MATERNAL OBESITY REMODELS THE MATERNAL INTESTINAL MICROBIOTA AND IS ASSOCIATED WITH ALTERED MATERNAL INTESTINAL AND PLACENTAL FUNCTION

MATERNAL OBESITY REMODELS THE MATERNAL INTESTINAL MICROBIOTA AND IS ASSOCIATED WITH ALTERED MATERNAL INTESTINAL AND PLACENTAL FUNCTION

By

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ABSTRACT

The prevalence of overweight and obesity have risen to epidemic proportions. Overweight and obesity are prominent risk factors for the development of chronic disease including diabetes, cardiovascular disease and cancer. Especially pronounced in women of reproductive age and children, the obesity epidemic represents a major threat to global health. Maternal obesity is a key predictor of childhood obesity and diseases of metabolic origin in adulthood. Previous work has demonstrated that the exposure to early life adversity, in the context of maternal obesity, is associated with an increased risk of metabolic disease and obesity in the offspring later in life. Although the mechanisms outlining the relationship between maternal and offspring obesity remain unclear, the intestinal microbiota has come forth as a promising area of research. To understand the factors involved in the maternal intestinal microbial shifts with healthy pregnancy, the preliminary study focused on investigating whether female sex-steroid hormones mediate maternal intestinal microbial shifts in non-pregnant, regularly cycling female mice. We have identified that intestinal microbial shifts are not associated with sex-steroid hormone fluctuations. The second study examined whether maternal intestinal microbial shifts that occur during obese pregnancy were associated with altered inflammatory signaling and function of the maternal intestine and placenta at a critical period of development; embryonic (E) day 14.5. Females fed a high fat diet (HFD) were significantly heavier at mating and throughout gestation compared to CON. At E14.5, High fat (HF) dams displayed increased adiposity, hyperglycemia, hyperinsulinemia, hyperleptinemia and were insulin resistant. Pregnancy and maternal obesity resulted in shifts in the maternal intestinal microbiota, where the most significant increase in microbial relative abundance was exhibited by the mucin degrading genus, *Akkermansia*. At

E14.5, maternal intestinal microbial shifts were associated with higher maternal intestinal NFκB activity in all sections of the maternal intestine, most notably in the maternal colon. Maternal obesity was associated with increased Muc5ac mRNA levels and a modest increase in CD3^{+} T cells in the maternal colon at E14.5. However, maternal intestinal permeability was unchanged between groups. In the placenta, mRNA levels of key signaling components in the pro-inflammatory toll-like receptor 4 (TLR4) pathway; TRAF6, NFκB and potent pro-inflammatory cytokine TNF-α were increased and in HF females. Maternal obesity was associated with an increase in CD3^{+} T cells in the junctional zone (JZ), but not in the labyrinth zone (LZ) of the placenta at E14.5. These findings were associated with increased mRNA levels of critical nutrient transporters; glucose transporter 1 (GLUT1) and sodium-coupled neutral amino acid transporter 2 (SNAT2) and a modest increase in glucose transporter 3 (GLUT3) in HF placentae compared to CON. These data identify the mechanistic signaling pathways and cell types involved in modulating the intrauterine environment, thus contributing to the current literature devoted to the investigation of the developmental origins of obesity.

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LIST OF ABBREVIATIONS

16S rRNA – 16S ribosomal RNA
17-β estradiol – 17-Beta estradiol
AC – Abdominal circumference
AGRP – Agouti-related peptide
Akt – Protein kinase B
AMPK – Adenosine monophosphate (AMP)-activated protein kinase
ARC – Arcuate nucleus
β-Actin – Beta actin
β-Cell – Beta cell
β-diversity – Beta diversity
β-Glucuronidase – Beta Glucuronidase
BMI – Body mass index
CD – Control diet
CD3 – Cluster of differentiation 3
CD14 – Cluster of differentiation 14
CD36 – Cluster of differentiation 36
CD68 – Cluster of differentiation 68
CDC – Centre for disease control
CI – Confidence interval
CON – Control
CRP – C-reactive protein
CVD – Cardiovascular disease
D - Diestrous
DIO – Diet-induced obesity
DNAJA2 – DNAJ heat-shock protein member A2
DOHaD – Developmental origins of health and disease
E – Estrus
E0.5 – Embryonic day 0.5
E6.5 – Embryonic day 6.5
E10.5 – Embryonic day 10.5
E14.5 – Embryonic day 14.5
ERβ – Estrogen receptor beta
EtOH – Ethanol
F4/80 – EGF-like module-containing mucin-like hormone receptor-like 1
FABP4 – Fatty acid binding protein 4
FATP – Fatty acid transport protein
FATP1 – Fatty acid transport protein 1
FATP4 – Fatty acid transport protein 4
Fiaf – Fasting-induced adipose factor
FITC-dextran – Fluorescein isothiocyanate-dextran
G-CSF – Granulocyte-colony stimulating factor

GF – Germ free
GDM – Gestational diabetes mellitus
GH – Growth hormone
GLUT1 – Glucose transporter 1
GLUT3 – Glucose transporter 3
GLUT4 – Glucose transporter 4
GM-CSF – Granulocyte-macrophage colony stimulating factor
Gpr41 – G protein-coupled receptor
GPT2 – Glutamic pyruvate transaminase 2
GWG – Gestational weight gain
HBSS – Hanks balanced salt solution
HC – Head circumference
HDAC1 – Histone deacetylase 1
HDL – High-density lipoprotein
HEK – Human embryonic kidney
HF – High fat
HFD – High fat diet
HOMA-IR – Homeostatic model assessment of insulin resistance
HPA – Hypothalamic pituitary adrenal
HPRT – Hypoxanthine-guanine phosphoribosyltransferase
HR – Hazard ratio
IFN-γ – Interferon gamma
IGF – Insulin-like growth factor
IGF-1 – Insulin-like growth factor-1
IGF-2 – Insulin-like growth factor-2
IGFBP-1 – Insulin-like growth factor binding protein-1
IKK – IκB kinase
IL-1α – Interleukin-1 alpha
IL-1β – Interleukin-1 beta
IL-2 – Interleukin-2
IL-3 – Interleukin-3
IL-4 – Interleukin-4
IL-5 – Interleukin-5
IL-6 – Interleukin-6
IL-8 – Interleukin-8
IL-9 – Interleukin-9
IL-10 – Interleukin-10
IL-12 – Interleukin-12
IL-13 – Interleukin-13
IL-17A – Interleukin-17A
IRβ – Insulin receptor beta
IRS2 – Insulin receptor substrate 2
LBW – Low birth weight

LGA – Large for gestational age
LPS – Lipopolysaccharide
MCP-1 – Monocyte chemoattractant protein-1
MCR4 – Melanocortin receptor 4
MD2 – Lymphocyte antigen 96
MIP-1α – Macrophage inflammatory protein-1 alpha
MIP-1β – Macrophage inflammatory protein-1 beta
MS – Metabolic syndrome
MUC1 – Mucin 1
MUC2 – Mucin 2
MUC4 – Mucin 4
MUC5AC – Mucin 5ac
MyD88 – Myeloid differentiation marker 88
NFκB – Nuclear factor kappa B
NIH – National institute of health
NO – Nitric oxide
Npas2 – Neuronal PAS domain protein 2
NPC – Non-pregnant control
NPY – Neuropeptide Y
OB-Rb – Leptin long receptor
OGTT – Oral glucose tolerance test
OR – Odds ratio
OTU – Operational taxonomic unit
P - Proestrous
PCoA – Principle coordinate analysis
PCR – Polymerase chain reaction
PERMANOVA – Permutational multivariate analysis of variance
POMC – Pro-opiatemelanocortin
PPARγ – Proliferator-activated receptor gamma
PVH – Paraventricular nucleus
PYY – Peptide YY
QIIME – Quantitative insights into microbial ecology
RANTES – Regulated on activation of normal T cell expressed and secreted
Rdh12 – Retinol dehydrogenase 12
RFU – Relative fluorescent unit
SCFA – Short-chain fatty acid
SGA – Small for gestational age
SNAT2 – Sodium coupled amino acid transporter 2
STAT3 – Signal transducers and activator of transcription 3
T1D – Type 1 diabetes
T2D – Type 2 diabetes
TG - Triglycerides
TLR2 – Toll-like receptor 2

TLR4 – Toll-like receptor 4
TNFα – Tumor necrosis factor alpha
TRAF6 – TNF receptor-associated factor 6
UK – United Kingdom
USSR – Union of Soviet Socialist Republics
VEGF – Vascular endothelial growth factor
WHO – World Health Organization

DECLARATION OF ACADEMIC ACHIEVEMENT

The present study was designed, executed, analyzed and written by the author of this thesis, under the supervision and guidance of Dr. Deborah M. Sloboda with the following exceptions:

All animal work was completed jointly by Tatiane Ribeiro and Jessica G. Wallace. Katherine Kennedy assisted with maternal serum insulin and leptin measurements. Laura Rossi assisted with bacterial 16S rRNA V3 sequencing. Wajiha Gohir, Tatiane Ribeiro, Brendan Patterson, Nina Varah, Fei Fei Xia, Patrycja Jazwiec and Lyn Li assisted with primer design and validation. F4/80 and CD3 positive cells were immunolocalized in the maternal colon and placenta by the McMaster Immunology Research Centre John Mayberry Histology Facility. Danielle Penney and Justine Mayne assisted with the semi-quantitative analysis of F4/80 positive macrophages and CD3 positive T cells respectively, in the maternal colon and placenta. Nina Varah assisted with the semi-quantitative analysis of goblet cell numbers in the maternal colon. Dr. Deborah M. Sloboda contributed significantly to the study design, analysis and interpretation of the data. Dr. Dawn Bowdish, Dr. Jonathan Schertzer and Dr. Michael Surette contributed significantly to the analysis and interpretation of the experimental results.

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1.0 INTRODUCTION

The worldwide prevalence of obesity is now recognized to have reached epidemic proportions. The World Health Organization (WHO) reports that globally at least 1.9 billion adults are overweight while 600 million are clinically obese [1]. The WHO and the National Institutes of Health (NIH) define overweight and obesity as abnormal or excessive fat accumulation that may impair health. Overweight is classified as a body mass index (BMI) of 25-29.9 kg/m^2 and obesity as a BMI > 30 kg/m^2. Obesity is further divided into three sub-categories; class I (BMI 30-34.9), class II (35-39.9) and class III (>40) and is classically defined as a combination of three of the following five complications; raised blood pressure, central adiposity, raised serum triglycerides, lowered serum high-density lipoprotein (HDL) cholesterol and fasting hyperglycemia [2]. Most concerning is the rising rates of obesity in children and women of reproductive age [3, 4].

Maternal obesity is directly associated with an increase in maternal complications during pregnancy [5]. Early in pregnancy, the obese woman is at an increased risk for miscarriage, while later pregnancy complications include gestational hypertension, preeclampsia, gestational diabetes mellitus (GDM) and preterm delivery. A positive linear regression exists between the incidence of gestational hypertension and preeclampsia with increasing maternal body mass index (BMI) (odds ratio (OR) 4.21; 95% confidence interval (CI) 3.45, 5.13 preeclampsia and OR 3.66; 95% CI 3.23, 4.15 for gestational hypertension) [6]. For each unit increase in pre-pregnancy BMI, preeclampsia risk is reported to increase by 8% [7]. Consistent investigations demonstrate a significant reduction in preeclampsia risk with declining BMI [8]. Pregnancy risk for these aforementioned complications is reported to be elevated threefold for morbidly obese women [6]. In addition to the increased risk of antenatal obstetric outcomes, obese women are

also at an increased risk for Caesarian delivery. A linear trend has been observed between pre-pregnancy maternal BMI and risk for both elective and unplanned Caesarian section [9, 10]. This risk is amplified with excess gestational weight gain (GWG) ; defined as weight gain during pregnancy which exceeds the guidelines implemented by the Institute of Medicine based on pre-pregnancy BMI (Table 1.0) [11] during pregnancy [12].

Pre-Pregnancy BMI	BMI (kg/m^2)	Total Weight Gain Range (lbs)	Rate of Weight Gain*
Underweight	< 18.5	28 – 40	1 (1 – 1.3)
Normal weight	18.5 – 24.9	25 – 35	1 (0.8 – 1)
Overweight	25.0 – 29.9	15 – 25	0.6 (0.5 – 0.7)
Obese	≥ 30.0	11 - 20	0.5 (0.4 – 0.6)

Table 1.0 Recommendations for Total and Rate of Weight Gain during Pregnancy, by Pre-Pregnancy BMI. * Calculations assume a 1.1 – 4.4 lb weight gain in the first trimester. Data adapted from [11]

1.1 Impact of obesity on the mother

1.1.1 Type 2 Diabetes Mellitus

Obesity and overweight significantly increase the risk of developing metabolic disease including type 2 diabetes and cardiovascular disease. Diabetes mellitus is a chronic metabolic disease caused by a decrease in the production of insulin or sensitivity to insulin, where type 1 diabetes (T1D) is defined as the destruction of insulin producing cells in the pancreas and type 2 diabetes (T2D) as resistance to insulin and insufficient insulin production [13]. Diabetes prevalence in women of reproductive age is on the rise; in the United Kingdom (UK), diabetes prevalence

increased from 2.34 to 5.09 per 1000 pregnancies between 1995 and 2008; and further increased

to 10.62 per 1000 pregnancies between 2009 and 2012 [13]. Established diabetes mellitus (T1D

or T2D) is a common pre-existing medical condition in pregnant women [14, 15]. The United

States Centres for Disease Control (CDC) reports that *pre-gestational* diabetes occurs in 2-5

women per 1000 pregnancies [16]. Globally, women of reproductive age show a particularly

high prevalence of T2D (3.2% of the population).

1.1.2 Gestational Diabetes Mellitus (GDM)

Not only does obesity and overweight increase the risk of T2D prior to pregnancy, these

conditions also increase maternal risk of developing gestational diabetes mellitus (GDM). GDM

is defined as carbohydrate intolerance of variable severity with onset or first recognition during

pregnancy [17, 18]. The definition applies irrespective of whether or not insulin is used for

treatment or the condition persists following gestation. Approximately 3-15% of women develop

GDM during pregnancy [17]. Although many predisposing factors have been identified

including, age, ethnicity, parity, family history, previous GDM diagnosis and degree of

hyperglycemia during pregnancy, obesity is a strong independent risk factor for developing

GDM with a risk of approximately 20%. Many epidemiological studies in various geographical

locations have examined the relationship between obesity and the risk of developing GDM. One

large study examining 287,213 pregnancies in the UK, found that maternal obesity was

associated with a 2-fold increase in the risk of developing GDM (Odds Ratio (OR) 1.68; 95% CI:

1.53-1.84) [19]. Similarly, studies investigating morbid obesity (BMI > 40) during pregnancy

show a GDM incidence rate of 24.5% [20] and a 3-fold increase in GDM risk [21] compared to

lean women. A population-based study of 96,801 singleton births found that not only obese

(BMI > 30), but also overweight women (BMI 25.0-29.9) also show a marked increase in GDM

risk (OR; 5.0 and 2.4, respectively) [22]. Although direct associations have been drawn between glucose screening categories, obesity, and GDM risk [23], conflicting evidence exists to suggest that the establishment of normoglycemia with insulin therapy in overweight and obese patients diagnosed with GDM reduces the risk of developing subsequent metabolic syndrome[24].

1.1.3 Cardiovascular Disease (CVD)

In addition to T2D, maternal obesity is also characterized by increased arterial blood pressure, hemoconcentration and poor cardiac function [19, 20]. The risk of pregnancy-induced hypertension or preeclampsia are significantly greater if the mother is overweight; where maternal BMI >30, is associated with a 2-3-fold increased risk of preeclampsia [19, 25-28]. Up to 16 weeks gestation, pregnancy-induced hypertension and preeclampsia are reported to be correlated with antenatal waist circumference; data collected from 1142 pregnant women show that a waist circumference > 80 cm was associated with a 2-fold greater (OR 1.8; CI 95% 1.1-2.9) and 3-fold greater (OR 2.7; CI 95% 1.1-6.8) risk in developing hypertension and preeclampsia respectively [19, 27]. Studies suggest that these associations appear to be sensitive to the degree of obesity during pregnancy [20, 21]. A retrospective study examining the impact of morbid obesity (BMI > 35; class II and III) on hypertensive complications during gestation show a 4-fold increase in preeclampsia risk relative to lean subjects [21]. A second study, evaluating the impact of BMI > 40 demonstrated similar findings of an increase in hypertensive complications 28.8% in the obese compared to 2.9% in the non-obese group [20]. Consistent with this, the only risk factors associated with the development of severe preeclampsia were obesity (OR 3.5; 95% CI 1.6-7.4) and a history of preeclampsia in multiparous women (OR 7.2; 95% CI 2.7-18.7) [29]. Preeclampsia risk has also been strongly correlated with pre-pregnancy

BMI where each 5-7 kg/m^2 increase in pre-pregnancy BMI doubled the risk of preeclampsia [30].

1.1.4 Metabolic Syndrome (MS)

In 1988 it was proposed that resistance to insulin-stimulated glucose uptake and secondary hyperinsulinemia are involved in the etiology of three major, related diseases: T2D, hypertension and cardiovascular disease (CVD) [31]. Originally, this triad was termed "Syndrome X". This term has since been modified to describe a group of clinical metabolic presentations including resistance to insulin stimulated glucose uptake, glucose intolerance, hyperinsulinemia, increased circulating triglycerides (TG), decreased HDL and hypertension and is referred to as the Metabolic Syndrome (MS) [32].

Obesity has been identified as the number 1 risk factor for developing MS. Several studies have investigated the relationship between obesity, GDM and subsequent MS. In obese women, an increased risk of developing MS has been identified as early as 3 months post-partum [33, 34] and has been observed to persist up to 11 years after delivery [35]. In a Spanish study investigating early MS onset, 3.7% of 788 Caucasian women with GDM were diagnosed with overt diabetes 3-6 months after delivery. The area under the post-partum glucose curve was found to be positively correlated with maternal BMI, waist circumference, waist/hip ratio, triglycerides, and systolic and diastolic blood pressure [33]. A similar trend has been observed with mild glucose intolerance during gestation and the subsequent development of MS. A prospective cohort study studying 487 women revealed that MS prevalence progressively increased from women with normal glucose tolerance (10%), to impaired glucose intolerance during pregnancy (17.6%), to women with gestational diabetes (20.0%) [34]. These results have

been supported by similar studies of longer duration [35], to examine the long-term effects of GDM on maternal metabolic status. In a group of 81 participants, women with prior gestational hyperglycemia displayed elevated BMI, waist, blood pressure, serum glucose, insulin, C-peptide, homeostatic model assessment (HOMA), fibrinogen and lower levels of HDL after a mean of 8.5 years from the index pregnancy. Prevalence of the MS and its components was 2-4-fold higher in women with prior GDM and 10-fold higher if comorbid obesity entering pregnancy existed relative to normoglycemic women. Cox proportional hazard analysis predicted a hazard ratio (HR 4.26 and 1.21) for gestational hyperglycemia and pregnancy BMI respectively after adjustment for age and pre-pregnancy BMI. Participants in the highest quartile of fasting serum glucose at the oral glucose tolerance test (OGTT) of the index pregnancy was significantly correlated with the incidence of MS and its components [36]. These observations have been replicated by a larger study consisting of 207 women (106 diagnosed with GDM and 101 healthy controls) where follow up after 11 years post-delivery was still predictive of subsequent MS [35]. Epidemiological studies have shown a relationship between pregnancies complicated by preeclamsia and an increased risk of maternal coronary heart disease later in life. The increase in the relative risk of death from ischemic heart disease in association with a history of preeclampsia is 2-fold [30].

1.2 Maternal obesity and impacts on fetal development

It is well recognized that maternal obesity and its associated obstetrical complications not only impact the health of the mother [37, 38], but also have ramifications for the offspring [39]. Maternal obesity and elevated GWG, during pregnancy are associated with altered fetal development and pregnancy outcomes [6, 40-46]. These include delivering a baby that is large for gestational age (LGA, birth weight > 90th percentile after adjusting for sex and gestational

age), fetal macrosomia (birth weight > 4.5 kg at any gestational age), neonatal hypoglycemia,

neural tube defects and cardiovascular defects [42]. The incidence of high birth weight babies is

positively associated with increasing maternal BMI, where both obese and morbidly obese

women show a 2-fold greater risk of delivering a baby weighing greater than 4000 grams (OR

2.01 (95% CI 1.83, 2.22) and OR 2.62 (95% CI 2.30, 2.98), respectively) [6]. Studies suggest

that macrosomia is more common in obese non-diabetic mothers relative to lean non-diabetic

mothers [41]. These studies suggest that additional factors influence fetal growth *in utero* aside

from the original Pedersen hypothesis, which suggested that maternal hyperglycemia results in

fetal overgrowth stimulated by increased glucose transport across the placenta and subsequent

hypertrophy of islets due to insulin-hypersecretion [46]. It is now recognized that in the absence

of GDM, accelerated fetal growth in response to hyperinsulinemia is driven not only by elevated

maternal glucose, but also by maternal triglyceride levels [47] where circulating triglyceride

concentrations have been shown to be positively correlated with birth weight [48] independent of

maternal BMI, weight gain, and plasma glucose concentrations [49] and amino acid turnover.

Maternal obesity has been associated with elevated fetal adiposity, insulin resistance and

increased cord leptin levels *in utero* [43]. Strong positive correlations have been observed

between maternal pre-gravid BMI and fetal insulin resistance, fetal adiposity and fetal insulin

resistance, and cord leptin levels and fetal insulin resistance [43]. Although maternal obesity has

been reported to protect against small for gestational age (SGA, birthweight <10th adjusted

centile), a number of obese women are at risk for both actual [44] and relative fetal growth

restriction [45]. Other studies suggest that the additive effects of maternal comorbidities during

pregnancy may impact fetal metabolism; at 28 weeks gestation, a fivefold risk of fetal abdominal

circumference (AC) > 90th percentile (adjusted relative risk 2.05 (95% CI 1.37-3.07)) and a

threefold risk of head circumference (HC) to AC ratio < 10th percentile 1.97 (1.30-2.99) is

observed in obese women diagnosed with GDM. Furthermore, fetal AC > 90th percentile at 28

weeks is associated with a fourfold risk of being large for gestational age at birth.

1.3 Maternal obesity and long term ramifications to the offspring

Short-term impacts of maternal obesity on fetal development are associated with long-term

consequences to offspring health and disease risk [50, 51]. Maternal obesity [52, 53] and excess

GWG [54] are independently associated with an increased risk of childhood obesity, defined by

elevated BMI [55], percent adiposity [56], waist circumference [57] and the metabolic syndrome.

These associations have been observed to extend into adulthood, where maternal obesity has

been associated with lifelong obesity risk and metabolic syndrome in the offspring characterized

by hyperglycaemia, increased insulin resistance, hypertension, dyslipidemia [53] and all-cause

mortality after adjustment for confounding variables including diet and lifestyle [58].

1.4 The Developmental Origins of Health and Disease (DOHaD)

Maternal obesity remains the strongest predictor of offspring obesity independent of genetic and

lifestyle determinants [5]. Cross-sectional [59] and retrospective [60] studies of inheritance

collectively show a stronger correlation of child to maternal BMI compared to paternal BMI after

controlling for multiple factors including birth weight, birth year and gender of the infant along

with the mothers age, ethnicity, education level, parity, weight gain and environmental exposures

[59-61]. These data suggest that in addition to genetic factors, the *in utero* environment may

strongly contribute to obesity development in the offspring. Thus, the investigation of

environmental factors that may influence disease risk have concentrated on the early life

environment. Today as an experimental field, this research is referred to as the Developmental Origins of Health and Disease (DOHaD).

A considerable proportion of disease risk is established well before birth. The developing organism is plastic and is able to adapt to its environment, thus, a single genotype can give rise to a range of different physiological or morphological states in response to differing environmental conditions during development [62]. From an evolutionary perspective, it is hypothesized that this plastic period enables the production of phenotypes that are best matched to their predicted postnatal environment to maximize organism fitness [63-71]. This critical period where the organism makes predictive adaptive responses is followed by a loss of plasticity and a fixed functional capacity [62]. For many mammals, this plastic period occurs *in utero* although many studies show that plasticity extends from preconception and is embedded in the germ cells up to early childhood [64-68, 70-72].

The Developmental Origins of Health and Disease (DOHaD) theory suggests that environmental factors interact with genotype during the phase of developmental plasticity and allow the organism to adapt to its predicted postnatal environment [73, 74]. Under circumstances where *in utero* adaptations are mismatched to the postnatal environment, disease risk increases [73]. Evidence from both epidemiological and more recently a growing number of experimental animal studies demonstrate that obesity and metabolic disease have developmental origins.

1.4.1 The Origins of DOHaD: Epidemiological data

Past orthodoxy accepted that the origin of metabolic disease was a product of genetic inheritance combined with an unhealthy lifestyle [73]. However this view left the prevalence and geography of metabolic disease studied in the UK largely unexplained. Steep increases in the prevalence of

metabolic diseases including cardiovascular disease and ischemic heart disease was associated

with rising prosperity [75, 76]. However, the prevalence of cardiovascular disease and ischemic

heart disease were demonstrated to be highest in poorer regions and lower-income populations in

the UK [74, 77, 78]. Biochemical and physiological measurements in adulthood have been

shown to correlate with the incidence of coronary heart disease [79]. However, even when

combined with these biochemical risk factors, adult lifestyle predicts the coronary heart disease

incidence with limited ability [80].

Geographical studies provided the first answer to this paradox suggesting that classical "lifestyle-

associated" disease may have origins during intrauterine development [73, 81, 82]. This

indication arose from a study that observed a parallel relationship between mortality rates from

cardiovascular disease in adults and newborn infants in England and Wales [73, 83]. Mortality

rates calculated from death certificates from men and women between 1968 and 1978 revealed

that the concentration of low mortality from coronary heart disease in the south and east

contrasted with the high mortality in the poor rural areas in the north and west of England and

Wales [84]. Death from cardiovascular disease was found to be twice as high in lower income

populations in poorer regions of England and Wales [84, 85]. Early epidemiological studies were

conducted using archived epidemiological data sets collected by health visitors and midwives in

three locations; Hertfordshire [82, 86], Preston [87] and Sheffield [88] in the United Kingdom

(UK) and were the first to suggest that weight at birth was associated with risk of developing

diseases of metabolic origin later in life. Data obtained from the Medical Research Council of

Britain between the years 1911 and 1930 [82] included detailed measurements of growth in

infancy as well as birthweight [81]. Using a correlative strategy, Dr. David JP Barker focused on

examining the relationship between weight at birth, identified as an established marker of fetal

growth *in utero*, and the later life development of metabolic disease including type 2 diabetes

mellitus (T2D) and cardiovascular disease [81, 82, 89].

1.4.1.1 Historical correlations between birthweight with adulthood disease risk

Between the years 1935 and 1943 standardized measures of infant birthweight, length from

crown to heel, head circumference and placental weight collected from women admitted to the

Sharoc Green Hospital and comprise the Preston records [90]. Investigations of these records

showed that thinness at birth measured by a low ponderal index (birthweight/length^3) was

associated with impaired glucose tolerance, elevated blood pressure, and altered lipid

metabolism evident at 7 years of age [91]. These findings have been supported by subsequent

investigations and found to persist into adulthood [90, 92]. Studies conducted in other

geographical locations in the UK support the early observations from the Preston records, further

suggesting that low birthweight (LBW) infants display insulin resistance in the first few years of

life [93, 94], evidenced by a poor response to glucose challenge. Correlative studies from the

Hertfordshire records, comprised of measures of birthweight and weight at 1 year of age from 10

636 men born between 1911 and 1930 revealed that death rates from coronary heart disease fell

progressively between individuals weighing < 5.5 to 9.5 lbs at birth. Birth weight was also found

to predict diabetes risk, where both extremes of the normal birth weight spectrum (< 5lbs and

>9.5lbs at birth) were found to exhibit the highest risk of diabetes incidence in adulthood,

producing a reversed "J shaped" relationship (Figure 1.4.1.1.1) [81]. Since the early

epidemiological observations in the UK, these associations have been replicated among men and

women in North America, Europe and India [95-100].
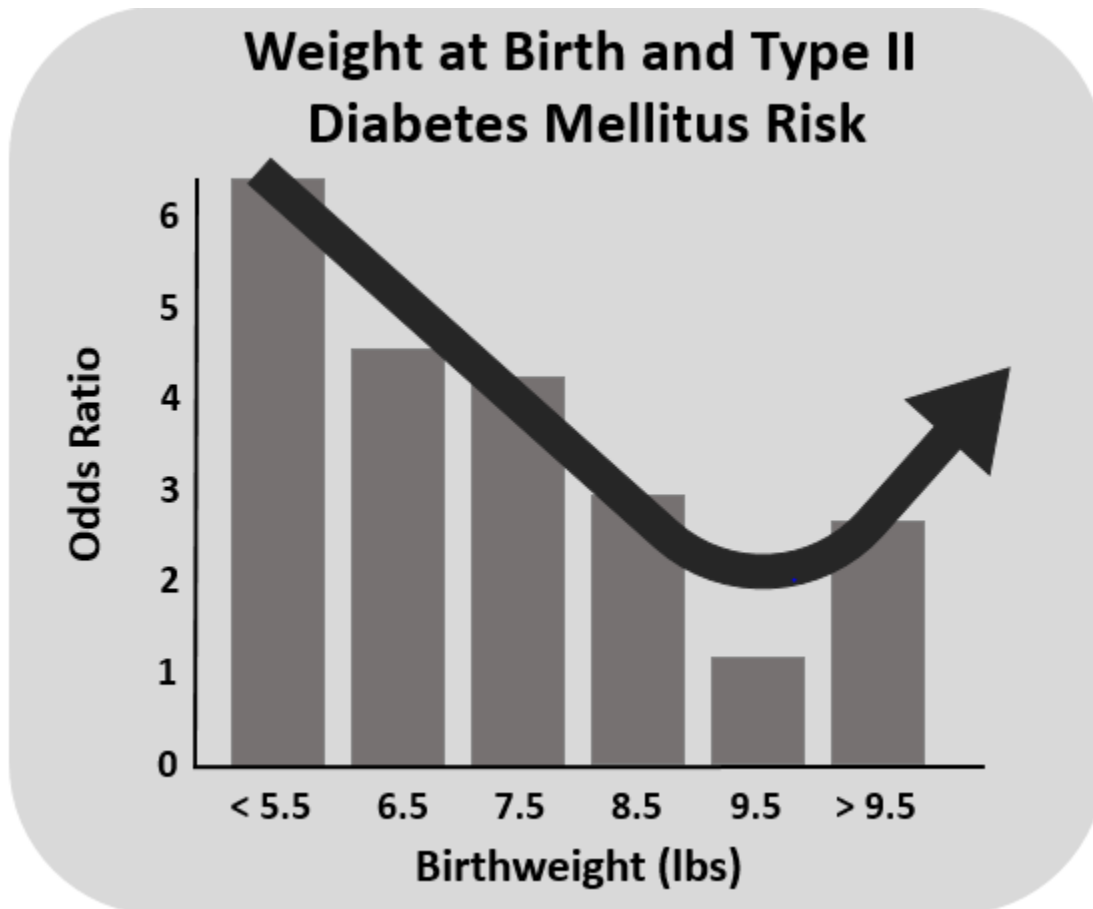
Figure 1.4.1.1.1 Birth weight predicts type 2 diabetes risk in adulthood. A schematic representation of the reversed "J shaped" relationship between age-adjusted weight at birth and risk of developing Type II Diabetes Mellitus in adulthood. Data adapted from [81].

The largest of these follow up studies, the Nurses' Health Study, was established in 1976 when

121,700 female registered nurses aged 30 to 55 responded to questionnaires requesting

information about their medical history and lifestyle. Initially designed to investigate the use of

oral contraceptives, cigarette smoking, and risk of major diseases in women, this prospective

cohort study was broadened to include the evaluation of health consequences of many lifestyle

factors including diet and physical activity [101]. Data from this study has contributed

significantly to the early associations of birthweight with adulthood disease risk [97, 102].

Retrospective self-reports of birth weight among participants was found to correlate with risk of

metabolic disease in adulthood. Following adjustment for adult BMI and maternal history of

diabetes, an inverse association was observed across the entire birth weight spectrum but was not

altered by adjustment for ethnicity, childhood socioeconomic status or adult lifestyle factors.

Compared with the reference group, the relative risks by ascending birth weight category were

1.83 (95% CI, 1.55-2.16) for birthweight < 5 lb, 1.76 (1.49-2.07) for birth weight 5 lb to 5.5 lb,

1.23 (1.11-1.37) for birth weight 5.6 lb to 7 lb, 0.95 (0.82-1.10) for birth weight 8.6 lb to 10 lb

and 0.83 (0.63-1.07) for birth weight > 10 lb (Figure 1.4.1.1.2) [102]. Another study

investigating the incidence of non-fatal CVD in the same cohort demonstrated that higher birth

weight was protective against non-fatal CVD events including myocardial infarction, coronary

revascularization and stroke. Relative risks adjusted for several cardiovascular risk factors were

1.49 (95% CI 1.05-2.10) for birth weight < 5 lbs, 1.25 (0.98-1.61) for birth weight 5 lb to 5 lb 8

oz, 1.12 (0.98-1.27) for birth weight > 5 lb 8 oz to 7 lb, 1.00 for birth weight > 7 lb to 8 lb 8 oz,

0.96 (0.80-1.15) for birth weight > 8 lb 8 oz to 10 lb and 0.68 (0.46 to 1.00) for birth weight > 10

lb [97]. Together, these data suggest that individuals at the lowest and highest weights of the

normal birth weight spectrum are at an increased risk of developing metabolic disease in

adulthood.

Relative Risk of Non-Fatal Coronary Heart Disease and Stroke
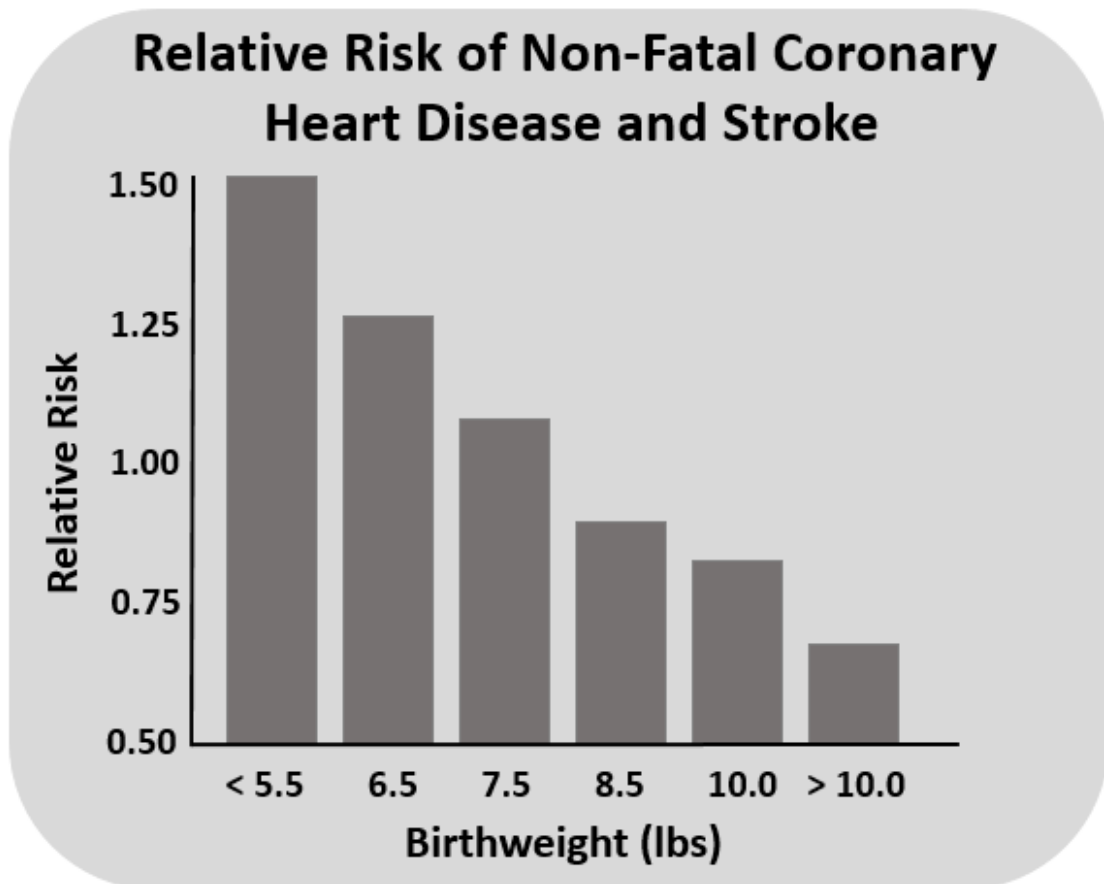
Figure 1.4.1.1.2 Birth Weight and Non-Fatal Cardiovascular Disease are Inversely Associated in Adult Women. A schematic representation of the inverse relationship between birth weight and non-fatal cardiovascular disease in adult women from the American Nurses' Health Study. Data adapted from [97].

