure 3.2.A) while at GD18 higher density of these cells was observed within the interstitial regions of the mesometrial triangle (Figure 3.2.E). The semi-quantitative analysis demonstrated statistically significant differences in total numbers of IL-10 positive cells present in the mesometrial triangle between diet groups and between gestational ages (Figure 3.2.I). We observed a main effect of gestational age where the GD18 mesometrial triangle demonstrated a decreased number of IL-10 positive cells compared to GD15 ($p=0.013$). In addition we also observed a main effect of diet where HFD-fed rats demonstrated a lower number of IL-10 positive cells in the mesometrial triangle compared to CD-fed rats ($p=0.017$); there was no significant interaction effect.

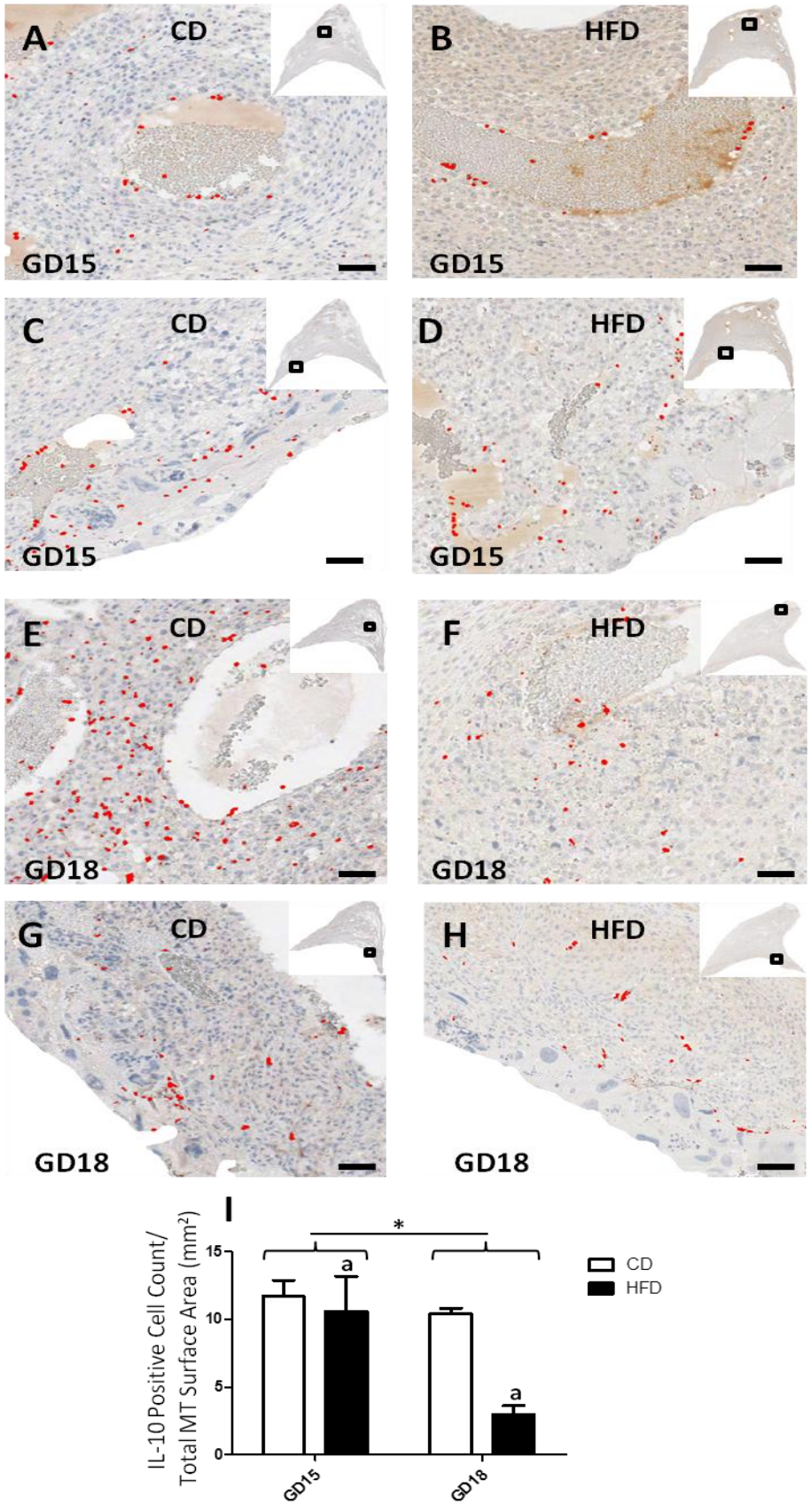

A
CD
GD15
B
HFD
GD15
C
CD
GD15
D
HFD
GD15
E
CD
GD18
F
HFD
GD18
G
CD
GD18
H
HFD
GD18
I
IL-10 Positive Cell Count/
Total MT Surface Area (mm²)
15
10
5
0
*
a
a
GD15
GD18
CD
HFD

Figure 3.2 IL-10 positive cell distribution in the mesometrial triangle of CD- and HFD-fed rats at GD15 and GD18. IHC of the mesometrial triangle (top right images) using anti-IL-10 as primary antibody. (A-D) are representative images of boxed areas of the rat mesometrial triangle at GD15. (A and C) are from CD and (B and D) are from HFD. (E-H) are representative images of boxed areas of the rat mesometrial triangle at GD18. (E and G) are from CD and (F and H) are from HFD. For better visualization Image J imaging software was used to enhance IL-10 positive cells by adjusting color threshold (see methods for details). Scale bar for (A-D) are 40μm and for (E-H) are 50μm. (I) Graphical representation of IL-10 positive cells counts in the entire mesometrial triangle divided by the total surface area (mm^2) of the mesometrial triangle (MT) at GD15 and GD18 from CD and HFD fed rats. All data represent the mean +\- SEM (standard error of the mean). A total of six animals per group and one mesometrial triangle cross section per animal was analyzed (n=6). There was a significant main effect of both diet (HFD <CD, (a) p=0.017) and gestational age (GD18<GD15, (*) p=0.013); however there was no significant interaction between diet and age (p=0.066).

3.3.2 TNFα, IL-1β and IL-10 quantification

There was a main effect of diet to alter TNFα levels in the decidual/mesometrial tissue; HFD-fed animals exhibited increased levels of TNFα compared to the CD-fed animals ($p=0.014$) (Figure 3.3.A). There was also a main effect of gestational age; higher levels of TNFα were present in decidual/mesometrial tissue at GD18 compared to GD15 ($p=0.002$). There was no significant interaction between diet and gestational age therefore we could not compare differences between the diet groups within a specific gestational age. There was no significant main effect of either diet or gestational age on levels of IL-1β. There was a significant interaction effect ($p=0.043$) (Figure 3.3.B) but in post-hoc testing there were no significant differences between groups (all $P>0.05$). There was no effect of diet, gestational age or an interaction on the levels of IL-10 (Figure 3.3.C).

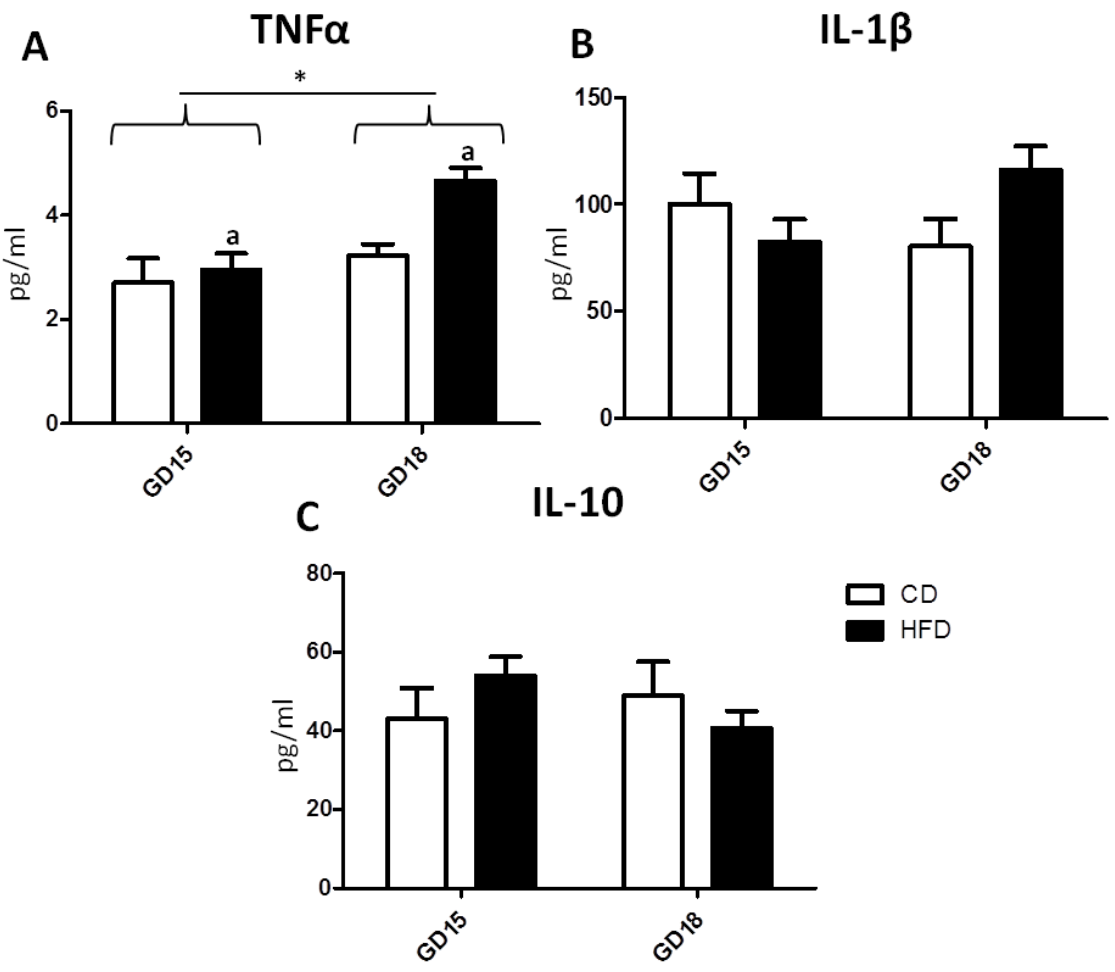


Figure 3.3 Quantification of TNFα, IL-1β and IL-10 in the uterine tissue of CD- and HFD-fed rats at GD15 and GD18. TNFα (A), IL-1β (B) and IL-10 (C) were quantified from total protein extracts of decidual/mesometrial tissue by EMD Millipore custom Multiplex immunoassay. Total protein from the uterine tissue was collected from a minimum of six animals per group and a maximum of eight (n=6 to 8). All data represent the mean +\- SEM (standard error of the mean). For TNFα, there was a significant main effect of both diet (HFD>CD, (a) p=0.014) and gestational age (GD18>GD15, (*) p=0.002); however there was no significant interaction between diet and gestational age (p=0.07). There was no effect of diet or gestational age on the levels of IL-1β. There was a significant interaction effect (p=0.043); however post-hoc testing did not identify any significant differences between the groups (all P>0.05). IL-10 levels were not different between any treatment groups (diet p=0.845, gestational age p=0.594, interaction p=0.182).

3.3.3 HFD-Fed Dams Show Evidence of Systemic Inflammation During Pregnancy

Since increased inflammatory stimuli are known to impact trophoblast invasion (Jovanovic & Vicovac, 2009; Jovanovic et al., 2010) we assessed levels of MCP-1, as a marker of systemic inflammation, in serum from CD- and HFD-fed dams (Figure 3.4). At GD15, MCP-1 levels were 1.4-fold higher in plasma from HFD-fed dams, while at GD18 MCP-1 levels were 1.7-fold higher in plasma from HFD-fed dams (all $P<0.01$).

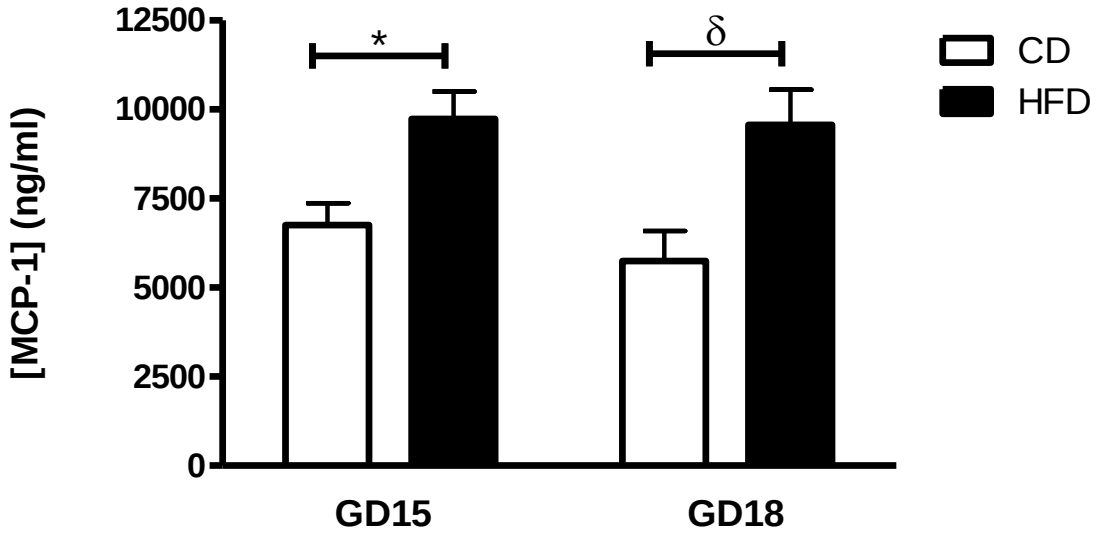


Figure 3.4 MCP-1 is elevated in the serum of HFD-fed dams at GD15 and GD18. MCP-1 was measured using a commercially available ELISA according to the manufacturer's instructions. ($p<0.01$) by two-way ANOVA. *=significantly different from GD15 CD-fed group; δ=significantly different from GD18 CD-fed group.

3.4 Discussion

As obesity trends continue to increase so do maternal obesity associated pregnancy complications such as FGR and fetal demise. Emerging evidence suggests that altered local and/or systemic pro-inflammatory cytokine production may form a link between maternal obesity, adverse utero-placental development and adverse pregnancy outcomes. For example independent studies demonstrate increased incidence of FGR pregnancy outcomes from obese women (Radulescu et al., 2013), and increased placental inflammation in obese pregnancy (Challier et al., 2008) and in FGR pregnancies with placental insufficiency (Bartha et al., 2003). Pro-inflammatory cytokines can inhibit trophoblast invasion (Huber et al., 2006; Renaud et al., 2007) and reduced trophoblast invasion is observed in FGR pregnancies (Khong et al., 1986). Modulatory cytokines TNFα, IL-1β and IL-10 relevant to utero-placental development have been shown to be involved in the regulation of trophoblast invasion (Huber et al., 2006; Prutsch et al., 2012; Renaud et al., 2007). However no studies have directly demonstrated maternal obesity induced increase uterine inflammatory cytokines in association with FGR pregnancy outcomes. In our study we focused on TNFα, IL-1β and IL-10 since evidence suggests a role of these cytokines in regulating trophoblast invasion (Huber et al., 2006; Otun et al., 2011; Prutsch et al., 2012; Renaud et al., 2007). Although the levels of other cytokines such as IL-18, IL-6 and IFNγ have demonstrated to be altered in obese non-pregnant individuals and/or during healthy pregnancy (Challier et al., 2008; Christian & Porter, 2014; Zhu et al., 2010), they have yet to be characterized in obese pregnancy. In addition, their role in regulating trophoblast invasion remains to be determined (Champion et al., 2012; Otun et al., 2011).

In our studies, we demonstrate that the HFD-fed animals had a significantly higher intrauterine TNFα concentration. This increased concentration however did not appear to influence the distribution of TNFα-positive cells within the utero-placental tissue. Comparable to humans, increased expression of TNFα mRNA was also observed from macrophages isolated from term placenta tissue of obese women (Challier et al., 2008). Our high fat diet induced rat model therefore demonstrates an increased uterine inflammatory environment similar to that observed in obese pregnant women. In addition we also demonstrated a significant increase in TNFα levels in the uterine tissue with increasing gestational age. In humans longitudinal studies of normal human serum during pregnancy have also demonstrated increased TNFα concentrations as pregnancy progresses (Christian & Porter, 2014; F. M. Stewart et al., 2007; Winkler et al., 2002).

IL-1β quantification demonstrated a significant interaction effect suggesting that IL-1β levels were affected by both diet and gestational age but with post-hoc testing there were no statistically significant differences between individual groups. Therefore obesity or gestational age does not appear to influence IL-1β levels in the rat uterine environment.

The immunoregulatory role of IL-10 within the uterine environment has the potential to regulate the levels of TNFα (Royle et al., 2009). In our study IL-10 concentrations within the mesometrial triangle were not influenced by diet or gestational age. In contrast we did observe a main effect of diet and gestational age on the number of IL-10-positive cells in the mesometrial triangle; where we observed a decreased number of IL-10-positive cells at GD18 compared to GD15, and a decreased number of IL-10-positive cells in the HFD-fed animals compared to the CD-fed animals. These

results indicate that both advancing gestational age and HFD contribute towards decreasing the number of IL-10 positive cells in the mesometrial triangle. Furthermore these results suggest that the fewer IL-10 cells in the mesometrial triangle in the HFD-fed animals and in late gestation are producing more IL-10 however to fully characterize this further investigation is required. Interestingly, the reduced IL-10 positive cells did coincide with increased TNFα levels in late gestation and in the HFD-fed animals. As described above IL-10 has been shown to inhibit macrophage activation and production of TNFα and indirectly reduce trophoblast invasion *in vitro* (Renaud et al., 2007). Whether fewer IL-10-positive cells could have an impact on the regulation of the immune balance and/or TNFα levels in specific regions of the mesometrial triangle remains to be determined.

Adipose tissue of obese individuals has also demonstrated increased secretion of MCP-1 (Dahlman et al., 2005). We have confirmed in our rat model that obesity during pregnancy significantly increases systemic levels of the pro-inflammatory cytokine MCP-1. While we did not directly quantify MCP-1 in utero-placental tissue, one study has reported significantly elevated levels of placental MCP-1 in early third trimester of a non-human primate model of maternal obesity (Frias et al., 2011). In addition, we also attempted to quantify the levels of TNFα in maternal circulation however these levels in the HFD- and CD-fed rats were below our assays detectable levels (data not shown). Interestingly, leukocyte-free first trimester decidual cells increased expression and secretion of MCP-1 in response to TNFα treatment (Lockwood et al., 2006). Therefore the increased circulating MCP-1 concentrations may be a result of increased TNFα observed in our obese rat model and in obese pregnant women (Friis et al., 2013).

Furthermore the elevated circulating MCP-1 may potentially be used as an indicator of altered utero-placental immune response and poor fetal development in the first trimester.

In conclusion, we have made progress in defining the influence of maternal obesity on the rat systemic and utero-placental immune states. However, additional research is required if we are to link these obesity associated altered immune states as a potential cause for perinatal complications. The importance of these studies is evident since obesity represents a leading cause of perinatal morbidity and mortality (Gardosi et al., 2009).

Chapter 4

Trophoblast Invasion and Blood Vessel Remodeling Are Altered in a Rat Model of Lifelong Maternal Obesity

Preface:

Chapter Four is a composition of currently published data and additional data currently unpublished. For details on the authors' contribution please refer to Statement of contribution on page VI. The published data are shown in section 4.3.2 to 4.3.4 of the results. These published data are presented as in the manuscript accepted to the journal Reproductive Sciences.

Hayes, E. K., Tessier, D. R., Percival, M. E., Holloway, A. C., Petrik, J. J., Gruslin, A., & Raha, S. (2014). Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity. *Reproductive Sciences (Thousand Oaks, Calif.), 21*(5), 648-657. doi:10.1177/1933719113508815

The additional unpublished data are shown in section 4.3.1 and 4.3.5 of the results, in which we assessed the influence of maternal obesity on rat utero-placental morphology and on *in vitro* human trophoblast invasion, respectively.

4.1 Introduction

Obese women have an increased risk of a number of pregnancy complications, including preeclampsia, miscarriage, stillbirth, as well as delivering both large for gestational age and small for gestational age babies (Norman & Reynolds, 2011; Perlow et al., 1992; Rajasingam et al., 2009). Small for gestational age or growth restricted fetuses are at higher risk of fetal demise, poor neonatal health and adult onset diseases (Wu et al., 2012). At this time, the mechanisms involved in the development of these complications in the context of maternal obesity are not well understood. Utilizing a previously characterized rat model of lifelong maternal obesity, which mimics many of the adverse pregnancy outcomes, observed in human populations, we can begin to elucidate the link between obesity and these pregnancy complications. The adverse outcomes in our animal model include increased fetal death and impaired fetal growth in association with altered placental development (Hayes et al., 2012).

Altered trophoblast invasion and aberrant spiral artery remodeling have been implicated in many of the pregnancy complications associated with obesity, including preeclampsia, fetal growth restriction, and stillbirth (Ball et al., 2006; Khong et al., 1986). Trophoblast cells are stimulated to invade into the maternal *decidua*/myometrium through a complex interaction of cytokines, growth factors and other signaling molecules in the local environment (Knofler, 2010; Tanaka et al., 2007). In the rat, the onset of such trophoblast invasion occurs at approximately GD15 and peaks at approximately GD18 (Vercruysse et al., 2006). The extent of this invasion into the mesometrial compartment follows a similar pattern as that observed in the human myometrium (Soares, et al. 2012a), making the rat an ideal model in which to study this process. The invasive

trophoblasts migrate/invade into maternal uterine tissue to reach the spiral arteries through either counter current intraluminal (achieved by ENVT) or uterine interstitial (achieved by IST) migratory pathways and contribute to the remodeling of these arteries (Cartwright et al., 2002; Wallace et al., 2012). This remodeling process is essential for converting the spiral arteries into high-capacity, low resistance vessels to increase blood supply to the placenta (Pijnenborg et al., 2006). In addition to trophoblasts, recent evidence demonstrates that uNK cells are important for the remodeling process (Robson et al., 2012). In normal pregnancy the progressive disruption of the smooth muscle cell layer surrounding the spiral arteries is an important component in the remodeling of these arteries. Although it is accepted that the failure to disrupt the VSMC layer surrounding the spiral arteries can lead to a number of pregnancy related pathologies (Helwig & Le Bouteiller, 2007), however the mechanisms by which this process may be affected in obese pregnancies is not well understood. Importantly, the disorganization of the smooth muscle cell layer surrounding the spiral arteries plays an important role in the remodeling of these arteries in both rats and humans (Soares et al., 2012a).

Obesity is associated with a state of chronic systemic inflammation (Schmatz et al., 2010). Obesity during pregnancy can contribute to altered levels of circulating pro-inflammatory cytokines in the maternal circulation as well as in the utero-placental tissues (Roberts et al., 2011). Interestingly, *in vitro* trophoblast invasion has been shown to be affected by a number of these pro-inflammatory cytokines. Some attenuate invasion (e.g., TNFα, 8-isoprostaglandin F2α) and others increase invasion (e.g., interleukin 6 (IL-6) and IL-8) (Bauer et al., 2004; Jovanovic & Vicovac, 2009; Jovanovic et al., 2010; Pavan et al., 2004; Staff et al., 2000). In addition, adipocyte-derived factors

have been shown to affect VSMC proliferation and inflammation (Nguyen Dinh Cat et al., 2011). Though experiments detailing the effects of individual cytokines on these processes have been documented *in vitro*, the effects of obesity on trophoblast invasion and spiral remodeling have yet to be determined *in vivo.*

Therefore the goal of these experiments was to assess changes, trophoblast invasion and vascular smooth muscle remodeling associated with maternal obesity in the established rat model of life-long obesity (Hayes et al., 2012).

4.2 Material and Methods

4.2.1 Animals

All animal procedures for this study were approved by the McMaster University Animal Research Ethics Board (AUP # 070740). Fifty female Sprague-Dawley rats, aged 21 days (84-100g), were purchased from Charles River Laboratories (Wilmington, MA). Rats were maintained under controlled lighting (12-hr light – dark cycle) and temperature (22°C) with *ad libitum* access to food and water. Dams were randomly assigned to receive either standard rat chow (CD) (16% kcal fat, 3.82 kcal/g; Harlan Teklad, Madison, WI) or a HFD (45% kcal fat, 4.70 kcal/g; Research Diets, New Brunswick, NJ). Twenty dams were assigned to the CD group and thirty to the HFD-fed (due to a diet induced reduction in fertility (Hayes et al., 2012)). Dams were maintained on their respective diets for 16 weeks before being mated with age-matched Sprague-Dawley males fed the CD diet. One female and one male per cage were paired until copulation was confirmed by visualization of sperm in a vaginal flush. The presence of sperm indicated day 0 of pregnancy GD0. HFD and CD-fed dams underwent laparotomy at either GD15 (early trophoblast invasion) or GD18 (invasion completed, spiral artery remodeling completed). These time points were selected based on the work of Pijnenborg and colleagues who demonstrated that the presence of invasive endovascular trophoblasts in the rat are observed at GD15 (Caluwaerts et al., 2005) while maximal invasion of interstitial trophoblasts can be seen at approximately GD18 (Vercruysse et al., 2006). Two entire conceptuses from the second position from the cervix, in each uterine horn, were removed and preserved in 10% neutral buffered formalin for immunohistological

analysis. The remaining placentas were removed from the uterus, and the underlying mesometrial triangles were excised and frozen at -80^{o} C for Western blot analysis.

4.2.2 Morphometric analysis

The morphometric analysis was performed on the middle cross section of the utero-placental unit as determined by the longest width measured from the chorionic plate to the basal plate. Standard hematoxylin and eosin (HE) staining was performed to visualize the placental cells and zones. Slide images were captured with the ScanScope™ by Aperio. Measurements and analysis were done with ImageScope™ software by Aperio. The specific zones, labyrinth, junctional zone and *decidua* of the utero-placental unit were identified and their surface areas were measured as previously described (Dokras et al., 2006). To eliminate any biased measurements the embedded conceptuses from the HFD and CD groups were randomly numbered. Once the analysis was completed the identity of the cross section was revealed and matched to its respective group. Data are expressed as mean +/- SEM.

4.2.3 Immunohistochemistry

Placental tissue was formalin-fixed and paraffin embedded. Placentas were embedded upright with the fetal side of the placental disc perpendicular to the cross-sectional plane. 5µm sections from the central area of the placenta containing a main spiral artery were cut and mounted on Superfrost Plus microslides (VWR, Mississauga, ON). Immunohistochemistry was performed to determine localization and expression of the protein or antigen of interest within placental and uterine tissue. Slides were

deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in methanol for 15 minutes at room temperature. For smooth muscle actin (SMA) antigen retrieval was achieved by immersing slides in 10 mM citrate buffer at 90^{o}C for 12 minutes. Antigen retrieval for cytokeratin analysis was achieved by microwaving slides just until boiling in a commercial citrate-based antigen unmasking solution (Vector Laboratories, Burlingame, CA),. Tissues were blocked using 1% (wt/vol) BSA with 10% (vol/vol) normal donkey serum for 1 hour at room temperature followed by incubation with anti-pan cytokeratin (a trophoblast marker; 1:300 dilution; Sigma-Aldrich, St Louis, MO), or anti-α-SMA (a vascular smooth muscle cell marker; 1:200 dilution; Sigma-Aldrich, St Louis, MO) overnight at 4ºC in a humidified chamber. Sections were washed in PBS and immunostaining was identified using anti-mouse biotinylated secondary antibody (Millipore, Billerica, MA) and the Vectastain kit (Vector Laboratories, Burlingame, CA) with diaminobenzadine (Sigma-Aldrich Canada Ltd., Oakville, ON) as the chromogen. Tissue sections were counterstained with Harris's hematoxylin, (Sigma-Aldrich, St Louis, MO), dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

4.2.4 Analysis of interstitial trophoblast invasion

Analysis of interstitial trophoblast invasion was carried out on cytokeratin-stained sections containing a cross-section of the central maternal artery by a single investigator who was blinded to the treatment groups, as previously described (Rosario et al., 2008; Vercruysse et al., 2006). The entire mesometrial triangle was scanned at 4X magnification and cytokeratin-positive cells within the mesometrial triangle were

selected using a threshold analysis after elimination of non-specific binding. The area covered by the mesometrial triangle was manually delineated and quantified and the extent of IST invasion (i.e., percentage of the mesometrial triangle with cytokeratin-positive cells) was reported as a percentage of the total area of the mesometrial triangle.

4.2.5 Analysis of endovascular trophoblast invasion

Analysis of endovascular trophoblast invasion was carried out on sections containing a cross-section of the central maternal artery, as previously described (Geusens et al., 2008). The entire mesometrial triangle was imaged at 4X magnification, and the arteries within the mesometrial triangle were counted and classified as 1) fully invaded; 2) partially invaded or 3) not invaded. Values were reported as the percentage of arteries in the mesometrial triangle that were invaded by endovascular trophoblasts (Geusens et al., 2008; Geusens et al., 2010; Verlohren et al., 2010).

4.2.6 Analysis of spiral artery remodeling

Pijnenborg and colleagues reported that spiral artery remodeling occurs primarily between gestational day 17 and 22 in rats (Caluwaerts et al., 2005). Therefore, we evaluated maternal spiral artery remodeling at GD18 on fetal-placental unit cross-sections containing a central maternal artery, as previously described in (Verlohren et al., 2010). Serial tissue sections were stained for α-SMA (Sigma-Aldrich, St. Louis MO) and scanned using the 4x objective. Spiral arteries in adjacent tissue sections were identified and analyzed for the presence of endovascular trophoblasts and SMA-positive cells.

The length of the blood vessel contour with SMA-positive cells was traced and quantified using NIS Elements Software (Nikon Canada, Mississauga ON) and the percentage of SMA coverage for each invaded (full and partially invaded) artery in the mesometrial triangle was calculated based on the total contour length of the same vessels. A minimum of three arteries per dam were quantified and the values were averaged to obtain the amount of SMA coverage for each animal in the CD and HFD groups.

4.2.7 Western blotting

Decidual tissue samples were homogenized in a 1:20 ratio of tissue to homogenization buffer (5 mM HEPES, 100 mM KCl, 70 mM sucrose, 220 mM mannitol, 1 mM EGTA; pH 7.2) containing protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis IN). Levels of MMP2 and MMP9 were quantified using Western blotting, as previously described (Robertshaw et al., 2008). Briefly, 10 µg of decidual homogenate was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA (MMP2; Roche, Indianapolis, IN) or 5% milk (MMP9) in Tris buffered saline (TBST: 137 mM NaCl, 2.7 mM KCl, 25 mM Tris-Cl, pH 8.0) supplemented with 0.1% Tween-20 overnight at 4°C. Membranes were then incubated with anti-MMP2 (1:5000 in 3% BSA in TBST; Abcam Inc, Cambridge, MA), or anti-MMP9 (1:20 000 in 3% milk in TBST; Abcam Inc, Cambridge, MA) for 2 hours and washed with TBST before incubation with anti-mouse IgG secondary antibody (1:5000; GE Healthcare, Mississauga ON) in 3% BSA or 3% milk in TBST. Blots were developed using enhanced chemiluminescence (Millipore, Billerica, MA) and

densitometric quantification was carried out using ImageJ software (ImageJ, Version 1.37, NIH, Bethesda, MD, USA).

4.2.8 Human Serum Samples

Banked first-trimester serum samples were obtained from a cohort of women receiving care between October 2002 and April 2009 at The Ottawa Hospital or Kingston General Hospital (Ottawa and Kingston [OaK] Birth Cohort) (Walker et al., 2011). Patient groups included: 1) obese women (BMI >30 kg/m^2) who delivered an average for gestational age (AGA) (>10th and <90th centiles) infant; 2) obese women who delivered an infant with FGR (<5th centile); 3) women with a normal (18.5 to 24.9 kg/m^2) BMI who delivered an AGA infant; and 4) women with a normal BMI who delivered an FGR infant (Table 1). Additional serum sample selection criteria were matched based on gestational age at serum sample collection as well as maternal age. In addition women in all groups were non-smokers, non-diabetic, had no prior history of FGR, pre-eclampsia or hypertension, and no history of stillbirth. In addition, there were no fetal morphological or chromosomal abnormalities reported in any groups.

Table 4.1 Serum sample data categorized according to maternal BMI and fetal outcome.

Maternal Weight Category	Fetal Outcome	Maternal BMI (Kg/m^2) (Range)	Mean BMI (Kg/m^2)	Mean Maternal Age (years)	Mean Gestational Age of Sampling (weeks)	Mean Fetal Weight (Kg)
Normal BMI	AGA	20.6-21.4	21.0 (0.16)	34 (1.86)	12.7 (0.22)	3.740 (0.099)
Normal BMI	FGR	20.9-21.2	21.0 (0.08)	31 (3.22)	12.4 (0.22)	2.437 [b] (0.264)
Obese BMI	AGA	29.7-36.3	32.0 [a] (1.56)	33 (2.74)	13.0 (0.34)	3.614 (0.151)
Obese BMI	FGR	31.8-34.3	32.9 [a] (0.53)	31 (2.83)	12.9 (0.27)	2.505 [b] (0.151)

Banked maternal serum was collected as part of the Ottawa and Kingston cohort (OaK Study)
Table values represented as mean +/- (SEM) from pooled serum samples of four women per group (n=4)
BMI: Body mass index, AGA: Average for gestational, and FGR: Fetal growth restriction
Statistical analysis was performed using two-way ANOVA grouped analysis using maternal BMI, maternal and fetal weight at birth as variables. Statistical significance was observed between; 1) maternal weight categories, normal BMI versus obese BMI, demonstrated by [a] and represented $P<0.0001$ and 2) fetal weight at birth, AGA versus FGR, demonstrated by [b] and represented $P<0.0001$.

4.2.9 In vitro human trophoblast invasion assay

For these experiments, the human HTR-8/SVneo trophoblast cell line was used (Graham et al., 1993). HTR-8/SVneo cells were maintained in culture with RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 5% fetal bovine serum. To examine the effects of human first-trimester serum on trophoblast invasion, BD BioCoat™ Matrigel™ invasion chambers were used according to the manufacturer's instructions. Briefly, trophoblast cells were seeded (25,000 cells/well) into transwell inserts (6.5-mm diameter polycarbonate membrane, 8-μm pore; Corning Costar, Lowell, MA) that were pre-coated with Matrigel (1 mg/mL; BD Biosciences, San Jose, CA); these were then placed into 24-well plates. The cells were allowed to invade through the reconstituted extracellular matrix for 24 h in the presence of RPMI 1640 medium supplemented with 0.5% maternal serum in the inner chamber and 5% maternal serum in the lower chamber. Invasion assays were repeated in five independent experiments. In each experiment, sera from four women from each group were pooled to normalize any inter-donor variability as previous described (Harris et al., 2009) (Table 1). Trophoblast cells located on the undersurface of the transwell membrane were fixed with ice-cold methanol and stained with hematoxylin. Brightfield images were obtained with an upright Zeiss Axioskop 40 microscope (Carl Zeiss Canada Ltd., ON, Canada). The number of invaded cells per membrane were counted using Image J freeware (National Institutes of Health). All cell counts were performed by one investigator who was blinded to the study group. To correct for variations between individual assays, invaded cell counts from each group were normalized to the average number of invaded cells of all four groups combined from that assay.

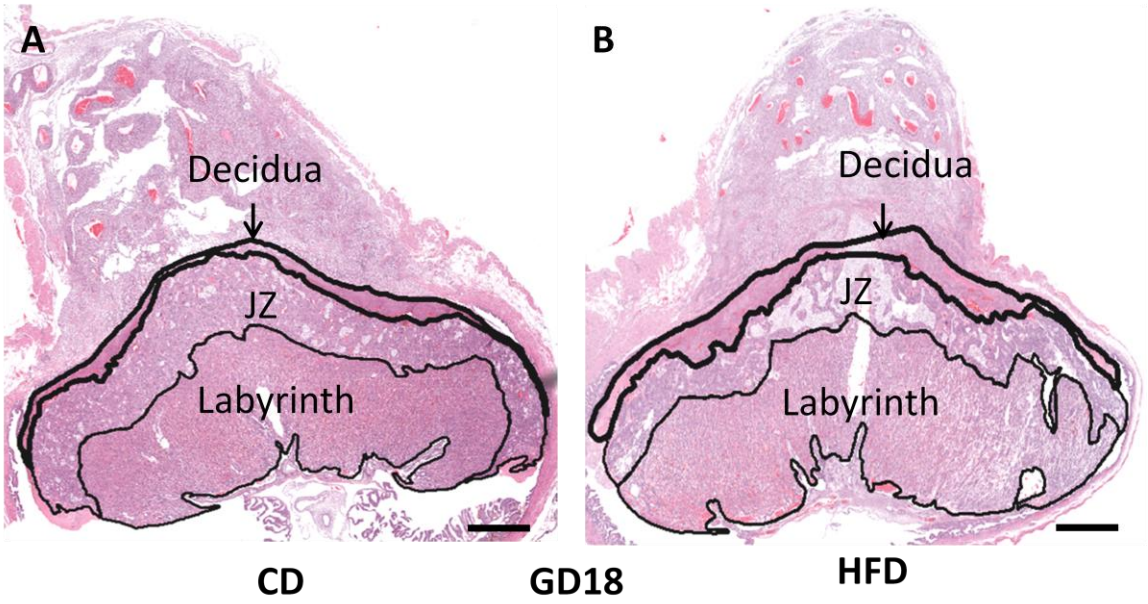
4.2.10 Statistical analysis

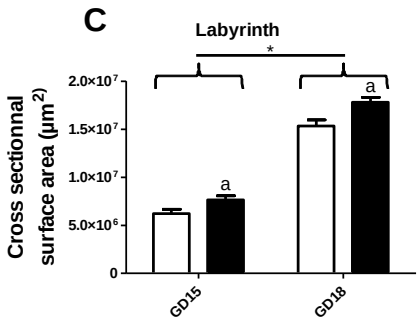
Statistical analyses were performed in GraphPad Prism v4.0 (GraphPad Software Inc, La Jolla, California). The morphometric analysis of the cross sectional surface of each utero-placental zone, IST invasion, and ENVT invasion were analyzed using a two-way ANOVA with diet and age as the independent variables; Bonferroni's post hoc testing was performed if a significant interaction effect of these variables was observed. The extents of spiral artery remodeling and Western blot data were analyzed using a 2-tailed Student t test. Statistics were performed using each dam as a single statistical unit. Results with P values of <0.05 were considered to be statistically significant. For invasion assays normalized cell counts were analyzed using two-way ANOVA, using maternal BMI and fetal outcomes (i.e., AGA or FGR) as independent variables. Bonferroni's post-hoc testing was performed if a significant interaction effect of these variables was observed. Outcomes with p values of <0.05 were considered to be statistically significant.

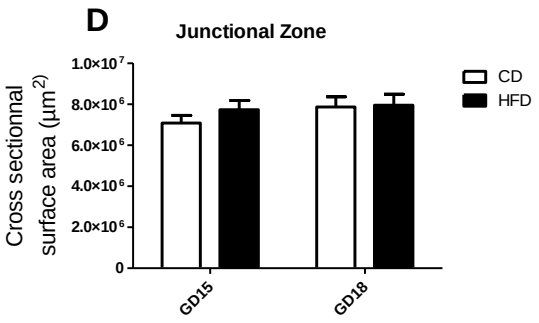
4.3 Results

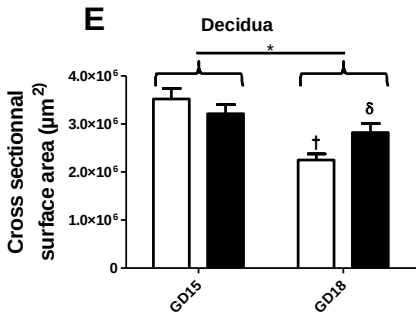
4.3.1 Altered utero-placental cross sectional surface areas are observed in HFD-fed animals

The morphometric analysis demonstrated a main effect of diet and gestational age on the cross sectional surface area of the labyrinth and the cross sectional surface area of all zones combined; HFD-fed animals exhibited increased cross sectional surface area compared to the CD-fed animals ($p<0.05$) and increased cross sectional surface area at GD18 compared to GD15 ($p<0.05$) (Figure 4.1.C and Figure 4.1.F). There was no significant interaction between diet and gestational age for the labyrinth zone or all zones combined. There was no significant main effect or interaction of either diet or gestational age on the cross sectional surface area of the junctional zone (Figure 4.1.D). There was a significant main effect of gestational age on the cross sectional surface area of the decidual zone; decreased cross sectional surface area at GD18 was observed compared to GD15 ($p<0.001$) (Figure 4.1.E). There was also significant interaction between diet and gestational age for the decidual zone; decreased cross sectional surface area in the CD-fed animals at GD18 compared to CD-fed animals at GD15 ($p<0.001$) and increased cross sectional surface area in the HFD-fed animals at GD18 compared to CD-fed animals at GD18 ($p<0.037$) (Figure 4.1.E).


A
Decidua
JZ
Labyrinth
B
Decidua
JZ
Labyrinth
CD
GD18
HFD


C
Labyrinth
Cross sectionnal surface area (µm2)
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
*
a
a
GD15
GD18


D
Junctional Zone
Cross sectionnal surface area (µm2)
1.0×10^7
8.0×10^6
6.0×10^6
4.0×10^6
2.0×10^6
0
CD
HFD
GD15
GD18


E
Decidua
Cross sectionnal surface area (µm2)
4.0×10^6
3.0×10^6
2.0×10^6
1.0×10^6
0
*
†
δ
GD15
GD18

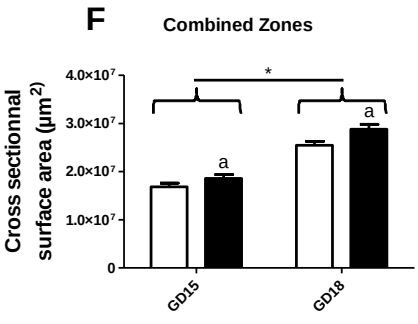

F
Combined Zones
Cross sectionnal surface area (µm2)
4.0×10^7
3.0×10^7
2.0×10^7
1.0×10^7
0
*
a
a
GD15
GD18

Figure 4.1 Morphometric analysis of the utero-placental zones at GD15 and GD18. (A-B) Representative images of a cross section at GD18, (A) CD (B) HFD. Scale bars for (A-B) are 600µm. (C-F) Graphical representation of the utero-placental zones' cross sectional surface areas. Utero-placental zones include (C) labyrinth, (D) junctional zone (JZ), (E) *decidua basalis* and (F) all three zones combined. All data are expressed as the mean +/- SEM. Minimum of 13 and maximum of 18 utero-placental units per group were analyzed by two-way ANOVA; P values were calculated; (*) $p<0.05$ and represents GD15 < GD18; (a) $p<0.05$ and represents HFD-fed group > CD-fed group; (†) $p<0.05$ represents GD18 CD-fed group < GD15 CD-fed group; (δ) $p<0.05$ represents GD18 HFD-fed group > GD18 CD-fed group.