1.4.1.2 Historical correlations between maternal nutrition during pregnancy and offspring disease risk

Following the early epidemiological studies investigating birth weight and the development of metabolic disease in adulthood, later epidemiological studies concentrated on investigating possible mechanisms underpinning this relationship. As nutrition is a key mediator of fetal growth, many studies began using historical data sets collected during times of famine to investigate the effect of poor maternal nutrition on the relationship between birthweight and subsequent adult disease risk. The most notable of these epidemiological studies, were the

investigations conducted using data compiled from *the Hongerwinter* [103-105], also known as

the Dutch Hunger Winter. During the winter of 1944 to 1945, the Nazi-occupied western region

of the Netherlands, including Amsterdam, experienced a great famine as a result of a German

blockade, which limited food transports to the densely populated western part of the country

[103, 105-108]. As a result, the western Netherlands failed to receive any food, fuel and medical

imports affecting all citizens of all social classes, including pregnant women. During the Dutch

Hunger Winter, the average daily caloric intake was limited to 500-750 calories/day compared to

the average of 1750 kcal/day in this region in the 4 years prior to the famine [106]. Thus, the

Dutch Hunger Winter serves as an exemplary epidemiological study to examine the effects of

early, mid and late intrauterine nutritional deprivation on subsequent health outcomes in the

offspring (Figure 1.4.1.1.3). Studies revealed that although exposure to the Dutch famine only

had a small impact on fetal growth [109], exposure during early gestation was associated with

the development of hypertension [103, 110], increased prevalence of coronary heart disease

[111], and obesity in women [105] and men [103]. Exposure during mid to late gestation was

associated with increased renal dysfunction [112]. Glucose intolerance in the offspring was

found to exist independent of the timing of exposure to famine [104, 107]. Furthermore, a

correlation between being small for gestational age (SGA) and onset of diabetes in adulthood has

been observed, particularly in females [108].
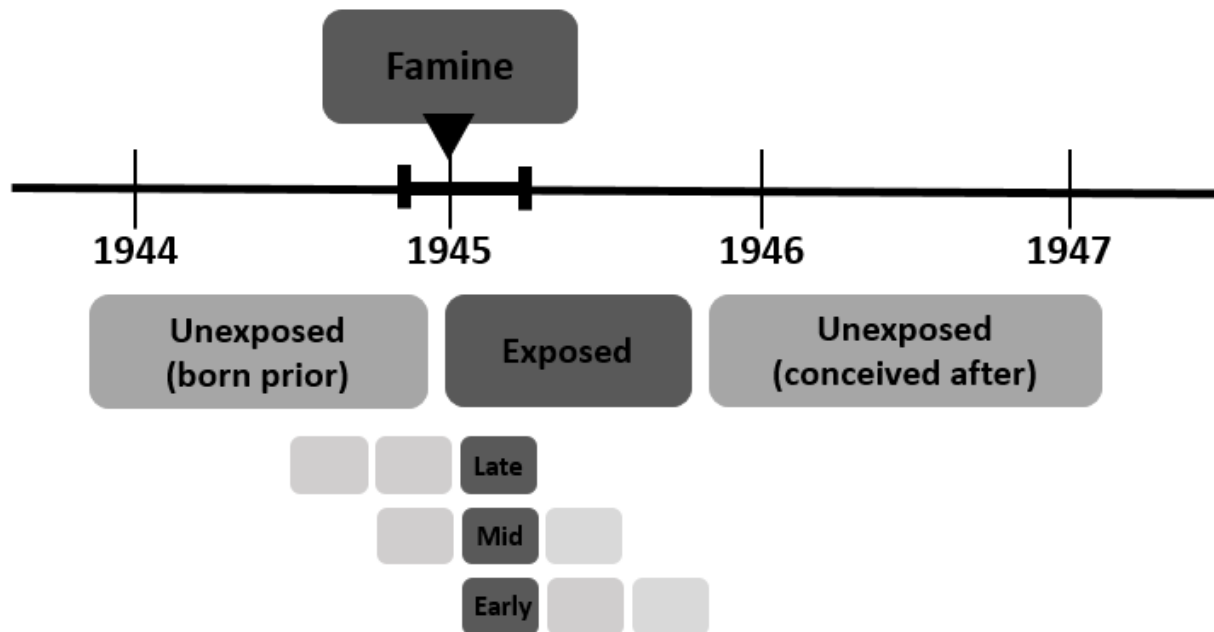
Figure 1.4.1.1.3 The Dutch Hunger Winter Birth Cohort. A schematic representation of famine exposure and birth in relation to the timing of *the Hongerwinter*. Data adapted from [106].

Although contested in the literature [113], hypotheses that concentrate on adaptations have been proposed to explain the associations between birthweight and metabolic disease in adulthood. The "thrifty phenotype hypothesis" proposed by Hales and Barker [65, 114] suggests that under conditions of poor maternal nutrition, adaptive responses are made by the fetus to maximize the uptake and conservation of any available nutrients, resulting in a conservative metabolism. Metabolic consequences arise when the postnatal environment consists of an adequate or plentiful nutrient intake which exceeds the range of the predictive adaptive responses [72]. The consequence of this "mismatch" is proposed to be the development of metabolic disease later in life. Following *the Hongerwinter*, nutrient and caloric intake were plentiful (Figure 1.4.2.2). Therefore, nutrition in the actual postnatal environment was higher than that predicted by the fetus *in utero*, resulting in metabolic complications in adulthood. Intrauterine growth restriction is considered to have large consequences on metabolism when infants are born into a postnatal

environment where nutrition is plentiful. These infants experience "postnatal catch-up growth" where rapid postnatal growth occurs in the first few months of life. Postnatal catch-up growth has been identified as a risk factor for the later development of metabolic disease [115].

Conversely, when intrauterine nutrient restriction is followed by subsequent exposure to a similar postnatal environment where nutrient intake is poor, programming of a thrifty phenotype is suggested to confer an adaptive advantage [65, 72]. This effect has been intensively studied with data collected from the Siege of Leningrad, Union of Soviet Socialist Republics (U.S.S.R) [116, 117]. Similar to the circumstances of the Dutch Hunger Winter, German forces besieged Leningrad, now known as St. Petersburg and prevented the transport of food supplies to the city centre from September 1941 to January 1944. As a result, approximately half of the city's population died due to starvation with the average daily caloric ration was approximately 300 calories [118]. Intrauterine nutrient restriction during the Siege of Leningrad was found to be associated with an average fall in birth weight [117], however follow up investigations of 549 children born during the siege concluded that there was no effect of maternal undernutrition on glucose intolerance, the incidence of T2D, hypertension or CVD in adulthood [116-118]. These results have been interpreted to be due to a matched average caloric intake before and after the siege of Leningrad, as the famine extended well into the postnatal period [116, 117]. Thus, intrauterine predictive adaptive responses for this population in the actual postnatal environment may have been appropriate for the actual postnatal environment. Consistent with the theory of predictive adaptive responses, data from other population-based studies suggest that intrauterine growth restriction has a lesser impact on long-term metabolic health when nutrition in the postnatal environment is compromised to a similar degree [119]. A range of follow up studies conducted in North America, South Africa [120], the Caribbean [121], India [96, 122], and

Australia [123] support the early observations from the Dutch Hunger Winter, Siege of Leningrad and the China famine and have identified the risk of developing metabolic disease to be greater in adults exposed to caloric restriction *in utero*.

In addition to maternal nutrient restriction, maternal nutrient excess during pregnancy influences offspring disease risk. Abundant evidence now exists linking high birth weight to increased obesity risk in adolescence and adulthood [52, 60, 124-126]. Large cohort studies such as the Nurses' Health Study [127] and the Health Professionals Follow-up Study [126] have shown that infants of a high birth weight show an increased risk for developing obesity (age-adjusted odds ratio (OR 1.62 95% CI, 1.38-1.90) and (OR 2.08 1.73-2.50) respectively. These investigations report a reversed J-shaped curve of the relationship between birth weight and obesity risk [126, 127] suggesting that similar to maternal undernutrition during pregnancy, individuals at the lowest and highest weights of the normal birth weight spectrum are at an increased risk of developing metabolic disease in adulthood when exposed to maternal nutrient excess *in utero*. A similar trend has been reported in LGA infants; defined as weight, length or head circumference that is above the 90th percentile for an infant's gestational age, born to obese mothers [52, 128]. A recent retrospective cohort study in low-income women demonstrated that maternal obesity in the first trimester of pregnancy (BMI > 30) was associated with the prevalence of childhood obesity (BMI > 95th percentile based on CDC criteria) at ages 2, 3 and 4 was 15.1, 20.6 and 24.1% respectively. After controlling for birth weight, birth year and gender of the infant and maternal age, ethnicity, education level, parity, weight gain and smoking the relative risk of childhood obesity was 2.0 (95% CI, 1.7-2.3) at 2 years of age and 2.3 (2.0-2.6) at 3 and 4 years of age. This result was between 2.4 and 2.7 times the prevalence of obesity observed in children born to mothers with a BMI within the normal range (BMI 18.5-24.9) [60]. These findings

support the hypothesis that early life exposure to adversity, for which birth weight is a marker, are associated with the development of metabolic disease later in life.

1.4.2 Animal models of the developmental origins of health and disease

Investigations using experimental animal models complement the knowledge drawn from early epidemiological studies and continue to be instrumental in advancing our understanding of the mechanisms underpinning the relationship between early life adversity and the risk of developing disease later in life. Gestational programming of obesity risk has previously been examined in a number of animal models, primarily in rodents, but also in larger animal models such as the sheep and pig [129-131]. These studies examine the impact of numerous factors capable of influencing the early life environment and include differing degrees, duration and critical timing of maternal macronutrient/caloric restriction and nutrient excess. In rodents, diet-induced maternal obesity often, but not always [132, 133], results in fetal growth restriction [134, 135], followed by postnatal catch up growth [132-135], and later life hyperinsulinemeia, hyperleptinemia, insulin resistance, pancreatic signalling and insulin secretion deficits [134, 136-139], and hepatic glucose and lipid handling dysfunction [135, 140-142]. Often, these results are sex specific [143-146] consistent with human data [146]. Fetuses from diet-induced obese ewes are macrosomic, hyperglycemic and hyperinsulinemic [147] and display decreased insulin signaling in skeletal muscle associated with increased intramuscular triglycerides and higher expression of fatty acid transporters [148]. Placentas of obese ewes show enhanced fatty acid transporter protein levels and transcript factors responsible for the regulation of lipid metabolism [135, 149]. Interestingly, metabolic compromise is often amplified in offspring born to high-fat mothers upon high-fat diet exposure following weaning [150, 151], suggesting an interaction between the pre and postnatal environment in the establishment of offspring metabolism and the

development of adult disease. These studies highlight the importance of the prenatal environment

in shaping the postnatal response to metabolic challenge [132-134, 152].

1.4.2.1 Maternal nutrient restriction

Early developmental origins studies focusing on the impact of maternal nutrition during

pregnancy concentrated on maternal nutrient/caloric restriction. Animal models of maternal

nutrient/caloric restriction have been instrumental in outlining the underlying mechanisms

resulting in the development of metabolic disease in adulthood. These models have been

predominantly undertaken using rodents [153-156] although studies have been conducted in the

sheep [68, 157-163] and primate [164-168]. Dietary manipulation by global caloric restriction,

reduction of dietary protein content, iron restriction or have been the focus of the majority of

studies. In these studies, maternal nutrient/caloric restriction ranges from mild (30% reduction in

caloric intake) [153] through moderate (50% reduction in caloric intake) [154] to severe (70%

reduction in caloric intake) [155, 156].

Phenotypically, offspring born to pregnant rodents exposed to caloric restriction during

pregnancy are growth restricted and as a result are born low birthweight [153-155, 169-171].

These offspring show impaired development and function of critical organs including the heart

[153, 154, 169], skeletal muscle [131], liver, kidney [169] and pancreas [171, 172]. Maternal

caloric restriction in rodents results in glucose intolerance [173] and hyperinsulinemia in

adulthood of offspring. Moderate nutrient restriction is observed to induce growth restriction and

promote insulin secretion resulting in elevated circulating insulin levels [170, 173]. In nutrient

restricted rats, dams are shown to lose a significant amount of weight during gestation relative to

controls [155]. Maternal plasma concentrations of insulin-like growth factor (IGF) -1 are

significantly reduced while insulin-like growth factor binding protein (IGFBP) -1,-2 and -3 are shown to be significantly increased. Consistently, plasma IGF-1 levels have been observed to be elevated in offspring of nutrient restricted dams from delivery to the first few days of life [155]. Reductions in IGF-1 levels suggest that maternal nutrient restriction impairs later fetal development, characterized by rapid growth. Impaired vascular function and hypertension have been proposed to manifest as a result of multiple factors including altered renin-angiotensin system, stress-induced stimulation of the hypothalamic pituitary adrenal (HPA) axis [153] and endothelial dysfunction [154]. The leading candidate resulting in endothelial dysfunction in the offspring of food restricted animals is the reduction of endothelial synthesis of nitric oxide (NO). Studies suggest that a reduction in NO synthesis occurs in response to hyperglycemia and hypoinsulinemia [154]. Impairments to pancreatic development are most often observed with maternal nutrient restriction late in pregnancy [171, 172], as the majority of β-cell proliferation and differentiation occurs within the late fetal period. These deficits are commonly observed to be sex-specific [143, 145].

Dietary nutrient restriction in sheep is commonly achieved with global caloric restriction. The main focus of developmental studies in ovine models have focused on the consequences of maternal nutrition to fetal physiology. This is due to the ability to catheterize fetal sheep, permitting the study of the intrauterine environment directly on fetal development [158-161]. 50% global nutrient restriction during the first half of pregnancy does not compromise neonatal lamb weight [163] but is found to alter the development of critical organ systems at birth including the liver [162] and heart [131, 158-160, 162] and skeletal muscle [131]. Studies examining hepatic gene expression during maternal nutrient restriction early in pregnancy show reduced mRNA levels of growth hormone (GH), prolactin, IGF-2 and hepatocyte growth factor

in the livers of nutrient restricted offspring [174-176]. This finding is consistent with studies conducted in the baboon, where maternal nutrient restriction did not alter offspring liver weight but was found to reduce fetal hepatic IGF-1, -2 and insulin-like growth factor binding protein-1 (IGFBP-1) mRNA levels. Consistently this study demonstrated reduced levels of IGF-1, -2, IGF-1 and -2 receptor protein in offspring of nutrient restricted baboons. In this study, maternal nutrient restriction increased caspase-3 and protein kinase B (Akt) protein levels indicative of blunted nutrient sensing [165]. Detriment to these organs is hypothesized to be influential in the development in of later life metabolic disease.

1.4.2.2 Maternal nutrient excess

Similar to models of maternal nutrient/caloric restriction, animal models of nutrient excess have been used to study the effects of maternal nutrition during pregnancy on offspring development. These models have been primarily undertaken in rodents, including experimental animal models in the mouse [141, 177-179] and rat [132, 180-185] but have also been conducted in larger animals such as the sheep [68, 147-149] and primate [186-188]. Maternal nutrient excess during gestation has been most commonly modeled using consumption of a diets high in sugar [136] or fat [142, 178, 189] or a "cafeteria diet" [190] designed to mimic the Western diet. Maternal obesity modeled by the consumption of a high fat diet (HFD), referred to as diet-induced obesity (DIO) has been the focus of the majority of studies examining the developmental origins of obesity. In these models, maternal nutrient excess in the form of dietary fat ranges depending on the experimental animal model under study. In rodent models, maternal nutrient excess ranges from low (20% kcal derived from fat) to moderate (45% kcal derived from fat) to high (60% kcal derived from fat) where the fat component varies between animal-derived fats including lard or

beef tallow and plant oils including corn or safflower oil and replaces another macronutrient component; carbohydrate or protein in the diet [191, 192].

Maternal exposure to HFD results in elevated maternal body weight [132, 179-181], adiposity [132, 177, 181, 193], fasting glucose levels [177, 179, 180], circulating insulin [132, 177, 178, 180], and leptin levels [132, 177, 181], and serum TG [178, 180]. Thus, HFD consumption is accepted as a valid model for generating the signatures of the MS [192]. Although maternal nutrient excess was hypothesized to most often result in fetal overgrowth, studies have shown that this is not always the case. Maternal HFD has been reported to have variable effects on offspring birth weight; with some studies reporting no effects [132, 184, 185, 194-196], and others reporting either decreased [197, 198] or increased birth weights [141] and are hypothesized to be mediated by changes in the placenta [177]. Long-term intrauterine exposure to maternal nutrient excess has been shown to impact offspring glucose homeostasis [182, 187], lipid metabolism [183], hypothalamic regulation [196, 199], and influence the development of insulin resistance [141, 190, 200], leptin resistance [201], hepatic steatosis [202], non-alcoholic liver disease (NALD) [141, 187], obesity [200, 203], hypertension [200] and cardiovascular disease [185] later in life. Experimental animal models have been instrumental to understanding the underlying mechanisms associated with intrauterine exposure to nutrient excess and the developmental of obesity later in life. The leading mechanisms in the mother include disrupted placental morphology, cell proliferation, inflammation [204] and altered placental nutrient transport [177, 193].

Experimental animal models of maternal obesity have consistently shown that exposure to HFD induces changes in placental growth, development and function. Maternal HFD in the mouse resulting in offspring born of a higher birth weight, is associated with an increase in critical

nutrient transporters; glucose transporter 1 (GLUT1) and sodium coupled neutral amino acid

transporter (SNAT2) protein expression in placentae at term [177]. Studies in heterozygous

GLUT4 knockout (GLUT4 +/-) mice reveal increases in fetal weight and crown rump length at

term when exposed to HFD during pregnancy [205]. In the rat, maternal obesity is associated

with gross morphological changes to the junctional, but not labyrinth zone of the placenta [206].

In this study, these morphological changes were not accompanied by any alterations to critical

markers of vascular development in the placenta; peroxisome proliferator-activated receptor

gamma (PPARγ) and vascular endothelial growth factor A (VEGFa). However, models of

maternal obesity in the sheep, which have investigated the impact of HFD on placental vascular

development, have shown elevated mRNA and protein levels of fatty acid transport protein 1

(FATP1), FATP4 and fatty acid translocase cluster of differentiation 36 (CD36). These changes

were found to be accompanied by, and were attributed to, increased mRNA and protein levels of

PPARγ, a regulator of FATP expression in placentae of obese ewes [149]. Although placental

size and weight are commonly unchanged in the non-human primate [187, 188, 207], changes in

other placental parameters including increased placental thickness [193] and fetal placental

hemodynamics have been demonstrated [186]. Primate models of maternal obesity have been

central to the investigation of the mechanisms underlying placental inflammation during

maternal obesity, a study examining 24 young adult Japanese macaques showed that

consumption of a HFD (32% kcal from fat) for 4 years increased placental expression of pro-

inflammatory toll-like receptor 4 (TLR-4), and pro-inflammatory cytokines interleukin-1β (IL-

1β) and monocyte chemoattractant protein-1 (MCP-1) mRNA levels in placentae of HF fed

primates [186]. Although a similar study in baboons failed to demonstrate increased signaling

through the TLR-4 pathway, a higher number of cluster of differentiation 68 (CD68)

macrophages were reported in placentae of obese animals [193]. A chronic catheterization model in rhesus monkeys demonstrated that infusion of lipopolysaccharide (LPS) into the amniotic cavity at 128 to 147 days gestation (mid gestation) in the presence of a TLR-4 antagonist (TLR-4A) significantly reduced IL-8 and TNF-α levels in the amniotic fluid [208]. Together, these data suggest that maternal obesity has adverse effects on the growth, development and function of the placenta and highlight *in utero* inflammatory mechanisms involved in the increased risk of the development of obesity and metabolic disease in the offspring later in life.

Programmed mechanisms acting in the offspring resulting from intrauterine exposure to maternal obesity include altered production of the adipokine leptin [136, 137, 196], changes in the hypothalamic regulation of genes involved in appetite control and energy balance [203] and the epigenetic histone modification of chromatin structure in metabolic tissues [207]. Studies investigating the impacts of maternal obesity on offspring hypothalamic leptin sensitivity arose from a key observation that rats born to obese mothers displayed a preference for diets high in fat, sugar and salt at the expense of a protein rich diet [209]. Several studies indicate that maternal nutrient excess during pregnancy may lead to permanent alterations in food intake due to developmental plasticity in the hypothalamus [199, 210-212]. Altered food preferences and intake patterns have been attributed to permanent modifications to the hypothalamic centres including the arcuate nucleus (ARC) and paraventricular nucleus (PVH) responsible for the regulation of appetite [213]. Studies in hyperphagic (*ob/ob*) mice have demonstrated a neurotropic role for leptin in the development of projections from the ARC and PVH [214]. Studies in rats show that offspring born to obese mothers exhibited diminished signal transducer and activator of transcription 3 (STAT3) phosphorylation in response to leptin administration [199] suggesting hypothalamic leptin resistance. Consistently, decreased STAT3 and insulin

receptor substrate 2 (IRS2) protein levels have been found in HFD exposed offspring. These changes were accompanied by increased mRNA levels of insulin receptor β (IRβ) and leptin long receptor (OB-Rb) and hypothalamic peptides including increased neuropeptide Y (NPY), Agouti-related peptide (AGRP) pro-opiatemelanocortin (POMC) and melanocortin receptor 4 (MCR4) [215]. These findings indicate potential mechanisms involved in hyperphagia and increased adiposity seen in offspring exposed to HFD during intrauterine development.

Similar to findings in the mouse [216] and rat [217], epigenetic modifications to the fetal genome have been observed in the non-human primate model as a molecular mechanism driving the developmental origins of obesity. In Japanese macaques, intrauterine exposure to nutrient excess (35% kcal from fat) was shown to induce epigenetic modifications in hepatic genes of the offspring responsible for liver metabolism [207]. This study identified significant alterations of hepatic histone H3 acetylation markers including; hyperacetylation at H3K14 with a trend towards an increase in acetylation at H3K9 and H3K18 in exposed offspring compared to control. Subsequent analysis of key determinants of H3 acetylation markers revealed that maternal obesity is associated with a depletion in fetal histone deacetylase 1 (HDAC1) mRNA and protein levels and *in vitro* functional activity in offspring born to obese primates [207]. These results were accompanied by postnatal changes in mRNA levels of offspring born to high fat fed animals including an up-regulation in glutamic pyruvate transaminase 2 (GPT2), DNAJ heat shock protein member A2 (DNAJA2) and retinol dehydrogenase 12 (Rdh12) and a down-regulation of neuronal PAS domain protein 2 (Npas2), a peripheral circadian regulator [207].

Together, these data suggest that maternal obesity has adverse effects on offspring development *in utero*. Remarkably, there is evidence for the programming of certain features of metabolic disease from both maternal nutrient restriction and maternal nutrient excess. This suggests a

common mechanism between the two nutritional exposures and highlights the importance of a balanced maternal diet during pregnancy to promote offspring development and health in adulthood.

1.5 The Intestinal microbiota: Impacts on host physiology

Despite the great advances in our understanding of the molecular and physiological pathways that govern the relationship between the early life environment and long-term disease risk, the fundamental mechanisms underpinning the link between maternal and childhood obesity remain unclear. As a result, the search to identify novel intervention targets is an area of budding research. Emerging evidence has identified the intestinal microbiota and its relationship to host physiology as a promising target [218].

Within the human gut reside 100 trillion (10^{14}) microbes [219]. Collectively these microbes constitute the microbiota, and the genes they encode along with the environment they inhabit are known as our microbiome [220]. Coined our "forgotten organ", commensal bacteria live symbiotically with the host, having coevolved with vertebrates over many millennia [221]. The intestinal commensals play an essential role in the maintenance of host health as they are responsible for the metabolism of indigestible polysaccharides, the production of essential nutrients, the regulation of energy and fat storage [222], and protection from colonization with pathogenic bacteria [223].

Germ-free (GF) mice are those born and reared without exposure to microbes and provide a powerful tool for understanding the effects of the gut microbiota on host physiology. Colonization of GF mice with the microbiota harvested from conventional counterparts resulted in the induction of adiposity in the absence of altered food intake [222]. Consistent with this

27

observation, colonization of GF mice with the microbiota harvested from the ceca of genetically

obese (*ob/ob*) and diet-induced obese (DIO) mice results in a greater increase in body adipose

relative to colonization with lean microbiota [218]. The regulation of host adiposity and

metabolism by the intestinal microbiota has been demonstrated to be a result of several linked

mechanisms including; microbial fermentation of indigestible polysaccharides, intestinal

absorption of monosaccharides and short-chain fatty acids (SCFAs), the conversion of SCFAs to

lipids in the liver, and microbial regulation of genes that promote lipid deposition in adipocytes

[218, 222]. Conventionalization of GF mice has been shown to suppress the expression of

fasting-induced adipose factor (Fiaf) also known as angiopoietin-like protein 4 in differentiated

villous epithelial cells in the ileum [222]. In a second experiment, conventionalization of Fiaf

knockout (Fiaf -/-) mice displayed a 10% increase compared to a 55% increase in total adiposity

in WT counterparts. Similarly, conventional and GF G protein-coupled receptor 41 (Gpr41)

knockout (Gpr41 -/-) mice are significantly leaner and weigh less than WT (Gpr41 +/+)

littermates. These differences are not evident when GF Gpr41 -/- and GF WT littermates are

compared [224]. Gpr41 deficiency has also been associated with reduced expression of peptide

YY (PYY), an enteroendocrine cell-derived hormone that functions to inhibit intestinal motility,

thus increasing intestinal transit rate and limiting energy harvest from the diet [224].

Colonization studies have also revealed a persistent activation of the enzyme adenosine

monophosphate (AMP)-activated protein kinase (AMPK) in the liver and skeletal muscle of GF

mice relative to conventional mice [222]. Collectively, these experiments highlight the role of

the intestinal microbiota in the regulation of host adiposity and metabolism.

Aside from regulating host metabolism, commensal bacteria and their metabolic products are

essential for the normal development and function of the mammalian immune system [225, 226]

and the maintenance of intestinal epithelial barrier integrity [227, 228]. GF mouse studies have

demonstrated that the intestinal microbiota is essential for the growth and development of the

gastrointestinal system, as these animals display reduced intestinal weight, surface area, and poor

nutrient absorption [229]. GF animals display impairments in the development of intestinal-

associated lymphoid tissue including fewer and smaller Peyer's patches, germinal centres,

isolated lymphoid follicles, mesenteric lymph nodes and a thinner lamina propria [225, 226]. To

reinforce this concept, colonization studies have shown that the formation of these structures can

be induced by the introduction of intestinal bacteria, suggesting a dynamic relationship between

the host immune system and the intestinal microbiota [225, 226].

The intestinal microbiota is modulated by several host-related factors including diet [230-232],

weight [222], age [233, 234], geography [234] and health status [231, 233, 235]. Of the many

shifts in microbial genera with diet and weight, *Akkermansia muciniphila* has been more recently

a target of intense investigation. The intestinal microbiota of genetically obese (*ob/ob*) mice have

been shown to display reduced levels of *Akkermansia*. A study examining the effect of prebiotic

administration to *ob/ob* mice resulting in an increase in *Akkermansia muciniphila* abundance was

reported to correlate with an improved metabolic profile; measured by reductions in fat mass,

metabolic endotoxemia and insulin resistance [236]. Consistent data has been observed in human

studies [237-239] where weight loss has been positively correlated with *Akkermansia* abundance

[239] and caloric restriction in overweight and obese subjects demonstrated an inverse

relationship between *Akkermansia muciniphila* abundance and fasting glucose, waist-to-hip ratio

and subcutaneous adipocyte diameter [238]. Furthermore, subjects with increased *Akkermansia*

muciniphila abundance exhibited the healthiest metabolic status measured by fasting plasma

glucose, plasma triglycerides, body fat distribution and displayed a greater improvement in

insulin sensitivity following caloric restriction [238]. These studies highlight host metabolism as a potential link between *Akkermansia* abundance and body weight. There is growing evidence that in addition to body weight correlations, *Akkermansia* is associated with gut health and age, where fewer *Akkermansia* counts have been observed in mucosal samples isolated from ulcerative colitis and Crohn's disease patients [240, 241] in the elderly [242] along with an inverse correlation of species richness reported with type I diabetes, body weight, and markers of inflammation [243-245].

Despite the accumulating evidence for the involvement of *Akkermansia* in intestinal and metabolic health, the basic mechanisms governing its interaction with the host have received little attention. New studies now show that *Akkermansia muciniphila's* role in promoting metabolic health may be related to its association with intestinal barrier function. The intestinal epithelium is the interface between the host and the intestinal microbiota, and provides a critical barrier between the host and its environment. This epithelial cell monolayer barrier is enhanced by the presence of a mucus layer, the composition of which includes a network of heavily glycosylated proteins, mucins, which are connected via cross-linking of disulfide bridges [246] and are produced by intestinal goblet cells. Although mucins function as a barrier to toxins, acids and bacterial invasion, they also a source of oligosaccharides for mucin degrading bacteria, including *Akkermansia muciniphila* [247]. Recently, it has been suggested that this mucosal barrier is vulnerable to dietary influences, where a 46% reduction in mucus layer thickness was shown in male mice challenged with a high fat diet and was reversed upon colonization with viable, but not heat-*killed Akkermansia muciniphila* [236]. Indeed, intestinal bacterial shifts can impact the integrity of the epithelial cell monolayer of the intestine [189, 204, 232] in addition to their interaction with the mucus layer. In male mice, a high fat diet induced increase in the

Firmicutes to Bacteroidetes ratio has been shown associated with intestinal inflammation [232] and increase intestinal permeability [204], and it has been shown that *Akkermansia muciniphila* can adhere to colonic enterocytes and strengthen the integrity of the enterocyte monolayer *in vitro* [248]. Due to the close relationship between diet, weight, metabolism, host health and intestinal microbial shifts, research in the field of developmental programming has been directed to investigate whether the same microbial and metabolic interactions observed in male mice occur during pregnancy, and whether this relationship influences long-term disease risk in the offspring.

1.5.1 Pregnancy: Healthy maternal microbial adaptations

Although it is well established that metabolic, cardiovascular, endocrine [249], immune [250], and skeletomuscular physiological adaptations accompany pregnancy, the investigation of a maternal intestinal microbial adaptation is a nascent area of research. Recent evidence from experimental animal [189] and clinical studies [249] suggest that pregnancy is characterized by shifts in the maternal intestinal microbiota. The biological importance of this shift may be to facilitate maternal metabolic adaptation to pregnancy or to promote fetal growth and development, but direct evidence of a maternal intestinal microbial influence on fetal development is weak. Nonetheless, studies are currently investigating the mechanisms mediating pregnant intestinal microbial shifts, how these occur, and what their purpose is.

Little information exists regarding normal physiological shifts in the maternal intestinal microbiome, how it occurs and what role it plays in maternal and fetal physiology. Recent work in pregnant mice has demonstrated that pregnancy induces shifts in the gut microbiota of lean female mice [189] where significant differences in the relative abundance of 21 genera were

observed including significant elevations in *Akkermansia*, *Bifidobacteria*, *Clostridium*, and *Bacteroides* [189]. Of the significant elevations in the 4 abundant genera with pregnancy, the genus *Akkermansia* is observed to exhibit the largest increase in abundance [189]. At conception, significant elevations in *Akkermansia* abundance was observed and, consistent with other reports [251], its levels remained elevated throughout gestation relative to non-pregnant females [189]. These data are consistent with an early study examining gut microbial shifts over the course of pregnancy conducted in women [251]. Differences have also been observed with advancing gestation where a significant increase in the total counts of *Akkermansia*, *Bifidobacteria*, *Clostridium*, *Bacteroides* comparing third to first trimester intestinal fecal microbial populations in pregnant women [249]. These shifts are consistent with studies investigating the relationship between the intestinal microbiota and host metabolism conducted in male mice where, elevated numbers of *Akkermansia* [252] and *Bifidobacteria* [253] have been positively correlated with weight loss, fasting glucose, improved glucose tolerance and glucose-induced insulin secretion [238]. While increased counts of *Clostridium* and *Bacteroides* have been associated with enhanced energy storage and weight gain [254]. In a prospective randomized mother-infant nutrition study in Finland, the relative abundance of Proteobacteria and Actinobacteria showed an increase from first to third term, occurring in 69.5% and 57% of lean women respectively [249].

Collectively, experimental animal and clinical studies have suggested that the maternal gut microbiota is remodelled over the course of an otherwise physiologically normal pregnancy. Together, the observed shifts in specific genera associated with metabolic outcomes, suggest that during pregnancy, maternal gut microbes may shift to promote efficient energy extraction, while working to maintain a healthy maternal body weight through modulating maternal metabolism to

support the health of the mother and developing fetus. These speculations remain to be

investigated and studies have yet to reveal the mechanisms explaining how these shifts occur at

the onset of pregnancy and their relationship, if any, to maternal metabolic indices and to fetal

development.

1.2.1.1 Mediators of maternal gut microbial shifts during pregnancy

Pregnancy is reliant on dramatic shifts in sex-steroid hormones estrogen (17-β estradiol) [255-

259] and progesterone [256, 258, 259], thus it has been hypothesized that female reproductive

steroid hormones may mediate gut microbial shifts through indirect mechanisms. In non-

pregnant females, estradiol is predominantly produced by the ovaries [255] with small amounts

produced by the adrenal glands, while during pregnancy it is produced by the placenta [260].

Metabolism of estrogens occurs in peripheral organs where in the liver [261], conjugated

estrogens are excreted in the bile as estriol-3-sulfate-16-glucuronide. Following passage through

the distal intestine they become activated through deconjugation by β-glucuronidase or sulfatase

[262]. Estrogen metabolites are subsequently reabsorbed through the intestinal mucosa and enter

the circulation as biologically active molecules [263, 264] following which they are reconjugated

into estriol-16-glucuronide and returned to the liver [265]. Steroids undergoing enterohepatic

circulation are exposed to bacterial metabolism. *E. coli* and *Bacteroides* species specialize in

glucuronidase production [266], while *Clostridium*, *Lactobacillus*, *Eubacterium*, *Peptococcus*

and *Bacteroides* are steroid-desulfating bacteria [267]. *Bacteroides* and *Clostridia* have been

reported to be very active in bile acid metabolism [268] and thus, it has been hypothesized that

these genera may be involved in the enterohepatic circulation of estrogen and progesterone

[269].