4.3.2 Invasion of interstitial and endovascular trophoblast cells is increased early in gestation in HFD-fed dams

We assessed trophoblast invasion through both the IST and ENVT pathways at GD15 and GD18. GD15 is thought to represent the initial stages of invasion (Caluwaerts et al., 2005) and we observed approximately two fold greater invasion by IST cells at this stage in implantation sites from HFD-fed dams (Figure 4.2; $P<0.05$) compared to the CD-fed dams. When comparing the progression of invasion from GD15 to GD18 in CD-fed dams, the mesometrial triangle of GD18 dams exhibited approximately 43% greater cross sectional surface area coverage by interstitial trophoblasts ($P<0.05$). However, when comparing GD15 to GD18 HFD-fed dams, the mesometrial triangle of GD18 dams exhibited only approximately 26% more cross sectional surface area coverage. Interestingly, at GD18, the peak of the trophoblast invasion process, HFD-fed dams had reduced cross-sectional surface area relative to CD-fed dams ($P<0.05$).

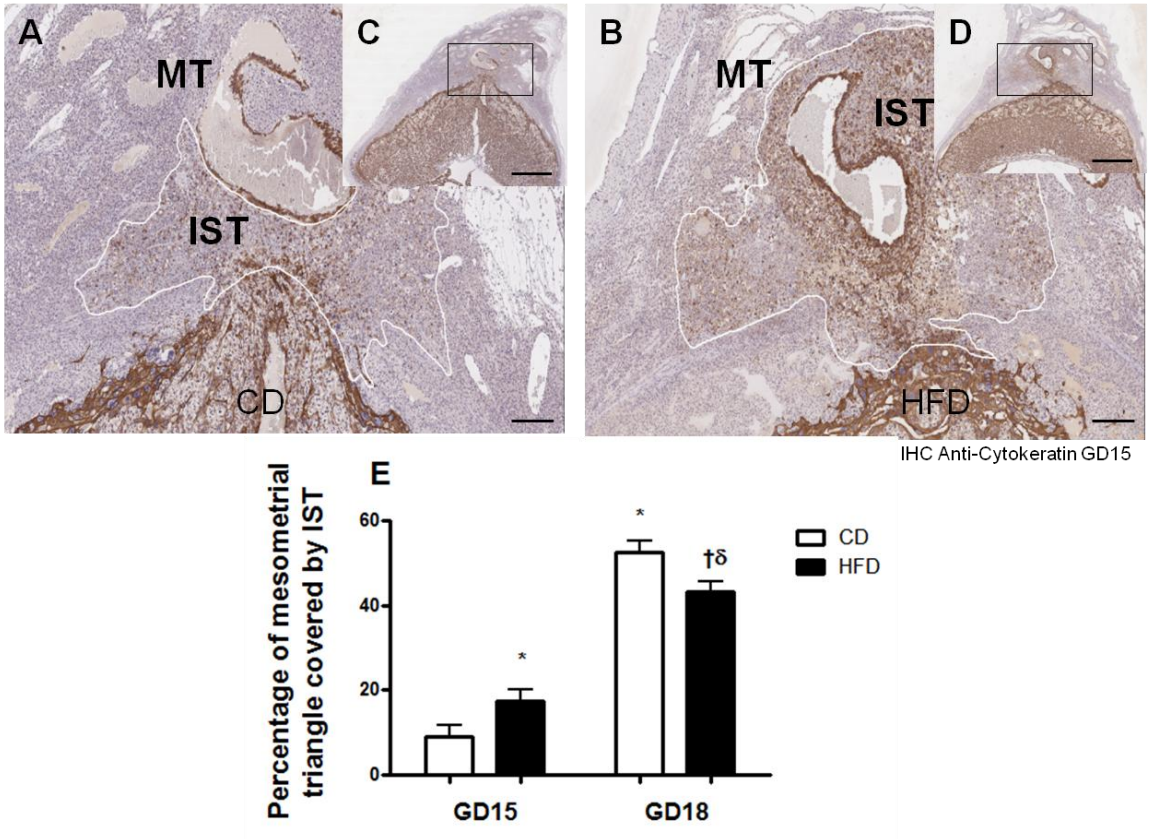


Figure 4.2 Percentage of cross sectional surface area covered by interstitial trophoblast cells in the mesometrial triangle is increased at GD15 and reduced at GD18 in HFD-fed dams. Central slides (5 µm thick) were stained with anti-pan cytokeratin. Cytokeratin staining (brown) indicates the presence of trophoblast cells. IST = interstitial trophoblast, MT = mesometrial triangle. Representative images of GD15 utero-placental unit from CD-fed (C) and HFD-fed dams (D) are shown; scale bar = 600 µm. (A and B) are magnified images of boxed region of (C and D) respectively; scale bar = 150 µm. (E), Graphical representation of the percentage of the mesometrial triangle invaded by interstitial trophoblast cells at GD15 and GD18. ($P<0.05$) by two-way ANOVA; *=significantly different from GD15 CD-fed group; †=significantly different from HFD-fed GD15 group; δ=significantly different from CD-fed GD18 group; one implantation site was examined per dam, with a minimum of 5 dams per group.

Trophoblast invasion through the endovascular pathway was also quantified by assessing the percentage of blood vessels in the mesometrial triangle invaded either partially or fully by endovascular trophoblast cells. Examples of fully, partially and invaded blood vessels within the mesometrial triangle are illustrated in Figures 4.3.A and 4.3.B and compared to blood vessels which have not been invaded (Figure 4.3.C). Analysis by two-way ANOVA demonstrated that the presence of ENVT in spiral arteries was significantly altered by gestational age (main effect: $P<0.05$) but there was no main effect of diet ($P>0.05$). There was, however, a significant interaction between gestational age and diet. The percentage of vessels invaded by ENVT was more than 2-fold higher in implantation sites from HFD-fed dams compared to CD-fed dams (Figure 4.3.E; $P<0.05$) at GD15. However, no significant difference in the percentage of vessels covered by ENVT was observed at GD18 between diet groups. In addition there was a significant effect of gestational age within the CD-fed group ($P<0.05$); an effect which was absent in the HFD-fed group.

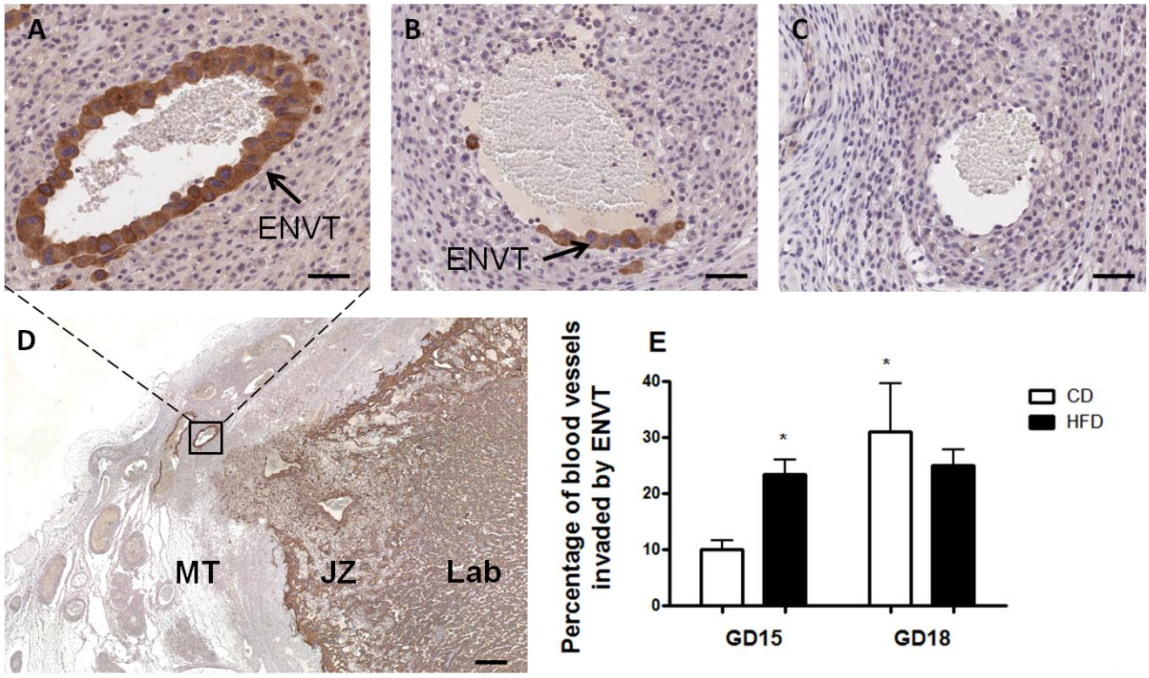


Figure 4.3 Percentage of blood vessels invaded by endovascular trophoblast cells in the entire mesometrial triangle is higher in HFD-fed dams at GD15 but not GD18. Central slides (5 μm thick) were stained with anti-pan cytokeratin. All blood vessels in the mesometrial triangle were traced and vessels fully invaded (A), partially invaded (B), and not invaded (C), by endovascular trophoblast (brown positive staining) were counted. Scale bars = 50 μm. (D) Shows a lower magnification of the mesometrial triangle (MT) from which the image of the fully invaded spiral artery (A) was taken, JZ= Junctional zone Lab= labyrinth, scale bar = 250μm. (E) Graphical representation of the percentage of total blood vessels that were fully or partially invaded was calculated for each implantation site before being averaged. ($P<0.05$) by two-way ANOVA; *=significantly different from CD-fed group; one implantation site was examined per dam, with a minimum of 4 dams per group.

4.3.3 The increased invasion early in gestation in HFD-fed dams is associated with an increase in matrix metalloproteinase 9 levels in the mesometrial triangle

We measured the protein expression of MMP2 and MMP9 in *decidua* tissue homogenates, as these proteins are known to be important mediators of invasive trophoblast cells (Luo, Qiao, & Yin, 2011). We observed a 2-fold increase in MMP9 levels in decidual tissue homogenates from HFD-fed dams at GD15 ($P<0.05$) (Figure 4.4.B). The mean MMP2 level was also higher at GD15, although this did not reach statistical significance ($p=0.21$) (Figure 4.4.A). Both MMP9 and MMP2 protein expression levels at GD18 were not significantly different between CD and HFD groups (data not shown).

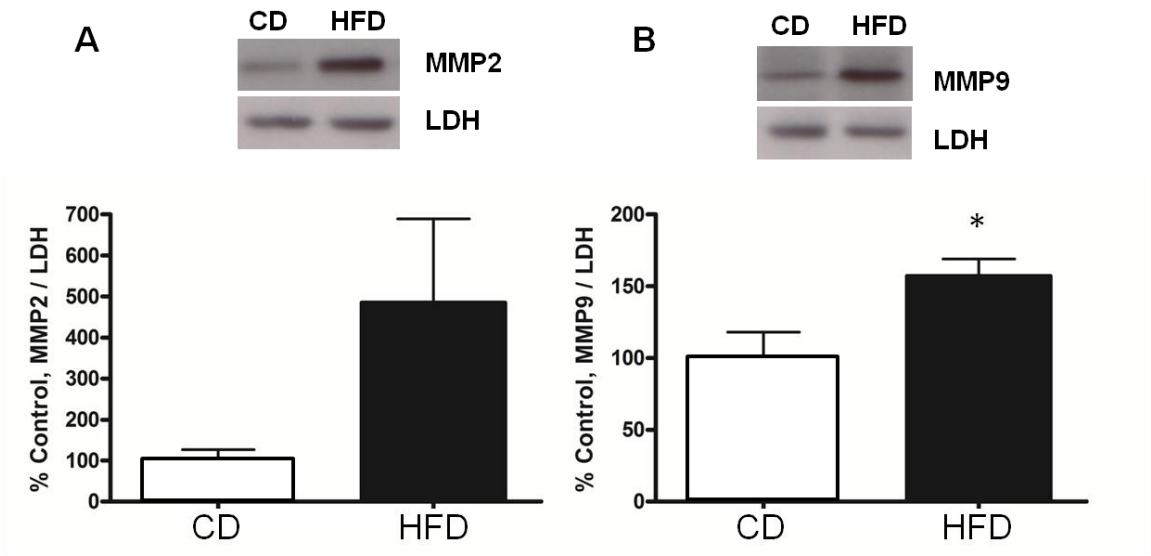


Figure 4.4 Matrix metalloproteinase 9 levels are elevated in the mesometrial triangle of HFD-fed rats at GD15. (A) MMP2 and (B) MMP9 protein levels were measured by Western blot and normalized to cellular levels of lactate dehydrogenase (LDH). 10 μg of mesometrial triangle homogenates were separated on a 10% polyacrylamide gel, transferred to nitrocellulose and incubated in 3% milk with anti-MMP9 monoclonal antibody or anti-MMP2 monoclonal antibody. n=7 per group. All values represent mean +/-SEM; P values were calculated using two-tailed Student's T-test; *P <0.05.

4.3.4 Remodeling of spiral arteries is compromised in implantation sites from HFD-fed dams at GD18

SMA-positive vascular smooth muscle cells, situated around the periphery of the spiral arteries, are lost when these vessels are converted to high capacity low resistance vessels. In addition, the proportion of the spiral artery that is SMA positive decreases with the normal remodeling seen during pregnancy (Caluwaerts et al., 2005; Harris et al., 2006). Therefore, we assessed spiral artery remodeling by examining the loss of SMA-positive VSMCs in invaded spiral arteries at GD18 (Figure 4.5). The percentage of the total contour of partially and fully invaded arteries that were covered by VSMCs was analyzed (Figure 4.5.A-C). At GD18, the arteries in the mesometrial triangle of HFD-fed dams were covered by significantly greater amounts of VSMCs compared to that seen in CD-fed dams (Figure 4.5.D; $P<0.05$).

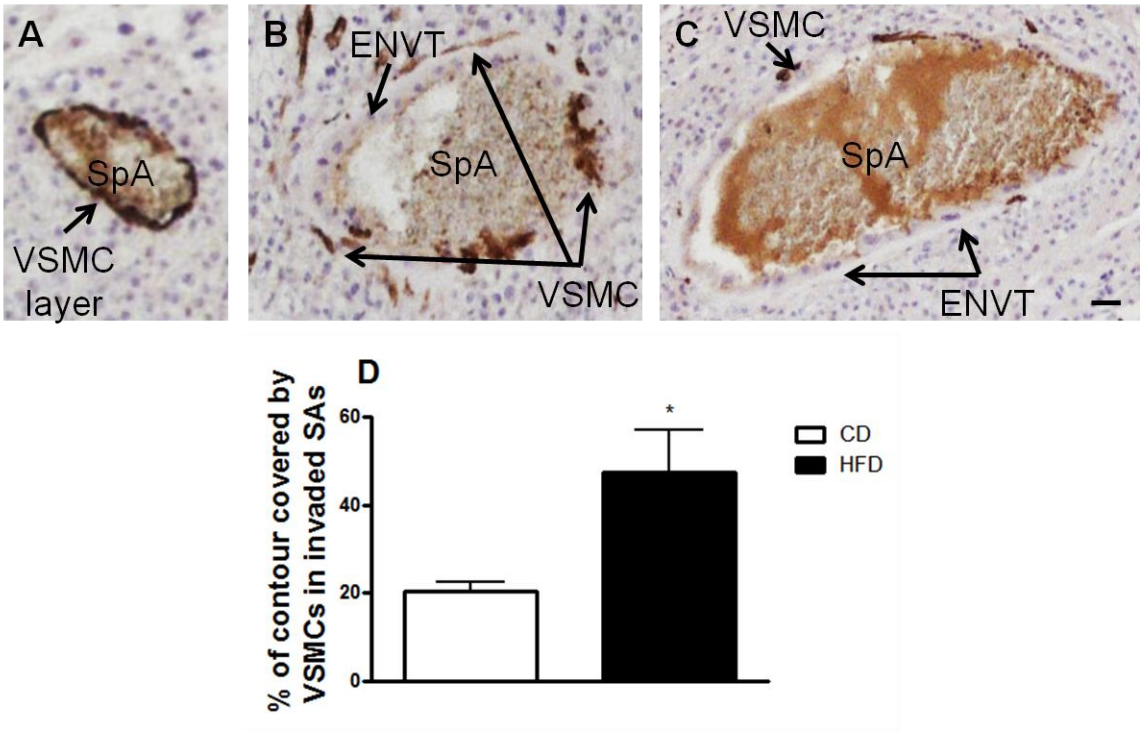


Figure 4.5 Vascular smooth muscle cell layer remodeling in invaded spiral arteries is compromised in HFD-fed dams at GD18. (A-C) Representative images of slides stained with anti-SMA. The vessels that were partially or fully invaded by endovascular trophoblasts (ENVT) were analyzed. A representative blood vessel of a non remodeled spiral artery (SpA) (A), a partially remodeled artery (B) and an almost fully remodeled artery (C) scale bar = 50um. (D) The percentage of contour length covered by SMA-positive vascular smooth muscle cells (VSMCs) was quantified in relation to the entire artery contour of fully or partially invaded arteries. Sections containing 3 to 6 spiral arteries were analyzed in each animal and an average value per animal was obtained; two-tailed Student's T-tests was performed on these averages. n= at least 5 dams per group; *P<0.05.

4.3.5 Serum from obese women inhibits trophoblast invasion in vitro

There was a significant (p=0.0008) main effect of serum from maternal obese women (i.e., BMI >30 kg/m^2) to inhibit trophoblast invasion compared to serum from normal BMI women (Figure 4.6). There was no impact on trophoblast invasion when considering fetal outcomes as a main variable. In addition, no interaction was observed between maternal obesity and fetal growth.

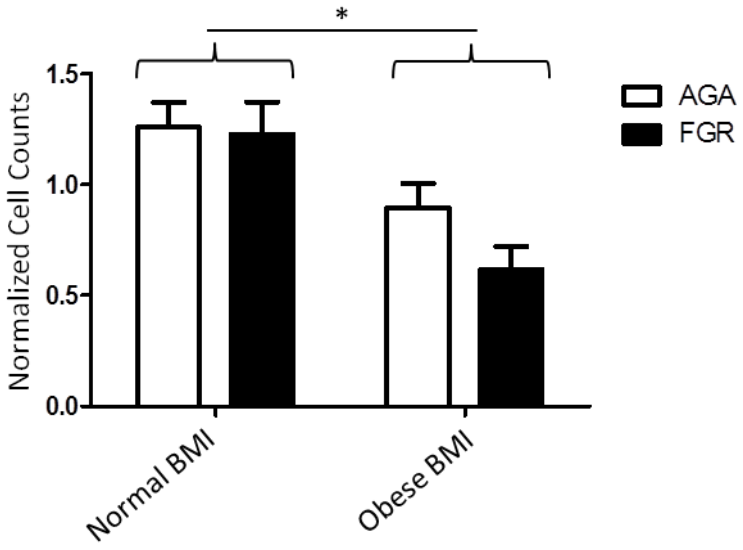


Figure 4.6 First trimester serum from obese mothers inhibits trophoblast invasion *in vitro*. Standard two chambered invasion assay was performed using HTR-8/SVneo cells on matrigel. Study groups consisted of pooled 1st trimester serum from different pregnant groups: 1) normal BMI women with AGA fetus, 2) normal BMI women with FGR fetus, 3) obese BMI women with AGA fetus or 4) obese BMI women with FGR fetus. All data represent the mean +\- SEM (standard error of the mean). Invasion assays were repeated five times (n=5) for all groups. There was a significant main effect of maternal BMI (obese BMI<normal BMI, (*) p=0.0008), there was no main effect of fetal outcome and no significant interaction between maternal BMI and fetal outcome on invasion.

4.4 Discussion

We developed a rat model of lifelong maternal obesity that results in increased fetal and neonatal death as well as reduced birth weight (Hayes et al., 2012). In the human population, obese women are more likely to develop complications including preeclampsia, as well as delivering small for gestational age babies (Norman & Reynolds, 2011; Perlow et al., 1992). These complications have been linked with reduced trophoblast invasion and incomplete spiral artery remodeling (Ball et al., 2006; McCarthy & Bennett, 2000) and the current literature suggests that this may be partially mediated by inflammatory signals (Whitley & Cartwright, 2010). The parallels in outcome between our model and what is observed in the human population prompted a focus on the consequences of obesity during pregnancy and its impact on pregnancy-induced uterine modifications.