Early observations in GF mouse models including increased difficulty in generating colonies, [270] smaller litter size [271], and a reduction in progesterone levels on day 1 of diestrus were the first indicators that a relationship may exist between microbial presence and reproductive hormones [269]. Colonization of GF mice has been shown to enhance reproductive capacity assessed by estrous cyclicity, copulation rate and implantation rate. Since this observation, few studies have examined the relationship between the concentrations of reproductive hormones with bacterial abundance in the host. In postmenopausal women, total levels of urinary estrogens have been correlated with measures of fecal microbiome richness and alpha diversity [263] . Strains of bacteria associated with the gastrointestinal tract, including *Pseudomonas aeruginosa* and *Staphylococcus* have been reported to metabolize estrogens and estrogen metabolites *in vitro* [272]. Similarly, systemic measures of non-ovarian estrogens via enterohepatic circulation have shown that circulating estrogen is significantly associated with fecal *Clostridia* and genera belonging to the *Ruminococcaceae* family [263]. More convincingly, a follow up study examining the associations of the fecal microbiota with urinary estrogen and 13 hydroxylated estrogen metabolites conducted in postmenopausal women revealed the ratio of metabolites to parents was directly associated with whole-tree phylogenetic diversity and the relative abundances of the order Clostridales and the genus *Bacteroides* independent of age and BMI [273]. Collectively, these results suggest that postmenopausal women with increased intestinal microbial diversity exhibit a higher ratio of hydroxylated estrogen metabolites to parent estrogen and highlight a potential link between particular bacterial genera and estrogen metabolism.

Molecular studies in estrogen receptor β (ERβ) knockout (ERβ$^{-/-}$) mice suggest that ERβ, the most abundance estrogen receptor found in the colon [274], mediates changes in the composition of the gut microbiota in response to diet, where Proteobacteria, Bacteroides and Firmicute phyla

were observed to be altered as a function of ERβ status [275]. Human studies in premenopausal women examining estrogen levels in relation to microbial abundance are inconsistent. No association was found to exist between systemic estrogen levels and the fecal microbiome [263], however other studies show shifts in the vaginal microbiome over the course of the female reproductive cycle [276], while others do not [277, 278]. To date, the impact of reproductive sex hormones on the intestinal microbiome is still unclear, with studies showing inconsistent results. Although it is possible that sex steroids play a role in mediating shifts in the gut microbiome, further studies are needed to elucidate whether endocrine mediated changes in microbial populations of the female gut can explain pregnancy-associated shifts in the gut microbiome.

1.5.2 Maternal obesity: A distinct microbial adaptation to pregnancy

Like pregnancy, obesity has been associated with changes in gut microbial ecology [218, 231, 235, 279]. Comparisons of the distal gut microbiota harvested from genetically obese *ob/ob* mice and their lean counterparts [231] as well as those of obese and lean humans [218, 235, 280] have shown that obesity is accompanied by changes in the relative abundance of the two dominant bacterial phyla in the intestine, Firmicutes and Bacteroidetes. Reciprocally, weight loss, through fat or carbohydrate restricted diets, is correlated with elevations in Bacteroidetes in animal models, suggesting that this genera might be responsive to diet/macronutrient intake [279]. A similar effect is observed in humans who show weight loss following Roux-en-Y gastric bypass surgery, where levels of Bacteroides and Prevotella were negatively correlated with energy intake and adiposity [281]. Although the mechanisms underpinning this observation are unknown, it is clear that a relationship exists between intestinal microbial population shifts and improved metabolism after weight loss achieved by dietary and surgical interventions.

To understand the relationship between maternal obesity and the maternal gut microbiota, Gohir *et al.* subjected female mice to a high-fat diet (HFD) challenge prior to and throughout pregnancy and examined the female gut microbiota [189]. This study showed that pregnancy-induced changes in the maternal gut microbiota were vulnerable to modulation by diet. Contrary to reports in women [251, 282], these microbial shifts were independent of maternal weight gain during gestation. Eleven genera were significantly different between non-pregnant and pregnant HF-fed females predominantly characterized by an increased ratio of Firmicutes to Bacteroidetes [189], consistent with male mouse models of genetic obesity (*ob/ob)* [235], leptin deficiency (*db/db)* and diet-induced obesity [231, 283]. In HF pregnancies, these changes were accompanied by elevations in the genus *Akkermansia* [189]. *Akkermancia* abundance peaks at the onset of pregnancy and remains elevated throughout gestation in HF relative to CON dams fed a CON diet [189]. These data suggest that pregnancy-induced shifts in the abundance of *Akkermansia* depend on maternal diet prior to and during gestation. Models of DIO in rats support the observations from mouse models, where maternal obesity has been associated with a shift in the maternal gut microbiota [284-286]. Dams fed a high-fat and high-sucrose diet show an elevation in *Akkermansia*, *Clostridium* and *Rosburia* and a reduction in the abundance of *Bifidobacterium* [284]. To further understand the relationship between specific intestinal bacteria and maternal metabolism during maternal obesity, selective alteration of the obese maternal gut microbiota has been achieved with the administration of prebiotics [284]. Similar to studies in male rodents [287-289], dietary supplementation with oligofructose has demonstrated a reduction in maternal energy intake, decreased gestational weight gain and adiposity [284, 286]. This result was accompanied by increased circulating concentrations of satiety hormones and relative abundance of the beneficial intestinal microbe, *Bifidobacterium* [284]. Gut bacterial

shifts resulting in an increased Firmicutes to Bacteroidetes ratio have been shown to impact the

integrity of the epithelial cell monolayer of the intestine and induce intestinal inflammation [204,

232], while elevations in *Akkermansia* have been shown to strengthen intestinal barrier integrity

[248]. The impact of the elevated Firmicutes to Bacteroidetes ratio on maternal intestinal

inflammation and permeability, and the influence of elevated *Akkermansia* abundance during

maternal obesity however remains to be investigated.

Contrary to early studies that reported that the fetus is sterile during development, recent studies

suggest that the fetus is exposed to bacteria before birth. Bacterial DNA known to be associated

with the intestinal tract identified in the placenta [290, 291], fetal membranes [292], amniotic

fluid [291], umbilical cord blood [293] and meconium [294] suggest that the fetus is exposed to

maternal microbes *in utero*. Studies since these observations, have now focused on

understanding the role of prenatal microbial exposure on fetal development. In a randomized,

double-blind, placebo-controlled trial, supplementation of *Bifidobacterium lactis* and

Lactobacillus rhamnosus 14 days prior to elective caesarian section was found to result in

detectable levels of microbial DNA in the amniotic fluid and placenta. *Lactobacillus* DNA in the

amniotic fluid was found to be associated with changes in TLR2 and 3 mRNA levels in the fetal

intestine, while in the placenta *Lactobacillus* DNA significantly increased TLR5 and 6 mRNA

[291]. These investigations suggest that selective prenatal exposure to bacteria associated with

the maternal intestine is associated with fetal innate immune adaptations *in utero*. These

observations identify a potential link between the components of the maternal intestinal

microbiota and intrauterine development. Although the impact of the maternal intestinal

microbial shifts that occur during maternal obesity on fetal development remain to be studied,

these investigations strengthen the argument that intrauterine exposure to an altered maternal

intestinal microbial profile during maternal obesity may serve as a mechanism influencing the fetal origins of adult disease.

1.6 Maternal obesity, placental inflammation and impacts to placental function

Early studies observed altered circulating levels of pro inflammatory cytokines including tumor necrosis factor (TNF) alpha [295], interleukin (IL-6) [296] and C-reactive protein (CRP) [297] in overweight and obese adults. Consistently, elevated circulating TNF-α and IL-6 have been strongly linked to the development of obesity-related comorbid diseases insulin resistance, metabolic dysfunction, and cardiovascular disease risk [295, 298]. Although early reports focused on inflammation in adipose tissue, multiple metabolic organs including the liver [298], pancreas [299], brain [300] and skeletal muscle [301] have been reported to be inflamed during obesity. Interestingly, during maternal obesity, inflammation has also been reported in the placenta [302].

1.6.1 Placental inflammatory signalling pathways during maternal obesity

During pregnancy, the placenta of obese women display an increased expression of IL-1, TNF-α and IL-6 similar to other metabolic tissues and the systemic circulation. Consistently, reports in obese women show an elevated infiltration of CD68^{+} and CD14^{+} immune cells in placentas indicative of elevated infiltration of differentiated, mature, pro-inflammatory macrophages [302]. Cellular activation by microbial ligands via members of the Toll-like receptors (TLRs) initiates a signaling cascade resulting in the translocation of NF-κB [303-308] and the induction of an inflammatory response through the production of TNF-α and IL-6. In particular, TLR2 and 4 are involved in innate immune cellular activation and are located on the surface of macrophages. TLR overexpression experiments using human embryonic kidney (HEK293) cells have been

instrumental in the identification of functional microbial product ligands which stimulate specific TLR signaling cascades. These studies in addition to experimental animal genetic knockout studies [307, 309] have shown that TLR4 is the exclusive signal transducer of LPS present in the cell wall of gram-negative bacteria [310, 311]. In addition to being activated by bacterial products, TLR4 has been shown to be activated by fatty acids [312, 313]. The interaction of TLR4 and its co-receptors CD14 and MD-2 with LPS ligation results in the recruitment of adapter molecule myeloid differentiation marker 88 (MyD88), the phosphorylation of the IL-1 receptor-associated kinase, and recruitment of the adaptor molecule TNF receptor associated factor (TRAF)6 [314, 315]. Recruitment of TRAF6 results in the phosphorylation of IκB kinase (IKK) and the release of NFκB a master regulator of inflammation. NFκB is then free to translocate to the nucleus and initiates the transcription of pro-inflammatory genes (Figure 1.6.1.1). Placental size, morphology, blood flow and nutrient transport functions primarily determine the growth trajectory of the fetus [157] and at term placental and fetal weights are strongly correlated [316]. Poor placental function is reported as the primary cause of fetal growth restriction and placental dysfunction is central to many common pregnancy complications [317]. The ratio of fetal to placental weight (or its inverse) is used as an endpoint measure of placental nutrient transport efficiency across species [318, 319]. Abnormal ratios at both extremes have been associated with an increased risk of adverse neonatal outcomes. Although research has begun investigating the cell types and the mechanistic signaling pathways associated with maternal obesity in the placenta, the impacts to placental function are largely unknown.
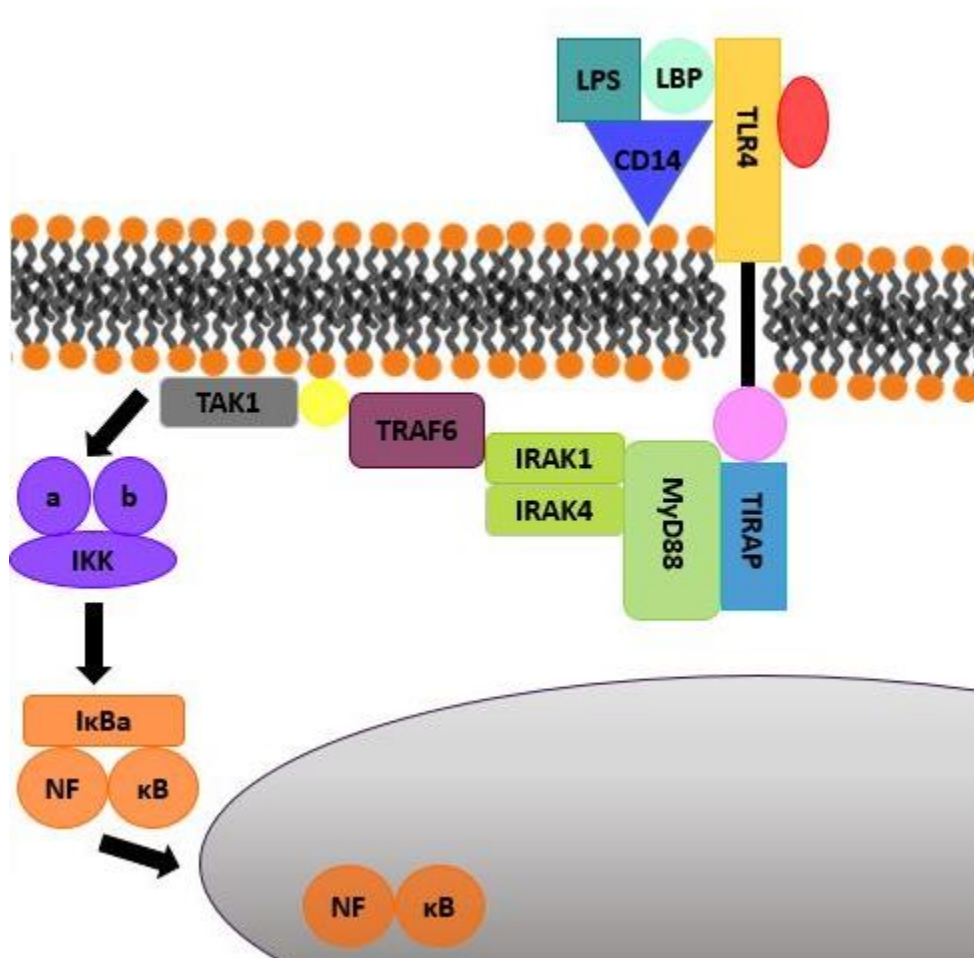
Figure 1.6.1.1 A schematic representation of the activation of TLR-4 by LPS and the signal transduction cascade resulting in the activation of NFκB. Abbreviations: LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; CD14, cluster of differentiation 14; TLR4, Toll-like receptor 4; MD2, Lymphocyte antigen 96; TIRAP, TIR adapter protein; MyD88, Myeloid differentiation primary response 88; IRAK1, Interleukin receptor-associated kinase 1; IRAK4, Interleukin receptor-associated kinase 4; TRAF6, TNF receptor-associated factor 6; TAK1, TGF-beta activated kinase 1; IKK, IκB kinase; NFκB, Nuclear factor kappa B.

1.7 Rationale

Obesity during pregnancy is now a common occurrence, where maternal obesity is a strong predictor of offspring obesity [134]. Obesity during pregnancy is associated with an increase in both maternal and fetal complications [37] and has short- and long-term ramifications for the offspring [38]. Disease risk is established early in life where offspring responses to the postnatal environment are modulated by intrauterine adaptations [72]. However, the biological mechanisms that underpin these adaptations and resultant phenotypes are not completely understood. Fetal adaptations to maternal obesity are believed to be in part mediated by the placenta [320, 321]. Although the mechanisms linking maternal and offspring obesity with compromised placental function are still under investigation, the gut microbiota has come forth as a novel factor contributing to the fetal origins of adult disease [218, 322].

Our lab and others have shown that bacterial communities change over the course of healthy pregnancy [189, 249] and are impacted by obesity [189, 282]. These shifts occur immediately at the onset of pregnancy, after mating and continue throughout gestation [189]. The mechanisms responsible for this rapid and prolonged intestinal microbial shift have not been studied. To date, the impact of reproductive sex hormones on the intestinal microbiome is still unclear, with studies showing inconsistent results. Further studies are needed to elucidate whether endocrine mediated changes in microbial populations of the female gut can explain pregnancy-associated shifts in the gut microbiome.

Like pregnancy, obesity is associated with changes in gut microbial ecology [218, 231, 235, 279]. During HF pregnancy, our lab reports an increase in the Firmicutes to Bacteroidetes ratio

[189] similar to that observed in genetically obese (*ob/ob*) [231], leptin deficient, and DIO mouse models and in obese humans [218]. This shift is accompanied by elevations in the genus *Akkermansia* [189]. It has been suggested that gut microbial shifts impact the integrity of the epithelial cell monolayer of the intestine. In male mice, HFD challenge resulting in an increased Firmicutes to Bacteroidetes ratio has been shown to promote gut inflammation [232] and increase intestinal permeability [204]. The impact of maternal intestinal microbial shifts on maternal intestinal inflammation and permeability however, remains to be investigated.

Contrary to early studies reporting that the fetus is sterile during development, recent studies suggest that the fetus is exposed to bacteria before birth. Bacterial DNA known to be associated with the intestinal tract have been identified in the placenta [290, 291], fetal membranes [292], amniotic fluid [291], umbilical cord blood [293] and meconium [294]. The role of prenatal microbial exposure is only beginning to be understood. Whether the intestinal microbial shifts, which accompany maternal obesity, play a role in fetal development through modifications to maternal pregnancy adaptations involving the maternal intestine or placenta remains to be investigated.

1.8 Hypothesis

General Hypothesis:

Maternal obesity is associated with shifts in the maternal intestinal microbiota and compromised maternal intestinal permeability contributing to maternal intestinal and placental inflammation, which ultimately compromises placental function at embryonic (E) day 14.5.

1.9 Aims

Overall Aim:

To understand how maternal obesity impacts the maternal intestinal microbiota, intestinal inflammation and barrier function, and placental inflammatory and nutrient signalling pathways at embryonic (E) day 14.5.

Specific Aims:

1. Investigate whether female intestinal microbial shifts are mediated by female sex-steroid hormones.

2. Examine the effects of maternal diet-induced obesity and pregnancy on: the composition of the maternal intestinal microbiota and maternal intestinal inflammation and barrier function.

3. Determine whether maternal obesity is associated with an increase in placental pro-inflammatory cytokine signalling and altered nutrient sensing.

2.0 MATERIALS AND METHODS

2.1 Female reproductive cycle study

2.1.1 Animal experimental design

All animal procedures for this study were approved by the McMaster University Animal

Research Ethics Board (Animal Utilization Protocol 12-10-38) in accordance with the guidelines

of the Canadian Council of Animal Care. Ten 6 week-old female C57Bl/6J mice were purchased

from the Jackson Laboratory (Bar Harbor, ME, Strain 000664). All mice were maintained in the

same room with a constant temperature of 25 ºC and a 12-h light, 12-h dark cycle. All females

were fed a control purified standard diet (CD) (17% fat, 29% protein, 54% carbohydrate, 3

kcal/g; Harlan 8640 Teklad 22/5 Rodent Diet), provided water *ad-libitum* and were individually

housed in hanging metal cages to prevent corprophagic gut microbial transfer between females

[323] and within each female between reproductive cycle stages.

2.1.2 Reproductive cycle determination and fecal collection

Following a 1 week acclimation period to the McMaster Central Animal Facility, a vaginal

smear and a time-matched fecal sample were collected daily from each individually housed

female for a total of 3 complete reproductive cycles (15 days). Fecal samples collected from

females were used to investigate gut microbial communities and were stored at -80°C until

bacterial sequencing. Reproductive cyclicity was determined by vaginal epithelial cell

modifications with estrous stage and each estrous stage was used as a marker of female steroid

hormone fluctuations. Vaginal epithelial cells were collected using a saline moist cotton swab,

prepared on microscope slides, and cells were stained with Haematoxylin Stain 2-Gill Method

(Fisher Chemical, CS-401-1D, Ottawa, Canada) for 5 minutes, removed from Haematoxylin and allowed to drip-dry for 10 minutes at ambient temperature before cellular morphological evaluation. The relative abundance of leukocytes, nucleated vaginal epithelial cells, and cornified epithelial cells in each smear was observed under a light microscope.

Based on cell type predominance [324], each vaginal epithelial cell preparation was assigned to 1 of the 4 stages of the murine estrous cycle (diestrous, proestrous, estrus and metestrous). Reproductive stage was characterized based on the following properties of the observed cell types in each smear: a preparation primarily consisting of leukocytes was deemed to be in *diestrous*, a preparation consisting predominantly of nucleated epithelial cells was deemed to be in *proestrous*; a preparation characterized predominantly by anucleated cornified epithelial cells was deemed to be in *estrus*, and a preparation characterized by an equal proportion of leukocytes, nucleated epithelial and anucleated cornified epithelial cells was deemed to be *metestrous* Females were considered to be cycling regularly if exhibiting diestrous for a maximum of 3 consecutive days, proestrous for a maximum of 1 day, estrus for a maximum of 3 consecutive days and metestrous for a maximum of 1 day. Irregular reproductive cyclicity was identified based on prolonged stage duration or the absence of an estrous stage. Females displaying more than 3 consecutive days in estrus were identified as having persistent estrus. Females displaying prolonged diestrous lasting more than 3 consecutive days were eliminated from the study due to the possible confounding of pseudopregnancy. Females displaying irregular reproductive cyclicity or persistent estrus were omitted from further analyses. Following staging, time-matched fecal samples for three consecutive estrous cycles were selected from regularly cycling females and processed for bacterial 16S rRNA V3 sequencing.

2.2 Genomic DNA extraction and 16S rRNA gene sequencing

Genomic DNA was extracted from fecal samples. Briefly, 0.2 g of fecal material was mechanically lysed at 4.5 m/s for 60 seconds using a homogenizer (MP Fast Prep -24 Tissue Homogenizer) in a 2.0 mL screwcap tube containing 2.8 mm ceraminc beads (MoBio Laboratories Inc., Carlsbad, CA, USA) in 800 μl of 200 mM sodium phosphate monobasic (pH 8) and 100 μl guanidinium thiocyanate EDTA *N*-lauroylsarkosine buffer (50.8 mM ethylenediaminetetraacetic acid and 34 mM *N*-lauroylsarcosine). A further mechanical and enzymatic lysis was performed using 0.2 g of 0.1-mm glass beads (MoBio Laboratories Inc., Carlsbad, CA, USA), 50 μl lysozyme (100 mg/mL) and 10μl RNase A (10 mg/mL) and incubated for 1 hour at 37 ℃. A second enzymatic lysis was performed using 25 μl 25% sodium dodecyl sulfate, 25 μl proteinase K and 62.5 μl 5 M NaCl and was incubated for 1 hour at 65℃. The resulting solution was centrifuged (12,000 g for 5 minutes) and supernatant removed and combined with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) in a new microcentrifuge tube. The sample was vortexed and centrifuged (12,000 g for 10 minutes). The aqueous phase was removed and combined with 200 μl DNA binding buffer (Zymo Research, Irvine, CA, USA). This mixture was passed through a Clean and Concentrator-25 column (Zymo Research, Irvine, CA, USA) according to kit directions and incubated at room temperature 5 minutes prior to elution. Elution was performed with 20 μl of ultrapure water. DNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Canada) using NanoDrop 2000/2000c software (Thermo Scientific, Canada). The ratio of absorbance at 260 nm to 280 nm (A260/A280) and at 260 nm to 230 nm (A260/A230) for each sample was > 1.5 and >

0.60 respectively. All DNA samples were stored at -80ºC until PCR amplification of the 16S

rRNA gene.

PCR amplification of the bacterial 16S rRNA gene V3 region tags was performed on extracted

DNA independently. Briefly, each reaction contained 5 pmol of primer, 200 mM of dNTPs, 1.5ul

50 mM MgCl$_2$, 2 μl of 10 mg/mL bovine serum albumin (irradiated with a transilluminator to

eliminate contaminating DNA) and 0.25ul Taq polymerase (Life Technologies, Canada) for a

total reaction volume of 50 μl. To allow multiplexing of samples, 341F and 518R rRNA primers

were used, modified to include adapter sequences specific to the Illumina technology and 6-base

pair barcodes were used. The PCR program used was as follows: 94º C for 2 minutes followed

by 30 cycles of 94º C for 30 seconds, 50º C for 30 seconds and 72º C for 30 seconds, with a final

extension step at 72º C for 10 minutes. DNA products of the PCR amplification were

subsequently sequenced using the Illumina MiSeq platform, resulting in 150bp paired-end reads.

2.3 Sequence processing and data analyses

Custom in-house Perl scripts were developed to process resultant FASTQ files [325]. In this

pipeline, Cutadapt [326] was used to trim any reads exceeding the length of the V3 region of the

bacterial 16S rRNA gene, PANDASeq [327] to align paired-end reads, AbundantOTU+ [328] to

group reads into Operational Taxonomic Units (OTUs) based on 97% similarity, and the RDP

Classifier [329] as implemented in Quantitative Insights into Microbial Ecology (QIIME) [330]

against the Feb 4 2011 release of the Greengenes reference database [331] to assign a taxonomy

to each OTU. Any OTU not assigned to the bacterial domain were culled, as was any OTU to

which only 1 sequence was assigned. This processing resulted in a total of 3981373 reads (mean 66356 reads per sample; range: 402-124660) and 1157 OTUs.

Analyses of these data were completed using various open source software. Taxonomic summaries were computed using R Studio. Measures of β-diversity, were computed using Phyloseq's [332] implementation of the Bray Curtis distance metric, and tested for whole community differences across groups using vegans implementation of permutational multivariate analysis of variance (PERMANOVA) in the adonis command. These results were visualized via Principal Coordinate Analysis (PCoA) ordination. Calculations of genera which differed significantly between groups were computed using DESeq2 [333] and considered significant if the p value was < 0.01 after adjustment for multiple testing via DESeq2's implementation of the Benjamini-Hochberg multiple testing adjustment procedure) and visualized using ggplot2 package [334].

2.4 Maternal obesity study

2.4.1 Animal experimental design

All animal procedures for this study were approved as in section 2.1. Twenty 3-week-old female C57Bl/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, Strain 000664). All animals were maintained in standard mouse cages in the same room with a constant temperature of 25ºC and a 12-h light, 12-h dark cycle, fed a control purified standard diet (CD) (17% kcal fat, 29% kcal protein, 54% kcal CHO, 3 kcal/g; Harlan 8640 Teklad 22/5 Rodent Diet) and provided water *ad-libitum*. Following a 1 week acclimation period to the McMaster Central Animal Facility, a baseline record of weight and a fecal sample were collected from each female, which were then randomly assigned to 1 of 2 nutritional groups: CONTROL: 150 g Control purified

standard diet (CD) (17% kcal fat, 29% kcal protein, 54% kcal CHO, 3 kcal/g; Harlan 8640

Teklad 22/5 Rodent Diet) (Con n = 20) or HIGH FAT DIET: 150 g High-fat diet (HFD) (60%

kcal fat, 20% kcal protein, 20% kcal carbohydrate, 5.24 kcal/g, Research Diets Inc., D12492)

(Figure 2.4.1.1). All females were maintained on their respective diet for a total of 6 weeks.

Body weight and food intake were recorded weekly during this time.

D12492 Formula		
Macronutrient	**g (%)**	**kcal (%)**
Protein	26	20
Carbohydrate	26	20
Fat	35	60
Total (kcal/g)	**5.24**	
Ingredient	**g (%)**	**kcal (%)**
Casein, 30 Mesh	200	800
L-Cystine	3	12
Maltodextrin 10	125	500
Sucrose	68.8	275
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0
Total	**773.85**	**4057**

Table 2.4.1.1 Research Diets D12492 Formula. Macronutrient content and ingredients represented in grams (g) and kilocalories (kcal).

After 6 weeks of dietary intervention, females were housed with a control fed C57BL/6J male obtained from the Jackson Laboratory (Bar Harbor, ME, Strain 000664) overnight. Mating was confirmed the next morning by the observation of a vaginal plug and designated embryonic (E) day 0.5. Pregnant females were housed individually and provided free access to 150 g of their respective diet and water *ad libitum*. Throughout gestation maternal weight and food intake were recorded. Maternal fecal samples were collected at E0.5, E6.5, E10.5 and E14.5 (Figure 2.4.1.1) and stored at -80°C until bacterial DNA extraction and sequencing.
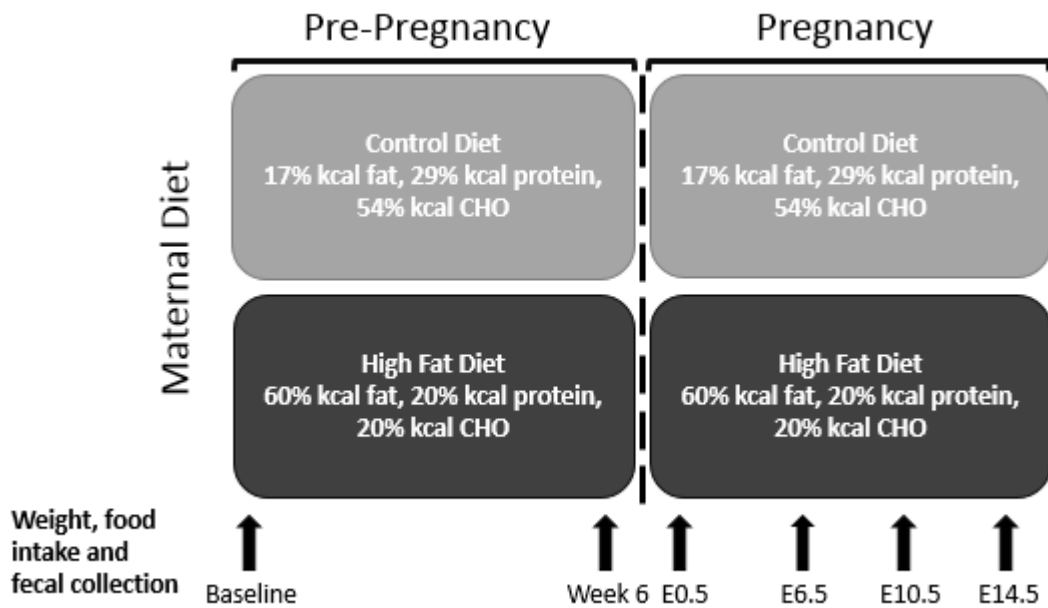
Figure 2.4.1.2 Mouse model of maternal obesity during pregnancy. Abbreviations: CHO: Carbohydrate. A schematic representation of the experimental animal design. Four-week old C57Bl/6 females were randomly assigned to 1 of 2 nutritional groups: Control diet (CON n = 10 17% kcal fat, 29% kcal protein, 54% kcal CHO, 3 kcal/g) or High fat diet (HF n = 10 60% kcal fat, 20% kcal protein, 20% kcal CHO, 5.24 kcal/g) fed for 6 weeks and then mated with 8 week old males. Pregnancy was identified at the observation of a vaginal plug and designated as embryonic day (E) 0.5 in both CON (n = 7) and HF (n = 9) groups. Fecal samples were collected from control females prior to nutritional randomization (baseline), after 6 weeks of randomization diet (Week 6) and throughout gestation (E0.5, E6.5, E10.5 and E14.5).

2.5 *In vivo* intestinal barrier integrity

Non-pregnant female *in vivo* intestinal barrier integrity was measured in each CON (n = 10) and

HF (n = 10) female prior to mating and pregnancy in order for each female to serve as her own

internal non-pregnant control (NPC) following 6 weeks of dietary intervention. Following

mating, pregnant *in vivo* maternal intestinal barrier integrity was assessed at at E14.5 in CON

and HF females.

In vivo intestinal barrier integrity was assessed by measuring the appearance of fluorescein

isothiocyanate-dextran (FITC-dextran) (molecular mass 4kDa; Sigma Aldrich, St Louis, MO,

USA) in maternal plasma after oral gavage. Animals were fasted for 6 hours after which a

baseline blood sample was collected via tail bleed using 70 µl micro-hematocrit capillary tubes

(Fisherbrand, Ottawa, Canada). The sample was centrifuged at 5,000 rpm for 10 minutes to

isolate plasma. This baseline plasma sample was stored at 4ºC and was protected from light until

analysis. Each female was then administered 12mg of 80mg/mL FITC-dextran by oral gavage

(diluted in sterile PBS). Four hours following gavage, a second blood sample was collected,

centrifuged and plasma collected. Each plasma sample was diluted in PBS (1:4) and fluorescence

intensity as measured using a plate reader at a wavelength of 530 nm (BioTek®, Oakville,

Canada). Baseline fluorescence values were subtracted from 4-hour post gavage values in order

to obtain fluorescence values that were representative of maternal intestinal barrier integrity.

Permeability was expressed as relative fluorescence units (RFU) between CON and HF dams.

2.6 Collection of maternal and fetal tissue

At E14.5 pregnant dams were fasted for 6 hours. Maternal body weight was recorded. Maternal whole blood glucose was measured with a glucometer (Precision Xtra, Abbott Diabetes Inc.), and a blood sample was collected via tail vein bleed using 70 µl micro-hematocrit capillary tubes (Fisherbrand, Ottawa, Canada) immediately placed on ice, incubated at room temperature for 30 minutes, centrifuged at 7500 rpm for 5 minutes and serum isolated and stored at -80ºC for further biochemical analyses. Following blood collection, all pregnant mice were sacrificed by cervical dislocation and placed into a sterile fume hood for organ dissection. Maternal mesenteric and gonadal fat pads were collected and weights recorded as a proxy measure of maternal adiposity. A hysterectomy was performed, via sterile caesarian section, and the uterus placed in a sterile petri dish (Fisherbrand, Ottawa, Canada) containing Hanks Balanced Salt Solution (HBSS) (Life Technologies, Canada) and transferred to a second sterile fume hood for organ dissection, to minimize bacterial transfer from mother to fetus. Fetuses were removed from the uterus, the amniotic sac was opened and the placenta and umbilical cord were detached. Placental and fetal weights were recorded. Placentae were separated from the maternal decidual tissue, and umbilical cords removed. Half of the recovered placentae from each litter were snap frozen in liquid nitrogen and the remaining half were placed in a tissue cassette and fixed with 4% paraformaldehyde for 48 hours and transferred to 70% ethanol (EtOH) for long-term storage prior to processing for immunohistochemistry. Using a dissection microscope (Nikon Eclipse NI-S-E, 960122, Japan) fetal liver, small and large intestine were collected and snap frozen in liquid nitrogen. One cm sections of the maternal duodenum, jejunum, ileum, and colon were collected, flash frozen in liquid nitrogen and stored at -80ºC. One cm sections of each maternal gut section

were also placed in a tissue cassette and fixed with 10% formalin for 48 hours and transferred to 70% EtOH for long-term storage.

2.7 Fetal genotyping

2.7.1 Genomic DNA extraction

Genomic DNA was extracted from fetal tails to determine fetal sex. Briefly, tails were immersed in 497.5 μl lysis buffer and 2.5 μl proteinase K and incubated at 37 ºC overnight. Following incubation, fully digested tails were centrifuged at 13, 000 rpm for 20 minutes at ambient temperature, the supernatant removed and combined with 500 μl isopropanol and tubes were gently inverted to precipitate DNA. Samples were centrifuged at 13, 000 rpm for 15 minutes at ambient temperature, isopropanol removed and DNA was washed twice in 700 μl EtOH (centrifuged at 13, 000 rpm for 10 minutes at ambient temperature) and DNA pellet air dried for 10 minutes prior to elution with 20 μl ultrapure water. Samples were then incubated at 55 ºC for 20 minutes and DNA concentration determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Canada; NanoDrop 2000/2000c software) and integrity determined via the ratio of absorbance at 260 nm to 280 nm (A260/A280) and at 260 nm to 230 nm (A260/A230); which for each sample was > 1.5 and > 0.60 respectively. All DNA samples were stored at 4ºC until PCR amplification.

2.7.2 PCR amplification of the Sry gene

PCR amplification of the Sry gene was conducted on extracted DNA from fetal tails to determine fetal sex. Briefly, each reaction contained 10X reaction buffer (Roche, Canada), 5 μM of forward

and reverse Sry primer, 10 mM of dNTPs, 0.25ul Taq polymerase (Life Technologies, Canada)

and the required volume of ultrapure water to bring the total reaction volume to 20 μl. The PCR

program used was as follows: 94º C for 2 minutes followed by 30 cycles of 94º C for 30 seconds,

50º C for 30 seconds and 72º C for 30 seconds, with a final extension step at 72º C for 10

minutes. DNA products of the PCR amplification were subsequently loaded onto a RedSafe gel

by combining 2 μl loading dye and 8 μl of each sample and gel electrophoresis was run at 120 V

for 15 minutes to detect the presence (male) or absence (female) of the Sry gene. Amplified

DNA stained with RedSafe DNA detection dye was visualized using a ChemiDoc gel imaging

system (Bio-Rad, ChemiDoc[TM] MP System, 1708280, Mississauga, Canada) to detect the

presence of the Sry gene (visible as a 300 base pair band).

2.8 Fecal genomic DNA Extraction and 16S rRNA sequencing

Fecal genomic DNA was extracted as in section 2.3 on non-pregnant and pregnant fecal samples

at E0.5, E6.5, E10.5 and E14.5 of control and high-fat females to investigate the impact of

pregnancy and maternal diet on the maternal gut microbiota.

2.9 Sequence processing and data analyses

Sequence processing and data analyses was performed as in section 2.4. This processing resulted

in a total of 7214096 reads (mean 110986 reads per sample; range: 60669-155790) and 2423

OTUs. Analyses of these data were completed using various open source software. Taxonomic

summaries were computed using R Studio. Measures of β-diversity, were computed using

Phyloseq's [332] implementation of the Bray Curtis distance metric, and tested for whole

community differences across groups using vegans implementation of permutational multivariate

analysis of variance (PERMANOVA) in the adonis command. These results were visualized via

Principal Coordinate Analysis (PCoA) ordination. Calculations of genera which differed

significantly between groups were computed using DESeq2 [333] and considered significant if

the p value was < 0.01 after adjustment for multiple testing via DESeq2's implementation of the

Benjamini-Hochberg multiple testing adjustment procedure) and visualized using ggplot2

package [334]

2.10 Fixed tissue processing

All E14.5 fixed maternal gut sections and placentae were processed by a fully automated tissue

processor (Leica Biosystems, TP1020, Concord, Canada) with the following processing steps:

70% EtOH (2 x 1 hour), 90% EtOH (2 x 1 hour), 100% EtOH (3 x 1 hour) and xylene (5 x 1

hour). Processed tissues were then paraffin-embedded using a standard tissue embedding centre,

dispenser and hot plate (Leica Biosystems EG1160, Concord, Canada). Maternal E14.5 paraffin-

embedded colons (5 μm) and E14.5 placentae (4 μm) were sectioned using a Microm HM325

(Thermo Scientific) microtome. All sections were placed in a 37ºC paraffin section flotation bath

(Electrothermal, MH8517, Staffordshire, UK) and placed on Superfrost™ Plus microscope slides

(Fisherbrand, 12-550-15, Ottawa, Canada), dried overnight at 37ºC and stored at ambient

temperature until future analyses.

2.10.1 Localization of intestinal mucins

2.10.1.1 Alcian blue stain

Maternal colon sections were stained for Alcian blue (binds to acidic mucins) as a marker of

goblet cell localization. Staining was performed by the McMaster Immunology Research Centre

John Mayberry Histology Facility. Briefly, colon sections were rinsed in distilled water and

placed in 3% acetic acid (pH 2.5) for 3 minutes. Sections were placed in alcian blue solution for

30 minutes. Slides were washed in running tap water for 2 minutes and rinsed in distilled water.

A counterstain was performed with nuclear fast red solution for 5 minutes. Sections were washed

in running tap water for 1 minute prior to cover-slipping with Permount^{TM} Mounting Medium

(Fisher Chemical, SP-15-100, Ottawa, Canada).

2.10.2 Maternal intestinal and placental localization of immune cell markers

To investigate macrophage and T cell infiltration, maternal colon and placental sections were

immunostained for F4/80 (equivalent to CD68 in humans, a macrophage cell surface marker) and

CD3 (T cell receptor complex located on mature T lymphocytes). Immunohistochemistry was

performed by the McMaster Immunology Research Centre John Mayberry Histology Facility.

Colonic and placental staining was performed under the following Leica Bond protocols; F4/80

with Citrate Buffer antigen retrieval on the Leica Bond RX secondary protocol, CD3 with high

pH antigen retrieval on the Leica Bond RX Rabbit protocol and Ly6G with high pH antigen

retrieval on the Leica Bond RX secondary protocol.

2.10.2.1 Computerized image analysis of intestinal and placental immune cell markers

Immunopositive F4/80 stained cells in E14.5 maternal colon and placental sections were

visualized using the Nikon Eclipse NI microscope (Nikon Eclipse NI-S-E, 960122, Japan) and

images captured using the Nikon DS-Qi2 Colour Microscope Camera. The total number of

colonic and placental cells positively stained for F4/80 was quantified by counting positive cells.

The total area of colonic and placental cells positively stained for F4/80 was quantified by

dividing the area positively stained for F4/80 by the total area of the tissue section, resulting in a

ratio of positive F4/80 expressing cells. This was determined in 2 colonic sections and 1

placental section per dam. All counts were performed with the experimenter blinded to the

nutritional group.

2.10.2.2 Computerized image analysis of intestinal and placental T cells

Immunopositive staining for CD3 in E14.5 maternal colon and placentae was visualized using

the Nikon Eclipse NI microscope (Nikon Eclipse NI-S-E, 960122, Japan) and images were

captured using the Nikon DS-Qi2 Colour Microscope Camera. The total number of colonic and

placental cells positively stained for CD3 were counted in each colonic and placental section.

This was determined in 2 colonic sections per dam and 1 placental section for 1 male and 1

female placenta from each litter. To eliminate bias between groups cross sections from the HF

and CON groups are blinded. All counts were performed with the experimenter blinding to the

nutritional group.

2.11 Evaluation of mRNA levels of key signalling molecules in the maternal intestine and placenta

2.11.1 Maternal intestinal and placental RNA extraction

Total RNA was extracted from frozen maternal duodenum, jejunum, ileum and colon using

TRIzol®-Reagent (50mg tissue/mL) following the manufacturer's instructions. Briefly, 45 mg of

frozen maternal intestine and placenta were homogenized in 900 μl TRIzol®-Reagent (ambion,

Life Technologies, 15596018, Canada) and 0.2 g of glass beads (Sigma-Aldrich, G4649-100G,

St Louis, MO, USA). Total RNA was purified using RNeasy columns (Qiagen RNeasy ® Mini

Kit, 74136 as per the manufacturer's specifications). RNA was eluted from the column following

a 15 minute incubation with 20 μl of RNase free water heated to 55ºC and was collected in a

fresh 1.5mL Eppendorf. RNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Canada) using NanoDrop 2000/2000c software (Thermo Scientific, Canada). The ratio of absorbance at 260 nm to 280 nm (A260/A280) and at 260 nm to 230 nm (A260/A230) for each sample was > 1.7 and > 1.72 respectively. All RNA samples were stored at -80ºC until cDNA synthesis.

2.11.2 Complementary DNA (cDNA) synthesis

2000 ng of total RNA was used for first strand cDNA synthesis using the SuperScript® VILO™ cDNA synthesis kit (Life Technologies, Canada) according to the manufacturer's instructions. Briefly, each reaction contained 4 µl of 5X VILO™ Reaction Mix, 2 µl of 10X SuperScript® Enzyme Mix, 2000 ng of total RNA and the required volume of molecular grade PCR water to bring the reaction volume to a total of 20 µl. Tube contents were gently mixed and placed into a thermocycler (Bio-Rad Laboratories, C1000 Touch, Mississauga, Canada) as follows: incubation at 25ºC for 10 minutes, 42ºC for 1 hour and 85ºC for 5 minutes. Complementary DNA samples were stored at -20ºC until quantitative PCR assay.

2.11.3 Primer design

Primer sets were designed using Primer-BLAST software available at the National Centre for Biotechnology Information Website (blast.ncbinlm.nih.gov,NCBI). Primers were manufactured by Invitrogen, Life Technologies, Canada). Optimal primer conditions were adjusted for the following cycling conditions: product size 50-150 base pairs; primer melting temperature 58ºC to 62ºC; exon junction span, all primers spanned exon-exon junctions. Only primer sets producing a single dissociation curve peak were used for quantification in unknown samples (see Table 2.11.3.1 for full primer set details).

Table 2.11.3 Primer Sequences and Amplicon Sizes for Genes of Interest				
Gene	Forward Primer	Reverse Primer	Amplicon length (bp)	GenBank Accession Number
Housekeepers				
β-actin	AGATCAAGATCATTGCTCC	ACGCAGCTCAGTAACAGTC	174	NM_007393.5
Cyclophilin	CTTCGAGCTGTTTGCAGACA	TGGCGTGTAAAGTCACCAC	147	NM_008907.1
HPRT	CAGTCCCAGCGTCGTGATT	TCGAGCAAGTCTTTCAGTCC	142	NM_013556.2
Intestinal Mucins				
MUC1	CAAGTTCAGGTCAGGTCCG	TGACTTCACGTCAGAGGCAC	74	NM_013605.2
MUC2	GAAGCCAGATCCCGAAACCA	GAATCGGTAGACATCGCCGT	81	NM_023566.3
MUC4	AGATGGCTCTGAACCTAAGTATGC	AGGTGGTAGCCTTTGTAGCC	67	NM_080457.3
MUC5AC	CTGCCTGTACAATGGGACACT	AACATGTGTTGGTGCAGTCAGT	68	NM_010844.1
Placental Nutrient Transport				
GLUT1	GGCTTGCTTGTAGAGTGACG	TGTAGAACTCCTCAATAACCTTCTG	275	NM_011400.3
GLUT3	AATAGGTAGGCTGGGCTTCG	AGAGATGGGGTCACCTTCGTT	184	NM_011401.4
SNAT2	GCAGTGGAATCCTTGGGCTT	TAAAGATCCTCCTTCGTTGGCAG	140	NM_175121.3
VEGF	CTGGACCCTGGCTTTACTGC	GCAGCCTGGGACCACTTG	54	NM_001025257.3
FABP4	AAGCTGGTGGTGGAATGTGTTA	CCTCTTCCTTTGGCTCATGC	77	NM_024406.2
Inflammation				
TLR-2	AAGGAGGTGCGGACTGTTT	CCTCTGAGATTTGACGCTT	156	NM_011905.3
TLR-4	TCTGGGGAGGCACATCTTC	TGCTCAGGATTCGAGGCTT	72	NM_021297.3
TRAF6	GCACGGAAACTTGGGTCTT	CTCTGTTGTCAGTCGACTTG	105	NM_009424.3
NFκB	CTGCTCAGGTCCACTGCTTG	TTGCGGAAGGATGTCTCCA	112	NM_008689.2
Adgre1	TTGTGGTCCTAACTCAGTCTGC	AGACACTCATCAACATCTGCG	141	NM_010130.4
MCP-1	CCACAACCACCTCAAGCACT	AGGCATCACAGTCCGAGTCA	75	NM_011333.3
TNF-α	CAGACCCTCACACTCAGACTA	GGCTACAGGCTTGTCACTCG	86	NM_013693.3
IL-6	GGGACTGATGCTGGTGACA	ACAGGTCTGTTGGGAGTGG	90	NM_001314054.1
IL-10	ACTTTAAGGGTTACTTGGGTTGC	CCTGGGGCATCACTTCTACC	75	NM_010548.2

Table 2.11.3.1 Primer sequences and amplicon sizes for genes of interest. Abbreviations: HPRT, Hypoxanthine-guanine phosphoribosyltransferase; MUC1, Mucin 1; MUC2, Mucin 2; MUC4, Mucin 4; MUC5AC, Mucin 5AC; GLUT1, Glucose transporter 1; GLUT3, Glucose transporter 3; SNAT2, Sodium-coupled neutral amino acid transporter 2; VEGF, Vascular endothelial growth factor; FABP4, Fatty acid binding protein 4; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4; TRAF6, TNF receptor associated factor 6; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; Adgre1, Adhesion G protein-coupled receptor E1; MCP1, Monocyte chemoattractant protein-1; TNF-α, Tumor necrosis factor alpha; IL-6, Interleukin-6; IL-10, Interleukin-10.

2.11.4 Quantitative real time Polymerase Chain Reaction (qPCR) assays and analyses

Quantification of maternal gut and placental mRNA levels of genes of interest and housekeeping genes was performed using a LightCycler 480 II (Roche, Canada) in 384-well plates (LightCycler 480 Multiwell Plate 384 White – Roche, 04729749001, Canada). For each assay, 1 well contained 2.5 µl of cDNA (1:10), 0.5 µl of 5 µM Forward Primer (1:20), 0.5 µl of 5 µM Reverse Primer (1:20), 1.5 µl PCR water and 5 µl of LightCycler 480 SYBR Green 1 Master (Roche, 04887352001, Canada). All qPCR assays were carried out with an initial denaturation at 95ºC for 10 seconds, followed by amplification of the gene product through 55 successive cycles of 95ºC for 10 seconds, 60ºC for 10 seconds and 72ºC. Dissociation curve began at 65ºC and ended at 95ºC. Each sample was run in triplicate. Negative control samples containing no cDNA were used to confirm absence of DNA contamination.

Quantification of maternal gut and placental gene expression was performed using a standard curve generated from the mean cycle threshold (Ct) of five standards (1:10 serial dilution) of pooled samples of colon or placental cDNA (as appropriate) in triplicate. Amplification and dissociation curves were generated for all standards and samples. To negate the effect of minor differences in the amount of RNA template used in cDNA synthesis, the geometric means of constitutively expressed housekeeping genes β-Actin, Cyclophilin and Hypoxanthine Phosphoribosyltransferase (HPRT) were used as a correction factor for the gene of interest results. The mean value for the genes of interest were expressed as a ratio to that of the animal-matched geomean of the housekeepers used. These corrected ratios, termed the relative levels of mRNA expression, were used in statistical analyses.

2.12 Serum biochemistry

2.12.1 Maternal metabolic indicators: serum insulin and leptin concentrations

Serum concentrations maternal insulin and leptin at E14.5 were measured using a Bio-Plex Pro™ Mouse Diabetes Standard 8-plex Assay kit (Bio-Rad Laboratories, 171F7001M, Mississauga, Canada). All serum samples were diluted four-fold (1:4 dilution) in Bio-Plex standard diluent. A standard curve was generated by reconstituting a single vial of diabetes standards (Bio-Rad Laboratories, 171-I70001, Mississauga, Canada) and preparing a fourfold (1:4) standard dilution series. Fifty μl of standards, samples and blanks were added to the plate and incubated with 1x insulin and leptin coupled beads for 1 hour at ambient temperature while shaking at 850 rpm. Following a wash step using Bio-Plex wash buffer, samples were incubated with 1x detection antibody for 30 minutes at ambient temperature shaking at 850 rpm. Following a second wash step in Bio-Plex wash buffer samples were incubated with 1x streptavidin-PE for 10 minutes at ambient temperature shaking at 850 rpm. Insulin and leptin coupled bead regions for the Bio-Plex Pro™ Mouse Diabetes Assay kit are 126 pg/mL and 16.07 pg/mL. Reading was performed using a Bio-Plex® 200 system (Bio-Rad Laboratories, 171-203001, Mississauga, Canada) using Bio-Plex Manager™ software (Bio-Plex Manager™ 6.1 © Copyright 2000 Bio-Rad Laboratories, Inc.) All standards, samples and blanks were analyzed in duplicate and the mean value was determined accounting for dilution factors where appropriate. Only the samples which fell between the linear range of the standard curve were included in analysis.

2.12.2 Maternal Inflammatory Indicators: Serum Inflammatory Mediators

Concentrations of Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin (IL) IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, monocyte chemoattractant protein-1 (MCP-1), monocyte inflammatory protein-1 alpha (MIP) (MIP-1α), MIP-1β, regulated on activation of normal T cell expressed and secreted (RANTES), and TNF-α were measured in maternal serum using a Bio-Plex Pro™ Mouse Cytokine 23-plex Assay kit (Bio-Rad Laboratories, M600009RDPD, Mississauga, Canada) following the manufacturer's instructions. Reading was performed using a Bio-Plex® 200 system (Bio-Rad Laboratories, 171-203001, Mississauga, Canada) using Bio-Plex Manager™ software (Bio-Plex Manager™ 6.1 © Copyright 2000 Bio-Rad Laboratories, Inc.).

2.13 Maternal intestinal and placental NFκβ p65 activity

NFκB activity was measured in intestinal and placental homogenates using the TransAM™ NFκB p65 Transcription Factor Assay Kit (Cedarlane Laboratories, 40096, Mississauga, Canada). Approximately 0.01 g of gut and placental tissue was lysed in 300 μl SBJ buffer and yielded protein lysate concentrations between 1 and 13.9 μg/μl. The concentration of both intestinal and placental lysate was standardized to 1 μg/μl by dilution with complete lysis buffer for use in the NFκB transcription factor assay. Briefly, to allow binding of NFκB to its consensus sequence, 30 μl of complete binding buffer and 20 μl diluted gut and placental lysate was added to each well on the provided 96 well plate. 2 μl of Jurkat nuclear extract diluted in 18 μl complete lysis buffer was used as a positive control and loaded into 1 well. 20 μl of complete lysis buffer was added to 1 well as a blank. Following a 1 hour incubation with mild agitation

(100 rpm) at ambient temperature each well was washed 3x with 200 µl wash buffer. To allow

binding of the primary antibody, 100 µl of NFκB antibody diluted in 1x antibody binding buffer

(1:1000) was added to each well. Following a 1 hour incubation at ambient temperature without

agitation, each well was washed 3x with 200 µl wash buffer. To allow binding of the secondary

antibody 100 µl HRP-conjugated antibody diluted in 1x antibody binding buffer (1:1000) was

added to each well. Following a 1 hour incubation at ambient temperature without agitation, each

well was washed 4x with 200 µl wash buffer. A colourimetric reaction was performed where 100

µl of developing solution was added to all wells and incubated for 5 minutes at ambient

temperature protected from light. The reaction was stopped by adding 100 µl stop solution to

each well. The absorbance of each sample was read using a plate reader at 450 nm followed by

an optional reading at a reference wavelength of 655 nm (BioTek®, Oakville, Canada). All

samples were analyzed in single with the exception of a triplicate blank. Data are presented as

mean with minimum and maximum.

2.14 Statistical analyses

In all analyses 1 litter is considered to be 1 biological replicate. Analyses of maternal gestational

weight and food intake was determined using a two-way repeated measures analysis of variance

(2-way ANOVA), with maternal diet and gestational day as factors. Analyses of placental

measures was conducted using a 2-way ANOVA with maternal diet and placental sex as factors.

Tukey's *post-hoc multiple comparison* analyses were used where the ANOVA was $p < 0.05$. All

other data were analyzed using a two-tailed unpaired Student's t-test where $p < 0.05$ was

considered statistically significant. With normally distributed data, outliers were removed using

the Grubb's test $\alpha = 0.05$. Statistical analyses for maternal and fetal phenotype, was performed

using GraphPad Prism 6.0 (GraphPad Prism 6.0 © Copyright GraphPad Software Inc. 1994-

2013) statistical software. All data are presented as mean with minimum and maximum showing

all raw experimental values.

3.0 RESULTS

3.1 Female reproductive cycle study

3.1.1 Non-Pregnant Control (NPC) female reproductive cyclicity

Ten non-pregnant control (NPC) females were evaluated for reproductive cyclicity where 7 of the 10 females exhibited regular cycles over the course of 3 reproductive (estrous) cycles (15 days). This is consistent with other reports of control female cyclicity [324, 335]. Three reproductive cycles were recorded and vaginal epithelial cells analyzed (Figure 3.1.1) within each NPC female to examine intra-animal variability in the gut microbiota between consecutive estrous cycles over time. In all cases, only those females whose vaginal epithelial cell analyses showed distinct cycle differences were used (Table 3.1.1.1).

Female ID	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
Control 1	D	E	E	D	P	E	E	E	D	P	E	E	D	P	E
Control 2	E	M	D	P	E	E	D	P	E	E	E	D	P	P	E
Control 3	D	D	P	E	E	E	M	D	D	P	E	E	D	P	E
Control 4	D	P	E	E	E	D	P	E	E	D	D	P	E	E	E
Control 5	P	E	E	D	P	E	E	E	D	P	P	E	E	D	D
Control 6	E	D	D	P	E	E	E	D	P	E	E	E	D	P	E
Control 7	E	E	D	P	E	E	D	D	P	E	E	D	D	P	E

Table 3.1.1.1 NPC female estrous cycles. Reproductive cycles of control females (n = 7) for a total of 3 full estrus cycles (15 days). D, diestrous; P, proestrous; E, estrous; M, metestrous. Reproductive stages that are highlighted in turquoise, maroon and lime represent the consecutive stages of diestrous, proestrous and estrous stages of the 3 consecutive reproductive cycles that were selected for gut microbial analysis via illumina sequencing of the V3 region of the 16S rRNA gene.
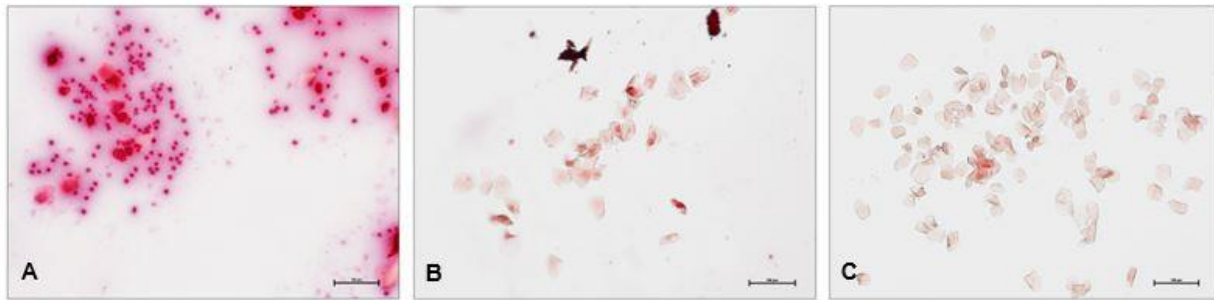
Figure 3.1.1.2 NPC female vaginal smears. Representative images of Haematoxylin stained vaginal epithelial cells from control females at: **A.** diestrous, predominantly consisting of leucocytes; **B.** proestrous, predominantly consisting of nucleated epithelial cells; and **C** estrus, consisting predominantly of cornified epithelial cells. Scale bar represents 100μm.

3.1.2 The NPC female intestinal microbiota does not shift across the reproductive cycle

No significant shifts in the NPC female intestinal microbiota were observed across reproductive stages. Figure 3.1.2.1A displays the taxonomic summaries of microbial relative abundance for the 25 most abundant taxa in the NPC female intestinal microbiome at diestrous, proestrous or estrus for three consecutive reproductive cycles. The two dominant phyla identified in the intestinal microbiota in regularly cycling NPC females were the Firmicutes and Bacteroidetes, consistent with other intestinal microbial profiles reported in mice [189, 218, 231, 235] and humans [218, 279, 280]. Principle Coordinate Analysis using the Bray Curtis distance metric showed no significant clustering of intestinal microbial communities at each estrus cycle stage (n = 6) (PERMANOVA p = 0.95) (Figure 3.1.2.1B). Boxplots of the top 25 most abundant taxa show the relative abundance of individual taxa at diestrous, proestrous and estrus for the three consecutive reproductive cycles (Figure 3.1.2.2). NPC female intestinal microbiota is not mediated by the known sex-steroid hormone fluctuations at diestrous, proestrous and estrus during the reproductive cycle. This analysis was performed in 6 regularly cycling females where 3 distinct regular reproductive cycles were analyzed. The seventh regularly

cycling control female was removed from analyses since only two consecutive estrous cycles

were collected.
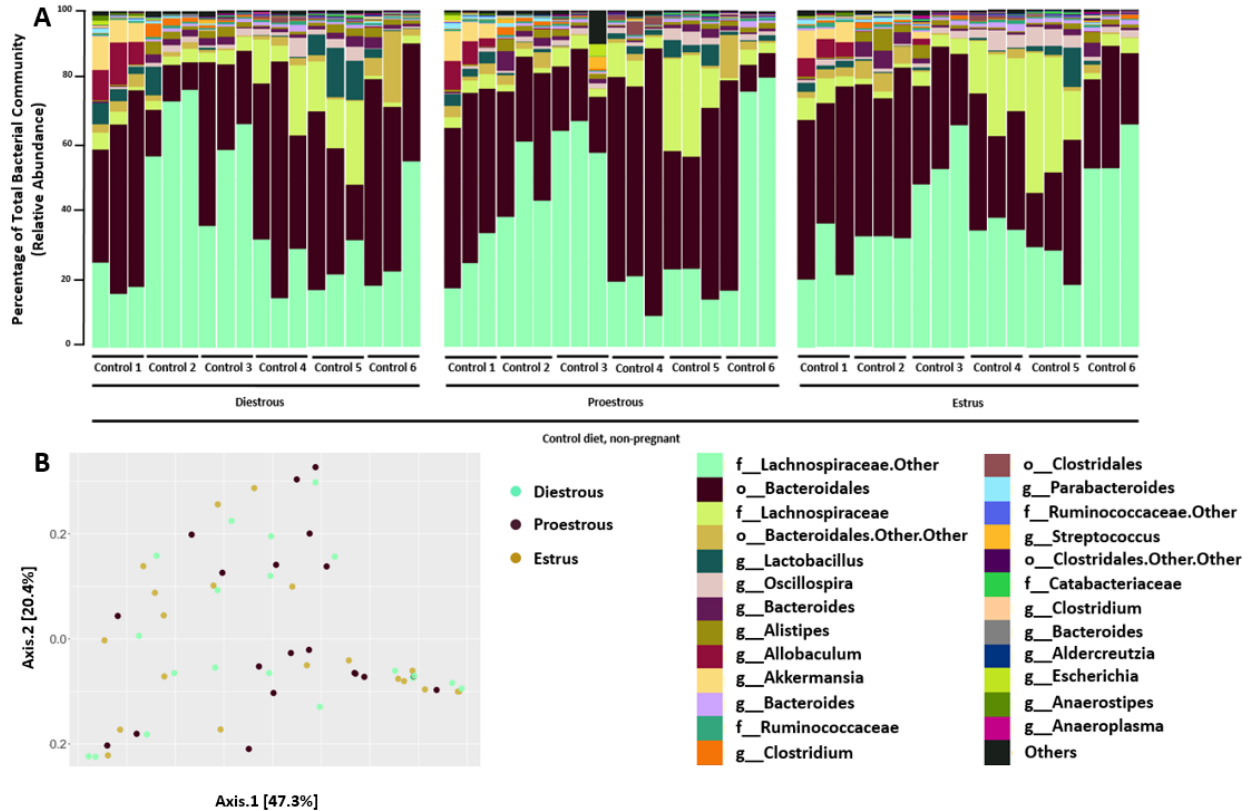
Figure 3.1.2.1 The NPC female intestinal microbiota does not shift during the reproductive cycle. A. Taxonomic classifications of the 25 most abundant taxa resolved to the order (o), family (f) and genus (g) level at diestrous, proestrous and estrus for three consecutive estrous cycles in NPC females (n = 6). **B.** Principle Coordinate Analysis using the Bray Curtis distance metric showed no significant clustering of intestinal microbial communities present at diestrous, proestrous or estrus in NPC females (n = 6).
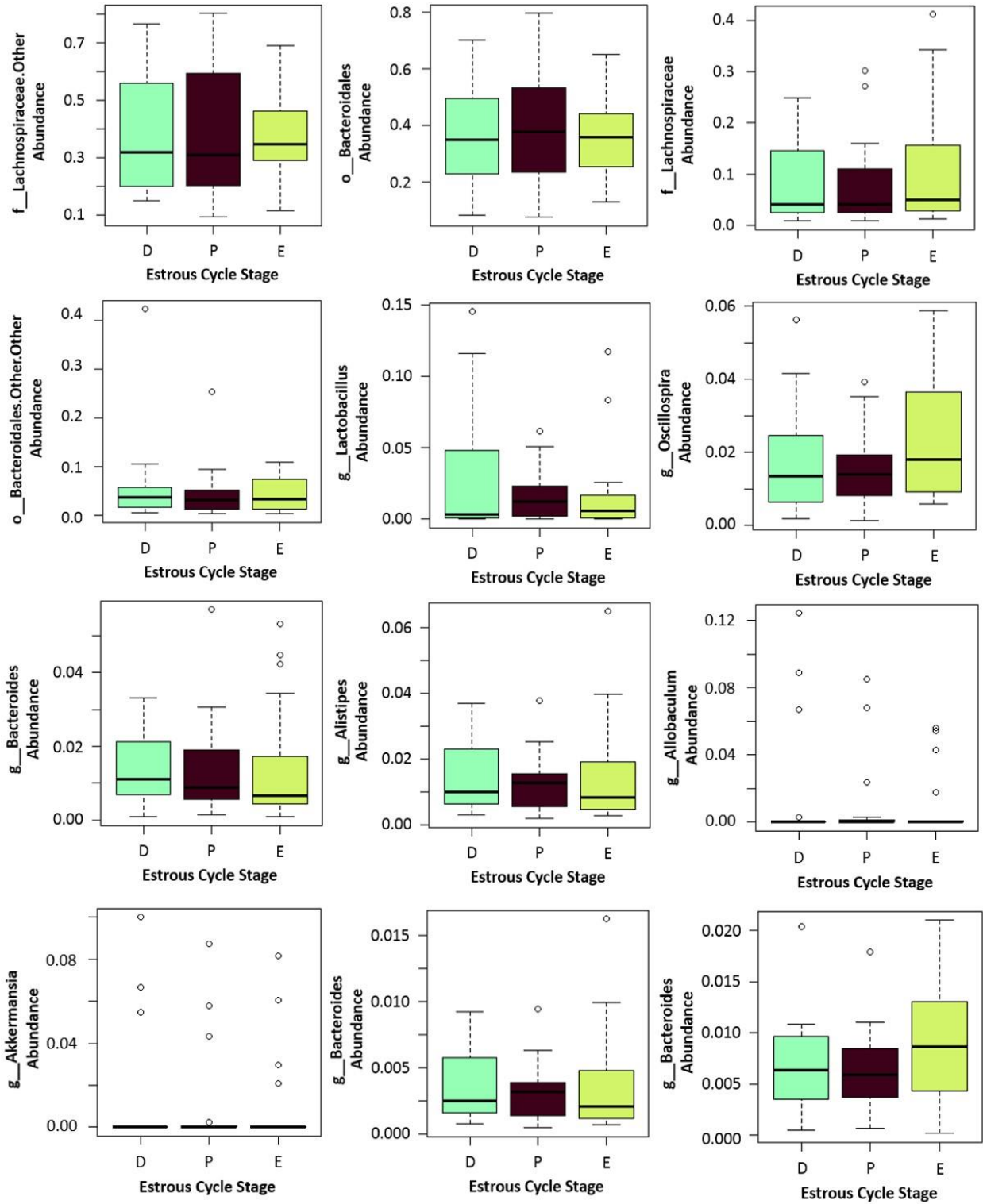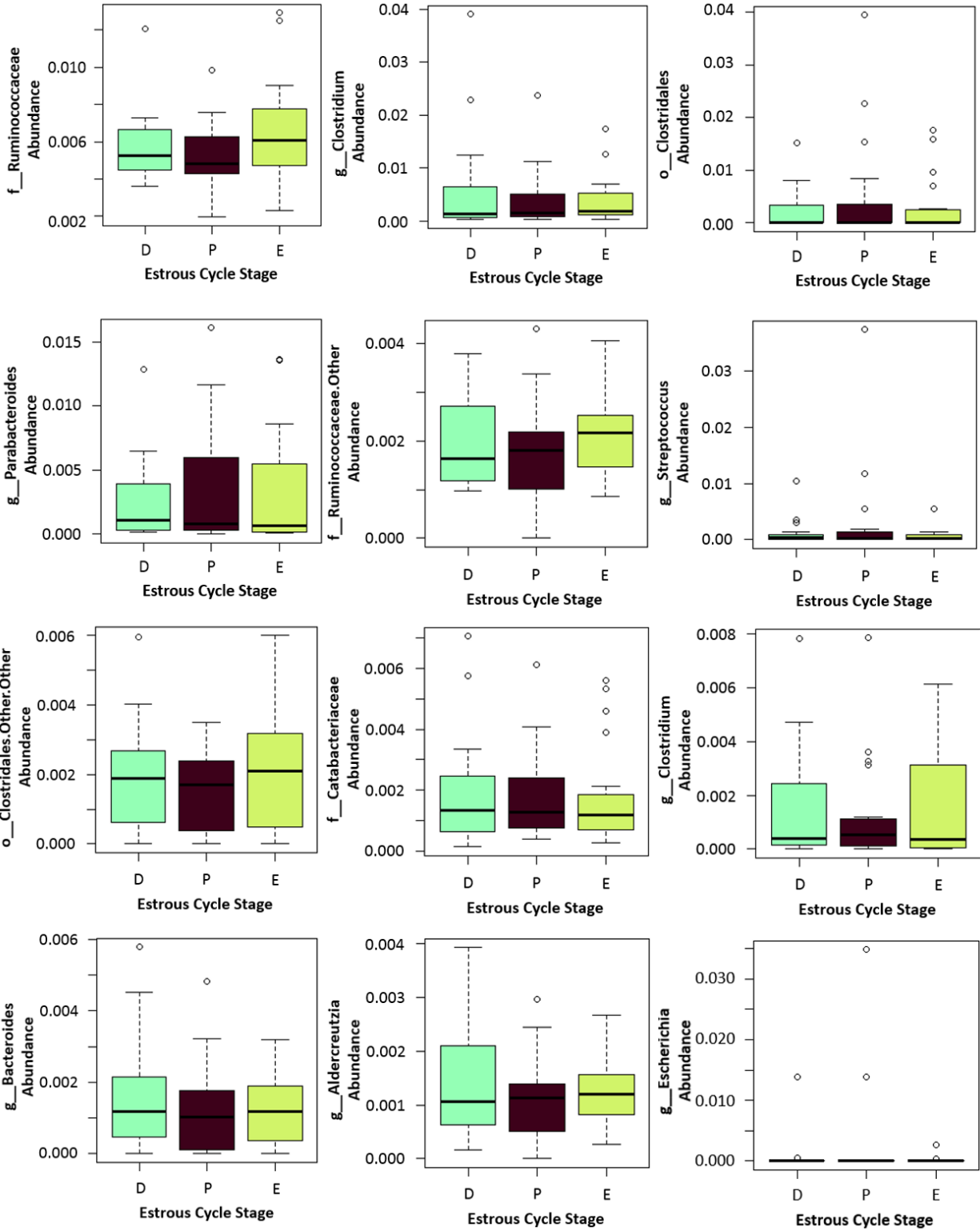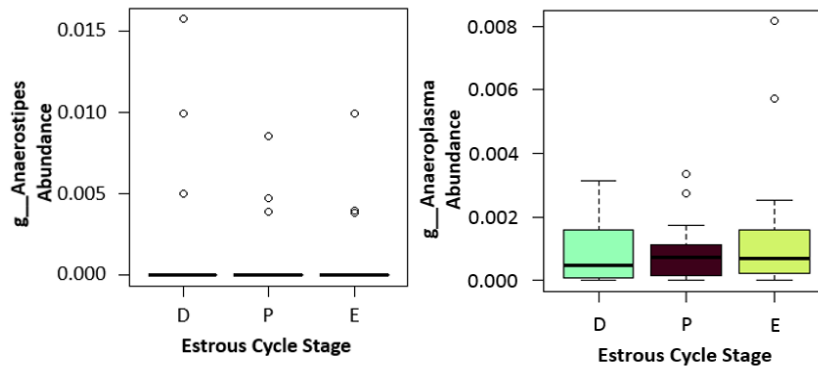
Figure 3.1.2.2 Individual taxa abundance at diestrous, proestrous and estrus in NPC females. The relative abundance of the 25 most abundant taxa resolved to the order (o), family (f) or genus (g) level classification for three consecutive estrus cycles at D; diestrous, P; proestrous and E; estrus in NPC females (n = 6).