The morphometric analysis of the CD utero-placental tissue has revealed an expected increase in the cross sectional surface area of the labyrinth zone as gestation progresses (GD15 to GD18) (Figure 4.1.C). However the HFD appears to influence a further increase of cross sectional surface area of the labyrinth zone compared to the CD-fed animals. We postulate two possible mechanisms for this HFD induced increased in labyrinth size: 1) altered growth patterns of the placental tissue is mediated by altered utero-placental immune response, in which either the change in the homeostatic balance of the immune response induces placental growth or there is a loss in the ability of the immune cell population to regulate the expansion of the placenta, 2) the increased labyrinth size is a compensatory mechanism to help, in part, to increase nutrient availability for the fetus as a result of decreased blood supply from decreased vascular

remodeling (as demonstrated in vascular remodeling analysis). A typical degeneration of the *decidua basalis* is observed as gestation progresses (Welsh & Enders, 1993). In our study we observed in the CD-fed animals a decrease in cross sectional surface area of the *decidua* from GD15 to GD18 which is consistent with this degeneration (Figure 4.1.E). However we observed an increased decidual cross sectional surface area in the HFD-fed animals at GD18 compared to the CD-diet fed animals at GD18, suggesting deficiencies in the late gestation mesometrial decidual degeneration process (Welsh & Enders, 1993). Other morphological studies have also reported a larger decidual zone in a high fat diet-fed mouse model and in a pre-eclamptic mouse model (Dokras et al., 2006; D. W. Kim, Young, Grattan, & Jasoni, 2014). In a hypertensive mouse model they described a deficiency in invasion by glycogen cells in late gestation as a potential cause, again consistent with our findings of decreased trophoblast invasion at GD18 in response to the HFD. This suggests that disruptions to trophoblast invasion may alter the normal progression of decidual degeneration with advancing gestation. Dokras et al., 2006 further revealed from their hypertensive mouse model, severe feto-placental abnormalities resulting in fetal morbidity/mortality at GD 14.5. Their morphometric analysis demonstrated similar abnormalities in utero-placental zones as our study as well as compromised utero-placental circulation due to vascular anomalies resulting in fewer viable feto-placental units at term. Moreover, the pups that survived gestation were smaller than normal litter sized pups similar to our HFD diet obese rat model.

In HFD-fed dams, we observed significantly increased interstitial and endovascular trophoblast invasion at GD15. By GD18, these dams exhibited decreased interstitial trophoblast invasion while there were no significant differences in the

endovascular trophoblast invasion between CD and HFD-fed animals. Taken together this demonstrates a dramatically altered progression of trophoblast invasion in the placenta of HFD-fed dams.

Since our study design focused on the analysis of two time points, GD15 (initiation) and GD18 (peak), we cannot delineate between two possible trajectories for the progression of invasion as a consequence of life-long HFD-diet. The first is a complete shift in the temporal regulation of trophoblast invasion (i.e. early initiation of invasion and an early retraction); and the second is a situation where early invasion is dramatically increased and is followed by halted progression (i.e. greater levels of initial trophoblast invasion into the mesometrial compartment that is inhibited before reaching the peak). In both of these scenarios the increased invasion at an earlier gestational time point results in an early arrival of an abundance of trophoblasts within proximity of the uterine spiral arteries. Comparison of the temporal relationship in trophoblast invasion profile for the CD-fed and HFD-fed dams demonstrated that while HFD-fed dams exhibited increased early invasion at GD15, there is not a significant difference in the extent of invasion between GD15 and GD18. In contrast CD-fed dams exhibited significant effect of gestational age for both ENVT and IST invasion; as demonstrated by a dramatic increase in the area occupied by ENVT and IST at GD18 compared to GD15.

We observed significantly greater staining for α-smooth muscle actin around the spiral arteries that had been invaded by ENVT in mesometrial triangle of HFD-fed dams. The increased levels of α-SMA staining, characteristic of VSMCs, suggest a deficiency associated with the normal loss of VSMCs which is important for the progression of vascular remodeling in pregnancy (Helwig & Le Bouteiller, 2007). Much of the early

evidence suggested that the remodeling of the spiral arteries might have been the result of trophoblast-mediated stimulation of apoptosis of the VSMCs (Fraser et al., 2012; Harris et al., 2006; Harris et al., 2007). More recent findings implicate VSMC migration, rather than apoptosis, as having a major contributory role in remodeling the spiral arteries (Bulmer et al., 2012). The mechanisms underpinning the removal of the VSMCs surrounding the spiral artery require further investigation. Our data demonstrate that in the HFD-fed dams, a premature increase in the presence of trophoblasts within the mesometrial triangle along with the deficiencies in the spiral artery remodeling process (i.e., the disruption of the uterine vascular smooth muscle cells layer surrounding the spiral arteries) are associated with an increased rate of stillbirths and a reduction in birth weight (P=0.05).

Increased trophoblast invasion in association with reduced fetal growth has also been observed in a rat model of preeclampsia (Geusens et al., 2008) as well as following maternal exposure to hypoxia during gestation (Chakraborty et al., 2011). Indeed, we previously reported evidence of increased placental hypoxia in placentas from HF-fed dams at GD15 (Hayes et al., 2012) . This was associated with reduced fetal growth and increased stillbirth in our model. This reduced level of oxygen may be the consequence of altered trophoblast invasion and poor remodeling of the spiral arteries. The low oxygen tension is likely an important contributor to the observed fetal outcomes (Hayes et al., 2012) which are similar to what has been reported in rat models which directly evaluate the consequences of exposure to reduced levels of oxygen during pregnancy on birth outcomes (Bourque et al., 2012). These observations are also consistent with a report by

Rosario et al., (2008) suggesting that HIF-1α , a well accepted marker of hypoxia, is an important regulator of trophoblast invasion (Soares et al., 2012b).

Coinciding with the increase in trophoblast invasion in mesometrial triangles from HFD-fed dams at GD15, we observed a 2-fold higher level of MMP9 protein expression (Figure 4.4). This protease is involved in extracellular matrix cleavage and is important for trophoblast invasion; it is secreted by invasive trophoblast cells but also by various cells of the *decidua* including decidual natural killer cells (dNK) (Isaka et al., 2003; Naruse et al., 2009; Staun-Ram et al., 2004). The important role of both MMP2 and MMP9 in placental development is supported by the observation that the inhibition of these gelatinases is associated with a reduction in trophoblast invasion (Verlohren et al., 2010). Additionally, the up regulation of MMP9 (and MMP2) has also been associated with obesity and may contribute to the increased invasiveness of cancers such as oesophagal adenocarcinoma (Allott et al., 2013). The increased MMP levels in obesity may be a consequence of increased dietary fat consumption, including oleate (Soto-Guzman et al., 2010) or of increased levels of adipocytokines such as resistin (Mu et al., 2006). In addition, the expression of these enzymes is also known to be potentiated under conditions of hypoxia (Na et al., 2012; Soares et al., 2012b). Under short-term hypoxic conditions (i.e., 24 hours) Lash et al., (2007) reported that HTR8/SVneo cells (immortalized and invasive first trimester human trophoblasts (Graham et al., 1993)) exhibit increased invasion *in vitro*, a result which in part supports our findings at GD15. Hypoxia is also associated with oxidative stress and can result in production of reactive oxygen species and increased MMP production (Raha & Robinson, 2001; Pustovrh et al. 2005). Oxidative stress has been observed in numerous placental pathologies associated

with pre-eclampsia, reviewed by Burton et al., (2009a). Our obese rat model however did not demonstrate significant increase in placental protein carbonyls (marker for oxidative damage) (Hayes et al., 2012). Notably, oxidative stress was not assessed in the rat uterine tissue of our HFD-fed rats, therefore, whether increases in MMP-9 and premature trophoblast invasion is a result of uterine oxidative stress remains to be determined.

In contrast to the increased expression of MMP9 at GD15, levels of both MMP9 and MMP2 were not significantly different at GD18 suggesting that the decreased trophoblast invasion observed at this time point maybe the result of HFD induced alterations of regulatory factors other than MMPs. Interestingly, prolonged exposure to reduced levels of oxygen (72h) results in reduced trophoblast invasion (Lash et al., 2007). These seemingly divergent observations regarding the effects of hypoxia on invasion may be due, in part, to the role of paracrine factors such as VEGF-A_{165} whose increased release as a consequence of prolonged exposure to hypoxia has been shown to inhibit HTR8/SVneo invasion (Lash et al., 1999). These *in vitro* studies provide a potential explanation to the altered invasion patterns observed at both gestational ages in our model. It should be borne in mind that while *in vitro* models provide valuable mechanistic information on individual factors affecting trophoblast function, they do not accurately address the influences of multiple cell types present in the mesometrial triangle during the process of spiral artery remodeling. One such cell type is the dNK cells.

The dNK cells play a central role in spiral artery remodeling. Indeed, Robson et al., 2012 demonstrated that conditioned culture medium from dNK cells induced vascular smooth muscle disorganization in human myometrial arteries. This disorganization

process is important in that it provides the invading IST cells access to the underlying spiral artery endothelium layer. Furthermore, *in vitro* studies have demonstrated that the interaction between trophoblasts and dNK cells may attenuate the vascular remodeling capability of the dNK cells (Robson et al., 2012). Additionally, factors secreted from dNK cells have also been shown to impact the ability of trophoblasts to form a network of vascular tubes and exhibit endothelium-like markers such as VEGF-C (Hu et al., 2010). The importance of the interaction between dNK cells and trophoblasts is also supported by *in vivo* evidence. Fraser et al., (2012) demonstrated that dNK isolated from pregnancies with poor spiral artery remodeling secreted reduced levels of pro-invasive factors and could not induce remodeling changes in VSMC in culture. While the full extent of the interaction between dNK cells and trophoblasts has yet to be appreciated, this may be an important regulatory point that is impacted in obese dams. The changes in the temporal progression of the trophoblast invasion process observed in the HFD-dams at GD15 may impact this important intercellular relationship and alter time sensitive cellular functions.

Although the exact mechanism behind the altered progression of trophoblast invasion remains elusive, it is possible that cytokines may play an important role in this process. Cytokine levels are known to be altered in obese individuals (Lolmede et al., 2003) and such changes may impact both the progression of trophoblast invasion as well as the interaction between the trophoblasts and dNK cells and ultimately impact spiral artery remodeling. Cytokines such as IL-15 (Kim et al., 2006) and macrophage inflammatory protein-1β (Soto-Guzman et al., 2010) may be important for recruiting dNK cells during the initial stages of placentation. Bar et.al, (2012), have also reported

increased inflammatory placental lesions, which are characterized by multiple sites of neutrophil infiltration on the chorionic plate and extraplacental membrane (Kovo et al., 2012), in association with obesity during pregnancy and have attributed these lesions to pro-inflammatory cytokines (Bar et al., 2012). While *in vitro* experiments assessing the effects of inflammatory cytokines on trophoblast invasion have shown both stimulatory and inhibitory effects (Bauer et al., 2004; Jovanovic & Vicovac, 2009; Jovanovic et al., 2010), the mechanism(s) underlying these effects in the context of obesity are less clear.

Failure to remodel the uterine spiral arteries can lead to increased contractile function which can result in decreased placental perfusion and adverse pregnancy outcomes. In humans, a decreased luminal area of the arteries feeding the placenta can lead to increased blood pressure entering the exchange surface; this can cause rupture of the delicate placental villous tissue and may contribute to reduced fetal growth (Burton et al., 2009b) or fetal death. We have previously reported that our HF-fed dams have increased blood pressure at GD18 (Hayes et al., 2012); while Frias et al., (2011) have demonstrated that maternal obesity and the resulting increase in placental inflammation leads to reduced placental blood flow. In both cases, the changes in blood pressure/blood flow were associated with increased incidence of stillbirth.

Impaired vascular function and relaxation is associated with obesity in humans (Sivitz et al., 2007). In addition, obesity is associated with end organ dysfunction and vascular disease, which is characterized by endothelial dysfunction, resulting in vessel stiffness and narrowing of vessel diameters (Denison et al., 2010). Therefore, in obese pregnancies, these same impairments may be present and such impairments can complicate pregnancy (Myers et al., 2006). In the utero-placental tissue, poor vascular function may be associated with increased placental inflammation (Frias et al., 2011).

Furthermore, immune cells and more specifically cytokines secreted from these immune cells are also thought to play an important role in regulating the remodeling of the vascular smooth muscle cell wall of the spiral arteries (Whitley & Cartwright, 2009; Whitley & Cartwright, 2010). Recent evidence suggests the uNK cells may impact vascular remodeling and these cells isolated from pregnancies which are complicated by hypertension are less able to affect the remodeling process (Fraser et al., 2012). Such observations may help elucidate the connection between altered immune response and poor vascular function. Furthermore, understanding the defects in regulating the release of pro and anti-inflammatory signaling molecules may be central to placental dysfunction in a variety of pathologies including those arising from maternal obesity.

Results from Chapter Three demonstrated that our obese rat model is associated with a systemic and uterine pro-inflammatory state and adverse utero-placental development. In obese pregnant women a systemic inflammatory state is also observed. Maternal serum from obese pregnant women contains pro-inflammatory factors with the potential to alter trophoblast invasion such as TNFα (Madan et al., 2009; Renaud et al., 2007; Roberts et al., 2011). It is therefore plausible that changes in the level of these factors within the maternal circulation perfusing the placenta has the potential to adversely affect trophoblast physiology, resulting in altered utero-placental development. Even though the uterine tissue is highly vascularised, these associations remain to be determined and it is still not clear whether this systemic inflammation can influence the utero-placental environment. To explore this hypothesis, we demonstrated preliminary results where first-trimester serum from obese women had the capacity to inhibit the invasion of HTR-8/SVneo cells (first-trimester immortalized invasive cell line) (Figure 4.6). Interestingly, this inhibition was independent of whether or not the mother was

carrying an AGA or FGR fetus. These results suggest that factors in maternal serum from obese women, regardless of the fetal contribution, have the potential to alter trophoblast function. Further studies however are needed to characterize the circulating factors that could influence the uterine environment and more importantly trophoblast physiology.

Taken together our results suggest that lifelong exposure to a HFD-diet alters utero-placental morphology, the progression of trophoblast invasion into rat mesometrial triangle during pregnancy and this change may be an important contributor to the reduced remodeling of the vascular smooth muscle surrounding the spiral arteries. We propose that altered trophoblast invasion in this model may be responsible, in part, for the increased fetal death and decreased birth weight we have previously reported. Understanding the mechanisms by which maternal obesity can impact placental development will have profound implications for designing intervention strategies to improve pregnancy outcomes in obese women.

Chapter 5

General Discussion

The goal of this thesis was to elucidate the linkages between maternal obesity, systemic and uterine inflammation, aberrant utero-placental development and adverse pregnancy outcomes. We have demonstrated that adverse fetal outcomes such as reduced birth weights, increased incidence of stillbirths and poor neonatal health observed in our HFD induced obese rat model are associated with increased systemic (MCP-1) and uterine (TNFα) pro-inflammatory markers. In addition these increased pro-inflammatory markers were associated with aberrant utero-placental development (potentially leading to placental insufficiencies) such as altered trophoblast invasion profiles (premature invasion at GD15 and reduced invasion at GD18) and reduced spiral artery remodeling (Figure 5.1). Multiple studies in rodents demonstrate that a HFD either before pregnancy, during pregnancy or both results in variable fetal birth weight outcomes. These studies reported either no effect, decreased birthweights, or increased birthweight (Caluwaerts et al., 2007; Hartil et al., 2009; Howie et al., 2009; Khan et al., 2003; Khan et al., 2005; Samuelsson et al., 2008; Shankar et al., 2008; Taylor et al., 2003). Similarly to our study design, administration of a HFD both before and during pregnancy in rats by Howie et al., (2009) resulted in decreased fetal birth weights comparable to our obese rat model. These results suggest some degree of placental insufficiency; however the underlying mechanisms leading to placental insufficiencies still remain unknown. In addition, whether the HFD/obesity induced increase in pro-inflammatory markers are responsible for the altered utero-placental development and poor fetal development remains to be

elucidated. Nonetheless this thesis does provide clues to the potential mechanisms leading to obesity associated adverse pregnancy outcomes.

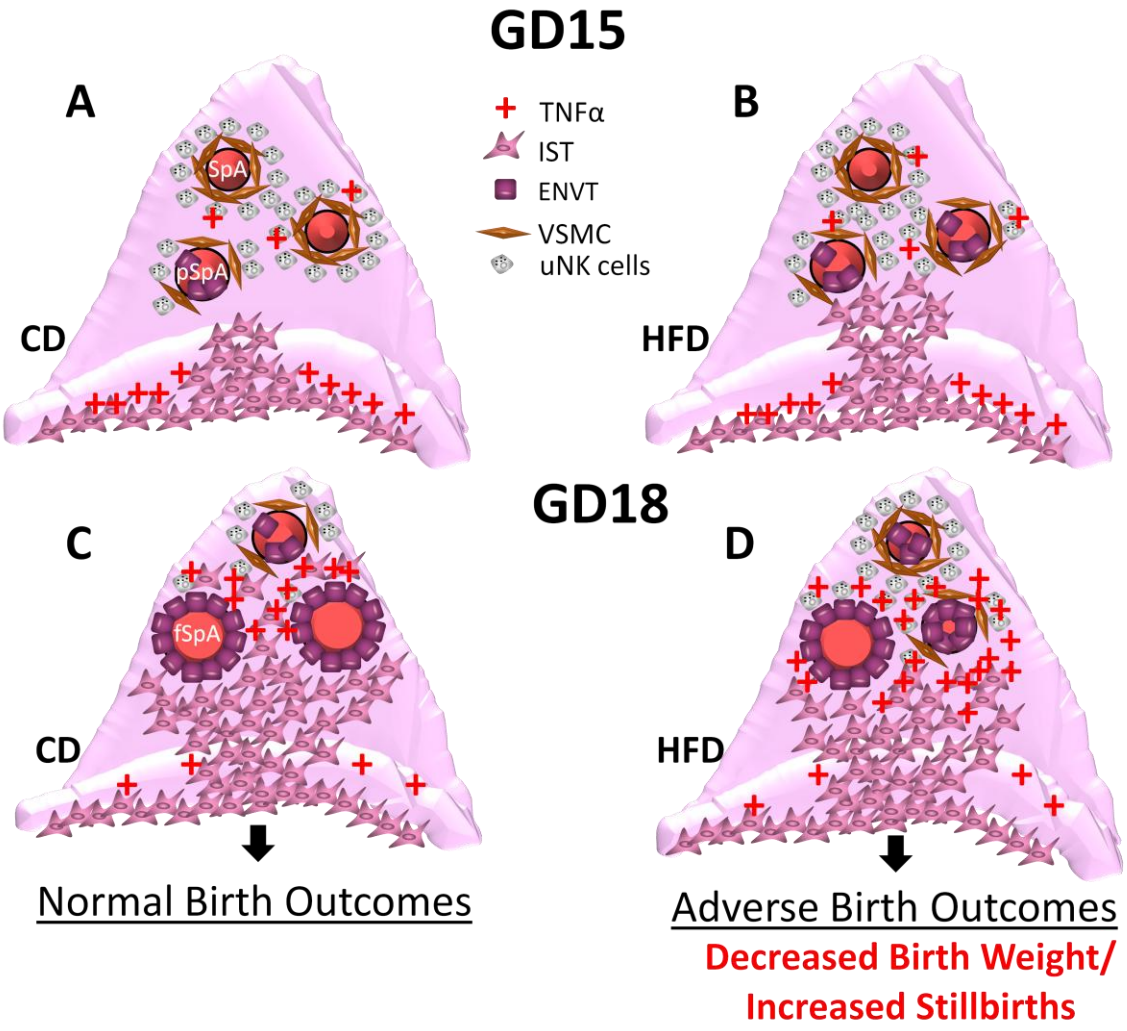


Figure 5.1 Summary diagram of maternal obesity's influence on the rat uterine immune response, trophoblast invasion and spiral artery remodeling. Schematic representation of a cross sectional view of the mesometrial triangle (MT) from rats fed a control diet (CD) or a high fat diet (HFD) at gestational day (GD) 15 and GD18 and with localization of tumor necrosis factor alpha (TNFα), interstitial trophoblast (IST), endovascular trophoblast (ENVT), vascular smooth muscle cells (VSMC), uterine natural killer cells (uNK) and spiral arteries (SpA). (A) GD15-CD: demonstrates unremodeled SpA and partially remodeled SpA (pSpA), shallow IST invasion, minimal presence of ENVT and a high density of TNFα at the base of the MT. (B) GD15-HFD: compared to GD15-CD demonstrates similar unremodeled SpA and pSpA, increased IST invasion, increased ENVT presence in SpA lumen and similar localization of TNFα at the base of the MT. (C) GD18-CD: demonstrates pSpA and fully remodeled SpA (fSpA), deep IST invasion, full coverage of ENVT in fSpA and high density of TNFα in the upper portions of the MT. (D) GD18-HFD: compared to GD18-CD demonstrates increased VSMC coverage of the SpA, decreased IST invasion, similar ENVT presence in the SpA lumen and increased concentration of TNFα in the MT.

Chapter 2: Characterization of immune cells and cytokine localization in the rat utero-placental unit during mid-late gestation

Many similarities exist between humans and rats in terms of crucial utero-placental development processes such as of deep trophoblast invasion and extensive uterine vascular remodeling (Soares et al., 2012a). Current evidence suggests that these processes are partially regulated by immune cells. Therefore by furthering our knowledge of immune cells and cytokines distribution in the rat uterine tissue we will hopefully provide clues to similar immune cell distribution and cytokine localization in humans. In addition, this knowledge may further validate the use of the rat model to study human related pregnancy complications.