3.1.3 The NPC female intestinal microbiota displays a high degree of intra-female variability

Taxonomic summarys displayed a high degree of intra-female variability, where the first NPC female dispalyed a unique gut microbiota characterized by a higher proportion of *Akkermansia* (phylum Verrucomicrobia) and *Allobaculum* (phylum Tenericutes). The second and third females displayed elevations in *Alistipes*, *Bacteroides* belonging to the Bacteroidetes phylum and *Clostridium* (phylum Firmicutes). The fourth and fifth female displayed a higher proportion of Clostridales, Lachnospiraceae, *Lactobacillus* and *Oscillospira* all belonging to the Firmicutes phylum. The sixth female showed elevations in Bacteroidales (phylum Bacteroidetes) only (Figure 3.1.3.1A). Similarly, significant clustering of intestinal microbial communities was found within individual animals and thus microbial shifts appear to be driven by animal-related differences rather than by reproductive cycle stage (PERMANOVA p=0.005) (Figure 3.1.3.1B). No clustering of was identified in each female at consecutive diestrous, proestrus and estrus stages of the reproductive cycle (PERMANOVA p=0.90) (Figure 3.1.3.1C).
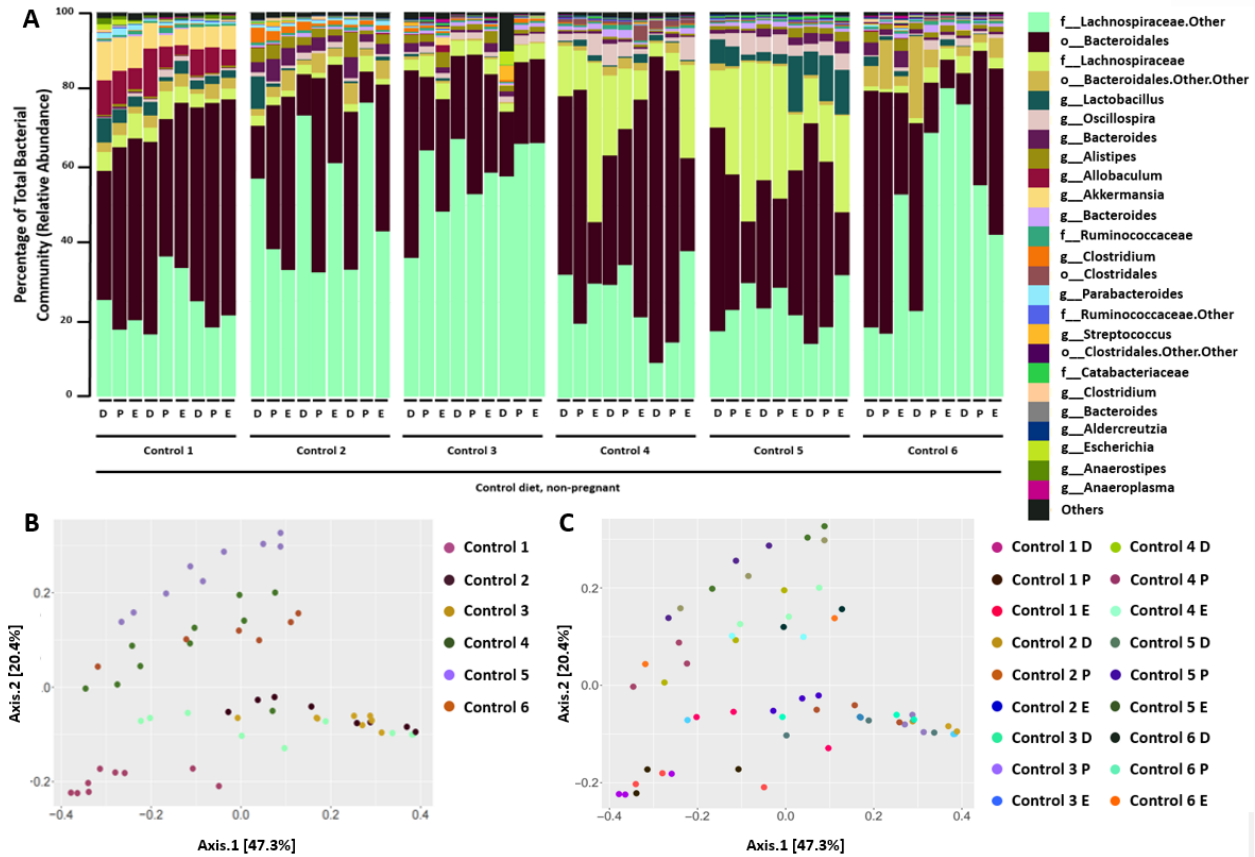
Figure 3.1.3.1 The intestinal microbiota is stable across the reproductive cycle in each NPC female.
A. Taxonomic classifications of the 25 most abundant taxa resolved to the order (o), family (f) and genus (g) level are displayed for three consecutive estrus cycles at D; diestrous, P; proestrous and E; estrous in NPC females (n = 6). **B.** Principle Coordinate Analysis using the Bray Curtis distance metric shows significant clustering of microbial communities present in each NPC female across three consecutive estrous cycles (n = 6). **C.** Principle Coordinate Analysis using the Bray Curtis distance metric shows no significant clustering of microbial communities present in each NPC female between the three consecutive D; diestrous, P; proestrous and E; estrus stages (n = 6).

3.2 Maternal obesity study

3.2.1 Maternal phenotypic characteristics

Consistent with other rodent models of maternal obesity [136, 137, 150, 177, 195, 215, 284], HF-fed females were significantly heavier than CON females at conception (E0.5) ($p < 0.0001$) and at all time points throughout gestation (E6.5, 10.5 and 14.5, $p <0.0001$) (Figure 3.2.1.1A). This is also represented by a significant elevation in overall change in weight during pregnancy in HF relative to CON dams ($p = 0.0011$) (Figure 3.2.1.1B). Caloric intake (kcal/g) was higher in HF fed pregnant mice early in pregnancy (E6.5 $p = 0.0008$) but similar between groups later in gestation (E10.5 $p = 0.5081$, E14.5 $p = 0.5999$) (Figure 3.2.1.1C). At E14.5, maternal gonadal fat mass ($p < 0.0001$) (Figure 3.2.1.1D), but not maternal mesenteric fat mass (Figure 3.2.1.1E) was significantly higher in HF dams compared to CON. Maternal whole blood glucose levels ($p < 0.0001$) (Figure 3.2.1.2A), serum insulin levels ($p = 0.0054$) (Figure 3.2.1.2.B), HOMA-IR ($p = 0.0004$) (Figure 3.2.1.2.C) and serum leptin levels ($p = 0.0002$) (Figure 3.2.1.2D) were significantly higher in HF dams compared to CON. Circulating levels of Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α were unchanged by maternal diet (Figure 3.2.1.3). This analysis was conducted in 7 CON and 9 HF dams.
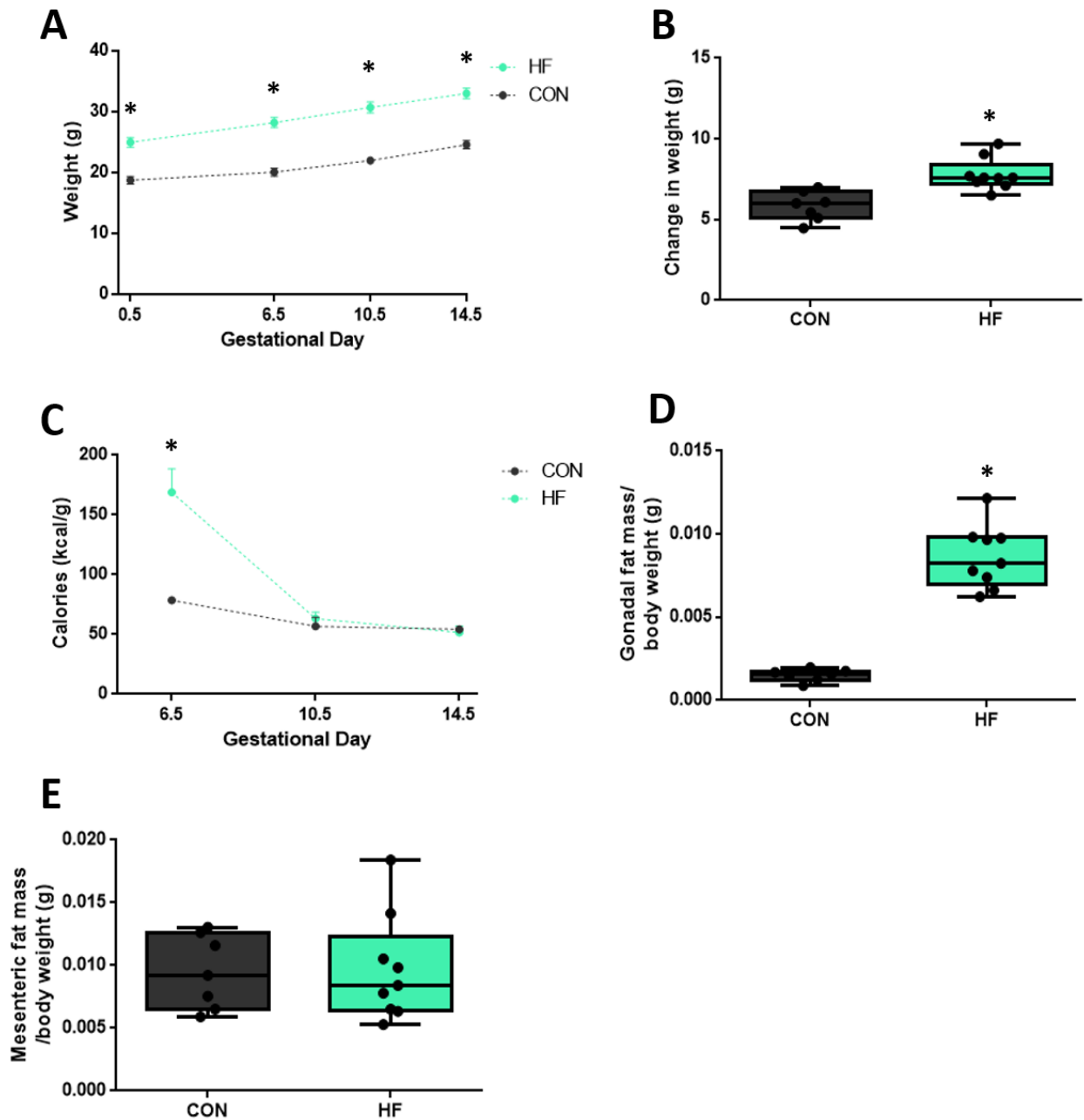
Figure 3.2.1.1 HF dams were heavier than CON at conception and throughout pregnancy to mid gestation, and displayed increased adiposity. A. Maternal body weight (g) during pregnancy in CON (n = 7) and HF (n = 9) animals. **B.** Overall change in body weight over gestation (maternal body weight at E14.5 – maternal body weight at E0.5) in CON (n = 7) and HF (n = 9) dams. **C.** Maternal food intake (kcal/g) during gestation in CON (n = 7) and HF (n = 9) dams. **D.** Gonadal fat mass (g) in CON (n = 7) and HF (n = 9) dams. **E.** Mesenteric fat mass (g) in CON (n = 7) and HF (n = 9) dams. Data are presented as mean ± minimum and maximum. Individual data points represent raw data values. *p < 0.05.
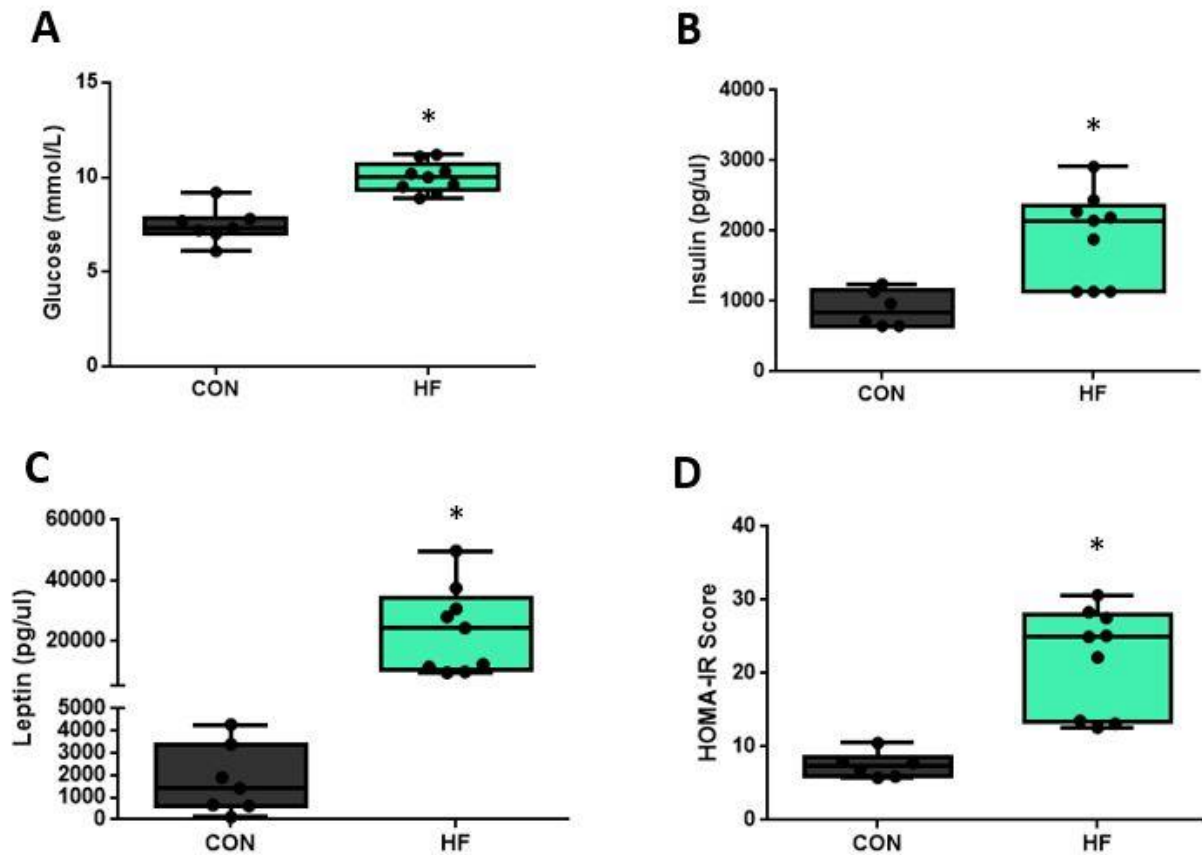
Figure 3.2.1.2 HF dams were hyperglycemic and displayed increased circulating insulin and leptin and HOMA-IR at E14.5 relative to CON. A. Glucose (mmol/L) in CON (n = 7) and HF (n = 9) dams at E14.5. **B.** Circulating insulin levels in CON (n = 6) and HF (n = 9) dams at E14.5. **C.** Circulating leptin levels in CON (n = 7) and HF (n = 9) dams at E14.5. **D.** HOMA-IR in CON (n = 7) and HF (n = 9) dams at E14.5. Data are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values. *p < 0.05.
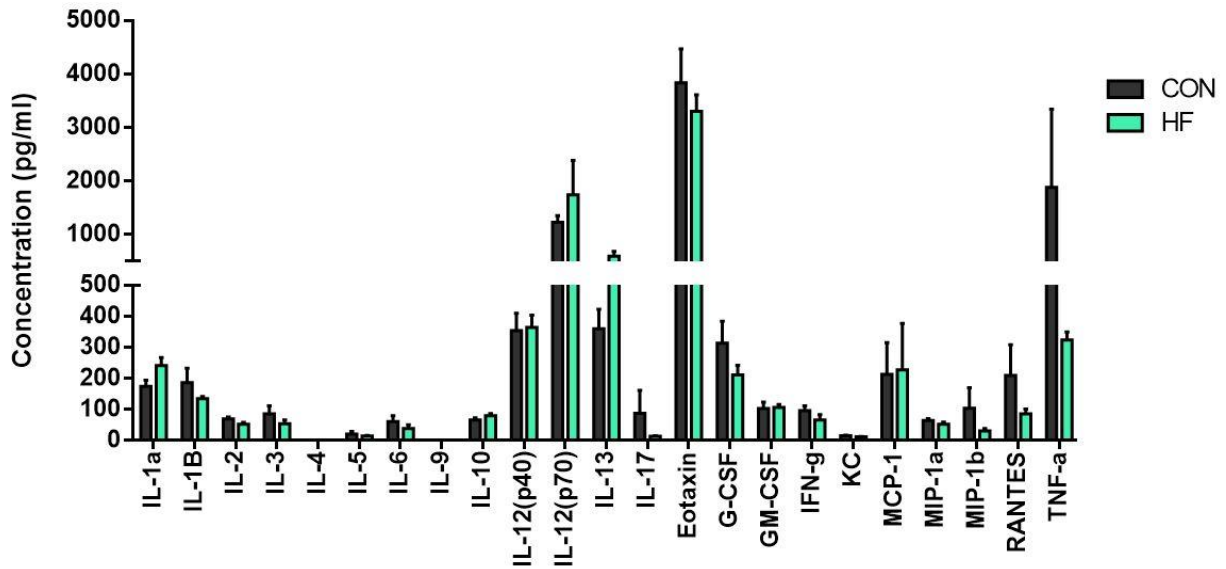
Figure 3.2.1.3 Circulating inflammatory mediators are unchanged by maternal obesity at E14.5. Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α (pg/ml) were unchanged between HF and CON dams at E14.5. Data are presented as mean $\pm$ SEM.

3.2.2 Fetal phenotypic characteristics

Overall fetal weight at E14.5 was similar between HF and CON (Figure 3.2.2.1A) (p = 0.9415) as well as fetal weight according to sex between groups (Figure 3.2.2.1B) (p_{diet} = 0.9820, p_{sex} = 0.3017). There was no significant difference between litter size (p = 0.3818) (Figure 3.2.2.1C) or sex ratio (male: female) (p = 0.3597) (Figure 3.2.2.1D) between groups.
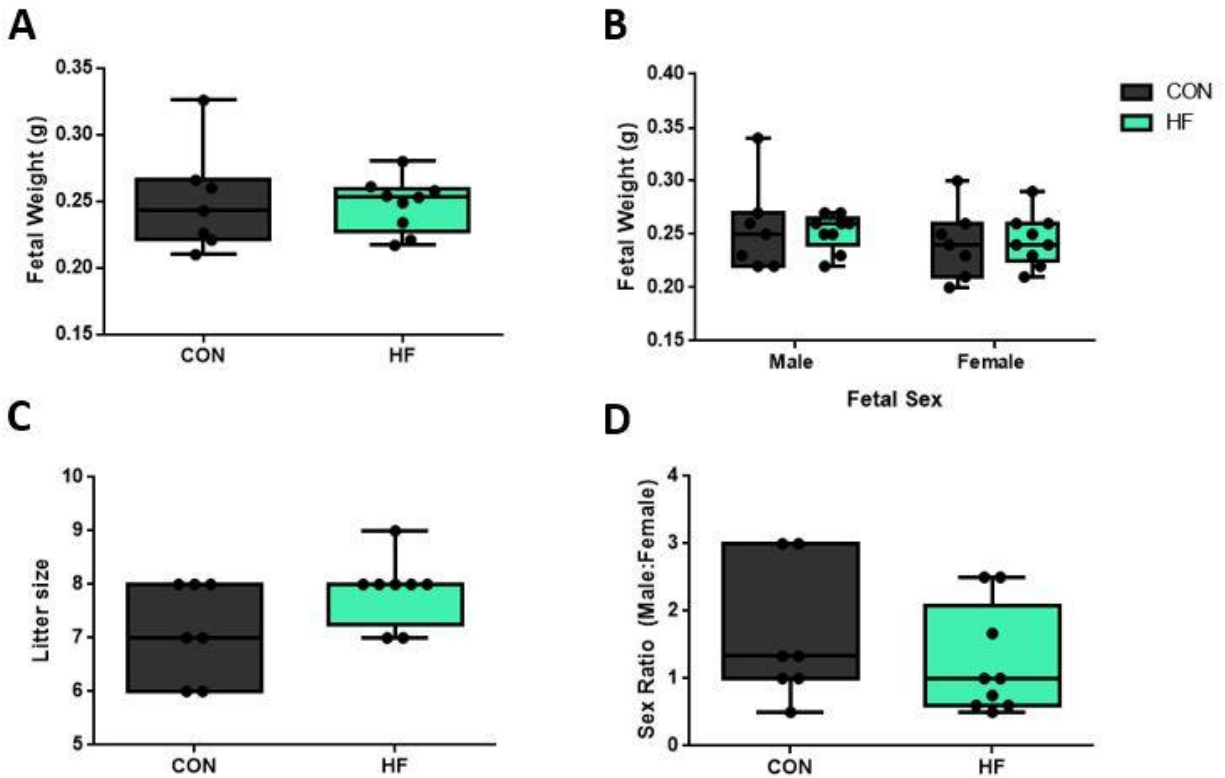
Figure 3.2.2.1 Maternal HF diet did not affect fetal weight, litter size or sex ratio at E14.5. A. Weight of CON (n = 7) and HF (n = 9) fetuses (males and females combined) **B.** Weight of CON (n = 7) and HF (n = 9) male and female fetuses. **C.** Litter size (number of fetuses/litter of CON (n = 7) and HF (n = 9) dams. **D.** Sex ratio (number of male:female fetuses) of CON (n = 7) and HF (n = 9) dams. Data are presented as mean ± minimum and maximum. Individual data points represent raw data values.

3.2.3 Placental characteristics

Placental weight at E14.5 was not significantly affected by maternal diet (Figure 3.2.3.1A) and remained unchanged when stratified by fetal sex ($p_{diet} = 0.5271$, $p_{sex} = 0.4241$) (Figure 3.2.3.1B). Maternal obesity did not impact placental efficiency (fetal body weight to placental weight ratio) ($p = 0.3656$) (Figure 3.2.3.1C) and remained unchanged when stratified by fetal sex ($p_{diet} = 0.1043$, $p_{sex} = 0.5023$) (Figure 3.2.3.1D).
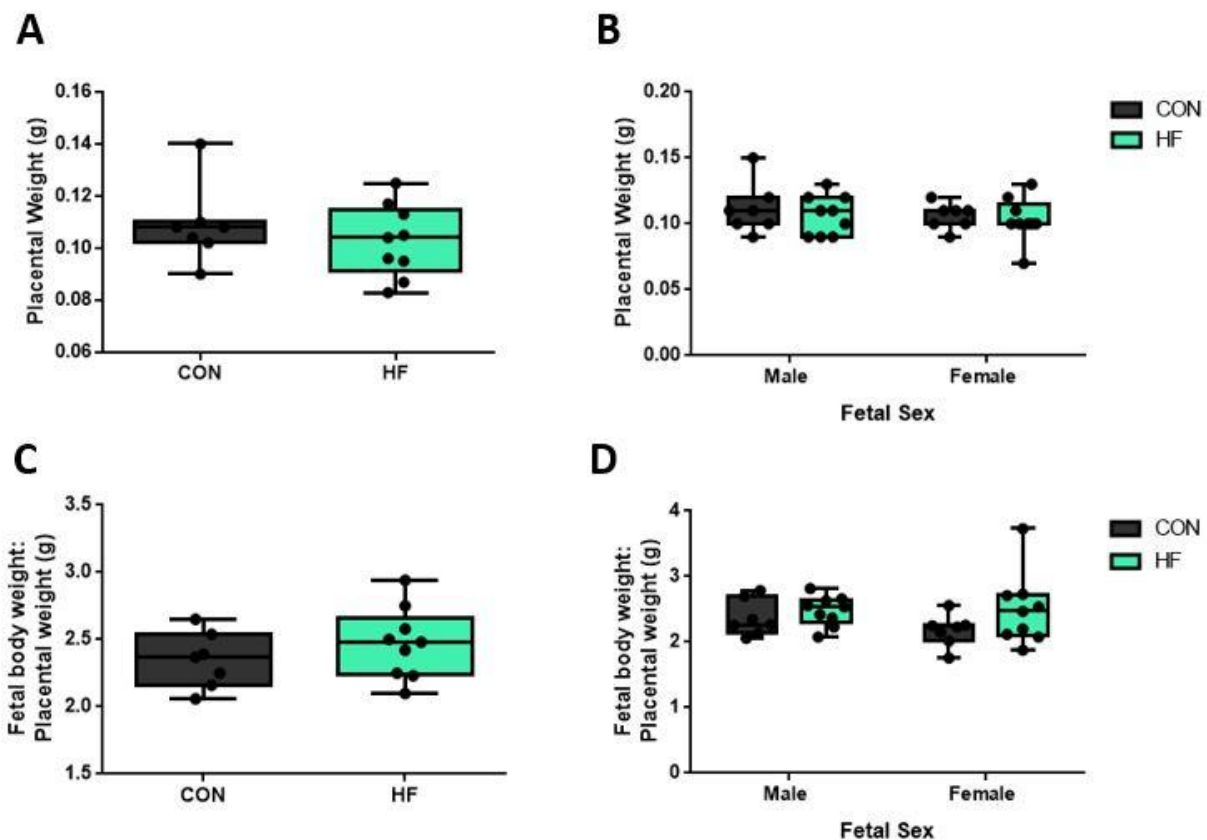
Figure 3.2.3.1 Maternal obesity did not impact placental weight or placental efficiency at E14.5. A. Placental weight of CON (n = 7) and HF (n = 9) (males and females combined). **B.** Placental weight of CON (n = 7) and HF (n = 9) male and female placentae. **C.** Placental efficiency (fetal body weight to placental weight ratio) in CON (n = 7) and HF (n = 9) pregnancies (males and females combined). **D.** Placental efficiency (fetal body weight to placental weight ratio) in CON (n = 7) and HF (n = 9) male and female fetuses. Data are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values.

3.2.4 Pregnancy induces a shift in the maternal intestinal microbiota of control-fed females

Pregnancy was characterized by an overall shift in the maternal intestinal microbiota (Figure 3.2.4.1A), where PERMANOVA demonstrated shifts were statistically significant at embryonic day (E) 10.5 (PERMANOVA p = 0.045) relative to non-pregnant controls, as previously reported [189]. Pregnancy induced significant differences in the relative abundance of 4 genera belonging to the Firmicutes phylum when comparing non pregnant and pregnant females (Figure 3.2.4.1B). With pregnancy, we observed a 4 fold increase in the abundance of bacteria belonging to the Catabacteriaceae family (order Clostridales) late in gestation at E10.5 and E14.5. The remaining 3 taxa were reduced late in gestation in pregnant females relative to non-pregnant females. Significant reductions were found in the genera *Lactobacillus* (order Lactobacillales, family Lactobacillaceae) and *Lactococcus* (order Lactobacillales, family Streptococcaceae). With pregnancy, reductions in both genera were observed as early as E0.5 and relative abundance remained lower than non-pregnant controls throughout gestation. Later in gestation, pregnancy is associated with a 3 fold reduction in *Clostridium* (order Clostridales, family Ruminococcaceae) abundance at E10.5 relative to non-pregnant controls. Between E10.5 and E14.5 the abundance of *Clostridium* increased slightly from 0.05% to 0.10% (Figure 3.2.4.2). This analysis was performed only in females where feces were collected at all pregnancy time points; prior to pregnancy, E0.5, E6.5, E10.5 and E14.5 and consisted of 5 CON and 9 HF females.

Figure 3.2.4.1 Pregnancy is characterized by a shift in the maternal gut microbiome in control fed females. A. Overall taxonomic summaries of microbial relative abundance resolved to the order (o), family (f), or genus (g) level classification between NPC females (n = 5) and CON pregnant females (n = 5). Taxa that showed significant shifts with pregnancy are labelled. **B.** Taxonomic summaries of microbial relative abundance resolved to the order (o), family (f), or genus (g) level classification between each control diet, female prior to pregnancy (n = 5) and at four time points during gestation (n = 5).

Figure 3.2.4.2 Pregnancy is associated with a shift in the relative abundance of 4 taxa in control fed females. The relative abundance of 4 taxa resolved to the genus (g) level classification in non-pregnant and pregnant females throughout gestation at E0.5, E6.5, E10.5 and E14.5 (n=5).

3.2.5 Periconceptional high-fat feeding modulates pregnancy-induced shifts in the female intestinal microbiota

Significant differences in the relative abundance of 8 genera were identified between non-pregnant and pregnant females fed a high-fat diet (Figure 3.2.5.1A). We report a significant shift with pregnancy, consistent with a previous report [189] at conception E0.5 (PERMANOVA p= 0.050) and throughout gestation (E6.5 (PERMANOVA p= 0.014), E10.5 (PERMANOVA p= 0.002) and E14.5 (PERMANOVA p= 0.013) (Figure 3.2.5.1B). Significant shifts in the maternal intestinal microbiota were observed to occur in taxa belonging to the Firmicutes, Bacteroidetes,

Verrucomicrobia and Tenericutes phyla. Consistent with previous reports, the Firmicutes to Bactroidetes ratio was significantly increased in HF compared to CON dams (PERMANOVA p = 0.001). Four taxa increased in abundance between non-pregnant and pregnant high-fat fed females. With pregnancy, the genus *Oscillospira* (increased from 5 to 10% relative abundance) and family Peptococcaceae (phylum Firmicutes) (increased from 0% to 1% relative abundance) were observed to exhibit a 2 fold increase in abundance identified as early as E6.5. Late in gestation, *Oscillospira* relative abundance exhibited a small reduction from 1% to 0.75% while Peptococcaceae relative abundance was observed to increase to E14.5. With pregnancy, *Alistipes* (phylum Bacteroidetes) was found to be elevated later in gestation where 2 fold to increase is observed at E10.5 and E14.5. Consistent with a previous report [189], high fat diet-induced obese pregnant mice demonstrated a 10-fold increase in *Akkermansia* (phylum Verrucomicrobia) compared to non-pregnant females fed a high-fat diet. This increase occurs as early at E0.5 (increased from 0% to 2% at E0.5) and continued to increase with advancing gestation (4% relative abundance at E6.5, 10% relative abundance at E10.5) to E14.5 (8% relative abundance).

In high fat fed mice, four taxa were significantly reduced with pregnancy, including 3 taxa belonging to the Firmicutes phylum; Clostridiaceae, *Lactobacillus* and *Lactococcus*. A significant reduction in abundance in these taxa was observed as early as E0.5 where Clostridiaceae decreased from 13% to 5% relative abundance, *Lactobacillus* decreased from 7% to 1% relative abundance and *Lactococcus* decreased from 10% to 5% relative abundance. With advancing gestation the relative abundance of Clostridiaceae, *Lactobacillus*, and *Lactococcus* continued to decrease to 3%, 0.5% and 1% relative abundance at E14.5 respectively. *Clostridium* (phylum Tenericutes) showed a 3 fold reduction identified at E0.5 (decreased from 3% to 1%