I hypothesized in Chapter Two that immune cells, uNK cells, neutrophils, and macrophages and relevant cytokines TNFα, INFγ and IL-10 would be specifically localized in the rat mesometrial triangle. Not only are the immune cells of interest present in the rat mesometrial triangle but we also observed the region specific distributions of uNK cells and neutrophils which coincided with the trophoblast invasion front and the spiral arteries. In addition, the distinct uNK cells and neutrophil distribution also revealed partial co-localization of uNK cells with TNFα and IFNγ and neutrophils with IL-10.

As described throughout this thesis, obesity is characterized by a chronic systemic low grade inflammatory state (Curat et al., 2006; Fantuzzi, 2005; Li et al., 2008). Adipose tissue of obese individuals demonstrates an increase in resident CD68 positive and/or CD14 positive macrophages proportionate to the individual's BMI (Curat et al., 2006; Spencer et al., 2010; Wozniak et al., 2009). These macrophages can exist in different activation states and undergo phenotypic switches depending on local

environmental factors. The M1 phenotype is characterized as the classical activation state which can be induced by inflammatory stimuli such as LPS and IFNγ. Once the M1 are activated, they produce multiple pro-inflammatory cytokines including TNFα, IL-1β and IL-6 (Gordon & Martinez, 2010). The second phenotype is achieved by an alternative activation (M2) and includes other subphenotypes, M2a, M2b and M2c. The M2a is characterized as an anti-inflammatory activation state which promotes tissue repair and remodelling. It can be induced by IL-4 and IL-13 (Nagamatsu & Schust, 2010). The M2c subphenotype is characterized as a wound healing, anti-inflammatory, fibrotic promoting macrophage (Spencer et al., 2010) which can be activated by IL-10. An activated M2c expresses TGFβ and IL-10 (Gordon & Martinez, 2010). Studies of human first trimester decidual tissue from normal pregnancies have revealed that resident monocytes/macrophages are predominantly polarized towards the M2 phenotype (M2 subphenotype remains to be determined) (Nagamatsu & Schust, 2010). In the rat uterine tissue however this remains to be determined. As described above, depending on the activation status, adipose tissue macrophages can produce different cytokines. For example M1 produce TNFα while M2c produce IL-10. As described in Chapter Three both these cytokines have demonstrated the potential to influence trophoblast invasion (Otun et al., 2011; Renaud et al., 2007). Collectively, these observations suggest that temporal regulation of the macrophages' activation status in the uterine tissue may be important in the regulation of trophoblast function. However, to date this area remains largely unexplored in the uterine environment of humans and rats.

In human and rat uterine tissue, CD68 has long been used as a generalized macrophage marker (Hamilton et al., 2012; Hazan et al., 2010; van Oostveen et al.,

1992). In our study, however, we question the use of CD68 as generalized macrophage marker in the rat uterine tissue since we observed potentially other cell types that are CD68 positive. Unfortunately, other generalized macrophage markers in the rat remain to be established. This increases the difficulty to assess the overall macrophage population in the rat uterine tissue. For these reasons we began to investigate the different macrophage phenotypes (M1 and M2). Our extensive literature search revealed no cell surface markers to define the M1 phenotype. The current methods to identify the M1 phenotype in adipose tissue is through assessment of specific enzymes/proteins expression such as inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokine TNFα (Sica & Mantovani, 2012). In the rat uterine tissue however these markers are not only expressed by macrophages. Skarzinski et al., (2009) demonstrated that iNOS is also expressed by interstitial and endovascular trophoblast cells while our data demonstrated that TNFα was partially co-localized with uNK. The multiple cell types expressing similar M1 macrophage markers again increased the difficulty to assess the M1 specific macrophage population in the rat uterine tissue. Human decidual macrophages on the other hand have revealed reliable M2 phenotype cell surface receptors CD163 and CD206 (Svensson et al., 2011). Although the subtypes were not demonstrated in this study, other studies have defined the CD206 as M2a marker and CD163 as M2c marker (as reviewed by Martinez et al., 2008). We have also conducted preliminary immunohistochemical studies using CD163 and CD206 and found cell specific staining of macrophage like cells in the rat mesometrial triangle (data not shown). With the lack of reliable generalized macrophage and M1 markers we did not pursue any further the influence of maternal obesity on the macrophage population or activation states in the rat

uterine tissue. The need for further research is evident if we are to characterize the macrophage population/subpopulations in the rat uterine tissue and in hopes of translating these findings into humans. In addition by furthering our knowledge of the macrophage population we will be better equipped to assess the influence of obesity/HFD on the uterine macrophage activation states during crucial utero-placental developmental events.

Chapter 3: Maternal obesity is associated with a systemic and uterine pro-inflammatory state in the rat

Obesity associated diseases include coronary heart disease, hypertension, and type 2 diabetes mellitus (Arendas et al., 2008). These diseases are the consequence of metabolic syndrome (disorder of energy utilization and storage). In addition, obesity associated diseases are believed to be a link between nutrient excess and an imbalance in the inflammatory response. This obesity associated imbalance of the inflammatory response leads to a metabolically triggered chronic low grade systemic inflammation, termed meta-inflammation (Hotamisligil, 2006; Lumeng & Saltiel, 2011). In non-pregnant individuals meta-inflammation negatively affects multiple organs including brain, pancreas, adipose tissue, and skeletal muscle and is associated with increased production of pro-inflammatory cytokines (reviewed by Frias & Grove, 2012). It is therefore plausible that obese pregnant women also display meta-inflammation which can negatively influence utero-placental development and ultimately fetal growth.

Notably normal weight pregnancies with favourable pregnancy outcomes are also characterized with a late onset low grade systemic inflammatory response (Stewart et al., 2007). In obese pregnant women this pro-inflammatory response appears to be present in

early pregnancy as demonstrated by increased circulating pro-inflammatory cytokines (Friis et al., 2013; Stewart et al., 2007). Included in these pro-inflammatory cytokines are TNFα, IL-6, IL-1β and MCP-1 (Challier et al., 2008; Christian & Porter, 2014; Friis et al., 2013; Madan et al., 2009; Roberts et al., 2011; Stewart et al., 2007; Winkler et al., 2002). Inconsistencies however have been observed in the quantification of these cytokines throughout pregnancy. Combined, these studies do demonstrate an increase in the overall levels of circulating pro-inflammatory cytokines in obese pregnant women, however between studies there are contrasting results in which some studies demonstrate no changes while other studies demonstrate increased levels of certain cytokines. Variations in the inclusion criteria of the patients and timing of sample collection and may account for these inconsistencies.

Human studies have also revealed obesity associated increases in placental inflammatory markers. Human placental studies conducted at term have demonstrated obesity was associated with increased placental cytokine expression of TNFα, IL-1β and MCP-1 (Challier et al., 2008; Roberts et al., 2011). Interpretation of these results however must be done with caution since the majority placental developmental events have already been completed at term. To better assess maternal obesity's influence on utero-placental inflammation and subsequent utero-placental developmental events crucial for appropriate fetal growth (trophoblast invasion and spiral artery remodeling), first trimester and early second trimester placenta and placental bed biopsies (uterine/placental tissue) from obese pregnant women should be analyzed. Uterine/placental tissues are however difficult to obtain from these time periods. Extensive literature search revealed no studies to date have investigated obesity associated uterine inflammation in early

pregnancy in humans. Because of the limitations of using human tissue we utilized the HFD-fed obese rat model to assess maternal obesity's influence on utero-placental inflammation. Furthermore we assessed this at GD15 and GD18 which coincides with major utero-placental developmental events (trophoblast invasion and spiral artery remodeling).

My hypothesis for Chapter Three was, since increased uterine tissue and systemic pro-inflammatory states are observed in obese pregnant women, our HFD induced obese rat model will also exhibit a uterine as well as a systemic pro-inflammatory state at both GD15 and GD18 compared to CD-fed rats. We did indeed observe an increase in the levels of uterine pro-inflammatory TNFα in response to the HFD, an indicator of a pro-inflammatory uterine state. Another pro-inflammatory cytokine, IL-1β, however did not reveal any differences in concentration between HFD-fed and CD-fed animals or between gestational ages. This increased TNFα and no difference in IL-1β suggest that the HFD potentially influences only specific pro-inflammatory responses.

Serum from HFD-fed rats demonstrated increased levels of the circulating pro-inflammatory cytokine MCP-1, which suggests a systemic pro-inflammatory state. This finding is consistent with increased circulating MCP-1 levels in the late first and early second trimester in obese pregnant women compared to normal weight pregnant women (Friis et al., 2013; Madan et al., 2009). Taken together we demonstrate that our HFD induced obesity rat model exhibits similar increases in pro-inflammatory markers as meta-inflammation in obese humans (Catalan et al., 2007; Zeyda et al., 2007) and that this meta-inflammation also appears present in the uterine tissue.

Human and animal studies have demonstrated that the potential driver of meta-inflammation is not obesity itself but the increase in circulating free fatty acids (FFAs) (Chen et al., 1987). Insulin's role in peripheral tissue is to stimulate storage of lipids into fat. In insulin resistant individuals (common in non pregnant obese women) an increase in FFAs in circulation occurs (Chen et al., 1987). An increase in circulating FFAs has demonstrated the potential to stimulate systemic and peripheral inflammatory responses through the Toll-like receptor 4 cellular signaling pathway inducing nuclear factor-κB activation. The activation of nuclear factor-κB results in production of pro-inflammatory cytokines, including TNFα and IL-6 (Lee et al., 2001; Volpe & Nogueira-Machado, 2013). More specifically in obese rats, the activation of this signaling pathway was observed with saturated free fatty acids as opposed to unsaturated fatty acids. TNFα in turn has also been demonstrated to further drive peripheral insulin resistance by further increasing FFAs in circulation, which further exasperates the inflammatory response (Hotamisligil et al., 1993). Interestingly neutralization of TNFα recovered insulin sensitivity in peripheral tissues (Hotamisligil et al., 1993). Therefore regulation of TNFα levels is emerging as a potential target for therapeutics for metabolic syndrome (Volpe & Nogueira-Machado, 2013). Moreover the use of anti-TNFα therapies may prove to be a valuable tool to regulate the systemic and peripheral immune responses and increase favourable pregnancy outcomes in obese women.

Chapter 4: Trophoblast Invasion and Blood Vessel Remodeling Are Altered in a Rat Model of Lifelong Maternal Obesity

Very few studies to date have investigated the link between an obesity-induced pro-inflammatory uterine environment and adverse utero-placental development and poor fetal outcomes. Frias et al., (2011) demonstrated that a maternal high-fat diet in non-human primates resulted in increased placental inflammation which was associated with reduced placental blood flow and increased incidence of stillbirths. Another study reported that a pro-inflammatory environment, induced by lipopolysaccharide-treatment in rats resulted in an increased maternal circulating TNFα which was associated with reduced trophoblast invasion and deficiencies in spiral artery remodeling as well as increased incidence of FGR (Cotechini et al., 2014). The disruption of trophoblast invasion in the first-trimester, a process that is important for proper utero-placental modifications to meet fetal demands, has the potential to adversely alter the fetal growth potential. For example altered trophoblast invasion can lead to insufficiencies in the remodeling of the uterine spiral arteries and in utero-placental morphology which can result in decreased placental perfusion and adverse pregnancy outcomes (Krishna & Bhalerao, 2011).

As described in the introduction, uterine natural killer cells (uNK) play a central role in the initiation of spiral artery remodeling. The VSMC disorganization process is important in that it provides the invading trophoblast cells access to the underlying spiral artery endothelium layer. *In vitro* studies have demonstrated that the interaction between trophoblasts and uNK cells may attenuate the vascular remodeling capability of the uNK cells (Robson et al., 2012). Therefore, the premature presence of trophoblast in the HFD-

dams at GD15 may impact early spiral remodeling events by interfering with uNK cell functions. Further investigation of the full extent of the interaction between uNK cells and trophoblasts is required if we are to elucidate the impact of maternal obesity on this interaction.

At GD18 we observed reduced trophoblast invasion in response to the HFD. This reduction in trophoblast invasion at GD18 was paralleled with increased α-smooth muscle coverage of the spiral arteries, an indicator of aberrant vascular remodeling. These findings are consistent with my hypothesis that the HFD negatively influences trophoblast invasion and spiral artery remodeling.

As described in the introduction, the contribution of invasive trophoblasts in later stages of arterial remodeling is highly dependent on their ability to migrate/invade into the decidual stroma. Trophoblast functions in spiral artery remodeling include replacement of the degraded endothelial cells lining the spiral artery, degradation of the extracellular matrix surrounding these vessels (Harris, 2011) and induction of migration/invasion of VSMCs away from the artery lumen (Bulmer et al., 2012). Therefore failure of trophoblast to reach the peak depth into the mesometrial triangle may severely compromise late spiral artery remodeling events.

Taken together our obese rat model demonstrated altered trophoblast invasion profiles at both gestation ages: at GD15 a premature presence of invasive trophoblast in the mesometrial triangle which has the potential to alter uNK cell function in the initial stages of spiral artery remodeling and, at GD18 a reduced invasion which has the potential to alter trophoblast functions in late stages of spiral artery remodeling. These

combined findings strongly suggest that the altered invasion profiles observed in our obese rat model are contributors to the reduced spiral artery remodeling.

Consequences of altered trophoblast invasion profiles and aberrant vascular remodeling in our obese rat model could also explain the altered morphology of the rat utero-placental unit. We demonstrated an increased labyrinth cross sectional surface at both gestational ages and an increased decidual cross sectional surface area at GD18 in our obese rat model. The increased cross sectional surface areas of the labyrinth suggest increased expansion of the placental tissue. This increased labyrinth cross sectional surface areas observed in late gestational may be a compensatory mechanism to increase the size and surface area of the labyrinth to facilitate nutrient exchange to meet the needs of the growing fetus as a result of reduced blood supply to the placenta from aberrant spiral artery remodeling (demonstrated in Chapter Four). In normal rat pregnancy, degeneration of the decidual zone occurs in late gestation (Welsh & Enders, 1993). In our study however we observed an increased *decidua* cross sectional surface area at GD18 in response to the HFD. The increased cross sectional surface area suggests deficiencies in the degeneration of this zone in response to the HFD. The decreased degeneration of the mesometrial *decidua* in late gestation could be a consequence of reduced trophoblast invasion observed in late gestation. This phenomenon is also observed in a pre-eclamptic mouse model, where reduced trophoblast invasion is associated with reduced degeneration of the *decidua* (Dokras et al., 2006).

The comparison of trophoblast invasion profiles between humans and rats in healthy pregnancy has revealed many similarities; first the depth of invasion and secondly the function of these invasive trophoblast in vascular remodeling. In hopes of

translating some of our findings from our obese rat model to humans we performed an *in vitro* human trophoblast invasion assay. Although our data are still preliminary our study demonstrated that potential factors in the serum from obese women can inhibit trophoblast invasion compared to serum from lean mothers. Unfortunately we exhausted our serum samples performing these invasion assays and therefore the identification of these potential factors in serum from obese pregnant women compared to normal weight women was not possible. If we were to continue these studies future sample collection would be required.

As described above, the obesity associated increased uterine TNFα levels has the potential to have adverse effects on trophoblast invasion within the uterine tissue however this increase in TNFα also has the potential to have deleterious effects on the actual development of the placenta and its structures. For example, Leisser et al., (2006) demonstrated that TNFα could decrease the secretion of human chorionic gonadotrophin from term placenta cytotrophoblasts by repressing the mRNA expression of the human chorionic gonadotrophin beta subunit. Human chorionic gonadotrophin was shown to play an important role in cell fusion during syncytialization of cytotrophoblast (Shi et al., 1993). This syncytialization is critical for adequate formation of the syncytium. Since the syncytium is in direct contact with maternal blood and is the primary site of exchange between mother and fetus, defects in syncytialization may have profound effects of the efficacy of this exchange and lead to inadequate nutrient supply for the fetus. In pre-eclamptic pregnancies such defects have been observed (Jones & Fox, 1981). In addition, pre-eclamptic pregnancies have also demonstrated increased levels of TNFα in term placentas (Wang & Walsh, 1996). Taken together, these results suggest that obesity

induced increases in TNFα could have deleterious effects on the formation of the syncytium and lead to pathological pregnancies. In our study however we did not characterize maternal obesity's influence on syncytialization and furthermore the levels of TNFα in maternal circulation which would be in direct contact with the syncytium in the HFD- and CD-fed rats were below our assays detectable levels (data not shown). Further investigation would therefore be required if we were to elucidate the effects of maternal obesity on placental development and the efficacy of nutrient exchange between mother and fetus leading to FGR pregnancy outcomes.

Future Directions

As obesity associated adverse pregnancy outcomes continue to rise in Canada and other industrialized countries, the need for further investigation on the causes or mechanisms responsible for poor fetal development is required. Our animal data suggest that obesity induced systemic and uterine pro-inflammatory responses are potentially responsible for adverse utero-placental development and poor fetal development. In chapter four we demonstrate preliminary evidence that factors in serum from obese pregnant women can inhibit trophoblast invasion. As described in Chapter Three, current studies have already begun to quantify altered expression patterns of immune regulatory cytokines in circulation and in the uterine tissue of obese pregnant women. However further investigation is warranted to quantify additional potential systemic and/or uterine inflammatory cytokines in the 1st trimester to determine altered expression profiles between obese pregnant women and normal weight pregnant women. To achieve this, 1st trimester serum samples and 1st trimester uterine tissue collection would be required. Notably, large scale cytokine arrays are readily available for these quantification studies. Once we have characterized obesity associated altered expression patterns, we could determine if specific cytokines could inhibit trophoblast invasion utilizing similar invasion assays as previously described. However instead of using the complete serum from obese mothers we would treat our trophoblast cell lines and 1st trimester extravillous explants with the individual cytokines.

The cytokine or cytokines that demonstrate the capacity to inhibit trophoblast invasion would be further be investigated. However, I would use the full maternal serum in addition to a neutralizing antibody to block the functions of the cytokine of interest.

Since serum from obese women demonstrated the capacity to inhibit trophoblast invasion, I hypothesize that in the presence of the obese serum with neutralizing antibody against the specific cytokine will restore the invasion profile comparable to 1st trimester serum from normal weight women. Notably, first and second trimester maternal serum demonstrated a positive correlation between TNFα levels and BMI (Gao et al., 2008). Therefore the neutralization experiment for TNFα would also be performed.

In our animal studies we demonstrated that adverse utero-placental development (reduced trophoblast invasion and impaired vascular remodeling in late gestation) and poor fetal outcomes are associated with an increased in uterine TNFα concentration in response to the lifelong exposure to a HFD. Whether increased uterine TNFα levels are the cause of these observed adverse utero-placental developmental processes remains to be determined. It would be interesting to test the effects of anti-TNFα therapies to manage the levels of TNFα *in vivo* in our HFD induced obesity rat model. Interestingly, anti-TNFα drugs, Infliximab (IFX) and adalimumab (ADA) are already being used during pregnancy to treat inflammatory bowel diseases (IBD). IFX and ADA are both classified as class B drugs according to the American Food and Drug Administration (Schnitzler et al., 2011). Class B classification indicates that, animal reproduction studies have failed to demonstrate a risk to the fetus but there are no adequate and well-controlled studies in pregnant women. Currently these drugs do not appear to impact fetal development. Indeed abortion/miscarriage rates were similar between women with IBD compared to healthy non-IBD women and women with IBD exposed to the anti-TNFα treatment (Schnitzler et al., 2011). Notably, neonates that were directly exposed to anti-TNFα treatment had a lower gestational age at delivery compared to children of healthy

women with no history of IBD that did not receive the anti-TNFα treatment. However there were no significant differences with respect to birth weight and growth retardation between children with direct exposure to anti-TNFα treatment during pregnancy and healthy controls. In addition no congenital malformations are observed with the use of IFX during pregnancy. Additional anti-TNFα drugs such as certolizumab pegol and golimumab, etanercept are also emerging to be safe for use during pregnancy and that would be of interest to examine using our HFD rat model, (N. Khan, Asim, & Lichtenstein, 2014).

Especially of interest is etanercept, which is also a class B anti-TNFα drug. This drug was administered to an LPS induced acute pro-inflammatory rat model. This model demonstrates increased circulating TNFα associated with reduced trophoblast invasion, reduced vascular remodeling and FGR pregnancy outcomes (Cotechini et al., 2014). Interestingly, the administration of etanercept before the onset of trophoblast invasion recovered the normal trophoblast invasion profile, adequate spiral artery remodeling and appropriate fetal development compared to non LPS treated rats. These results suggest that regulating the levels of TNFα has great potential in alleviating the obesity induced inflammatory response and promoting normal trophoblast function and spiral artery remodeling as well as appropriate fetal growth. Findings from such studies could then potentially be translated to humans and provide a valuable alternative to improve the success of pregnancy in obese women. In our rat model, however, we also observed an increase in trophoblast invasion at GD15. Therefore precise timing of the anti-TNF administration would be important to achieve the normal trophoblast invasion profiles within the mesometrial triangle of our obese rat model.