relative abundance) which remained stable throughout the remainder of gestation to E14.5
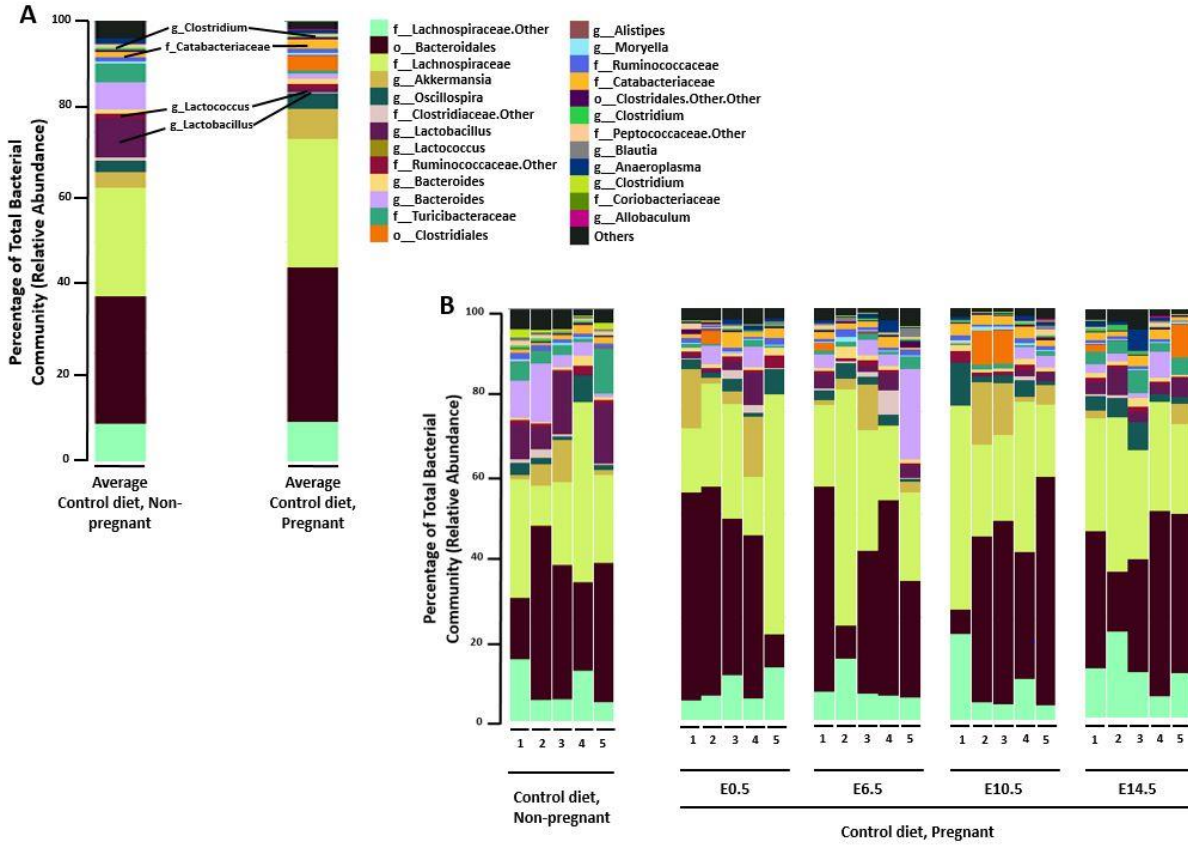
(Figure 3.2.5.2).
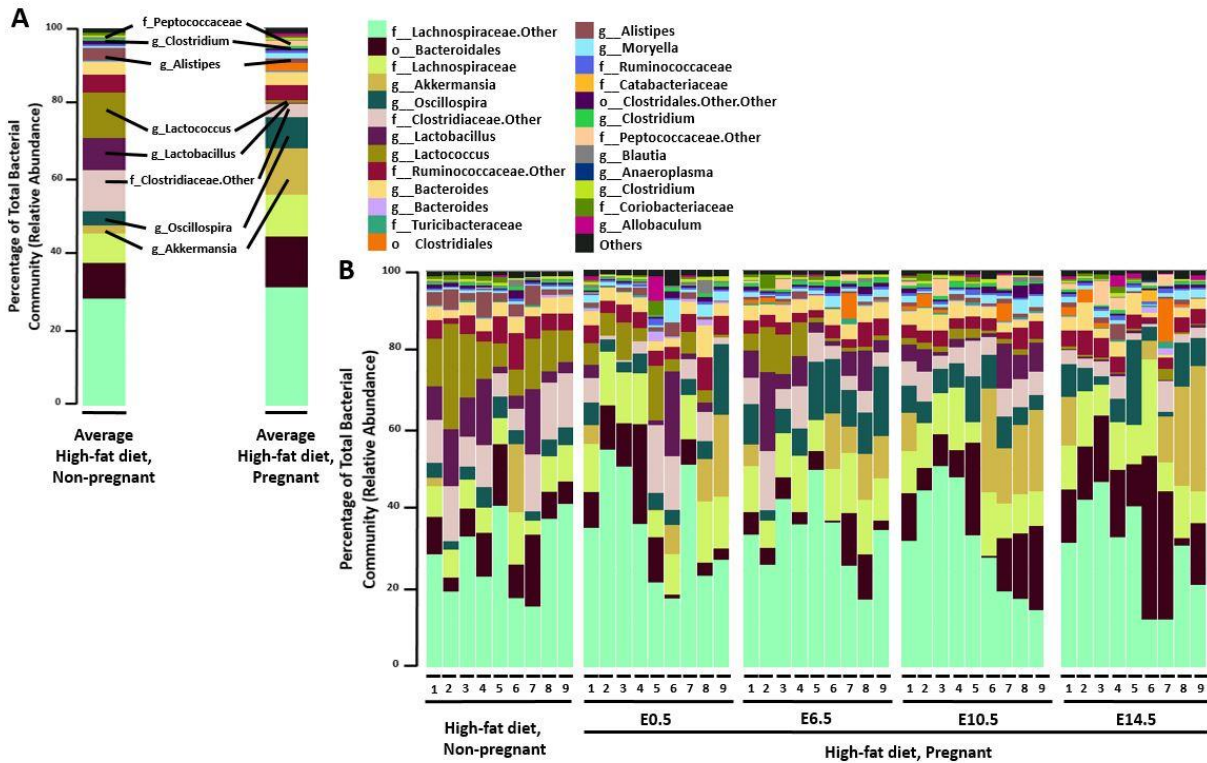
Figure 3.2.5.1 Pregnancy is characterized by a shift in the maternal intestinal microbiome in high-fat fed females. A. Average taxonomic summaries of microbial relative abundance resolved to the order (o), family (f), or genus (g) level classification between high-fat diet-fed, non-pregnant females (n = 9) and high-fat diet, pregnant females (n = 9). Taxa that showed significant shifts with pregnancy are labelled. **B.** Taxonomic summaries of microbial relative abundance resolved to the order (o), family (f), or genus (g) level classification between each high-fat diet, female prior to pregnancy (n = 9) and at four time points during gestation (n = 9).
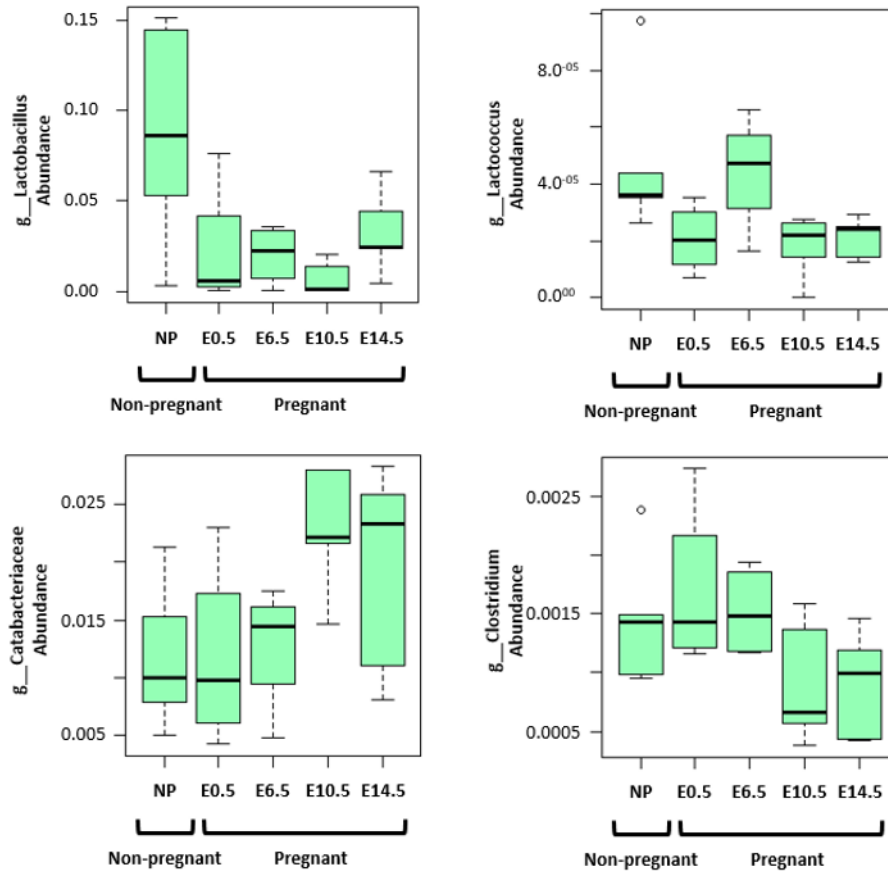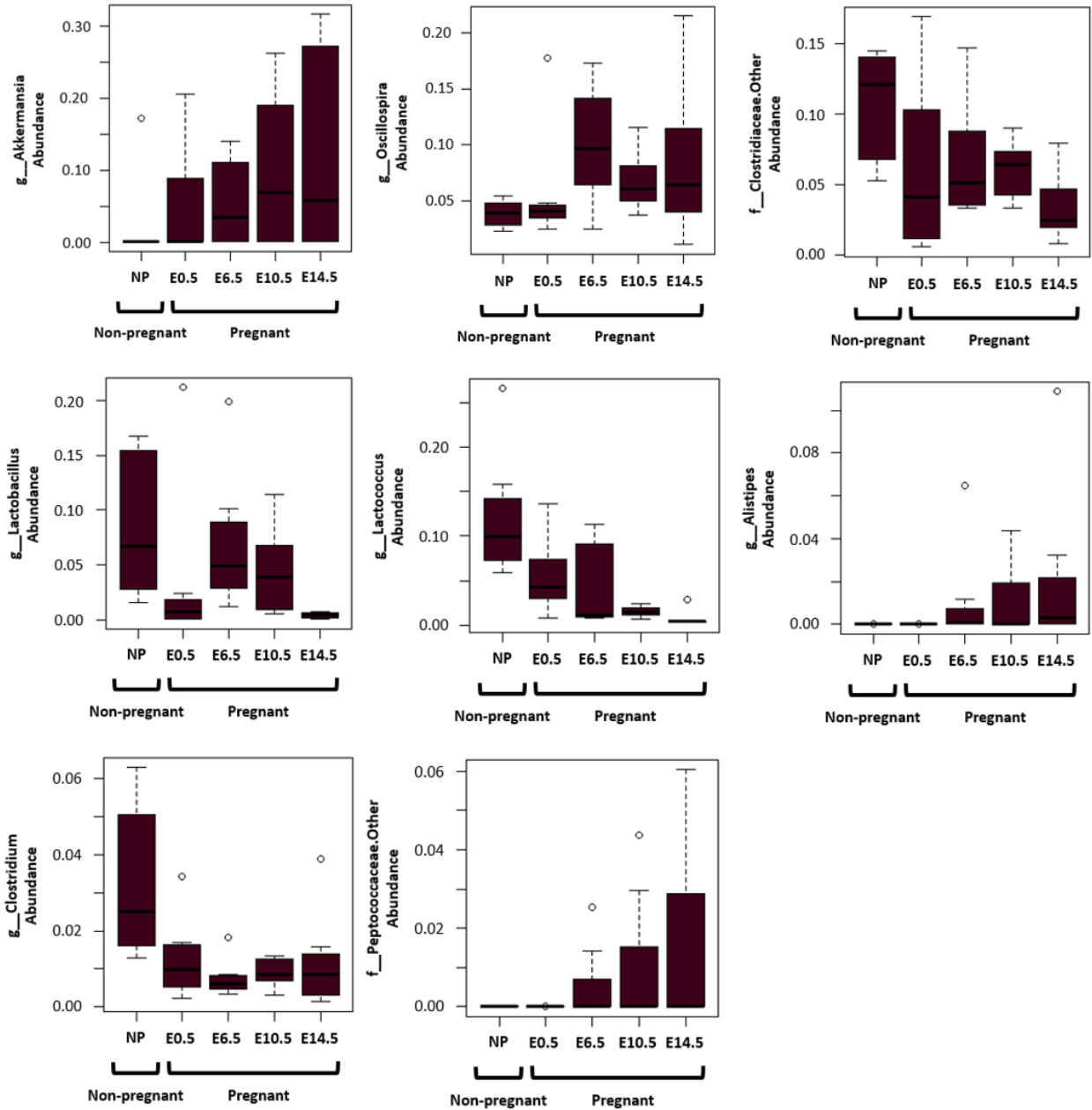
Figure 3.2.5.2 Pregnancy is associated with a shift in the relative abundance of 8 taxa in high-fat fed females. The relative abundance of 8 taxa resolved to the family (f) and genus (g) level classification in non-pregnant (NP) and pregnant high-fat fed females throughout gestation at E0.5, E6.5, E10.5 and E14.5 (n=9).

3.2.6 The interactive impacts of pregnancy and diet-induced obesity on the maternal intestinal microbiota

We observed a significant interaction between diet-induced obesity and pregnancy on microbial abundances, where eighteen taxa were significantly different between CON and HF pregnancies (Figure 3.2.6.1). This is greater than the 4 and 8 taxonomic differences that were altered exclusively with pregnancy in control and high-fat fed females respectively. The greatest number of the taxa observed to shift between control pregnant and high-fat pregnant females belong to the Firmicutes phylum, although others include the Bacteroidetes, Verrucomicrobia, Tenericutes and Actinobacteria. Consistent with intestinal microbial studies conducted in male mice fed a high-fat diet, we observe an increase in the Firmicutes to Bacteroidetes ratio between CON pregnant and HF pregnant females (PERMANOVA $p = 0.016$). The increase in the relative abundance of the Firmicutes phylum between CON and HF dams is driven by the order Clostridiales, where we observe an increase in Lacnospiraceae (order Clostridiales), *Oscillospira* (order Clostridiales, family Ruminococcaceae), *Bacteroides* (order Clostridiales, family Ruminococcaceae), *Moryella* (order Clostridiales, family Lachnospiraceae), *Clostridium* (order Clostridiales, family Lachnospiraceae) and *Clostridium* (order Clostridiales, family Ruminococcaceae). A significant elevation in the relative abundance of 1 member of the Actinobacteria phylum, Coriobacteriaceae (order Coriobacteriales) was also identified (Figure 3.2.6.2).

Significant reductions are also identified in a small number of taxa within the Firmicutes phylum including Lachnospiraceae (order Clostridiales) Catabacteriaceae (order Clostridiales) and a member within the Tenericutes phylum *Anaeroplasma* (order Anaeroplasmatales, family Anaeroplasmataceae). Together, these data demonstrated that an interaction between pregnancy

and diet-induced obesity on the composition of the maternal intestinal microbiota (Figure 3.2.6.2).

Measures of β diversity of intestinal microbial communities were analyzed with Principal Coordinates Analysis (PCoA) using the Bray Curtis distance metric. Significant separation was observed as a result of diet, between CON and HF-fed groups and further separation is displayed in each nutritional group as a result of pregnancy, between non-pregnant and pregnant females despite some inter group variation in gut microbial communities (Figure 3.2.6.3A). However, significant clustering of intestinal microbial communities was not identified between advancing stages of gestation; E0.5, E6.5, E10.5 and E14.5 (Figure 3.2.6.3B)
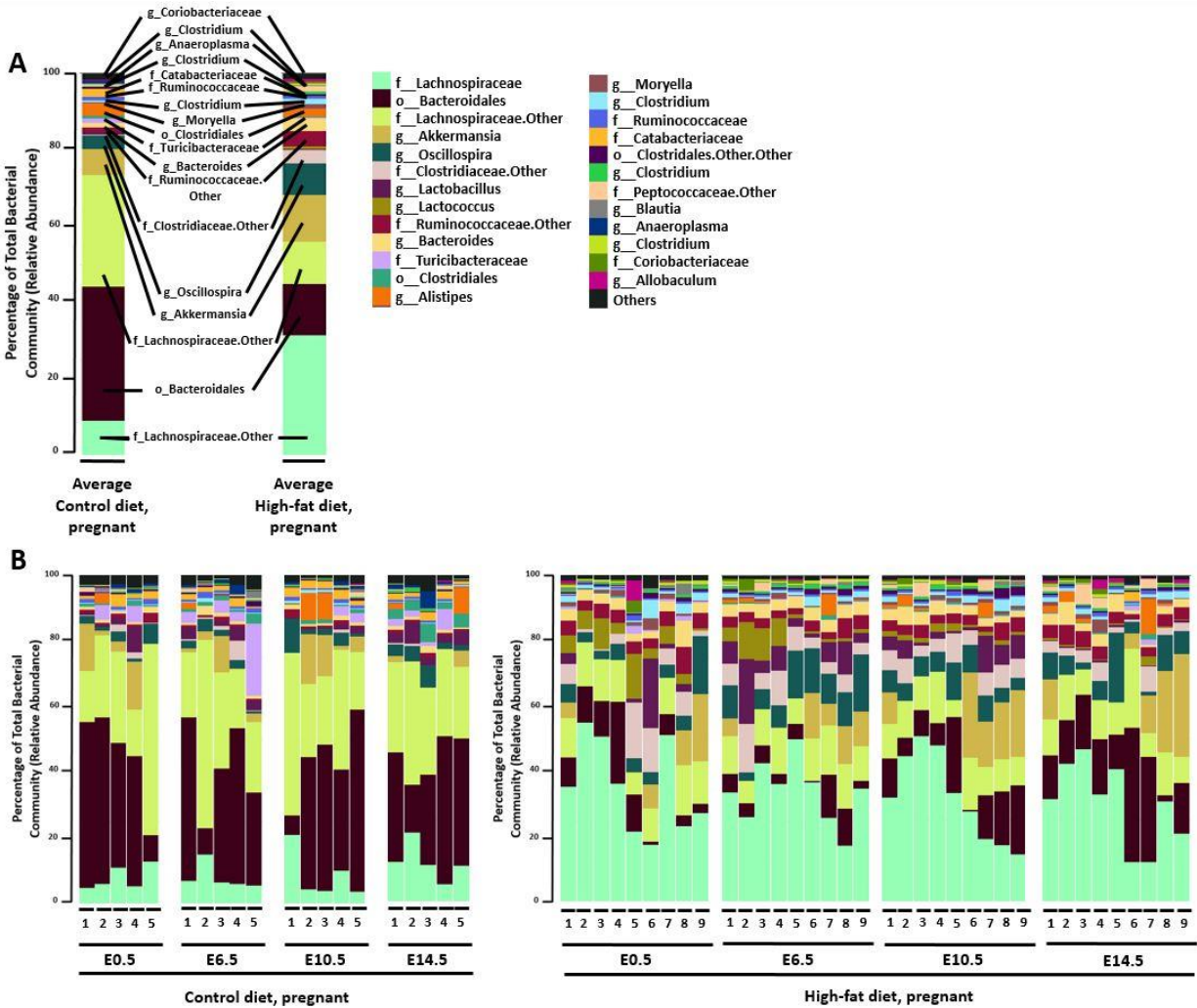
Figure 3.2.6.1 Maternal periconceptional diet modulates pregnancy-induced shift in the maternal gut microbiota. A. Average taxonomic summaries of microbial relative abundance resolved to the family (f) or genus (g) level classification between control fed pregnant (n = 5) and high-fat diet-fed pregnant females (n = 9) and high-fat diet. **B.** Taxonomic summaries of microbial relative abundance resolved to the family (f) or genus (g) level classification between each control (n = 5) and high-fat fed female (n = 9) prior to pregnancy and at four time points during gestation.
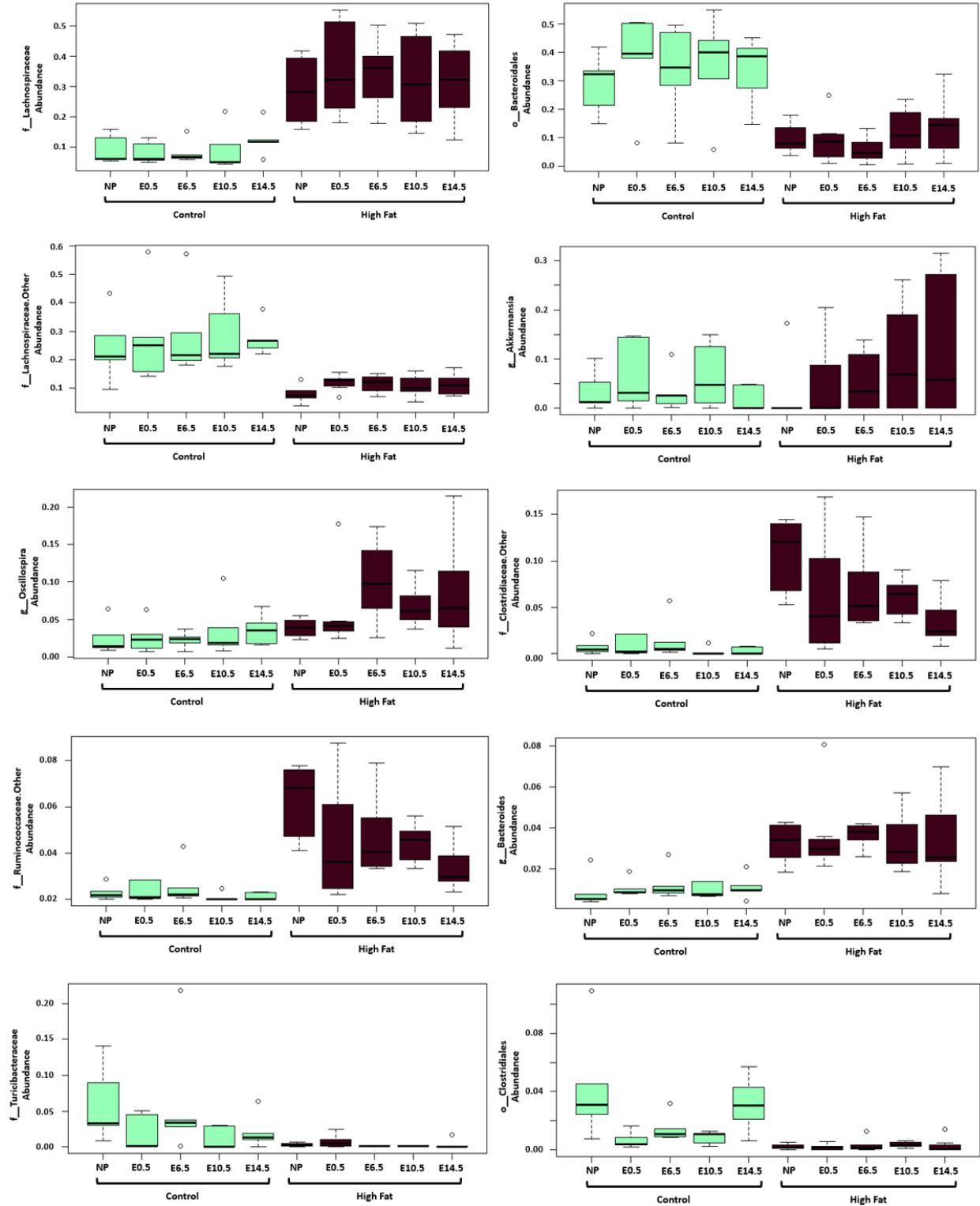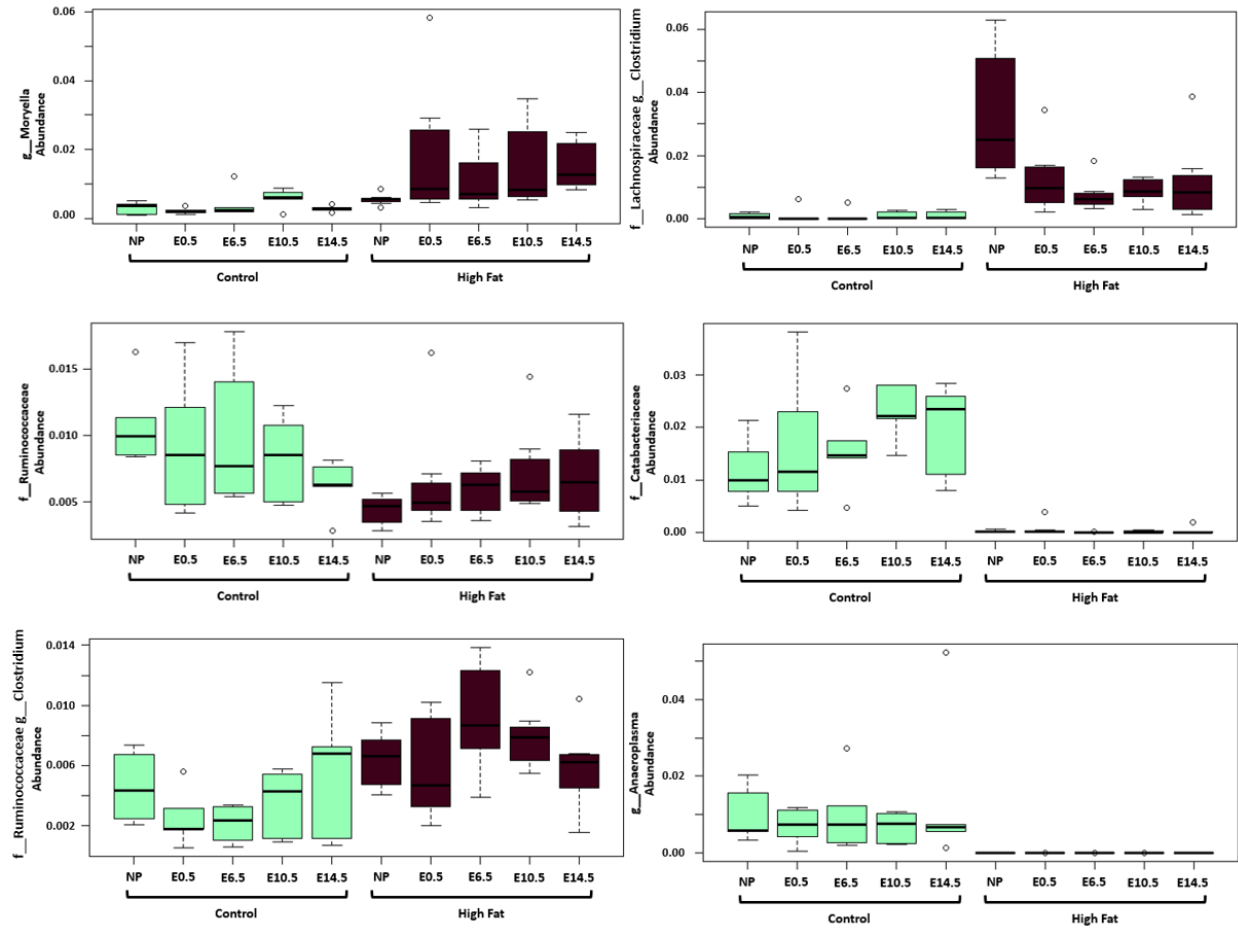
Figure 3.2.6.2 Control and high-fat pregnancy is associated with a shift in the relative abundance of 8 taxa. The relative abundance of 8 taxa resolved to the family (f) and genus (g) level classification in non-pregnant (NP) and pregnant control (n = 5) and high-fat (n = 9) females throughout gestation at E0.5, E6.5, E10.5 and E14.5 (n=9).
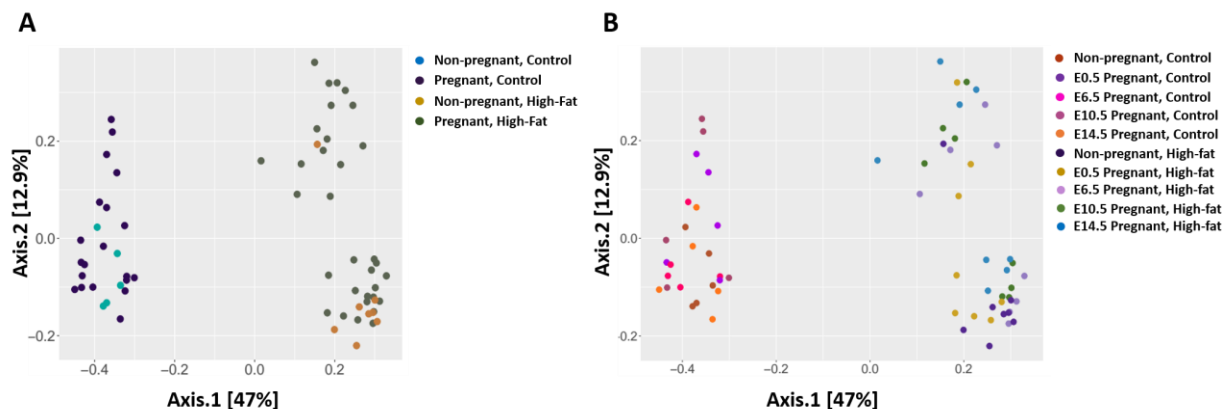
Figure 3.2.6.3 Gut microbial communities cluster according to maternal diet and pregnancy but do not cluster according to gestational time point. A. Principle Coordinate Analysis using the Bray Curtis distance metric displays the distinct separation of microbial communities in control females from females fed a high-fat diet. With pregnancy, further separation between each respective group is visualized in CON and HF groups. **B.** Principle Coordinate Analysis using the Bray Curtis distance metric displays no distinct separation of gut microbial communities in control and high-fat fed females at E0.5, E6.5, E10.5 and E14.5.

3.2.7 Relationships between maternal intestinal microbiota and metabolic indices

To understand the relationship between shifts in intestinal microbial abundances and maternal metabolic indices; whole blood glucose, serum insulin and leptin were correlated to taxa that were found to be different between CON and HF pregnant mice at E14.5. In CON and HF females, maternal whole blood glucose was correlated with the relative abundance of 8 taxa. At E14.5, maternal whole blood glucose was found to be positively correlated with 4 taxa belonging to the order Clostridiales of the Firmicutes phylum, namely, Lachnospiraceae ($R^2 = 0.320$, p = 0.025), Clostridaceae.Other ($R^2 = 0.595$, p = 0.001), Ruminococcaceae.Other ($R^2 = 0.402$, p = 0.012) and *Clostridium* ($R^2 = 0.277$, p = 0.037). A positive correlation was also observed between maternal whole blood glucose and the relative abundance of *Clostridium* (phylum Tenericutes) ($R^2 = 0.325$, p = 0.025) and Coriobacteriaceae (phylum Actinobacteria) ($R^2 = 0.267$, p = 0.041) at E14.5 (Figure 3.2.7.1A). Negative correlations were identified between maternal

whole blood glucose and the relative abundance of the family Lachnospiraceae.Other (phylum Firmicutes) ($R^2 = 0.293$, p = 0.032) and the order Bacteroidales (phylum Bacteroidetes) ($R^2 = 0.31$, p = 0.030) (Figure 3.2.7.1B).

In CON and HF females, maternal serum insulin was correlated with the relative abundance of 5 taxa, all members of the Firmicutes phylum. At E14.5, maternal serum insulin was found to be positively correlated with 2 taxa belonging to the order Clostridiales, including the family Ruminococcaceae.Other ($R^2 = 0.349$, p = 0.020) and *Bacteroides* ($R^2 = 0.243$, p = 0.050) (Figure 3.2.7.2A). Negative correlations were identified between 3 taxa, namely, the order Clostridiales ($R^2 = 0.315$, p = 0.027), the family Lachnospiraceae.Other ($R^2 = 0.575$, p = 0.002) and the family Catabacteriaceae ($R^2 = 0.414$, p = 0.010) at E14.5 (Figure 3.2.7.2B).

In CON and HF females, maternal serum leptin was positively correlated with the relative abundance of 4 taxa all belonging to the Firmicutes phylum. Positive correlations between the family Clostridiaceae.Other ($R^2 = 0.348$, p = 0.020) and the family Ruminococcaceae.Other ($R^2 = 0.355$, p = 0.019) were observed at E14.5 (Figure 3.2.7.3A). Negative correlations were found between maternal serum leptin and the relative abundance of the family Lachnospiraceae.Other ($R^2 = 0.483$, p = 0.005) and the family Catabacteriaceae ($R^2 = 0.347$, p = 0.020) at E14.5 (Figure 3.2.7.3B). Together, these data suggest that maternal glucose, insulin and leptin are factors which may be influence or may be influenced by the gut microbial shifts observed mediated by diet and pregnancy.
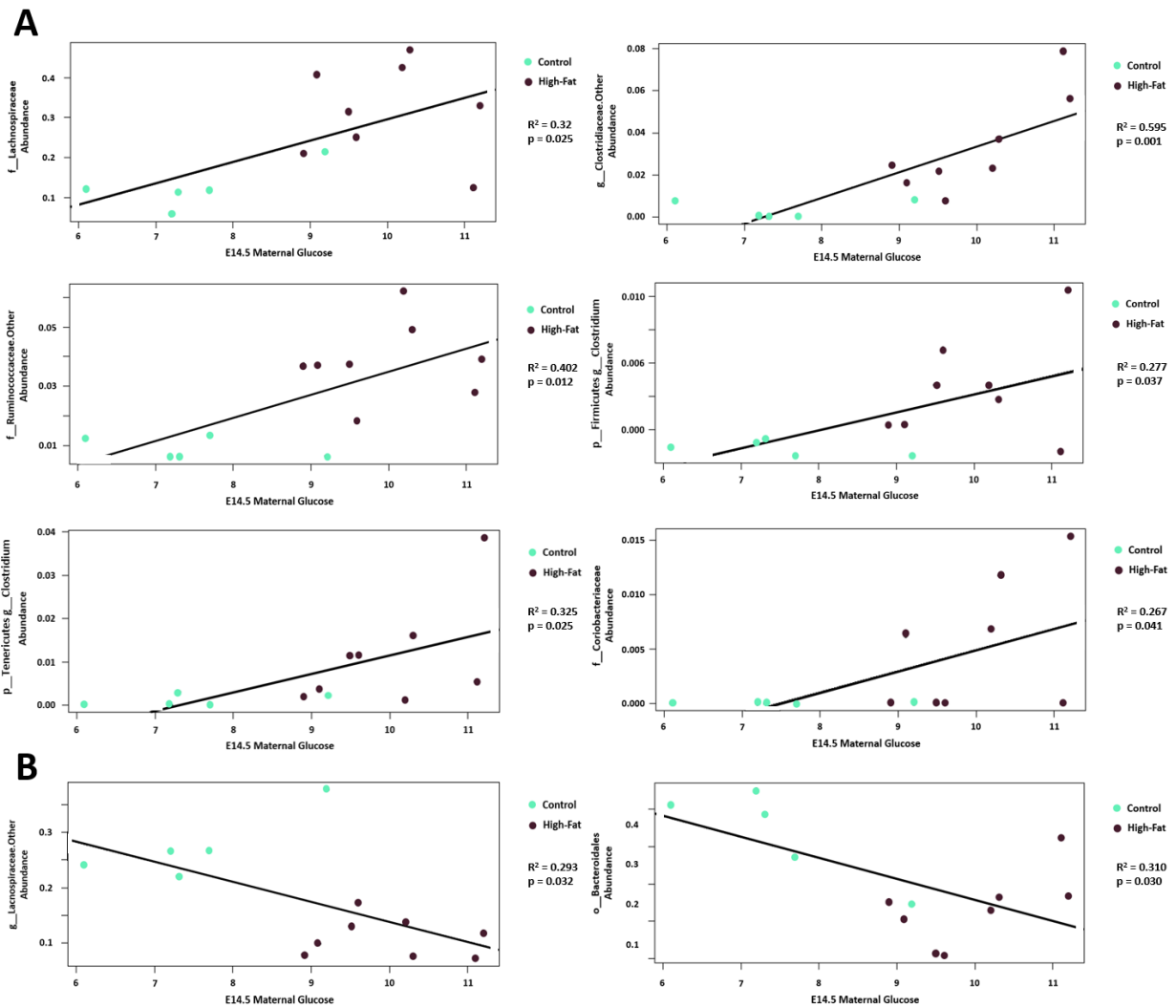
Figure 3.2.7.1 Maternal whole blood glucose is correlated with the relative abundance of 8 taxa at E14.5 in CON and HF females. A. Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification positively correlated with maternal whole blood glucose in CON (n = 5) and HF (n = 9) females at E14.5. **B.** Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification negatively correlated with maternal whole blood glucose levels in control (n = 5) and high-fat (n = 9) females at E14.5.
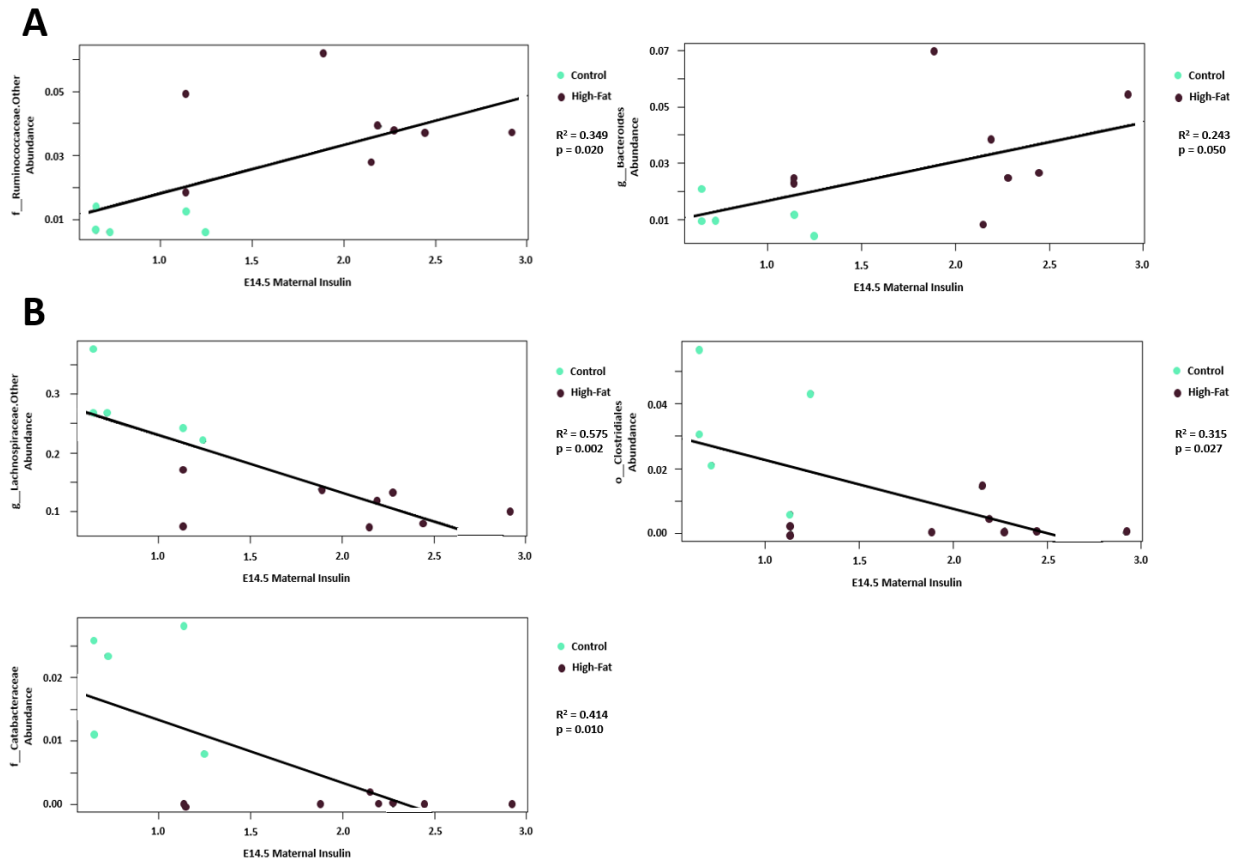
Figure 3.2.7.2 Maternal serum insulin is correlated with the relative abundance of 5 taxa at E14.5 in CON and HF females. A. Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification positively correlated with maternal insulin in CON (n = 5) and HF (n = 9) females at E14.5. **B.** Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification negatively correlated with maternal whole blood glucose levels in control (n = 5) and high-fat (n = 9) females at E14.5.
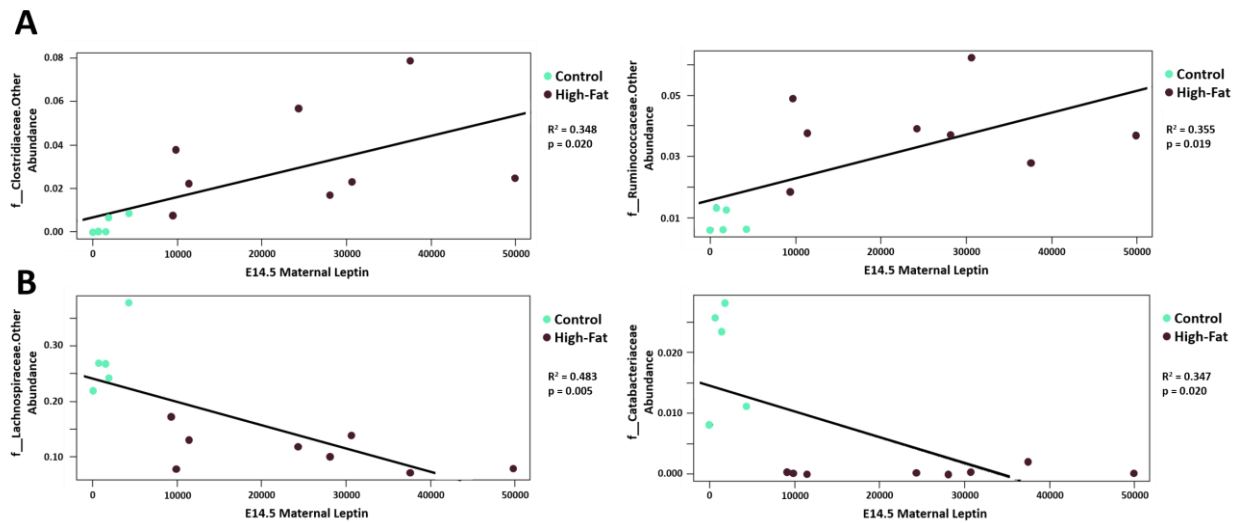
Figure 3.2.7.3 Maternal serum leptin is correlated with the relative abundance of 4 taxa at E14.5 in CON and HF females. A. Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification positively correlated with maternal insulin in CON (n = 5) and HF (n = 9) females at E14.5. **B.** Linear regression analyses of the relative abundance of the taxa resolved to the order (o), family (f), and genus level classification negatively correlated with maternal whole blood glucose levels in control (n = 5) and high-fat (n = 9) females at E14.5.