In addition to the future experimentation on the causes of adverse utero-placental development, better dissemination of information regarding the increased risk of adverse pregnancy outcomes associated to maternal pre-pregnancy obesity is required. Infants born large or small for gestational age from obese mothers have been demonstrated to be at higher risk of developing obesity in early childhood, in addition to being at higher risk of developing metabolic diseases within their lifetime (reviewed by Frias & Grove, 2012). While there are some purely genetic factors associated with the development of obesity, lifestyle influences and fetal metabolic programming appear to be primarily responsible (Savona-Ventura & Savona-Ventura, 2014). Included in lifestyle influences are a rich caloric diet and reduced physical activity during infancy and adolescence. These have been demonstrated to be strong predictors of obesity and obesity associated diseases. Fetal metabolic programming on the other hand occurs *in utero* and is only now emerging as an additional cause for this epidemic (Figure 5.2). Therefore to prevent future intergenerational programming of obesity, we need improved educational programs on healthy eating and physical activity prior to the onset of pregnancy. In addition, long term lifestyle intervention strategies are crucial if we are to prevent the current intergenerational cycle of obesity. Numerous websites and programs already provide an abundance of information on healthy eating; for example Canada's Food Guide (http://www.hc-sc.gc.ca/fn-an/food-guide-aliment/index-eng.php). Even though Canada's Food Guide is in place and is disseminated in schools, obesity trends continue to increase amongst Canadians and more importantly amongst women of childbearing age.

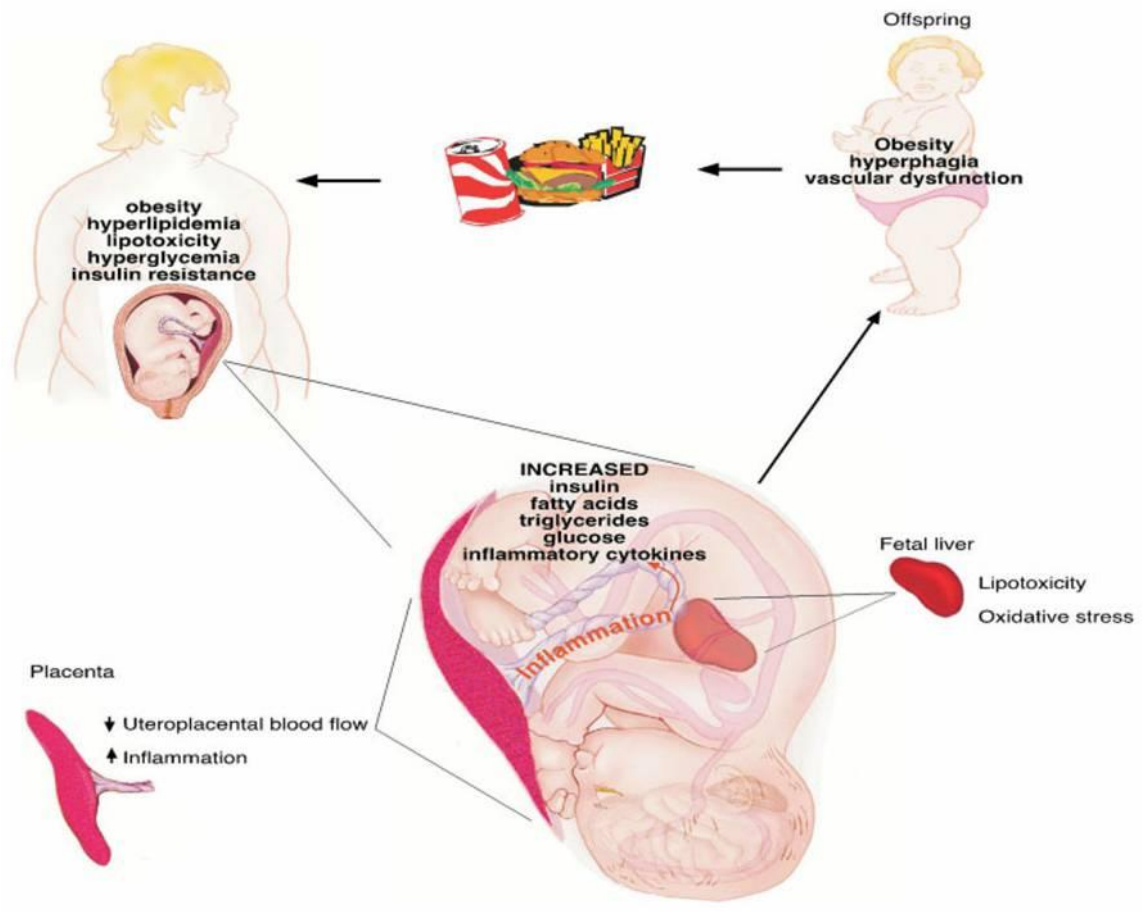


Figure 5.2 Maternal obesity and high-fat diet (HFD) consumption have an impact on the developing placenta and fetus. The fetus and placenta from a HFD-consuming mother experiences an environment characterized by elevated levels of glucose, insulin, fatty acids, triglycerides, and inflammatory cytokines. This environment leads to changes in placenta function/transport that result in systemic fetal inflammation, hyperinsulinemia, hyperlipidemia, and lipotoxicity, resulting in offspring at increased risk for obesity, diabetes, and vascular dysfunction (Frias & Grove, 2012).

In addition to preventative strategies the Society of Obstetricians and Gynecologists of Canada currently adhere to specific recommendations for counseling and management of obese women (Davies et al., 2010).

Whether these recommendations and implementation/adherences to these recommendations will be beneficial for obese pregnancy outcomes remains to be determined. Only time will tell. Unfortunately the creation and dissemination of guidelines/recommendations does not necessarily equate with their implementation (Grimshaw et al., 2004). Limitations to the implementation of these recommendations exist and include: 1) failure of obese women to consult a physician prior to the onset of pregnancy, 2) failure of obese women to follow recommendations or to acknowledge the health risk to themselves and/or to the fetus/infant prior to and during pregnancy, 3) clinicians fail to disseminate information to obese women about the potential risks, due to their lack of awareness of the recommendations, poor motivation and/or lack of time (McDonald et al., 2014). Although these recommendations are well designed with the current data available further research is required if we are to improve on them.

In conclusion, whether it is healthier eating and living, better management of potential pregnancy complications or effective therapeutics to improve the success of pregnancy in obese women, it is imperative that we continue our research and efforts to give our future generation a healthy start to life as well as better knowledge to maintain a healthy life.

References

Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C., & Cross, J. C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Developmental Biology, 250*(2), 358-373.

Ain, R., Canham, L. N., & Soares, M. J. (2003). Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: Novel endocrine phenotype and regulation. *Developmental Biology, 260*(1), 176-190.

Allott, E. H., Lysaght, J., Cathcart, M. C., Donohoe, C. L., Cummins, R., McGarrigle, S. A., Kay, E., Reynolds, J.V., Pidgeon, G. P. (2013). MMP9 expression in oesophageal adenocarcinoma is upregulated with visceral obesity and is associated with poor tumour differentiation. *Molecular Carcinogenesis, 52*(2), 144-154. doi:10.1002/mc.21840

Arendas, K., Qiu, Q., & Gruslin, A. (2008). Obesity in pregnancy: Pre-conceptional to postpartum consequences. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal d'Obstetrique Et Gynecologie Du Canada : JOGC, 30*(6), 477-488.

Ashkar, A. A., Di Santo, J. P., & Croy, B. A. (2000). Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *The Journal of Experimental Medicine, 192*(2), 259-270.

Ball, E., Bulmer, J. N., Ayis, S., Lyall, F., & Robson, S. C. (2006). Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. *The Journal of Pathology, 208*(4), 535-542. doi:10.1002/path.1927

Bar, J., Schreiber, L., Saruhanov, E., Ben-Haroush, A., Golan, A., & Kovo, M. (2012). Placental histopathological findings in obese and nonobese women with complicated and uncomplicated pregnancies. *Archives of Gynecology and Obstetrics, 286*(6), 1343-1347. doi:10.1007/s00404-012-2450-z

Bartha, J. L., Romero-Carmona, R., & Comino-Delgado, R. (2003). Inflammatory cytokines in intrauterine growth retardation. *Acta Obstetricia Et Gynecologica Scandinavica, 82*(12), 1099-1102.

Bauer, S., Pollheimer, J., Hartmann, J., Husslein, P., Aplin, J. D., & Knofler, M. (2004). Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. *The Journal of Clinical Endocrinology and Metabolism, 89*(2), 812-822. doi:10.1210/jc.2003-031351

Benirschke, K., Kaufmann, P., & Baergen, R. N. (2006). *Pathology of the human placenta* (Fifth Edition ed.). New York, NY: Springer.

Bosquiazzo, V. L., Durando, M., Varayoud, J., Ramos, J. G., Rodriguez, H. A., Munoz-de-Toro, M., & Luque, E. H. (2005). Macrophage density in the pregnant rat uterine cervix is modulated by mast cell degranulation. *Journal of Reproductive Immunology, 65*(2), 147-158. doi:10.1016/j.jri.2005.01.002

Bourque, S. L., Dolinsky, V. W., Dyck, J. R., & Davidge, S. T. (2012). Maternal resveratrol treatment during pregnancy improves adverse fetal outcomes in a rat model of severe hypoxia. *Placenta, 33*(5), 449-452. doi:10.1016/j.placenta.2012.01.012

Boutsikou, T., Mastorakos, G., Kyriakakou, M., Margeli, A., Hassiakos, D., Papassotiriou, I., Kanaka-Gantenbein, C., Malamitsi-Puchner, A. (2010). Circulating levels of inflammatory markers in intrauterine growth restriction. *Mediators of Inflammation, 2010*, 790605. doi:10.1155/2010/790605

Bulmer, J. N., Innes, B. A., Levey, J., Robson, S. C., & Lash, G. E. (2012). The role of vascular smooth muscle cell apoptosis and migration during uterine spiral artery remodeling in normal human pregnancy. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 26*(7), 2975-2985. doi:10.1096/fj.12-203679

Bulmer, J. N., & Lash, G. E. (2005). Human uterine natural killer cells: A reappraisal. *Molecular Immunology, 42*(4), 511-521. doi:10.1016/j.molimm.2004.07.035

Burton, G. J., Yung, H. W., Cindrova-Davies, T., & Charnock-Jones, D. S. (2009a). Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta, 30 Suppl A*, S43-8. doi:10.1016/j.placenta.2008.11.003

Burton, G. J., Woods, A. W., Jauniaux, E., & Kingdom, J. C. (2009b). Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta, 30*(6), 473-482. doi:10.1016/j.placenta.2009.02.009

Caluwaerts, S., Lambin, S., van Bree, R., Peeters, H., Vergote, I., & Verhaeghe, J. (2007). Diet-induced obesity in gravid rats engenders early hyperadiposity in the offspring. *Metabolism: Clinical and Experimental, 56*(10), 1431-1438. doi:10.1016/j.metabol.2007.06.007

Caluwaerts, S., Vercruysse, L., Luyten, C., & Pijnenborg, R. (2005). Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat. *Placenta, 26*(7), 574-584. doi:10.1016/j.placenta.2004.09.007

Cartwright, J. E., Kenny, L. C., Dash, P. R., Crocker, I. P., Aplin, J. D., Baker, P. N., & Whitley, G. S. (2002). Trophoblast invasion of spiral arteries: A novel in vitro model. *Placenta, 23*(2-3), 232-235. doi:10.1053/plac.2001.0760

Catalan, V., Gomez-Ambrosi, J., Ramirez, B., Rotellar, F., Pastor, C., Silva, C., Rotellar, F., Cienfuegos, J. A., Salvador, J., Fruhbeck, G. (2007). Proinflammatory cytokines in obesity: Impact of type 2 diabetes mellitus and gastric bypass. *Obesity Surgery, 17*(11), 1464-1474.

Chakraborty, D., Rumi, M. A., Konno, T., & Soares, M. J. (2011). Natural killer cells direct hemochorial placentation by regulating hypoxia-inducible factor dependent trophoblast lineage decisions. *Proceedings of the National Academy of Sciences of the United States of America, 108*(39), 16295-16300. doi:10.1073/pnas.1109478108

Challier, J. C., Basu, S., Bintein, T., Minium, J., Hotmire, K., Catalano, P. M., & Hauguel-de Mouzon, S. (2008). Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta, 29*(3), 274-281. doi:10.1016/j.placenta.2007.12.010

Champion, H., Innes, B. A., Robson, S. C., Lash, G. E., Bulmer, J. N. (2012). Effects of interleukin-6 on extravillous trophoblast invasion in early human pregnancy. *Mol.Hum.Reprod.*, 18(8), 391-400. doi: 10.1093/molehr/gas010

Charalambous, F., Elia, A., & Georgiades, P. (2012). Decidual spiral artery remodeling during early post-implantation period in mice: Investigation of associations with decidual uNK cells and invasive trophoblast. *Biochemical and Biophysical Research Communications, 417*(2), 847-852. doi:10.1016/j.bbrc.2011.12.057

Chen, Y. D., Golay, A., Swislocki, A. L., & Reaven, G. M. (1987). Resistance to insulin suppression of plasma free fatty acid concentrations and insulin stimulation of glucose uptake in noninsulin-dependent diabetes mellitus. *The Journal of Clinical Endocrinology and Metabolism, 64*(1), 17-21. doi:10.1210/jcem-64-1-17

Christian, L. M., & Porter, K. (2014). Longitudinal changes in serum proinflammatory markers across pregnancy and postpartum: Effects of maternal body mass index. *Cytokine,* doi:10.1016/j.cyto.2014.06.018

Correia-da-Silva, G., Bell, S. C., Pringle, J. H., & Teixeira, N. A. (2004). Patterns of uterine cellular proliferation and apoptosis in the implantation site of the rat during pregnancy. *Placenta, 25*(6), 538-547. doi:10.1016/j.placenta.2003.11.007

Cotechini, T., Komisarenko, M., Sperou, A., Macdonald-Goodfellow, S., Adams, M. A., & Graham, C. H. (2014). Inflammation in rat pregnancy inhibits spiral artery remodeling leading to fetal growth restriction and features of preeclampsia. *The Journal of Experimental Medicine, 211*(1), 165-179. doi:10.1084/jem.20130295

Craven, C. M., Morgan, T., & Ward, K. (1998). Decidual spiral artery remodelling begins before cellular interaction with cytotrophoblasts. *Placenta, 19*(4), 241-252.

Cross, J. C., Hemberger, M., Lu, Y., Nozaki, T., Whiteley, K., Masutani, M., & Adamson, S. L. (2002). Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta. *Molecular and Cellular Endocrinology, 187*(1-2), 207-212.

Croy, B. A., Chen, Z., Hofmann, A. P., Lord, E. M., Sedlacek, A. L., & Gerber, S. A. (2012). Imaging of vascular development in early mouse decidua and its association with leukocytes and trophoblasts. *Biology of Reproduction, 87*(5), 125. doi:10.1095/biolreprod.112.102830

Croy, B. A., & Kiso, Y. (1993). Granulated metrial gland cells: A natural killer cell subset of the pregnant murine uterus. *Microscopy Research and Technique, 25*(3), 189-200. doi:10.1002/jemt.1070250302

Curat, C. A., Wegner, V., Sengenes, C., Miranville, A., Tonus, C., Busse, R., & Bouloumie, A. (2006). Macrophages in human visceral adipose tissue: Increased accumulation in obesity and a source of resistin and visfatin. *Diabetologia, 49*(4), 744-747. doi:10.1007/s00125-006-0173-z

Dahlman, I., Kaaman, M., Olsson, T., Tan, G. D., Bickerton, A. S., Wahlen, K., Andersson, J., Nordstrom, E. A., Blomqvist, L., Sjogren, A., Forsgren, M., Attersand, A., Arner, P. (2005). A unique role of monocyte chemoattractant protein 1 among chemokines in adipose tissue of obese subjects. *The Journal of Clinical Endocrinology and Metabolism, 90*(10), 5834-5840. doi:10.1210/jc.2005-0369

Damoiseaux, J. G., Dopp, E. A., Calame, W., Chao, D., MacPherson, G. G., & Dijkstra, C. D. (1994). Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology, 83*(1), 140-147.

Davies, G. A., Maxwell, C., McLeod, L., Gagnon, R., Basso, M., Bos, H., Delisle, M. F., Farine, D., Hudon, L., Menticoglou, S., Mundle, W., Murphy-Kaulbeck, L., Ouellet, A., Pressey, T., Roggensack, A., Leduc, D., Ballerman, C., Biringer, A., Duperron, L., Jones, D., Lee, L. S., Shepherd, D., Wilson, K., Society of Obstetricians and Gynaecologists of Canada. (2010). Obesity in pregnancy. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal d'Obstetrique Et Gynecologie Du Canada : JOGC, 32*(2), 165-173.

de Bie, H. M., Oostrom, K. J., & Delemarre-van de Waal, H. A. (2010). Brain development, intelligence and cognitive outcome in children born small for gestational age. *Hormone Research in Paediatrics, 73*(1), 6-14. doi:10.1159/000271911

De Wolf, F., De Wolf-Peeters, C., Brosens, I., & Robertson, W. B. (1980). The human placental bed: Electron microscopic study of trophoblastic invasion of spiral arteries. *American Journal of Obstetrics and Gynecology, 137*(1), 58-70.

Delgado, S. R., McBey, B. A., Yamashiro, S., Fujita, J., Kiso, Y., & Croy, B. A. (1996). Accounting for the peripartum loss of granulated metrial gland cells, a natural killer cell population, from the pregnant mouse uterus. *Journal of Leukocyte Biology, 59*(2), 262-269.

Denison, F. C., Roberts, K. A., Barr, S. M., & Norman, J. E. (2010). Obesity, pregnancy, inflammation, and vascular function. *Reproduction (Cambridge, England), 140*(3), 373-385. doi:10.1530/REP-10-0074

Dijkstra, C. D., Dopp, E. A., Joling, P., & Kraal, G. (1985). The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology, 54*(3), 589-599.

Dokras, A., Hoffmann, D. S., Eastvold, J. S., Kienzle, M. F., Gruman, L. M., Kirby, P. A., Weiss, R. M., Davisson, R. L. (2006). Severe feto-placental abnormalities precede the onset of hypertension and proteinuria in a mouse model of preeclampsia. *Biology of Reproduction, 75*(6), 899-907. doi:10.1095/biolreprod.106.053603

Duc-Goiran, P., Mignot, T. M., Bourgeois, C., & Ferre, F. (1999). Embryo-maternal interactions at the implantation site: A delicate equilibrium. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 83*(1), 85-100.

Even, M. D., Laughlin, M. H., Krause, G. F., & vom Saal, F. S. (1994). Differences in blood flow to uterine segments and placentae in relation to sex, intrauterine location and side in pregnant rats. *Journal of Reproduction and Fertility, 102*(1), 245-252.

Fantuzzi, G. (2005). Adipose tissue, adipokines, and inflammation. *The Journal of Allergy and Clinical Immunology, 115*(5), 911-9; quiz 920. doi:10.1016/j.jaci.2005.02.023

Fraser, R., Whitley, G. S., Johnstone, A. P., Host, A. J., Sebire, N. J., Thilaganathan, B., & Cartwright, J. E. (2012). Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *The Journal of Pathology, 228*(3), 322-332. doi:10.1002/path.4057

Frias, A. E., & Grove, K. L. (2012). Obesity: A transgenerational problem linked to nutrition during pregnancy. *Seminars in Reproductive Medicine, 30*(6), 472-478. doi:10.1055/s-0032-1328875

Frias, A. E., Morgan, T. K., Evans, A. E., Rasanen, J., Oh, K. Y., Thornburg, K. L., & Grove, K. L. (2011). Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition. *Endocrinology, 152*(6), 2456-2464. doi:10.1210/en.2010-1332

Friis, C. M., Paasche Roland, M. C., Godang, K., Ueland, T., Tanbo, T., Bollerslev, J., & Henriksen, T. (2013). Adiposity-related inflammation: Effects of pregnancy. *Obesity (Silver Spring, Md.), 21*(1), E124-30. doi:10.1002/oby.20120

Furukawa, S., Hayashi, S., Usuda, K., Abe, M., Hagio, S., & Ogawa, I. (2011). Toxicological pathology in the rat placenta. *Journal of Toxicologic Pathology, 24*(2), 95-111. doi:10.1293/tox.24.95

Gao, X. L., Yang, H. X., & Zhao, Y. (2008). Variations of tumor necrosis factor-alpha, leptin and adiponectin in mid-trimester of gestational diabetes mellitus. *Chinese Medical Journal, 121*(8), 701-705.

Gardosi, J., Clausson, B., & Francis, A. (2009). The value of customised centiles in assessing perinatal mortality risk associated with parity and maternal size. *BJOG : An International Journal of Obstetrics and Gynaecology, 116*(10), 1356-1363. doi:10.1111/j.1471-0528.2009.02245.x

Georgiades, P., Ferguson-Smith, A. C., & Burton, G. J. (2002). Comparative developmental anatomy of the murine and human definitive placentae. *Placenta, 23*(1), 3-19. doi:10.1053/plac.2001.0738

Geusens, N., Hering, L., Verlohren, S., Luyten, C., Drijkoningen, K., Taube, M., Vercruysse, L., Hanssens, M., Pijnenborg, R. (2010). Changes in endovascular trophoblast invasion and spiral artery remodelling at term in a transgenic preeclamptic rat model. *Placenta, 31*(4), 320-326. doi:10.1016/j.placenta.2010.01.011

Geusens, N., Verlohren, S., Luyten, C., Taube, M., Hering, L., Vercruysse, L., Hanssens, M., Dudenhausen, J. W., Dechend, R., Pijnenborg, R. (2008). Endovascular trophoblast invasion, spiral artery remodelling and uteroplacental haemodynamics in a transgenic rat model of pre-eclampsia. *Placenta, 29*(7), 614-623. doi:10.1016/j.placenta.2008.04.005

Gordon, S., & Martinez, F. O. (2010). Alternative activation of macrophages: Mechanism and functions. *Immunity, 32*(5), 593-604. doi:10.1016/j.immuni.2010.05.007

Graham, C. H., Hawley, T. S., Hawley, R. G., MacDougall, J. R., Kerbel, R. S., Khoo, N., & Lala, P. K. (1993). Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental Cell Research, 206*(2), 204-211.

Grimshaw, J. M., Thomas, R. E., MacLennan, G., Fraser, C., Ramsay, C. R., Vale, L., Whitty, P., Eccles, M. P., Matowe, L., Shirran, L., Wensing, M., Dijkstra, R., Donaldson, C. (2004). Effectiveness and efficiency of guideline dissemination and implementation strategies. *Health Technology Assessment (Winchester, England), 8*(6), iii-iv, 1-72.

Hamilton, S., Oomomian, Y., Stephen, G., Shynlova, O., Tower, C. L., Garrod, A., Lye, S. J., Jones, R. L. (2012). Macrophages infiltrate the human and rat decidua during term and

preterm labor: Evidence that decidual inflammation precedes labor. *Biology of Reproduction, 86*(2), 39. doi:10.1095/biolreprod.111.095505
Harris, L. K. (2011). IFPA gabor than award lecture: Transformation of the spiral arteries in human pregnancy: Key events in the remodelling timeline. *Placenta, 32 Suppl 2,* S154-8. doi:10.1016/j.placenta.2010.11.018
Harris, L. K., Clancy, O. H., Myers, J. E., & Baker, P. N. (2009). Plasma from women with preeclampsia inhibits trophoblast invasion. *Reproductive Sciences (Thousand Oaks, Calif.), 16*(11), 1082-1090. doi:10.1177/1933719109341842
Harris, L. K., Keogh, R. J., Wareing, M., Baker, P. N., Cartwright, J. E., Aplin, J. D., & Whitley, G. S. (2006). Invasive trophoblasts stimulate vascular smooth muscle cell apoptosis by a fas ligand-dependent mechanism. *The American Journal of Pathology, 169*(5), 1863-1874. doi:10.2353/ajpath.2006.060265
Harris, L. K., Keogh, R. J., Wareing, M., Baker, P. N., Cartwright, J. E., Whitley, G. S., & Aplin, J. D. (2007). BeWo cells stimulate smooth muscle cell apoptosis and elastin breakdown in a model of spiral artery transformation. *Human Reproduction (Oxford, England), 22*(11), 2834-2841. doi:10.1093/humrep/dem303
Hartil, K., Vuguin, P. M., Kruse, M., Schmuel, E., Fiallo, A., Vargas, C., Warner, M. J., Durand, J. L., Jelicks, L. A., Charron, M. J. (2009). Maternal substrate utilization programs the development of the metabolic syndrome in male mice exposed to high fat in utero. *Pediatric Research, 66*(4), 368-373. doi:10.1203/PDR.0b013e3181b33375
Hauguel-de Mouzon, S., & Guerre-Millo, M. (2006). The placenta cytokine network and inflammatory signals. *Placenta, 27*(8), 794-798. doi:10.1016/j.placenta.2005.08.009
Hayes, E. K., Lechowicz, A., Petrik, J. J., Storozhuk, Y., Paez-Parent, S., Dai, Q., Samjoo, I. A., Mansell, M., Gruslin, A., Holloway, A. C., Raha, S. (2012). Adverse fetal and neonatal outcomes associated with a life-long high fat diet: Role of altered development of the placental vasculature. *PloS One, 7*(3), e33370. doi:10.1371/journal.pone.0033370
Hayes, E. K., Tessier, D. R., Percival, M. E., Holloway, A. C., Petrik, J. J., Gruslin, A., & Raha, S. (2014). Trophoblast invasion and blood vessel remodeling are altered in a rat model of lifelong maternal obesity. *Reproductive Sciences (Thousand Oaks, Calif.), 21*(5), 648-657. doi:10.1177/1933719113508815
Hazan, A. D., Smith, S. D., Jones, R. L., Whittle, W., Lye, S. J., & Dunk, C. E. (2010). Vascular-leukocyte interactions: Mechanisms of human decidual spiral artery remodeling in vitro. *The American Journal of Pathology, 177*(2), 1017-1030. doi:10.2353/ajpath.2010.091105
Head, J. R., Kresge, C. K., Young, J. D., & Hiserodt, J. C. (1994). NKR-P1+ cells in the rat uterus: Granulated metrial gland cells are of the natural killer cell lineage. *Biology of Reproduction, 51*(3), 509-523.
Helwig, J. J., & Le Bouteiller, P. (2007). Physiological smooth muscle cell apoptosis contributes to the uterine vascular remodeling in human early pregnancy. *Circulation Research, 100*(6), 754-756. doi:10.1161/01.RES.0000263394.59727.ca
Higgins, L., Greenwood, S. L., Wareing, M., Sibley, C. P., & Mills, T. A. (2011). Obesity and the placenta: A consideration of nutrient exchange mechanisms in relation to aberrant fetal growth. *Placenta, 32*(1), 1-7. doi:10.1016/j.placenta.2010.09.019
Honkanen, T., Mustonen, J., Kainulainen, H., Myllymaki, J., Collin, P., Hurme, M., & Rantala, I. (2005). Small bowel cyclooxygenase 2 (COX-2) expression in patients with IgA nephropathy. *Kidney International, 67*(6), 2187-2195. doi:10.1111/j.1523-1755.2005.00324.x
Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature, 444*(7121), 860-867. doi:10.1038/nature05485
Hotamisligil, G. S., Shargill, N. S., & Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science (New York, N.Y.), 259*(5091), 87-91.

Howie, G. J., Sloboda, D. M., Kamal, T., & Vickers, M. H. (2009). Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *The Journal of Physiology, 587*(Pt 4), 905-915. doi:10.1113/jphysiol.2008.163477

Hu, Y., Eastabrook, G., Tan, R., MacCalman, C. D., Dutz, J. P., & von Dadelszen, P. (2010). Decidual NK cell-derived conditioned medium enhances capillary tube and network organization in an extravillous cytotrophoblast cell line. *Placenta, 31*(3), 213-221. doi:10.1016/j.placenta.2009.12.011

Huber, A. V., Saleh, L., Bauer, S., Husslein, P., & Knofler, M. (2006). TNFalpha-mediated induction of PAI-1 restricts invasion of HTR-8/SVneo trophoblast cells. *Placenta, 27*(2-3), 127-136. doi:10.1016/j.placenta.2005.02.012

Hunt, J. S., Petroff, M. G., & Burnett, T. G. (2000). Uterine leukocytes: Key players in pregnancy. *Seminars in Cell & Developmental Biology, 11*(2), 127-137. doi:10.1006/scdb.2000.0158

Isaka, K., Usuda, S., Ito, H., Sagawa, Y., Nakamura, H., Nishi, H., Suzuki, Y., Li, Y. F., Takayama, M. (2003). Expression and activity of matrix metalloproteinase 2 and 9 in human trophoblasts. *Placenta, 24*(1), 53-64.

Jokhi, P. P., King, A., Sharkey, A. M., Smith, S. K., & Loke, Y. W. (1994). Screening for cytokine messenger ribonucleic acids in purified human decidual lymphocyte populations by the reverse-transcriptase polymerase chain reaction. *Journal of Immunology (Baltimore, Md.: 1950), 153*(10), 4427-4435.

Jones, C. J., & Fox, H. (1981). An ultrastructural and ultrahistochemical study of the human placenta in maternal essential hypertension. *Placenta, 2*(3), 193-204.

Jovanovic, M., Stefanoska, I., Radojcic, L., & Vicovac, L. (2010). Interleukin-8 (CXCL8) stimulates trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase (MMP)2 and MMP9 and integrins alpha5 and beta1. *Reproduction (Cambridge, England), 139*(4), 789-798. doi:10.1530/REP-09-0341

Jovanovic, M., & Vicovac, L. (2009). Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. *Placenta, 30*(4), 320-328. doi:10.1016/j.placenta.2009.01.013

Karmakar, S., & Das, C. (2002). Regulation of trophoblast invasion by IL-1beta and TGF-beta1. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 48*(4), 210-219.

Kaufmann, P., Black, S., & Huppertz, B. (2003). Endovascular trophoblast invasion: Implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biology of Reproduction, 69*(1), 1-7. doi:10.1095/biolreprod.102.014977

Keelan, J. A., & Mitchell, M. D. (2007). Placental cytokines and preeclampsia. *Frontiers in Bioscience : A Journal and Virtual Library, 12*, 2706-2727.

Khan, I. Y., Dekou, V., Douglas, G., Jensen, R., Hanson, M. A., Poston, L., & Taylor, P. D. (2005). A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 288*(1), R127-33. doi:10.1152/ajpregu.00354.2004

Khan, I. Y., Taylor, P. D., Dekou, V., Seed, P. T., Lakasing, L., Graham, D., Dominiczak, A. F., Hanson, M. A., Poston, L. (2003). Gender-linked hypertension in offspring of lard-fed pregnant rats. *Hypertension, 41*(1), 168-175.

Khan, N., Asim, H., & Lichtenstein, G. R. (2014). Safety of anti-TNF therapy in inflammatory bowel disease during pregnancy. *Expert Opinion on Drug Safety, 13*(12), 1699-1708. doi:10.1517/14740338.2014.973399

Khong, T. Y., De Wolf, F., Robertson, W. B., & Brosens, I. (1986). Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. *British Journal of Obstetrics and Gynaecology, 93*(10), 1049-1059.

Kim, C. S., Park, H. S., Kawada, T., Kim, J. H., Lim, D., Hubbard, N. E., Kwon, B. S., Yu, R. (2006). Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and

associated with obesity-related parameters. *International Journal of Obesity (2005), 30*(9), 1347-1355. doi:10.1038/sj.ijo.0803259

Kim, D. W., Young, S. L., Grattan, D. R., & Jasoni, C. L. (2014). Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse. *Biology of Reproduction, 90*(6), 130. doi:10.1095/biolreprod.113.117259

King, A., Burrows, T., Verma, S., Hiby, S., & Loke, Y. W. (1998). Human uterine lymphocytes. *Human Reproduction Update, 4*(5), 480-485.

Knofler, M. (2010). Critical growth factors and signalling pathways controlling human trophoblast invasion. *The International Journal of Developmental Biology, 54*(2-3), 269-280. doi:10.1387/ijdb.082769mk

Kovo, M., Schreiber, L., Ben-Haroush, A., Shor, S., Golan, A., & Bar, J. (2012). Intrapartum fever at term: Clinical characteristics and placental pathology. *The Journal of Maternal-Fetal & Neonatal Medicine : The Official Journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstetricians, 25*(8), 1273-1277. doi:10.3109/14767058.2011.629248

Krishna, U., & Bhalerao, S. (2011). Placental insufficiency and fetal growth restriction. *Journal of Obstetrics and Gynaecology of India, 61*(5), 505-511. doi:10.1007/s13224-011-0092-x

Lash, G. E., Cartwright, J. E., Whitley, G. S., Trew, A. J., & Baker, P. N. (1999). The effects of angiogenic growth factors on extravillous trophoblast invasion and motility. *Placenta, 20*(8), 661-667. doi:10.1053/plac.1999.0427

Lash, G. E., Hornbuckle, J., Brunt, A., Kirkley, M., Searle, R. F., Robson, S. C., & Bulmer, J. N. (2007). Effect of low oxygen concentrations on trophoblast-like cell line invasion. *Placenta, 28*(5-6), 390-398. doi:10.1016/j.placenta.2006.06.001

Lash, G. E., Otun, H. A., Innes, B. A., Kirkley, M., De Oliveira, L., Searle, R. F., Robson, S. C., Bulmer, J. N. (2006). Interferon-gamma inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 20*(14), 2512-2518. doi:10.1096/fj.06-6616com

Lea, R. G., McIntyre, S., Baird, J. D., & Clark, D. A. (1998). Tumor necrosis factor-alpha mRNA-positive cells in spontaneous resorption in rodents. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 39*(1), 50-57.

Lee, J. Y., Sohn, K. H., Rhee, S. H., & Hwang, D. (2001). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through toll-like receptor 4. *The Journal of Biological Chemistry, 276*(20), 16683-16689. doi:10.1074/jbc.M011695200

Leisser, C., Saleh, L., Haider, S., Husslein, H., Sonderegger, S., & Knofler, M. (2006). Tumour necrosis factor-alpha impairs chorionic gonadotrophin beta-subunit expression and cell fusion of human villous cytotrophoblast. *Molecular Human Reproduction, 12*(10), 601-609. doi:gal066

Li, Y., Zhang, Y., Dorweiler, B., Cui, D., Wang, T., Woo, C. W., Brunkan, C. S., Wolberger, C., Imai, S., Tabas, I. (2008). Extracellular nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism. *The Journal of Biological Chemistry, 283*(50), 34833-34843. doi:10.1074/jbc.M805866200

Lockwood, C. J., Matta, P., Krikun, G., Koopman, L. A., Masch, R., Toti, P., Arcuri, F., Huang, S. T., Funai, E. F., Schatz, F. (2006). Regulation of monocyte chemoattractant protein-1 expression by tumor necrosis factor-alpha and interleukin-1beta in first trimester human decidual cells: Implications for preeclampsia. *The American Journal of Pathology, 168*(2), 445-452.

Lolmede, K., Durand de Saint Front, V., Galitzky, J., Lafontan, M., & Bouloumie, A. (2003). Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *International Journal of Obesity and Related Metabolic Disorders : Journal of the International Association for the Study of Obesity, 27*(10), 1187-1195. doi:10.1038/sj.ijo.0802407

Lumeng, C. N., & Saltiel, A. R. (2011). Inflammatory links between obesity and metabolic disease. *The Journal of Clinical Investigation, 121*(6), 2111-2117. doi:10.1172/JCI57132

Luo, J., Qiao, F., & Yin, X. (2011). Impact of silencing MMP9 gene on the biological behaviors of trophoblasts. *Journal of Huazhong University of Science and Technology.Medical Sciences = Hua Zhong Ke Ji Da Xue Xue Bao.Yi Xue Ying De Wen Ban = Huazhong Keji Daxue Xuebao.Yixue Yingdewen Ban, 31*(2), 241-245. doi:10.1007/s11596-011-0260-3

Madan, J. C., Davis, J. M., Craig, W. Y., Collins, M., Allan, W., Quinn, R., & Dammann, O. (2009). Maternal obesity and markers of inflammation in pregnancy. *Cytokine, 47*(1), 61-64. doi:10.1016/j.cyto.2009.05.004

Martinez, F. O., Sica, A., Mantovani, A., & Locati, M. (2008). Macrophage activation and polarization. *Frontiers in Bioscience : A Journal and Virtual Library, 13*, 453-461.

McCarthy, N. J., & Bennett, M. R. (2000). The regulation of vascular smooth muscle cell apoptosis. *Cardiovascular Research, 45*(3), 747-755.

McDonald, S. D., Machold, C. A., Marshall, L., & Kingston, D. (2014). Documentation of guideline adherence in antenatal records across maternal weight categories: A chart review. *BMC Pregnancy and Childbirth, 14*, 205-2393-14-205. doi:10.1186/1471-2393-14-205

Moffett-King, A. (2002). Natural killer cells and pregnancy. *Nature Reviews.Immunology, 2*(9), 656-663. doi:10.1038/nri886

Mu, H., Ohashi, R., Yan, S., Chai, H., Yang, H., Lin, P., Yao, Q., Chen, C. (2006). Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovascular Research, 70*(1), 146-157. doi:10.1016/j.cardiores.2006.01.015

Myers, J., Hall, C., Wareing, M., Gillham, J., & Baker, P. (2006). The effect of maternal characteristics on endothelial-dependent relaxation of myometrial arteries. *European Journal of Obstetrics, Gynecology, and Reproductive Biology, 124*(2), 158-163. doi:10.1016/j.ejogrb.2005.05.017

Na, K. H., Lee, H. J., Choi, J. H., Eun, J. W., Nam, S. W., Yoon, T. K., & Kim, G. J. (2012). Dynamic alterations in integrin alpha4 expression by hypoxia are involved in trophoblast invasion during early implantation. *Journal of Cellular Biochemistry, 113*(2), 685-694. doi:10.1002/jcb.23398

Nagamatsu, T., & Schust, D. J. (2010). The contribution of macrophages to normal and pathological pregnancies. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 63*(6), 460-471. doi:10.1111/j.1600-0897.2010.00813.x

Naruse, K., Lash, G. E., Innes, B. A., Otun, H. A., Searle, R. F., Robson, S. C., & Bulmer, J. N. (2009). Localization of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitors for MMPs (TIMPs) in uterine natural killer cells in early human pregnancy. *Human Reproduction (Oxford, England), 24*(3), 553-561. doi:10.1093/humrep/den408

Nguyen Dinh Cat, A., Briones, A. M., Callera, G. E., Yogi, A., He, Y., Montezano, A. C., & Touyz, R. M. (2011). Adipocyte-derived factors regulate vascular smooth muscle cells through mineralocorticoid and glucocorticoid receptors. *Hypertension, 58*(3), 479-488. doi:10.1161/HYPERTENSIONAHA.110.168872

Norman, J. E., & Reynolds, R. M. (2011). The consequences of obesity and excess weight gain in pregnancy. *The Proceedings of the Nutrition Society, 70*(4), 450-456. doi:10.1017/S0029665111003077

Otun, H. A., Lash, G. E., Innes, B. A., Bulmer, J. N., Naruse, K., Hannon, T., Searle, R. F., Robson, S. C. (2011). Effect of tumour necrosis factor-alpha in combination with interferon-

gamma on first trimester extravillous trophoblast invasion. *Journal of Reproductive Immunology, 88*(1), 1-11. doi:10.1016/j.jri.2010.10.003

Parr, E. L., Chen, H. L., Parr, M. B., & Hunt, J. S. (1995). Synthesis and granular localization of tumor necrosis factor-alpha in activated NK cells in the pregnant mouse uterus. *Journal of Reproductive Immunology, 28*(1), 31-40.

Pavan, L., Hermouet, A., Tsatsaris, V., Therond, P., Sawamura, T., Evain-Brion, D., & Fournier, T. (2004). Lipids from oxidized low-density lipoprotein modulate human trophoblast invasion: Involvement of nuclear liver X receptors. *Endocrinology, 145*(10), 4583-4591. doi:10.1210/en.2003-1747

Peleg, D., Kennedy, C. M., & Hunter, S. K. (1998). Intrauterine growth restriction: Identification and management. *American Family Physician, 58*(2), 453-60, 466-7.

Peltier, M. R., Richey, L. J., & Brown, M. B. (2003). Placental lesions caused by experimental infection of sprague-dawley rats with mycoplasma pulmonis. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 50*(3), 254-262.

Perlow, J. H., Morgan, M. A., Montgomery, D., Towers, C. V., & Porto, M. (1992). Perinatal outcome in pregnancy complicated by massive obesity. *American Journal of Obstetrics and Gynecology, 167*(4 Pt 1), 958-962.

Picut, C. A., Swanson, C. L., Parker, R. F., Scully, K. L., & Parker, G. A. (2009). The metrial gland in the rat and its similarities to granular cell tumors. *Toxicologic Pathology, 37*(4), 474-480. doi:10.1177/0192623309335632

Pijnenborg, R., Bland, J. M., Robertson, W. B., & Brosens, I. (1983). Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta, 4*(4), 397-413.

Pijnenborg, R., Dixon, G., Robertson, W. B., & Brosens, I. (1980). Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta, 1*(1), 3-19.

Pijnenborg, R., & Vercruysse, L. (2006a). Mathias duval on placental development in mice and rats. *Placenta, 27*(2-3), 109-118. doi:10.1016/j.placenta.2005.01.009

Pijnenborg, R., Vercruysse, L., & Hanssens, M. (2006b). The uterine spiral arteries in human pregnancy: Facts and controversies. *Placenta, 27*(9-10), 939-958. doi:10.1016/j.placenta.2005.12.006

Platt, J. S., & Hunt, J. S. (1998). Interferon-gamma gene expression in cycling and pregnant mouse uterus: Temporal aspects and cellular localization. *Journal of Leukocyte Biology, 64*(3), 393-400.

Prud'homme, G. J. (2004). Altering immune tolerance therapeutically: The power of negative thinking. *Journal of Leukocyte Biology, 75*(4), 586-599. doi:10.1189/jlb.0803394

Prutsch, N., Fock, V., Haslinger, P., Haider, S., Fiala, C., Pollheimer, J., & Knofler, M. (2012). The role of interleukin-1beta in human trophoblast motility. *Placenta, 33*(9), 696-703. doi:10.1016/j.placenta.2012.05.008

Pustovrh, M. C., Jawerbaum, A., Capobianco, E., White, V., Martinez, N., Lopez-Costa, J. J., & Gonzalez, E. (2005). Oxidative stress promotes the increase of matrix metalloproteinases-2 and -9 activities in the feto-placental unit of diabetic rats. *Free Radical Research,39*(12), 1285-1293. doi:V8274463411V886L

Qiu, Q., Yang, M., Tsang, B. K., & Gruslin, A. (2004). EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction (Cambridge, England), 128*(3), 355-363. doi:10.1530/rep.1.00234

Radulescu, L., Munteanu, O., Popa, F., & Cirstoiu, M. (2013). The implications and consequences of maternal obesity on fetal intrauterine growth restriction. *Journal of Medicine and Life, 6*(3), 292-298.