3.2.8 In vivo maternal intestinal barrier integrity

Mucosal genes: Maternal diet induced obesity did not impact mucin mRNA levels in maternal small intestine at E14.5. Maternal small intestinal Muc1 (p = 0.203) (Figure 3.2.8.1A) and Muc4 (p = 0.760) (Figure 3.2.8.1B) mRNA levels were not statistically different in the duodenum, jejunum or ileum in HF dams relative to CON. However, a significant difference in Muc1 (p = 0.040) (Figure 3.2.8.1A) but not Muc4 (p = 0.320) (Figure 3.2.8.1B) expression is observed between maternal gut sections. Maternal obesity differentially alters mucin production at the transcriptional level in the maternal colon at E14.5. mRNA levels of Muc2 were not significantly altered by maternal diet (p = 0.091) (Figure 3.2.8.1C). However, mRNA levels of Muc5ac were significantly increased in HF dams compared to CON (p = 0.036) (Figure 3.2.8.1D).

Permeability measures: No significant differences were observed in intestinal permeability as measured by the appearance of FITC-dextran in maternal plasma in response to maternal diet (p = 0.2866), pregnancy (p = 0.3028), or a diet x pregnancy interaction (p = 0.1928) (Figure 3.2.8.1E) at E14.5.

Goblet cells: Alcian blue (pH 2.5) which stains acidic mucins is routinely used to localize goblet cells within intestinal tissue. Semi-quantitative analysis of acidic mucins in the maternal colon (Figure 3.2.8.1F) demonstrated that HF and CON had similar numbers of goblet cells (p = 0.6952) (Figure 3.2.8.1G). This analysis was performed only in a colonic sections where structural integrity of the colon was maintained and included CON (n = 6) and HF (n = 7) dams.
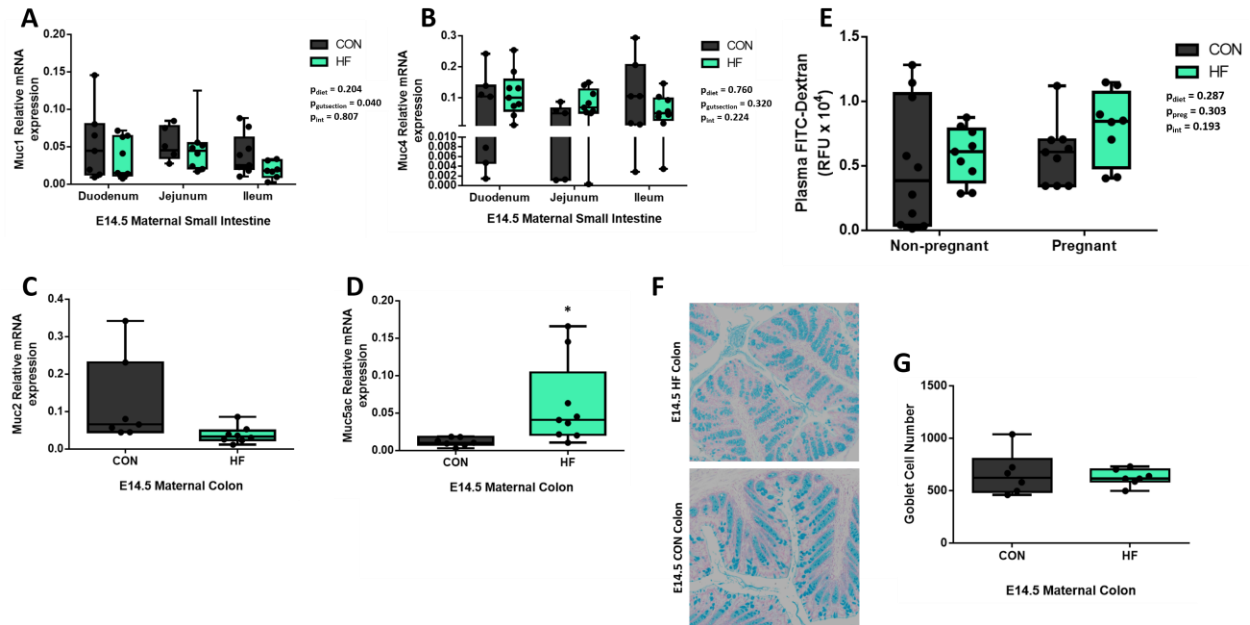
Figure 3.2.8.1 Diet and pregnancy do not impact maternal barrier integrity at E14.5. A. Muc1 mRNA expression in the maternal duodenum, jejunum and ileum in CON (n = 7) and HF (n = 9) dams. **B.** Muc4 mRNA expression in the maternal duodenum, jejunum and ileum in CON (n = 7) and HF (n = 9) dams. **C.** Muc2 mRNA levels in the maternal colon in CON (n = 7) and HF (n = 9) dams. **D.** Muc5ac mRNA levels in the maternal colon in CON (n = 7) and HF (n = 9) dams.   Data were calculated using a 2-way ANOVA and are presented as mean $\pm$ SEM relative to CON. **D.** Muc2 mRNA levels in the maternal colon in CON (n = 7) and HF (n = 8) dams. **E.** Plasma FITC-Dextran concentrations in NPC (n = 10), Non-pregnant High-Fat (NPHF) (n = 10), pregnant CON (n = 10) and pregnant HF (n = 10) females. **F.** Localization of goblet cells in representative CON and HF colons. **G.** Goblet cell number in the maternal colon in CON (n = 6) and HF (n = 7) dams. Data A, B and E were analyzed by 2-way ANOVA. Data C, D and G were analyzed using a Student's t-test. All data are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values. *p < 0.05.

3.2.9 Maternal intestinal inflammatory milieu

Maternal obesity significantly increased NFκB activity in the maternal gut at E14.5 (p = 0.0075).

NFκB activity was statistically different across maternal intestinal sections (p = 0.0003) and was

highest in the maternal jejunum and colon. There is no interaction effect observed between

maternal diet and maternal gut section (p = 0.6001) (Figure 3.2.9.1).
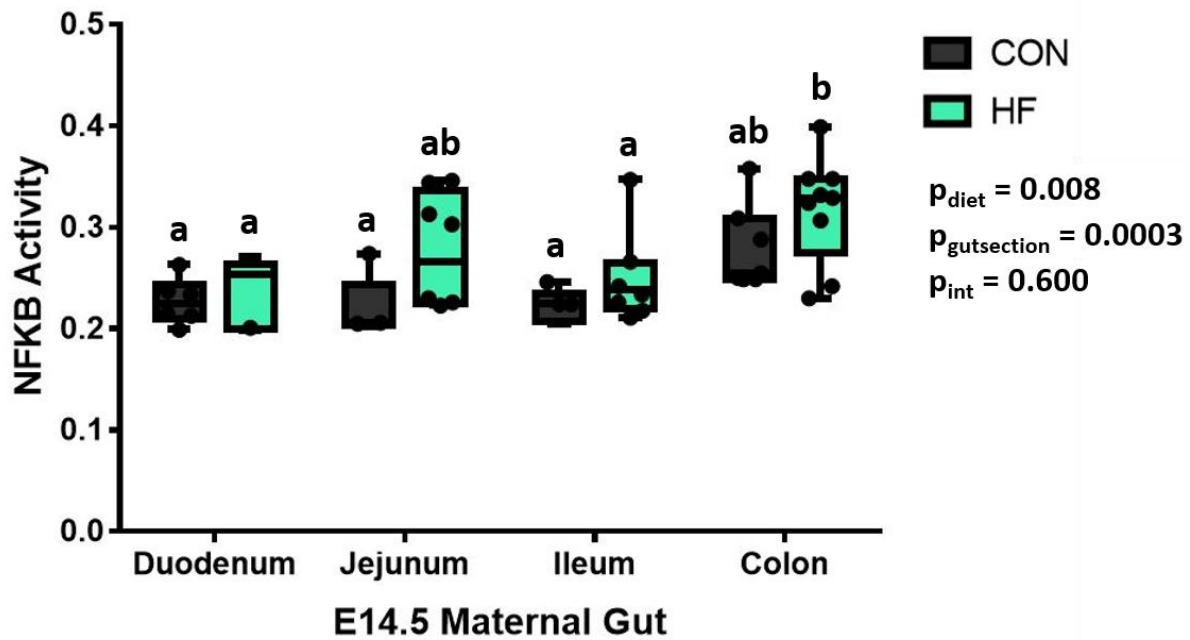
Figure 3.2.9.1 Maternal obesity is associated with elevated NFκB activity in the maternal intestine at E14.5. NFκB activity in CON (n = 7) and HF (n = 9) maternal colons at E14.5. Data was calculated using a 2-way ANOVA and are presented as mean ± minimum and maximum. Individual data points represent raw data values. Letters represent multiple comparisons tests where different letters represent statistically significant differences.

Semi-quantitative analysis of the murine macrophage marker F4/80 localized within CON and

HF colons at E14.5 showed that macrophage number in maternal colons was similar between

groups (p = 0.6095) (Figure 3.2.9.2). This analysis was performed only in a colonic sections

where structural integrity of the colon was maintained; CON (n = 6) and HF (n = 7) dams.
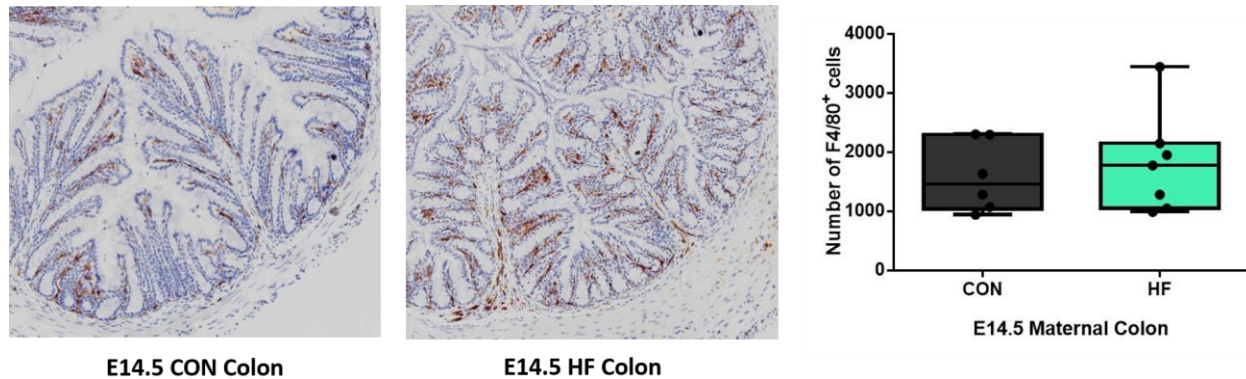
Figure 3.2.9.2 Maternal obesity does not impact the number of macrophages in the colon at E14.5.
Semi-quantitative analysis of immunopositive staining for F4/80 for CON (n = 6) and HF (n = 7) dams at
E14.5. Data are presented as mean ± minimum and maximum. Individual data points represent raw data
values.

Semi-quantitative analysis of murine T cell surface marker CD3 within CON and HF colons at

E14.5 revealed that colonic CD3 cell number was modestly higher in HF colons, although this

difference did not reach statistical significance (p = 0.0555) (Figure 3.2.9.3). This analysis was

performed only in a colonic sections where structural integrity of the colon was maintained and

included CON (n = 6) and HF (n = 7) dams.
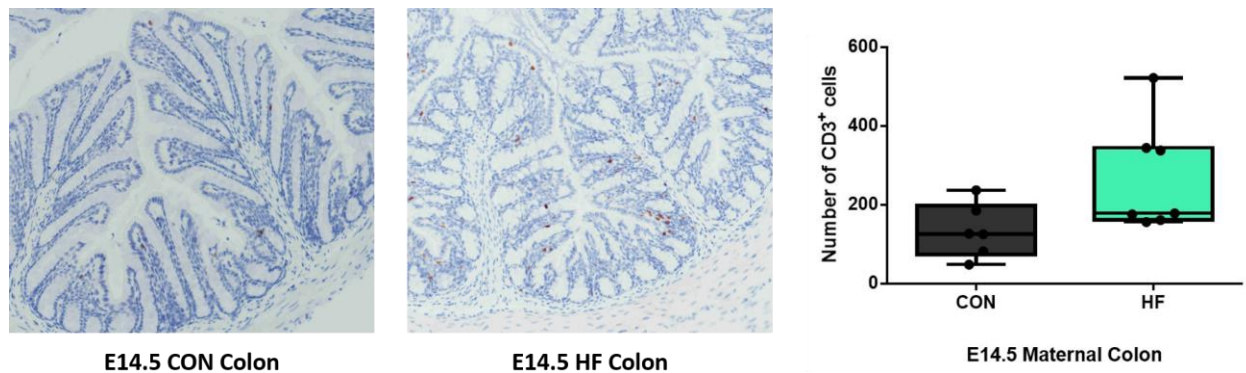
Figure 3.2.9.3 Maternal obesity does not impact the number of T cells in the colon at E14.5. Semi-quantitative analysis of immunopositive staining for CD3 for CON (n = 6) and HF (n = 7) dams at E14.5. Data are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values.

3.2.10 Placental inflammatory milieu

Maternal HF diet significantly increased placental pro-inflammatory pattern recognition receptor

TLR-2 mRNA levels (Figure 3.2.10.1A) but not TLR-4 mRNA levels (Figure 3.2.10.1B).

Similarly, TRAF6 mRNA (Figure 3.2.10.1C) and NFκB mRNA (Figure 3.2.10.1D) levels were

increased in HF placenta compared to CON, effects that were predominantly observed in female

placenta. These transcriptional changes in NFKB were not consistent with an increase in NFKB

activity as a result of maternal diet or fetal sex, however we did observe a modest interaction

between maternal diet and fetal sex, which appeared mainly due to a modest elevation in activity

in male placenta (Figure 3.2.10.1E).
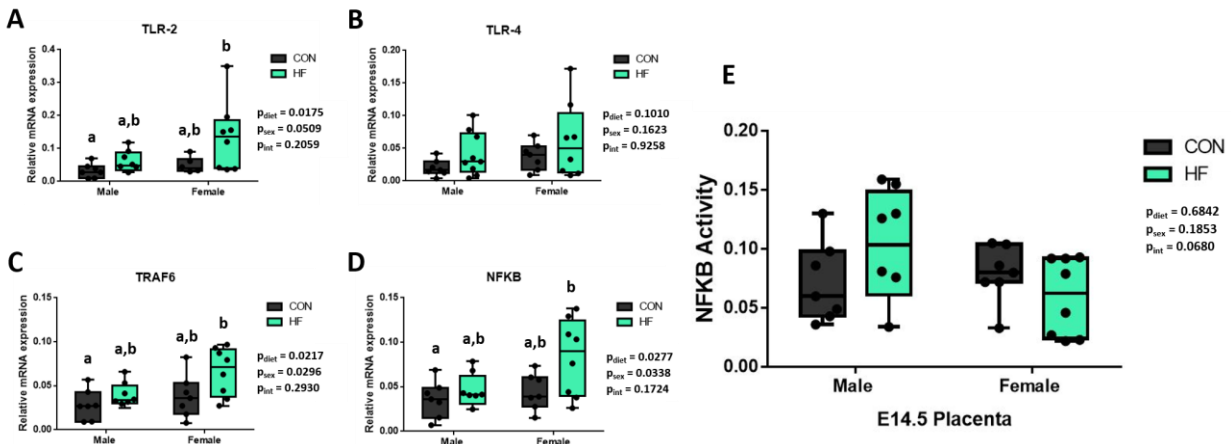
Figure 3.2.10.1 Maternal obesity is associated with elevated mRNA levels of key components of the pro-inflammatory TLR-4 signaling pathway but not NFκB activity in the placenta at E14.5. A. TLR-2 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **B.** TLR-4 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **C.** TRAF6 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **D.** NFκB mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **E.** NFκB activity in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. Data are presented as mean ± minimum and maximum. Individual data points represent raw data values. Letters represent multiple comparisons tests where different letters represent statistically significant differences.

Consistent with an upregulation of some components of pro-inflammatory signalling, HF placenta had elevated mRNA levels of the pro-inflammatory cytokine TNF-α (Figure 3.2.10.2A), but not IL-6 (Figure 3.2.10.2B) compared to CON placenta. These effects appeared equal in male and female placenta (no effect of sex). HF placenta showed increased mRNA levels of macrophage cell surface marker F4/80 (Figure 3.2.10.2C), monocyte chemoattractant protein MCP-1 (Figure 3.2.10.2D), but this did not appear to translate into an increased number of F4/80 positive macrophages in the placental tissue (Figure 3.2.10.2E).
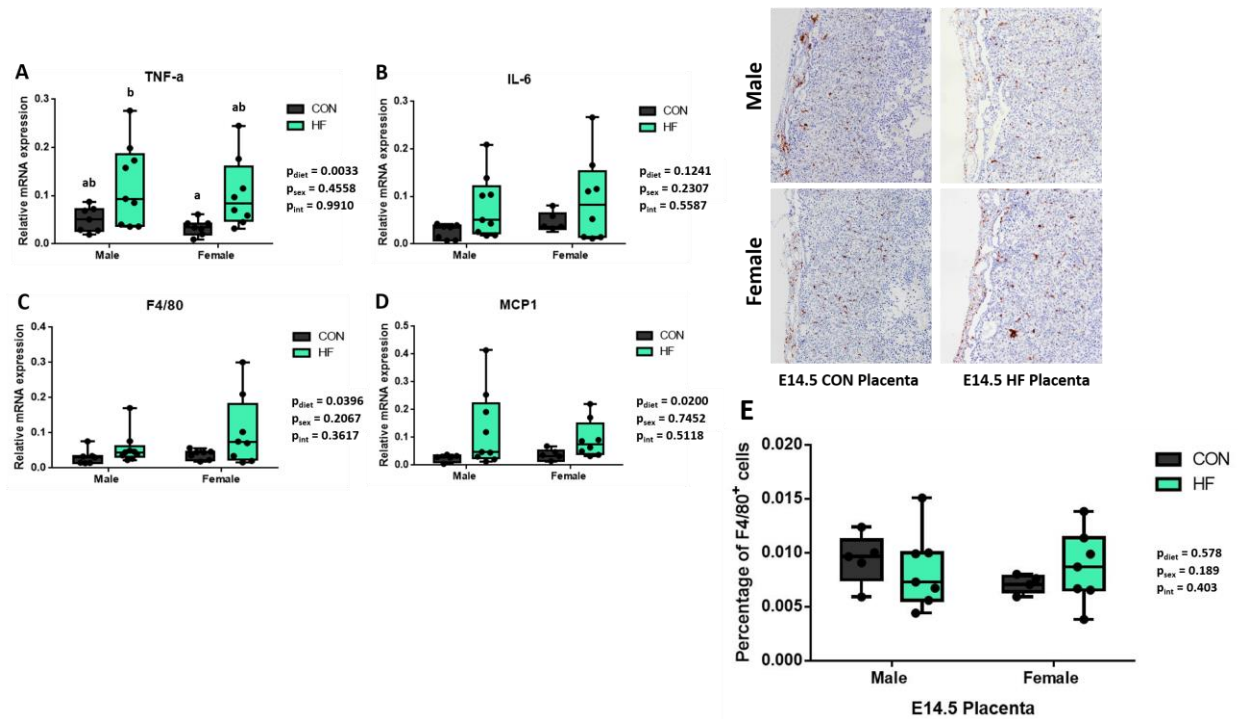
Figure 3.2.10.2 Maternal obesity is associated with elevated mRNA levels of F4/80, MCP1, and TNF-α but not IL-6 in the placenta at E14.5. A. TNF-α mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **B.** IL-6 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **C.** F4/80 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams.. **D.** MCP-1 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams.. **E.** Semi-quantitative analysis of immunopositive staining for F4/80 for CON (n = 6) and HF (n = 7) dams at E14.5. Data were calculated using a 2-way ANOVA and presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values. Letters represent multiple comparisons tests where different letters represent statistically significant differences.

Maternal HF modestly elevated total placental CD3 positive cells but this different did not reach statistical significance (p = 0.0957) (Figure 3.2.10.3A). However, when the different placental zones were analyzed separately, the number of CD3 positive cells was increased in the junctional zone in HF placenta (p = 0.0213) (Figure 3.2.10.3B) but not in the labyrinth zone (p = 0.9550) (Figure 3.2.10.3C). HF placentae showed an increase in IL-10 mRNA levels (Figure 3.2.11.3D).
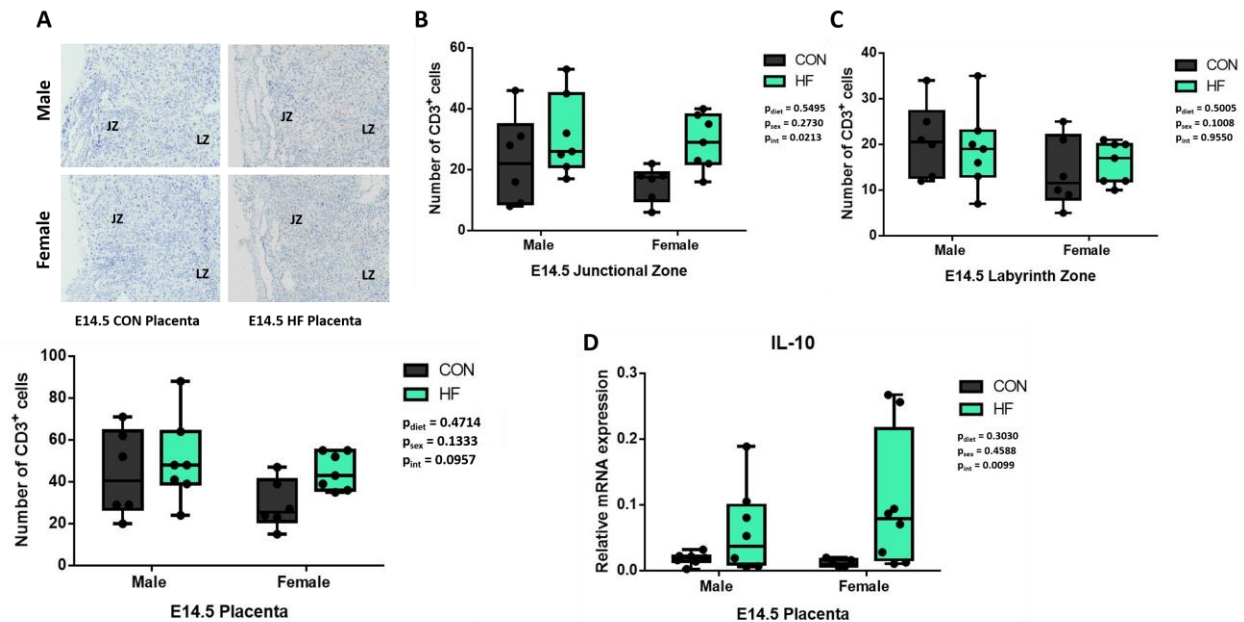
Figure 3.2.10.3 Maternal obesity impacts the number of CD3 positive cells in the junctional zone but not the labyrinth zone of the placenta at E14.5. A. Immunolocalization of CD3 positive cells in male and female sex-matched placentae and semi-quantitative analysis of CD3 cell number in the total placenta for CON (n = 6) and HF (n = 7) dams at E14.5. **B.** Semi-quantitative analysis of CD3 cell number in the junctional zone for CON (n = 6) and HF (n = 7) dams at E14.5. **C.** Semi-quantitative analysis of CD3 cell number in the labyrinth zone for CON (n = 6) and HF (n = 7) dams at E14.5. **D.** IL-10 mRNA levels in E14.5 placentae at E14.5 in CON (n = 7) and HF (n = 9) dams. Abbreviations: JZ, Junctional zone; LZ, Labyrinth zone. Data were calculated using a 2-way ANOVA and are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values.

3.2.11 Placental function

To understand the impact of elevated placental M1 mediated macrophage inflammation on placental function mRNA levels of critical nutrient transporters and growth factors were investigated. Placental GLUT1 (Figure 3.2.11.1A) and 3 (although modestly, p=0.058) (Figure 3.2.11.1B), and system N/A amino acid transporter SNAT2 (Figure 3.2.11.1C) were significantly elevated in HF pregnancies compared to CON, but mRNA levels of vascular endothelial growth factor (VEGF) (Figure 3.2.11.1D) and fatty acid binding protein 4 (FABP4) were similar between groups (Figure 3.2.11.1E).
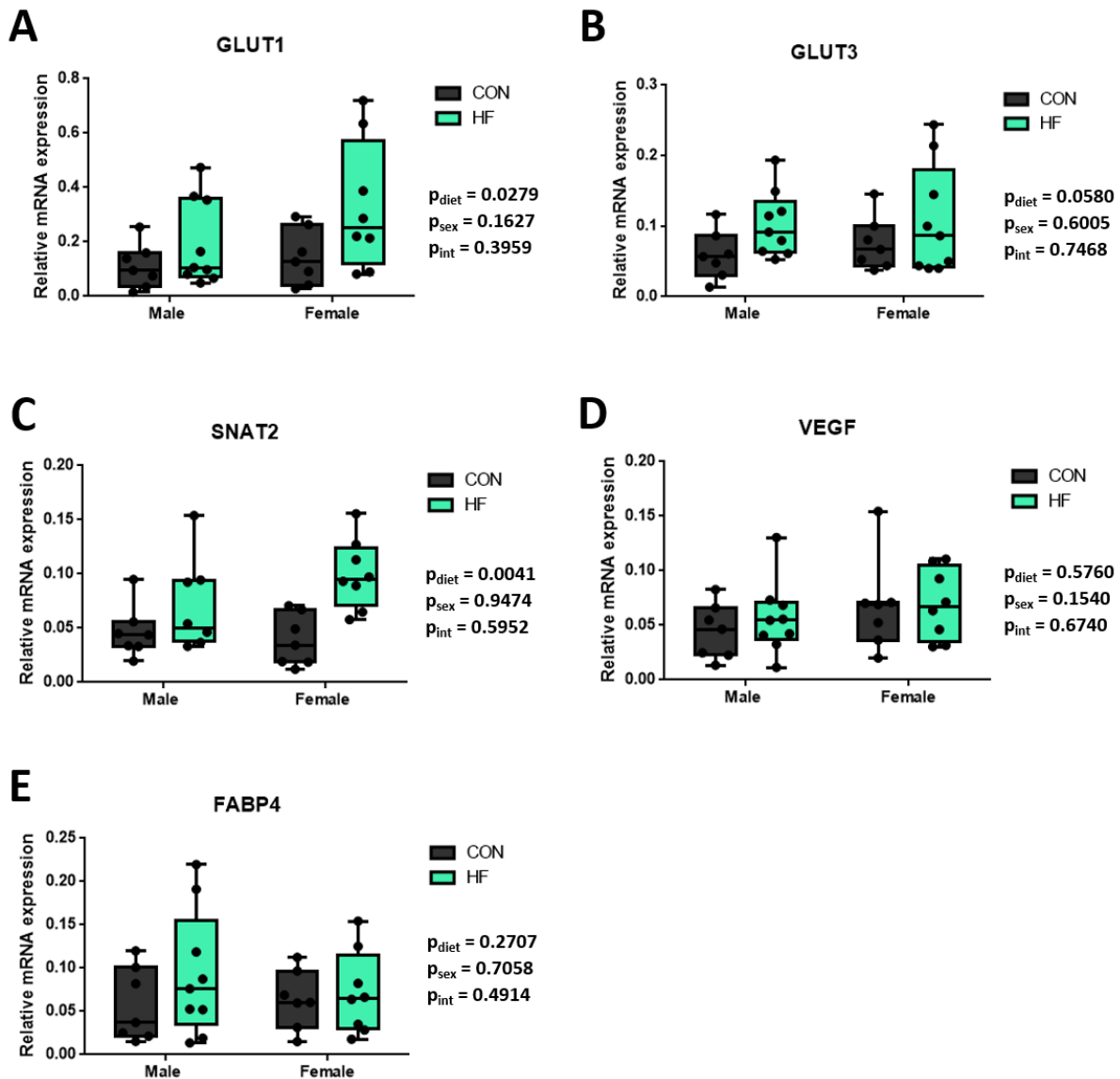
Figure 3.2.11.1 Maternal obesity is associated with increased mRNA levels of critical nutrient transporters and growth factors in the placenta at E14.5. A. GLUT1 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **B.** GLUT3 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **C.** SNAT2 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **D.** VEGF mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. **E.** FABP4 mRNA levels in E14.5 placentae in CON (n = 7) and HF (n = 9) dams. Data were calculated using a 2-way ANOVA and are presented as mean $\pm$ minimum and maximum. Individual data points represent raw data values.

4.0 DISCUSSION

This established murine model of maternal diet-induced obesity provides novel evidence that maternal obesity is characterized by altered maternal metabolic adaptation to pregnancy, distinct intestinal microbial shifts, increased maternal intestinal NFκB activity and increased placental mRNA levels of critical nutrient transporters at E14.5.

4.1 Maternal and fetal phenotype in the context of obesity

In this study, we demonstrated that the consumption of a HFD for 6 weeks prior to pregnancy is sufficient to induce maternal obesity. Consistent with previous reports [132, 136, 178-180, 336], at conception females fed a HFD were significantly heavier than control-fed females. Throughout gestation, HF dams displayed elevated GWG and were hyperglycemic, hyperleptinemic, hyperinsulinemic and displayed insulin resistance at E14.5. Together, these data suggest that our model of maternal obesity is associated with GDM at E14.5.

Hyperglycemia is often, but not always [136, 178] associated with maternal diet induced obesity (DIO), while hyperinsulinemia and hyperleptinemia are common signatures in experimental animal models of maternal obesity [136]. Known as the "satiety hormone", the adipokine leptin is synthesized by adipocytes and acts within the hypothalamus to regulate energy balance by inhibiting hunger and food intake [337, 338]. Both experimental animal models [339-343] and clinical trials [344-348] show that during healthy pregnancy, leptin levels rise. Early in gestation, leptin has been but is not always [349], positively correlated with maternal BMI in women [348, 350], consistent with the maternal anabolic state favouring the accretion of adipose [351]. Later in gestation, these levels plateau, consistent with the maternal metabolic switch to a state of catabolism to favour glucose transport to the developing fetus [351, 352]. Following delivery,

leptin levels drop significantly in the postpartum period [347-349]. Leptin regulates fetal growth and development [353-356], fetal and placental angiogenesis [357, 358], embryonic hematopoiesis [359-361] and hormone biosynthesis within the maternal-fetal interface [352]. Although fetal concentrations are lower than maternal levels, experimental animal models [362] and clinical trials [347, 348, 363, 364] show that at term, leptin is detectable in the fetal circulation and is commonly higher in females than in males [352, 365]. Some studies have shown a correlation between serum cord leptin concentrations and placental weight [356, 365-367], while others have not [355, 363, 368, 369]. Data showing a rapid decline in fetal leptin levels after birth [370] and higher leptin concentrations in umbilical veins than in umbilical arteries [369], have demonstrated that in addition to production by adipocytes, leptin is also produced by the placenta [345, 364, 370, 371]. Leptin in cord blood, originating from the placenta [364] and fetus [368, 372] have been proposed to mediate fetal growth through components of the insulin signaling pathway including, circulating IGF-1 levels [373] and the IGF-1/IGFBP 3 ratio [366, 374].

Despite the fact that we show significant alterations in maternal adaptation to pregnancy, fetal and placental weight were similar between groups. Therefore, it appears that maternal hyperglycemia, hyperinsulinemia and hyperleptinemia do not impact fetal or placental growth at E14.5. This is consistent with other reports where studies have shown that maternal HFD has variable effects on offspring weight at term; with some studies reporting no effects [132, 184, 185, 194-196], and others reporting either decreased [197, 198] or increased weights at term [141, 375]. Despite inconsistencies, long-term intrauterine exposure to maternal nutrient excess strongly influences offspring glucose homeostasis [182, 187], lipid metabolism [183], hypothalamic regulation [196, 199], the development of insulin resistance [141, 190, 200], leptin

resistance [201], hepatic steatosis [202], non-alcoholic liver disease (NALD) [141, 187], obesity [200, 203], hypertension [200] and cardiovascular disease [185] later in life. In this study, we did not investigate IGF-1 levels, or the IGF-1/IGFBP 3 ratio in fetuses. Thus, whether maternal obesity impacts important regulators of fetal growth and development at E14.5 in our model is unknown. Previous work in our lab, however, has demonstrated that fetal weight at term (E18.5) is also similar between groups (Gohir, Sloboda unpublished). These data suggest that factors including IGF or insulin signaling are either not altered, not altered for a long enough period of time, or are not altered to a large enough extent in our model to impact fetal growth. We also show that litter size and sex ratio (male:female) fetuses was unchanged between CON and HF litters at E14.5. These findings are consistent with other investigations in experimental animal models of maternal obesity [136, 375].

Pregnancy has been described as an inflammatory state [376] and this inflammation has been demonstrated to be enhanced when complicated by obesity [302, 377] Animal models of maternal obesity and work in obese women, show inconsistent changes in the levels of inflammatory mediators in the circulation during various stages of pregnancy [178, 302, 375-381]. Some reports have displayed elevated levels of pro-inflammatory cytokines [302, 376-378] including TNF-α [178, 375, 379], IL-1β [178, 375], IL-6 [375, 377], IL-17A [375], MCP-1 [379] in the maternal circulation. Circulating levels of CRP [380], TNF-α [379] and MCP-1 [379, 380] have been positively correlated with maternal BMI in women. Other studies have shown no change in circulating TNF-α levels between lean, obese and morbidly obese women at term [302, 377, 378, 380, 381]. In the present study, we do not observe any changes in maternal serum cytokine/chemokine levels at E14.5. During healthy pregnancy, serum levels of TNF-α in women have been shown to increase in the third trimester [178, 379], but not in the first [382].

Thus, it is possible that maternal obesity influences circulating cytokine levels later in gestation, however we did not test this. Although we observe higher TNF-α mRNA levels in the placentae of obese females at E14.5, studies have suggested that distinct from hormones like leptin, that enter the circulation from the placenta [383], TNFα production by placental macrophages remains in the placenta to illicit inflammatory cascades and is not released into the systemic circulation [384]. Thus, it is likely that in our study, placental TNF-α production remains localized in the placenta and may only exert local effects.

4.2 Maternal intestinal microbial shifts are not mediated by female sex-steroid hormone fluctuations

In this study we show that pregnancy is characterized by a shift in the maternal intestinal microbiota in control-fed females. With pregnancy, we observe a significant difference in the abundance of 4 taxa belonging to the Firmicutes phylum, namely, Catabacteriaceae, *Lactobacillus*, *Lactococcus*, and *Clostridium*. Data from the present study is similar to previous work in lean women, demonstrating that during pregnancy, the maternal intestinal microbiota changes with advancing gestation [249, 251]. Consistent with a previous report from our lab, we report that these shifts occur as early as 0.5 days of pregnancy and continue to shift throughout gestation [189]. Due to the immediate nature of these shifts, we hypothesized that intestinal microbial shifts may be linked to reproductive endocrine changes during the non-pregnant female estrous cycle. Early observations in experimental animal models of enhanced reproductive capacity following the colonization of GF mice [269, 270] and lower concentrations of circulating progesterone on day 1 of diestrus in GF mice [385] suggested a strong link between sex-steroid concentrations and microbial presence. Subsequent studies in postmenopausal women further supported a relationship between specific sex-steroid levels and

intestinal microbial shifts [263, 273]. Total urinary estrogens has been associated with fecal microbiome richness and alpha diversity while systemic measures of non-ovarian estrogens via enterohepatic circulation have been correlated with fecal Clostridia and Ruminococcaceae abundance [273]. Similarly, the ratio of estrogen to 13 hydroxylated estrogen has been correlated to the relative abundance of Clostridiales and *Bacteroides* [263]. This study did not show any intestinal microbial shifts with the estrous cycle. We thus conclude that our previously observed maternal microbial shifts at conception and throughout gestation [189] are not likely associated with estradiol or progesterone fluctuations in levels that occur during the estrous cycle in NPC female mice. These observations are similar to studies which suggest that the vaginal microbiome is stable across the menstrual cycle in non-human primates [276] and in healthy women of reproductive age [278, 386, 387].