Raghupathy, R. (2013). Cytokines as key players in the pathophysiology of preeclampsia. *Medical Principles and Practice : International Journal of the Kuwait University, Health Science Centre, 22 Suppl 1*, 8-19. doi:10.1159/000354200

Raha, S., & Robinson, B. H. (2001). Mitochondria, oxygen free radicals, and apoptosis. *American Journal of Medical Genetics, 106*(1), 62-70. doi:10.1002/ajmg.1398

Rajasingam, D., Seed, P. T., Briley, A. L., Shennan, A. H., & Poston, L. (2009). A prospective study of pregnancy outcome and biomarkers of oxidative stress in nulliparous obese women. *American Journal of Obstetrics and Gynecology, 200*(4), 395.e1-395.e9. doi:10.1016/j.ajog.2008.10.047

Red-Horse, K., Rivera, J., Schanz, A., Zhou, Y., Winn, V., Kapidzic, M., Maltepe, E. Okazaki, K., Kochman, R., Vo, K. C., Giudice, L., Erlebacher, A., McCune, J. M., Stoddart, C. A., Fisher, S. J. (2006). Cytotrophoblast induction of arterial apoptosis and lymphangiogenesis in an in vivo model of human placentation. *The Journal of Clinical Investigation, 116*(10), 2643-2652. doi:10.1172/JCI27306

Reister, F., Heyl, W., Kaufmann, P., & Rath, W. (1999). Trophoblast invasion in pre-eclampsia. [Die Trophoblastinvasion bei Praeklampsie] *Zentralblatt Fur Gynakologie, 121*(12), 587-590.

Renaud, S. J., Macdonald-Goodfellow, S. K., & Graham, C. H. (2007). Coordinated regulation of human trophoblast invasiveness by macrophages and interleukin 10. *Biology of Reproduction, 76*(3), 448-454. doi:10.1095/biolreprod.106.055376

Rivera, D. L., Olister, S. M., Liu, X., Thompson, J. H., Zhang, X. J., Pennline, K.,Azuera, R., Clark, D. A., Miller, M. J. (1998). Interleukin-10 attenuates experimental fetal growth restriction and demise. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 12*(2), 189-197.

Roberts, K. A., Riley, S. C., Reynolds, R. M., Barr, S., Evans, M., Statham, A., Hor, K., Jabbour, H. N., Norman, J. E., Denison, F. C. (2011). Placental structure and inflammation in pregnancies associated with obesity. *Placenta*, doi:10.1016/j.placenta.2010.12.023

Robertshaw, H. A., Raha, S., Kaczor, J. J., & Tarnopolsky, M. A. (2008). Increased PFK activity and GLUT4 protein content in McArdle's disease. *Muscle & Nerve, 37*(4), 431-437. doi:10.1002/mus.20947

Robson, A., Harris, L. K., Innes, B. A., Lash, G. E., Aljunaidy, M. M., Aplin, J. D., . . . Bulmer, J. N. (2012). Uterine natural killer cells initiate spiral artery remodeling in human pregnancy. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 26*(12), 4876-4885. doi:10.1096/fj.12-210310

Rosario, G. X., Konno, T., & Soares, M. J. (2008). Maternal hypoxia activates endovascular trophoblast cell invasion. *Developmental Biology, 314*(2), 362-375. doi:10.1016/j.ydbio.2007.12.007

Rossant, J., & Cross, J. C. (2001). Placental development: Lessons from mouse mutants. *Nature Reviews.Genetics, 2*(7), 538-548. doi:10.1038/35080570

Royle, C., Lim, S., Xu, B., Tooher, J., Ogle, R., & Hennessy, A. (2009). Effect of hypoxia and exogenous IL-10 on the pro-inflammatory cytokine TNF-alpha and the anti-angiogenic molecule soluble flt-1 in placental villous explants. *Cytokine, 47*(1), 56-60. doi:10.1016/j.cyto.2009.04.006

Rusterholz, C., Hahn, S., & Holzgreve, W. (2007). Role of placentally produced inflammatory and regulatory cytokines in pregnancy and the etiology of preeclampsia. *Seminars in Immunopathology, 29*(2), 151-162. doi:10.1007/s00281-007-0071-6

Saito, S., Nishikawa, K., Morii, T., Enomoto, M., Narita, N., Motoyoshi, K., & Ichijo, M. (1993). Cytokine production by CD16-CD56bright natural killer cells in the human early pregnancy decidua. *International Immunology, 5*(5), 559-563.

Salam, R. A., Das, J. K., & Bhutta, Z. A. (2014). Impact of intrauterine growth restriction on long-term health. *Current Opinion in Clinical Nutrition and Metabolic Care, 17*(3), 249-254. doi:10.1097/MCO.0000000000000051

Samuelsson, A. M., Matthews, P. A., Argenton, M., Christie, M. R., McConnell, J. M., Jansen, E. H., Piersma, A. H., Ozanne, S. E., Twinn, D. F., Remacle, C., Rowlerson, A., Poston, L.,

Taylor, P. D. (2008). Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: A novel murine model of developmental programming. *Hypertension, 51*(2), 383-392. doi:10.1161/HYPERTENSIONAHA.107.101477

Savona-Ventura, C., & Savona-Ventura, S. (2014). The inheritance of obesity. *Best Practice & Research.Clinical Obstetrics & Gynaecology*, doi:10.1016/j.bpobgyn.2014.07.023

Schmatz, M., Madan, J., Marino, T., & Davis, J. (2010). Maternal obesity: The interplay between inflammation, mother and fetus. *Journal of Perinatology : Official Journal of the California Perinatal Association, 30*(7), 441-446. doi:10.1038/jp.2009.182

Schnitzler, F., Fidder, H., Ferrante, M., Ballet, V., Noman, M., Van Assche, G., Spitz, B., Hoffman, I., Van Steen, K., Vermeire, S., Rutgeerts, P. (2011). Outcome of pregnancy in women with inflammatory bowel disease treated with antitumor necrosis factor therapy. *Inflammatory Bowel Diseases, 17*(9), 1846-1854. doi:10.1002/ibd.21583; 10.1002/ibd.21583

Sennikov, S. V., Injelevskaya, T. V., Krysov, S. V., Silkov, A. N., Kovinev, I. B., Dyachkova, N. J., Zenkov, A. N., Loseva, M. I., Kozlov, V. A. (2004). Production of hemo- and immunoregulatory cytokines by erythroblast antigen+ and glycophorin A+ cells from human bone marrow. *BMC Cell Biology, 5*(1), 39. doi:10.1186/1471-2121-5-39

Sennikov, S. V., Krysov, S. V., Silkov, A. N., Injelevskaya, T. V., & Kozlov, V. A. (2002). Production of IL-10, TNF-alpha, IFN-gamma, TGF-beta1 by different populations of erythroid cells derived from human embryonal liver. *Cytokine, 17*(4), 221-225. doi:10.1006/cyto.2001.0975

Shankar, K., Harrell, A., Liu, X., Gilchrist, J. M., Ronis, M. J., & Badger, T. M. (2008). Maternal obesity at conception programs obesity in the offspring. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 294*(2), R528-38. doi:10.1152/ajpregu.00316.2007

Shi, Q. J., Lei, Z. M., Rao, C. V., & Lin, J. (1993). Novel role of human chorionic gonadotropin indifferentiation of human cytotrophoblasts. *Endocrinology, 132*(3), 1387-1395. doi:10.1210/endo.132.3.7679981

Shibata, Y., Foster, L. A., Kurimoto, M., Okamura, H., Nakamura, R. M., Kawajiri, K., Justice, J. P., Van Scott, M. R., Myrvik, Q. N., Metzger, W. J. (1998). Immunoregulatory roles of IL-10 in innate immunity: IL-10 inhibits macrophage production of IFN-gamma-inducing factors but enhances NK cell production of IFN-gamma. *Journal of Immunology (Baltimore, Md.: 1950), 161*(8), 4283-4288.

Sica, A., & Mantovani, A. (2012). Macrophage plasticity and polarization: In vivo veritas. *The Journal of Clinical Investigation, 122*(3), 787-795. doi:10.1172/JCI59643

Sitras, V., Paulssen, R. H., Gronaas, H., Leirvik, J., Hanssen, T. A., Vartun, A., & Acharya, G. (2009). Differential placental gene expression in severe preeclampsia. *Placenta, 30*(5), 424-433. doi:10.1016/j.placenta.2009.01.012

Sivitz, W. I., Wayson, S. M., Bayless, M. L., Sinkey, C. A., & Haynes, W. G. (2007). Obesity impairs vascular relaxation in human subjects: Hyperglycemia exaggerates adrenergic vasoconstriction arterial dysfunction in obesity and diabetes. *Journal of Diabetes and its Complications, 21*(3), 149-157. doi:10.1016/j.jdiacomp.2005.12.003

Skarzinski, G., Khamaisi, M., Bursztyn, M., Mekler, J., Lan, D., Evdokimov, P., & Ariel, I. (2009). Intrauterine growth restriction and shallower implantation site in rats with maternal hyperinsulinemia are associated with altered NOS expression. *Placenta, 30*(10), 898-906. doi:10.1016/j.placenta.2009.07.014

Sladek, S. M., Kanbour-Shakir, A., Watkins, S., Berghorn, K. A., Hoffman, G. E., & Roberts, J. M. (1998). Granulated metrial gland cells contain nitric oxide synthases during pregnancy in the rat. *Placenta, 19*(1), 55-65.

Smith, S. D., Dunk, C. E., Aplin, J. D., Harris, L. K., & Jones, R. L. (2009). Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy. *The American Journal of Pathology, 174*(5), 1959-1971. doi:10.2353/ajpath.2009.080995

Soares, M. J., Chakraborty, D., Karim Rumi, M. A., Konno, T., & Renaud, S. J. (2012a). Rat placentation: An experimental model for investigating the hemochorial maternal-fetal interface. *Placenta, 33*(4), 233-243. doi:10.1016/j.placenta.2011.11.026

Soares, M. J., Chakraborty, D., Renaud, S. J., Kubota, K., Bu, P., Konno, T., & Rumi, M. A. (2012b). Regulatory pathways controlling the endovascular invasive trophoblast cell lineage. *The Journal of Reproduction and Development, 58*(3), 283-287.

Soto-Guzman, A., Navarro-Tito, N., Castro-Sanchez, L., Martinez-Orozco, R., & Salazar, E. P. (2010). Oleic acid promotes MMP-9 secretion and invasion in breast cancer cells. *Clinical & Experimental Metastasis, 27*(7), 505-515. doi:10.1007/s10585-010-9340-1

Spencer, M., Yao-Borengasser, A., Unal, R., Rasouli, N., Gurley, C. M., Zhu, B., Peterson, C. A., Kern, P. A. (2010). Adipose tissue macrophages in insulin resistant subjects are associated with collagen VI, fibrosis and demonstrate alternative activation. *American Journal of Physiology.Endocrinology and Metabolism*, doi:10.1152/ajpendo.00329.2010

Staff, A. C., Ranheim, T., Henriksen, T., & Halvorsen, B. (2000). 8-iso-prostaglandin f(2alpha) reduces trophoblast invasion and matrix metalloproteinase activity. *Hypertension, 35*(6), 1307-1313.

Staun-Ram, E., Goldman, S., Gabarin, D., & Shalev, E. (2004). Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. *Reproductive Biology and Endocrinology : RB&E, 2*, 59. doi:10.1186/1477-7827-2-59

Stewart, F. M., Freeman, D. J., Ramsay, J. E., Greer, I. A., Caslake, M., & Ferrell, W. R. (2007). Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers. *The Journal of Clinical Endocrinology and Metabolism, 92*(3), 969-975. doi:10.1210/jc.2006-2083

Stewart, I., & Peel, S. (1982). Changes in the cell population of the pregnant rodent uterus in relation to the differentiation of granulated metrial gland cells. *Journal of Anatomy, 135*(Pt 1), 111-118.

Svensson, J., Jenmalm, M. C., Matussek, A., Geffers, R., Berg, G., & Ernerudh, J. (2011). Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. *Journal of Immunology (Baltimore, Md.: 1950), 187*(7), 3671-3682. doi:10.4049/jimmunol.1100130

Tanaka, M., Nozaki, M., Fukuhara, A., Segawa, K., Aoki, N., Matsuda, M., Komuro, R., Shimomura, I. (2007). Visfatin is released from 3T3-L1 adipocytes via a non-classical pathway. *Biochemical and Biophysical Research Communications, 359*(2), 194-201. doi:10.1016/j.bbrc.2007.05.096

Tantbirojn, P., Crum, C. P., & Parast, M. M. (2008). Pathophysiology of placenta creta: The role of decidua and extravillous trophoblast. *Placenta, 29*(7), 639-645. doi:10.1016/j.placenta.2008.04.008

Taylor, P. D., Khan, I. Y., Lakasing, L., Dekou, V., O'Brien-Coker, I., Mallet, A. I., Hanson, M. A., Poston, L. (2003). Uterine artery function in pregnant rats fed a diet supplemented with animal lard. *Experimental Physiology, 88*(3), 389-398.

Tessier, D. R., Yockell-Lelievre, J., & Gruslin, A. (2014). Uterine spiral artery remodeling: The role of uterine natural killer cells and extravillous trophoblasts in normal and high-risk human pregnancies. *American Journal of Reproductive Immunology (New York, N.Y.: 1989)*, doi:10.1111/aji.12345

Tycko, B., & Efstratiadis, A. (2002). Genomic imprinting: Piece of cake. *Nature, 417*(6892), 913-914. doi:10.1038/417913a

Vadgama, J. V., Castro, M., & Christensen, H. N. (1987). Characterization of amino acid transport during erythroid cell differentiation. *The Journal of Biological Chemistry, 262*(27), 13273-13284.

van der Avoort, I. A., van der Laak, J. A., Paffen, A., Grefte, J. M., Massuger, L. F., de Wilde, P. C., de Hullu, J. A., Bulten, J. (2007). MIB1 expression in basal cell layer: A diagnostic tool to identify premalignancies of the vulva. *Modern Pathology : An Official Journal of the United States and Canadian Academy of Pathology, Inc, 20*(7), 770-778. doi:10.1038/modpathol.3800796

van der Meer, A., Lukassen, H. G., van Lierop, M. J., Wijnands, F., Mosselman, S., Braat, D. D., & Joosten, I. (2004). Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. *Molecular Human Reproduction, 10*(3), 189-195. doi:10.1093/molehr/gah032

van Oostveen, D. C., van den Berg, T. K., Damoiseaux, J. G., & van Rees, E. P. (1992). Macrophage subpopulations and reticulum cells in rat placenta. an immunohistochemical study. *Cell and Tissue Research, 268*(3), 513-519.

Vercruysse, L., Caluwaerts, S., Luyten, C., & Pijnenborg, R. (2006). Interstitial trophoblast invasion in the decidua and mesometrial triangle during the last third of pregnancy in the rat. *Placenta, 27*(1), 22-33. doi:10.1016/j.placenta.2004.11.004

Verlohren, S., Geusens, N., Morton, J., Verhaegen, I., Hering, L., Herse, F., Dudenhausen, J. W., Muller, D. N., Luft, F. C., Cartwright, J. E., Davidge, S. T., Pijnenborg, R., Dechend, R. (2010). Inhibition of trophoblast-induced spiral artery remodeling reduces placental perfusion in rat pregnancy. *Hypertension, 56*(2), 304-310. doi:10.1161/HYPERTENSIONAHA.110.153163

Volpe, C. M., & Nogueira-Machado, J. A. (2013). The dual role of free fatty acid signaling in inflammation and therapeutics. *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery, 7*(3), 189-197.

Vrachnis, N., Vitoratos, N., Iliodromiti, Z., Sifakis, S., Deligeoroglou, E., & Creatsas, G. (2010). Intrauterine inflammation and preterm delivery. *Annals of the New York Academy of Sciences, 1205*, 118-122. doi:10.1111/j.1749-6632.2010.05684.x

Wali, J. A., Thomas, H. E., & Sutherland, A. P. (2014). Linking obesity with type 2 diabetes: The role of T-bet. *Diabetes, Metabolic Syndrome and Obesity : Targets and Therapy, 7*, 331-340. doi:10.2147/DMSO.S51432

Walker, M. C., Finkelstein, S. A., Rennicks White, R., Shachkina, S., Smith, G. N., Wen, S. W., & Rodger, M. (2011). The ottawa and kingston (OaK) birth cohort: Development and achievements. *Journal of Obstetrics and Gynaecology Canada : JOGC = Journal d'Obstetrique Et Gynecologie Du Canada : JOGC, 33*(11), 1124-1133.

Wallace, A. E., Fraser, R., & Cartwright, J. E. (2012). Extravillous trophoblast and decidual natural killer cells: A remodelling partnership. *Human Reproduction Update, 18*(4), 458-471. doi:10.1093/humupd/dms015

Walschus, U., Hoene, A., Kochanowski, A., Neukirch, B., Patrzyk, M., Wilhelm, L., Schroder, K., Schlosser, M. (2011). Quantitative immunohistochemical examination of the local cellular reactions following implantation of biomaterials. *Journal of Microscopy, 242*(1), 94-99. doi:10.1111/j.1365-2818.2010.03445.x

Wang, Y., & Walsh, S. W. (1996). TNF alpha concentrations and mRNA expression are increased in preeclamptic placentas. *Journal of Reproductive Immunology, 32*(2), 157-169. doi:S0165-0378(96)00998-9

Watson, E. D., & Cross, J. C. (2005). Development of structures and transport functions in the mouse placenta. *Physiology (Bethesda, Md.), 20*, 180-193. doi:10.1152/physiol.00001.2005

Welsh, A. O., & Enders, A. C. (1993). Chorioallantoic placenta formation in the rat. III. granulated cells invade the uterine luminal epithelium at the time of epithelial cell death. *Biology of Reproduction, 49*(1), 38-57.

Whitley, G. S., & Cartwright, J. E. (2009). Trophoblast-mediated spiral artery remodelling: A role for apoptosis. *Journal of Anatomy, 215*(1), 21-26. doi:10.1111/j.1469-7580.2008.01039.x

Whitley, G. S., & Cartwright, J. E. (2010). Cellular and molecular regulation of spiral artery remodelling: Lessons from the cardiovascular field. *Placenta, 31*(6), 465-474. doi:10.1016/j.placenta.2010.03.002

Williams, P. J., Searle, R. F., Robson, S. C., Innes, B. A., & Bulmer, J. N. (2009). Decidual leucocyte populations in early to late gestation normal human pregnancy. *Journal of Reproductive Immunology, 82*(1), 24-31. doi:10.1016/j.jri.2009.08.001

Winkler, G., Cseh, K., Baranyi, E., Melczer, Z., Speer, G., Hajos, P., Salamon, F., Turi, Z., Kovacs, M., Vargha, P., Karadi, I. (2002). Tumor necrosis factor system in insulin resistance in gestational diabetes. *Diabetes Research and Clinical Practice, 56*(2), 93-99.

Wozniak, S. E., Gee, L. L., Wachtel, M. S., & Frezza, E. E. (2009). Adipose tissue: The new endocrine organ? A review article. *Digestive Diseases and Sciences, 54*(9), 1847-1856. doi:10.1007/s10620-008-0585-3

Wu, G., Imhoff-Kunsch, B., & Girard, A. W. (2012). Biological mechanisms for nutritional regulation of maternal health and fetal development. *Paediatric and Perinatal Epidemiology, 26 Suppl 1*, 4-26. doi:10.1111/j.1365-3016.2012.01291.x

Xu, J., Zhao, Y. H., Chen, Y. P., Yuan, X. L., Wang, J., Zhu, H., & Lu, C. M. (2014). Maternal circulating concentrations of tumor necrosis factor-alpha, leptin, and adiponectin in gestational diabetes mellitus: A systematic review and meta-analysis. *TheScientificWorldJournal, 2014*, 926932. doi:10.1155/2014/926932

Yelavarthi, K. K., Chen, H. L., Yang, Y. P., Cowley, B. D.,Jr, Fishback, J. L., & Hunt, J. S. (1991). Tumor necrosis factor-alpha mRNA and protein in rat uterine and placental cells. *Journal of Immunology (Baltimore, Md.: 1950), 146*(11), 3840-3848.

Zeyda, M., Farmer, D., Todoric, J., Aszmann, O., Speiser, M., Gyori, G., Zlabinger, G. J., Stulnig, T. M. (2007). Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *International Journal of Obesity (2005), 31*(9), 1420-1428. doi:10.1038/sj.ijo.0803632

Zhang, H., & Zhang, C. (2009). Regulation of microvascular function by adipose tissue in obesity and type 2 diabetes: Evidence of an adipose-vascular loop. *American Journal of Biomedical Sciences, 1*(2), 133-142.

Zhu, M. J., Du, M., Nathanielsz, P. W., & Ford, S. P. (2010). Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. *Placenta, 31*(5), 387-391. doi:10.1016/j.placenta.2010.02.002