It is possible that factors other than female reproductive hormones could be orchestrating maternal intestinal microbial shifts. It is possible that allocoprophagy of male feces during the mating period mediates changes in maternal microbiome at E0.5. During the mating period, each female mouse is co-housed with a male and thus the transfer of a female to a male cage introduces a confounding variable referred to as a "cage effect", where the female is exposed to the male cage microenvironment. Cage effects have been repeatedly identified as a powerful factor confounding studies investigating the intestinal microbiota [388-390]. Mice are copropagic mammals and the consumption of fecal matter is an important source of nutrition. Studies have demonstrated that the intestinal microbiota of individually housed mice converge to a similar profile over time following cohousing [390, 391]. Therefore, the intestinal microbial shift observed at conception may be a representation of the male gut microbiota induced by female allocoprophagy of the male feces, granted this was not measured in the current study.

Although we conclude that estrous-related sex steroids are not likely to be influencing the relative abundance of intestinal bacteria within the microbiome, it is important to recognize that non-pregnant female mice have different concentrations of circulating sex-steroids compared to pregnant females. In non-pregnant mice, the concentrations of the two main hormones that are largely responsible for the maintenance of pregnancy, 17-β estradiol and progesterone, may not adequately represent pregnancy patterns and concentrations. In pregnant mice, concentrations of 17-β estradiol exhibits two peaks; initially on the first day of gestation (39 pg/ml), while a second peak occurs at the end of pregnancy and is maintained until parturition, (60.8 - 112 pg/ml) [392]. In regularly cycling non-pregnant control female mice, 17-β estradiol levels exhibit one peak from proestrus to estrus (up to 70pg/ml) and levels decline again during metestrus and diestrus [393-395]. During early pregnancy, progesterone levels remain low (5ng/ml). In the second half of pregnancy when production is mediated by the corpus lutea [396] levels rise and peak (34 ng/ml) on E14 and again on E17. After day 17, levels fall (<5 ng/ml), correlating with luteal regression [397] prior to the onset of parturition [392, 398]. In regularly cycling non-pregnant control female mice, circulating progesterone exhibits two peaks; the first surge occurs at proestrus where levels rise to 64 ng/ml and the second surge occurs during metestrus where levels peak at 27 ng/ml [399]. It is possible that feces collected and sequenced in non-pregnant female mice did not correspond with the single peak of 17-β estradiol or the two peaks in progesterone during the estrous cycle. Thus to conclusively show that sex steroids do not influence maternal intestinal microbial shifts, serum concentrations of 17-β estradiol and progesterone could be directly measured at the time of fecal collection in future studies. Alternatively, the collection of feces following the exogenous administration of pregnancy-related concentrations of 17-β estradiol and progesterone to NPC females would elucidate

whether microbial shifts are sensitive to the sex-steroid hormone concentrations during pregnancy.

It is well established that the intestinal microbiota influence host metabolism through modulating energy harvest [218], the immune system [232], and lipid metabolism [400]. Studies in experimental animal models [401] and humans [402-408] have also demonstrated the regulation of intestinal bacteria by metabolic indices. Thus, it is possible that maternal intestinal microbial shifts may be mediated by key metabolic indices in our model. We have identified significant correlations between members of the order Clostridiales, of the Firmicutes phylum and maternal whole blood glucose levels at E14.5. Of these taxa, the family Lachnospiraceae, Clostridiaceae.Other, Ruminococcaceae.Other, and the genus *Clostridium* (family Lachnospiraceae) and *Clostridium* (family Ruminococcaceae) displayed a significant positive correlation, while *Bacteroidales* displayed a significant negative correlation with maternal whole blood glucose. An early investigation in GF *ob/ob* mice demonstrated that colonization with Lachnospiraceae was sufficient to induce significant increases in fasting blood glucose levels [401]. Although the mechanisms explaining this relationship remain unclear, this finding is consistent with the identification of GDM in our model at E14.5 and suggests a direct effect of intestinal Lachnospiraceae on the regulation of host glucose metabolism. Furthermore, a reduction in the relative abundance of taxa belonging to the Firmicutes phylum has been displayed following the administration of prebiotics to *ob/ob* mice and has been associated with lower fasting glycemia levels and improved glucose tolerance [252].

Studies in GF mice have demonstrated that glucose, insulin and leptin levels rise following conventionalization [222] and that the intestinal microbiota promotes the storage of triglycerides in adipocytes. Similar to Lachnospiraceae [407], bacteria belonging to the Ruminococcaceae

family are also commonly found in the intestinal microbiome of diabetics [404, 406-408]. In this study, the relative abundance of the family Ruminococcaceae.Other was identified as the only taxa that exhibited a positive correlation with maternal whole blood glucose, serum insulin and leptin in CON and HF females at E14.5. These data are consistent with the manifestation of GDM at E14.5 and highlight Ruminococcaceae.Other as an important microbial mediator of adiposity for future research.

In our study, we also observe that the order Bacteroidales (phylum Bacteroidetes, class Bacteroidia) exhibits a significant negative correlation with maternal whole blood glucose at E14.5. This finding is supported by work in men where lower Bacteroidia counts have been associated with impaired glucose tolerance and prediabetes [408] and suggests a beneficial role of the Bacteroidales order in mediating glucose homeostasis. Bacteria from the genera *Roseburia* [403, 404], *Bifidobacterium* [405] and *Akkermansia* [403, 406, 407] have all been implicated in glucose homeostasis and in the progression from normoglycemia to prediabetes and diabetes [407]. Although our model of maternal obesity is associated with a progressive increase in the relative abundance of the butyrate producing genus, *Akkermansia* throughout gestation and GDM at E14.5, we do not observe any correlations between the abundance of these genera and maternal metabolic indices.

Together, these data suggest that it is possible that maternal metabolic indices are an additional factor that may influence, or be influenced by the shifts in the relative abundance of intestinal bacteria during pregnancy and should be investigated in future work.

4.3 Maternal obesity induces a shift in the maternal intestinal microbiota

In this study, we show that maternal obesity is characterized by a shift in the maternal intestinal microbiota in high fat-fed females. With pregnancy, we observe a significant difference in the abundance of taxa belonging to the Firmicutes, Bacteroidetes, Verrucomicrobia and Tenericutes phyla in females fed a HFD. Similar to work in male mice [218, 231], and in humans [279, 282], we show that maternal obesity resulted in an increase in taxa belonging to the Clostridiales order of the Firmicutes phylum and a decrease in taxa belonging to the Bacteroidetes phylum. With HF pregnancy, we identified significant increases in the relative abundance of the Lachnospiraceae, Clostridiaceae.Other, Ruminococcaceae.Other families and the *Oscillospira*, *Bacteroides*, *Moryella*, *Clostridium* (family Lachnospiraceae) and *Clostridium* (family Ruminococcaceae) genera. Correspondingly, we observed a significant decrease in the relative abundance of one taxa belonging to the Bacteroidetes phylum, namely, the order Bacteroidales in HF pregnant females. Studies investigating the intestinal microbiota of lean and obese women during pregnancy present findings that are consistent with our experimental animal model. In obese women, members of the Firmicutes phylum including *Clostridium* counts are consistently increased [251, 282] and members of the Bacteroidetes phylum including *Bacteroides* are deacreased [282].

The regulation of host adiposity and metabolism by the intestinal microbiota has been demonstrated to be a result of several linked mechanisms including; microbial fermentation of indigestible polysaccharides, intestinal absorption of monosaccharides and short-chain fatty acids (SCFAs), the conversion of SCFAs to lipids in the liver, and microbial regulation of genes that promote lipid deposition in adipocytes [218, 222]. Metagenomic analyses of the intestinal microbial communities in obese male mice, have revealed an increase in the representation of

genes encoding proteins responsible for the synthesis of SCFAs from the fermentation of dietary

carbohydrates [218], utilized by the host for hepatic lipogenesis [222]. Whether this notion

applies to the shifts that occur in the maternal intestinal microbiota during maternal obesity is

unclear. Indeed, early pregnancy is characterized by a maternal anabolic state; where maternal

metabolic adaptations, mediated by sex-steroid hormones facilitates lipogenesis, glycogenesis

and adipocyte hypertrophy [351]. Thus, it is possible that the maternal intestinal microbiota may

shift during maternal obesity to promote energy extraction from the diet and to meet the

metabolic demands of pregnancy and should be examined in future work.

Consistent with intestinal microbial studies in obese male mice [219, 236] and humans [239], we

have shown that the relative abundance of *Akkermansia muciniphila* is decreased upon HFD

challenge in non-pregnant females. Interestingly, for the second time, we show that the genus

Akkermansia exhibits the largest increase in abundance with pregnancy in females fed a HFD.

This is inconsistent with investigations in pregnant women, where *Akkermansia muciniphila*

counts have been observed to be elevated in lean women compared to obese women [282].

Commonly associated with intestinal health [219, 236-239, 243], the biological significance of

this increase during HF pregnancy in our model remains unclear.

4.4 Maternal obesity induces intestinal Inflammation but does not impact intestinal permeability

It is well established that intestinal microbial shifts during obesity impact the integrity of the

epithelial monolayer of the intestine [189, 204, 232]. Studies in male mice show that

consumption of a HFD increases the Firmicutes to Bacteroidetes ratio and is associated with

increased intestinal inflammation [204] and permeability [189, 204, 232]. Therefore, we set out

to investigate whether the intestinal microbial shifts during maternal obesity were associated

with maternal intestinal inflammation and altered intestinal permeability at E14.5. In our model, consistent with an increase in taxa belonging to the Firmicutes phylum, we have demonstrated that maternal obesity is associated with elevated maternal intestinal NFκB activity, where activity is increased in all sections of the maternal intestine at E14.5, with a specific increase in the colon. This finding is of particular interest, as the colon is an intestinal site responsible for bacterial fermentation [409]. It may be that bacterial fermentation is altered during maternal obesity, although we did not specifically measure this. We do observe a significant decrease in the relative abundance of two prominent members of the Firmicutes phylum, with pregnancy in HF females. Both responsible for the fermentation of glucose, this observation supports the notion that maternal obesity may impair bacterial fermentation in the colon at E14.5. Previous work in our lab has shown that maternal caecal SCFA content is decreased at E18.5 (Gohir, Sloboda unpublished) and is consistent with this hypothesis.

Indeed, elevated NFκB activity has also been observed in the ileum [410] and colon [204] of male mice following HFD challenge. Increases in colonic NFκB activity have been associated with an increase in TLR-4 signaling and pro-inflammatory cytokine levels of TNF-α, IL-1β and IL-6 [204]. To evaluate whether we observe a corresponding increase in immune cells that produce pro-inflammatory cytokines, we investigated the numbers of F4/80 positive macrophages and CD3 positive T cells in the maternal colon at E14.5. The number of macrophages were not altered although a modest increase in T cells are observed in the maternal colon at E14.5. Multiple cell types have been identified to result in the activation of NFκB in the intestine in response to HFD challenge in male mice, among them is CD3 positive T cells [410]. Although we did not test whether CD3 positive T cells were required for this increase, it is

possible that elevated NFκB activation in the maternal colon at E14.5 is mediated by CD3 positive T cells.

To determine whether intestinal inflammation was associated with altered maternal intestinal physiology and function, we investigated intestinal mucus production and permeability at E14.5. At the level of transcription, we show that maternal obesity does not impact mucin mRNA levels in the small intestine, however, mRNA levels of colonic Muc5ac were increased. Although increased Muc5ac transcription did not correlate with an increase in the numbers of goblet cells in the colon, higher colonic Muc5ac production is consistent with our observation of increased *Akkermansia* relative abundance, a mucin degrading bacterium. A similar relationship between mucus layer thickness and *Akkermansia* presence has been demonstrated in male mice, where a reduction in mucus layer thickness, associated with consumption of a HFD, was reversed upon colonization with viable but not heat-killed *Akkermansia muciniphila* [236]. Although our model suggests that intestinal microbial shifts associated with maternal obesity stimulate intestinal inflammation at E14.5, maternal intestinal permeability is not altered. Interestingly, in the same experimental model, preliminary evidence from our lab suggests that at term, both pregnancy and maternal obesity result in an increase in maternal intestinal permeability. It is unlikely that intestinal permeability changes would precede metabolic adaptations, intestinal microbial shifts and the activation of an inflammatory response in the maternal intestine that we observe at E14.5. Therefore, an increase in intestinal permeability at term could manifest as a later consequence of a compromised intestinal microenvironment at E14.5, but remains to be directly investigated.

In addition to increased mRNA levels of colonic Muc5ac in the colon, increased maternal intestinal *Akkermansia* abundance may explain the maintenance of intestinal barrier integrity.

Commonly associated with intestinal health [236-243, 245], *Akkermansia muciniphila* has been shown to adhere to colonic enterocytes, strengthening the integrity of the enterocyte monolayer *in vitro* [248]. Whether *Akkermansia* is involved in maintaining epithelial monolayer integrity of the intestine in our model of maternal obesity is currently unknown.

4.5 Maternal obesity impacts placental nutrient transporter expression in the and is associated with placental inflammation

Experimental animal models of maternal obesity have consistently shown that exposure to HFD induces changes in placental growth, development and function [375], although often the exact components that are altered, is inconsistent. Animal models of maternal DIO and work in obese women, report conflicting findings regarding placental inflammation during various stages of pregnancy. In women, maternal obesity has been associated with elevated TNF-α [377], IL-1β [378], IL-6 [377], IL-8 [377, 378] , MCP-1 [377, 378] mRNA in the placenta. These findings have been often, but not always [378], associated with increased macrophage infiltration and accumulation in placentae of obese women [302]. Accordingly, studies examining multiple pregnancy time points in mice have demonstrated no change in placental inflammation at mid gestation (E15.5), but an up-regulation of placental TNF-α, IL-1β, IL-6, and IL-10 near term (E17.5) [375]. Although our study demonstrates an upregulation of F4/80 and MCP-1 mRNA in placentae of HF females, a corresponding increase in monocyte and macrophage populations was not demonstrated, consistent with other work [375]. Cells belonging to the monocyte and macrophage lineage have been shown to become activated and increase cytokine levels without proliferating [378, 411]. When examining macrophage activation state, studies which have not shown an increase in macrophage cell populations within the placenta were able to show that although levels of Cd11b, a marker of activated type 1 and 2 macrophages [412, 413], were

unchanged at mid gestation, Cd11b mRNA was elevated at term [375]. In this study, we did not assess macrophage activation state. Therefore, elevations in TNF-α, F4/80 and MCP-1 mRNA levels could be explained by investigating macrophage activation state, rather than abundance in placentae of HF and control females at mid gestation.

We demonstrate that maternal obesity impacts the mRNA levels of key components in the pro-inflammatory TLR-4 signaling pathway; TRAF6, NFκB and TNF-α in the placenta. The findings from animal models of maternal obesity and studies in obese women are consistent with our observations and show that TNF-α production is mediated through the pro-inflammatory TLR-4 signaling pathway through IκB kinase (IKK), downstream of TRAF6, in the placenta at mid gestation [414] and at term [302]. However, this was not associated with elevated NFκB activity. It is interesting that the upregulation of key components in the TLR-4 pathway is accompanied by increased mRNA levels of IL-10 in placentae during maternal obesity. This same result has been observed in a mouse model during pregnancy [375]. The exact role of IL-10 in the placenta is unknown. However, it is possible that levels of IL-10 are upregulated as a protective mechanism in response to obesity-driven placental inflammation. IL-10 has been identified to be expressed by trophoblast cells *in vitro* [415], suggesting that these cell types may respond to elevated levels of pro-inflammatory TNF-α.

Placental inflammation impairs tissue function [379, 414] and has been associated with altered transport of nutrients [177] and free fatty acids [149, 414] to the fetus, ultimately influencing fetal growth and development. In this study we demonstrate that maternal obesity results in transcriptional changes in key nutrient transporters responsible for regulating fetal growth and development. At E14.5, we show a significant elevation in placental GLUT1 and SNAT2 mRNA and a modest increase in GLUT3 mRNA in HF placentae, consistent with other experimental

animal models of maternal obesity [177, 320]. Consistent evidence has been drawn from other mouse models of maternal obesity where a 5-fold increase in GLUT1 protein expression and a 9-fold increase in SNAT2 protein expression has been shown at term [177]. Similar to our study where we observe only a modest increase in GLUT3 mRNA levels, GLUT3 protein expression was unchanged [177]. Although we did not investigate the mechanisms involved in altered nutrient transport in our model, previous *in vitro* studies have shown the capability of TNF-α [416-418], insulin and leptin to regulate nutrient transport systems.

In vitro, treatment of quiescent 3T3-L1 preadipocytes with TNF-α was shown to result in enhanced GLUT1 translocation to the cell surface. The maximum accumulation of GLUT1 mRNA was attributed to a 3-fold increase in GLUT1 mRNA stability, rather than transcription or translation, ultimately leading to enhanced protein production and glucose transport into cells [418]. Whether TNF-α exerts a similar effect in placental trophoblast cells is unknown, but would be consistent with our observed increases in GLUT1 and 3 mRNA levels. Recent work *in vitro* has shown an increase in SNAT1 and 2 protein through a mitogen-activated protein kinase (MAPK)-dependent mechanism in primary trophoblast cells treated with TNF-α [419]. Furthermore, maternal BMI and birth weight have been positively correlated with the phosphorylation of placental p38-MAPK [379], where the phosphorylation of p38-MAPK was found to be stimulated by MCP-1 and TNF-α. Thus it is possible that locally produced TNF-α in the placenta might directly influence placental nutrient transport by increasing levels of critical nutrient transporters through the p38-MAPK signaling cascade rather than through the phosphoinositide 3 kinase (PI3K) or IκB kinase (IKK) signaling cascades resulting in the translocation of NFκB during maternal obesity. First-trimester human trophoblast cells obtained early in pregnancy have been shown to upregulate GLUT1 mRNA levels in response to insulin

[420]. Similarly, insulin and leptin have been shown to stimulate the activity of SNAT2 in

primary villous fragments and cultured trophoblast cells [421, 422]. Therefore, it is possible that

elevated placental TNF-α, circulating maternal insulin or leptin may be involved in the

upregulation of critical nutrient transporters in the placenta during maternal obesity at E14.5.

5.0 STUDY LIMITATIONS AND FUTURE DIRECTIONS

The present study provides novel data regarding impacts of pregnancy and maternal obesity on the maternal intestinal microbiota, intestinal NFκB activity and placental mRNA levels of key inflammatory cytokines and nutrient transporters at E14.5.

The findings of the preliminary study investigating the relationship between fluctuating sex-steroid hormones during the estrous cycle in NPC female mice is limited by the approximation of circulating 17-β estradiol and progesterone levels according to estrous cycle staging founded on vaginal epithelial cell analysis. To further investigate the factors involved in the intestinal microbial shift in control-fed pregnant female mice, male feces should be collected and sequenced in future work to determine whether female allocoprophagy of the male feces occurs during mating and contributes to a shift in microbial populations at E0.5. To determine whether intestinal microbial shifts during pregnancy are sensitive to pregnancy-related 17-β estradiol and progesterone concentrations in the circulation, the levels of these hormones should be measured in the serum at the time of fecal collection. In more invasive experiments, to test the hypothesis that sex steroids influence maternal microbial shifts, exogenous administration of 17-β estradiol and progesterone concentrations that mimic circulating biological levels during pregnancy could be performed followed by a fecal time course collection.

Although we report significant shifts in a number of intestinal microbes with diet, pregnancy and a diet x pregnancy interaction, we recognize that all experimental findings drawn from this study are a result of the global changes, and not the shifts in individual taxa, in the maternal intestinal microbiome. Future studies should study the colonization of GF mice to understand the effects of individual taxonomic shifts during pregnancy and maternal obesity, on maternal and fetal health.

Furthermore, the characterization of the maternal intestinal microbiota in our pipeline, is limited by genus level classification. Future studies that are able to sequence to the species depth will contribute significantly to our knowledge of maternal intestinal microbial shifts during pregnancy and maternal obesity and their involvement in maternal and fetal health.

To understand the impacts of the intestinal microbial shifts that characterize pregnancy and maternal obesity on the function of the maternal intestine, we investigated maternal intestinal permeability using FITC-dextran. Although FITC-dextran is routinely used as a measure of *in vivo* intestinal permeability in animal models, our findings are limited to intestinal paracellular transport and do not account for intestinal transcellular transport of molecules across the entire intestinal epithelial monolayer. To circumvent these limitations, future intestinal permeability experiments should be conducted using an Ussing chamber, where the paracellular and transcellular transport of ions and nutrients can be measured across specific intestinal sections in the intestinal epithelium.

To elucidate the origins of elevated NFκB activity in the maternal intestine and to understand the cell types involved in the increased pro-inflammatory TLR-4 signaling pathway components in the placenta, we investigated F4/80 positive macrophages and CD3 positive T cells in the maternal intestine and placenta at E14.5. Our measures are limited to the number of immune cells present in each cross section of the intestine and placenta. Understanding the activation state of these cell types may provide meaningful evidence for their contribution to intestinal and placental inflammation and the functional impacts during maternal obesity. To further investigate the impacts of maternal intestinal microbial shifts during maternal obesity on maternal intestinal inflammation and placental inflammation, future investigations should concentrate on assessing the activation state of colonic and placental macrophages and T cells to understand the cell types

associated with the altered inflammatory signaling pathways to understand the mechanisms associated with impaired tissue function.

In this study, we identify that maternal obesity is associated with altered nutrient transport in the placenta at E14.5. Although critical nutrient transporters, GLUT1, GLUT3 and SNAT2 is observed to be elevated as a result of maternal obesity, these findings are based on transcriptional changes in mRNA levels and do not directly reflect the levels of functional protein in the placenta. To understand the functional impacts of maternal obesity on the placenta placental, the protein expression of GLUT1, GLUT3 and SNAT2 should be investigated in future work.

The final limitation of this study lies within the use of a rodent model. The single largest limitation of the rodent model arises from the fundamental differences between rodent and human development. Rodents are altricial animals and are born with a poorly developed central nervous and autocrine system. Additionally, due to the polycocial nature of rodent pregnancy, variable fetal and neonatal nutrient supply can occur to offspring within each litter resulting in different programming effects between fetuses [129]. Thus, the findings from this study must be interpreted within the biological context from which they are presented.

6.0 CONCLUSIONS

This is the first study to show that intestinal microbial shifts in control-fed non pregnant females are not mediated by reproductive stage dependant hormone fluctuations and suggests that maternal microbial shifts may be due to factors other than estradiol and progesterone. We speculate that other factors including allocoprophagy of the male feces during the mating period, concentration differences sex-steroids between non-pregnant and pregnant females or changes in maternal glucose, insulin and leptin throughout gestation may explain pregnancy-induced shifts. We show that diet and pregnancy induce shifts in the maternal intestinal microbiota and the relative abundance of *Ruminococcaceae* and *Lachnospiraceae* were significantly correlated with maternal circulating glucose, insulin and leptin. At E14.5, maternal intestinal microbial shifts were associated with increased NFκB activity in all sections of the maternal intestine in HF females, but this did not impact maternal intestinal barrier integrity. These data suggest that intestinal inflammation may precede compromised intestinal function in our model. At E14.5 despite no change in maternal serum cytokine levels, we show elevated mRNA levels of components in the pro-inflammatory TLR-4 pathway, TNF-α and markers of macrophage infiltration suggesting that local placental inflammation occurs in this model in the absence of systemic inflammation. This inflammatory response is also accompanied by elevated T cell activation in the placental junctional, but not the labyrinth zone. This finding is accompanied by elevated mRNA levels of critical nutrient transporters in the placenta at E14.5 and suggest that placental inflammatory signaling during maternal obesity is associated with altered nutrient sensing in the placenta at E14.5.

7.0 APPENDIX I

7.1 Awards

Excellence in Poster Presentation Award
McMaster Faculty of Health Science Research Plenary, McMaster University, Hamilton, ON.
May 2016.

7.2 Publications

Wallace, J.G., Gohir, W., & Sloboda, D.M. (2016). The impact of early life gut colonization on metabolic and obesogenic outcomes: What have animal models shown us? *Journal of the Developmental Origins of Health and Disease*, 7(01), 15-24.

7.3 Presentations

Oral presentation delivered at the 44th Annual Southern Ontario Reproductive Biology Meeting, Queen's University, Kingston, ON. May 2016.

Poster presentation delivered at the Faculty of Health Sciences Research Plenary, McMaster University, Hamilton, ON. May 2016.

1.   *Obesity and overweight*. 2015, World Health Organization.
2.   Wilson, P.W. and S.M. Grundy, *The metabolic syndrome: practical guide to origins and treatment: Part I.* Circulation, 2003. **108**(12): p. 1422-4.
3.   Hedley, A.A., et al., *Prevalence of overweight and obesity among US children, adolescents, and adults, 1999-2002.* Jama, 2004. **291**(23): p. 2847-50.
4.   Ogden, C.L., et al., *Prevalence of childhood and adult obesity in the United States, 2011-2012.* Jama, 2014. **311**(8): p. 806-14.
5.   Catalano, P. and S.H. deMouzon, *Maternal obesity and metabolic risk to the offspring: why lifestyle interventions may have not achieved the desired outcomes.* Int J Obes (Lond), 2015. **39**(4): p. 642-9.
6.   Wallace, J.M., G.W. Horgan, and S. Bhattacharya, *Placental weight and efficiency in relation to maternal body mass index and the risk of pregnancy complications in women delivering singleton babies.* Placenta, 2012. **33**(8): p. 611-8.
7.   Frederick, I.O., et al., *Adult weight change, weight cycling, and prepregnancy obesity in relation to risk of preeclampsia.* Epidemiology, 2006. **17**(4): p. 428-34.
8.   Villamor, E. and S. Cnattingius, *Interpregnancy weight change and risk of adverse pregnancy outcomes: a population-based study.* Lancet, 2006. **368**(9542): p. 1164-70.
9.   Graves, B.W., et al., *Maternal body mass index, delivery route, and induction of labor in a midwifery caseload.* J Midwifery Womens Health, 2006. **51**(4): p. 254-9.
10.   Barau, G., et al., *Linear association between maternal pre-pregnancy body mass index and risk of caesarean section in term deliveries.* Bjog, 2006. **113**(10): p. 1173-7.
11.   Institute of, M. and I.O.M.P.W.G. National Research Council Committee to Reexamine, *The National Academies Collection: Reports funded by National Institutes of Health*, in *Weight Gain During Pregnancy: Reexamining the Guidelines*, K.M. Rasmussen and A.L. Yaktine, Editors. 2009, National Academies Press (US)National Academy of Sciences.: Washington (DC).

12.   Seligman, L.C., et al., *Obesity and gestational weight gain: cesarean delivery and labor complications.* Rev Saude Publica, 2006. **40**(3): p. 457-65.
13.   Coton, S.J., I. Nazareth, and I. Petersen, *A cohort study of trends in the prevalence of pregestational diabetes in pregnancy recorded in UK general practice between 1995 and 2012.* BMJ Open, 2016. **6**(1): p. e009494.
14.   Feig, D.S. and V.A. Palda, *Type 2 diabetes in pregnancy: a growing concern.* Lancet, 2002. **359**(9318): p. 1690-2.
15.   Lapolla, A., M.G. Dalfra, and D. Fedele, *Pregnancy complicated by type 2 diabetes: an emerging problem.* Diabetes Res Clin Pract, 2008. **80**(1): p. 2-7.
16.   *From the Centers for Disease Control. Perinatal mortality and congenital malformations in infants born to women with insulin-dependent diabetes mellitus--United States, Canada, and Europe, 1940-1988.* Jama, 1990. **264**(4): p. 437, 441.
17.   *Gestational Diabetes Mellitus.* Diabetes Care, 2004. **27**(suppl 1): p. s88-s90.
18.   Buchanan, T.A. and A.H. Xiang, *Gestational diabetes mellitus.* J Clin Invest, 2005. **115**(3): p. 485-91.
19.   Sebire, N.J., et al., *Maternal obesity and pregnancy outcome: a study of 287,213 pregnancies in London.* Int J Obes Relat Metab Disord, 2001. **25**(8): p. 1175-82.

20. Kumari, A.S., *Pregnancy outcome in women with morbid obesity.* Int J Gynaecol Obstet, 2001. **73**(2): p. 101-7.

21. Bianco, A.T., et al., *Pregnancy outcome and weight gain recommendations for the morbidly obese woman.* Obstet Gynecol, 1998. **91**(1): p. 97-102.

22. Baeten, J.M., E.A. Bukusi, and M. Lambe, *Pregnancy complications and outcomes among overweight and obese nulliparous women.* Am J Public Health, 2001. **91**(3): p. 436-40.

23. Yogev, Y., et al., *Glucose screening in Mexican-American women.* Obstetrics & Gynecology, 2004. **103**(6): p. 1241-1245.

24. Langer, O., et al., *Overweight and obese in gestational diabetes: the impact on pregnancy outcome.* Am J Obstet Gynecol, 2005. **192**(6): p. 1768-76.

25. Sibai, B.M., et al., *Risk factors for preeclampsia in healthy nulliparous women: a prospective multicenter study. The National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units.* Am J Obstet Gynecol, 1995. **172**(2 Pt 1): p. 642-8.

26. Sibai, B.M., et al., *Risk factors associated with preeclampsia in healthy nulliparous women. The Calcium for Preeclampsia Prevention (CPEP) Study Group.* Am J Obstet Gynecol, 1997. **177**(5): p. 1003-10.

27. Sattar, N., et al., *Antenatal waist circumference and hypertension risk.* Obstet Gynecol, 2001. **97**(2): p. 268-71.

28. Barton, J.R. and B.M. Sibai, *Prediction and prevention of recurrent preeclampsia.* Obstet Gynecol, 2008. **112**(2 Pt 1): p. 359-72.

29. Stone, J.L., et al., *Risk factors for severe preeclampsia.* Obstet Gynecol, 1994. **83**(3): p. 357-61.

30. O'Brien, T.E., J.G. Ray, and W.S. Chan, *Maternal body mass index and the risk of preeclampsia: a systematic overview.* Epidemiology, 2003. **14**(3): p. 368-74.

31. Reaven, G.M., *Role of insulin resistance in human disease (syndrome X): an expanded definition.* Annu Rev Med, 1993. **44**: p. 121-31.

32. Alberti, K.G., P. Zimmet, and J. Shaw, *The metabolic syndrome--a new worldwide definition.* Lancet, 2005. **366**(9491): p. 1059-62.

33. Pallardo, F., et al., *Early postpartum metabolic assessment in women with prior gestational diabetes.* Diabetes Care, 1999. **22**(7): p. 1053-8.

34. Retnakaran, R., et al., *Glucose intolerance in pregnancy and postpartum risk of metabolic syndrome in young women.* J Clin Endocrinol Metab, 2010. **95**(2): p. 670-7.

35. Verma, A., et al., *Insulin resistance syndrome in women with prior history of gestational diabetes mellitus.* J Clin Endocrinol Metab, 2002. **87**(7): p. 3227-35.

36. Bo, S., et al., *Prior gestational hyperglycemia: a long-term predictor of the metabolic syndrome.* J Endocrinol Invest, 2004. **27**(7): p. 629-35.

37. Athukorala, C., et al., *The risk of adverse pregnancy outcomes in women who are overweight or obese.* BMC Pregnancy Childbirth, 2010. **10**: p. 56.

38. Crane, J.M., et al., *Maternal and perinatal outcomes of extreme obesity in pregnancy.* J Obstet Gynaecol Can, 2013. **35**(7): p. 606-11.

39. Portela, D.S., et al., *Maternal obesity, environmental factors, cesarean delivery and breastfeeding as determinants of overweight and obesity in children: results from a cohort.* BMC Pregnancy Childbirth, 2015. **15**: p. 94.

40. Institute of Medicine Committee on Nutritional Status During, P. and Lactation, in *Nutrition During Pregnancy: Part I Weight Gain: Part II Nutrient Supplements*. 1990, National Academies Press (US)Copyright (c) 1990 by the National Academy of Sciences.: Washington (DC).

41. Maresh, M., et al., *Factors predisposing to and outcome of gestational diabetes.* Obstet Gynecol, 1989. **74**(3 Pt 1): p. 342-6.

42. Stothard, K.J., et al., *Maternal overweight and obesity and the risk of congenital anomalies: a systematic review and meta-analysis.* Jama, 2009. **301**(6): p. 636-50.

43. Catalano, P.M., et al., *Fetuses of obese mothers develop insulin resistance in utero.* Diabetes Care, 2009. **32**(6): p. 1076-80.

44. Rajasingam, D., et al., *A prospective study of pregnancy outcome and biomarkers of oxidative stress in nulliparous obese women.* Am J Obstet Gynecol, 2009. **200**(4): p. 395.e1-9.

45. Higgins, L., et al., *Obesity and the placenta: A consideration of nutrient exchange mechanisms in relation to aberrant fetal growth.* Placenta, 2011. **32**(1): p. 1-7.

46. Pedersen, J., *Diabetes and pregnancy; blood sugar of newborn infants during fasting and glucose administration.* Nord Med, 1952. **47**(30): p. 1049.

47. Knopp, R.H., et al., *Relationships of infant birth size to maternal lipoproteins, apoproteins, fuels, hormones, clinical chemistries, and body weight at 36 weeks gestation.* Diabetes, 1985. **34 Suppl 2**: p. 71-7.

48. Di Cianni, G., et al., *Maternal triglyceride levels and newborn weight in pregnant women with normal glucose tolerance.* Diabet Med, 2005. **22**(1): p. 21-5.

49. Kitajima, M., et al., *Maternal serum triglyceride at 24--32 weeks' gestation and newborn weight in nondiabetic women with positive diabetic screens.* Obstet Gynecol, 2001. **97**(5 Pt 1): p. 776-80.

50. Catalano, P.M. and H.M. Ehrenberg, *The short- and long-term implications of maternal obesity on the mother and her offspring.* Bjog, 2006. **113**(10): p. 1126-33.

51. Drake, A.J. and R.M. Reynolds, *Impact of maternal obesity on offspring obesity and cardiometabolic disease risk.* Reproduction, 2010. **140**(3): p. 387-98.

52. Boney, C.M., et al., *Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus.* Pediatrics, 2005. **115**(3): p. e290-6.

53. O'Reilly, J.R. and R.M. Reynolds, *The risk of maternal obesity to the long-term health of the offspring.* Clin Endocrinol (Oxf), 2013. **78**(1): p. 9-16.

54. Sridhar, S.B., et al., *Maternal gestational weight gain and offspring risk for childhood overweight or obesity.* Am J Obstet Gynecol, 2014. **211**(3): p. 259.e1-8.

55. Parsons, T.J., C. Power, and O. Manor, *Fetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study.* Bmj, 2001. **323**(7325): p. 1331-5.

56. Sewell, M.F., et al., *Increased neonatal fat mass, not lean body mass, is associated with maternal obesity.* Am J Obstet Gynecol, 2006. **195**(4): p. 1100-3.

57. Reynolds, R.M., et al., *Maternal BMI, parity, and pregnancy weight gain: influences on offspring adiposity in young adulthood.* J Clin Endocrinol Metab, 2010. **95**(12): p. 5365-9.

58. Reynolds, R.M., et al., *Maternal obesity during pregnancy and premature mortality from cardiovascular event in adult offspring: follow-up of 1 323 275 person years.* Bmj, 2013. **347**: p. f4539.

59.   Danielzik, S., et al., *Impact of parental BMI on the manifestation of overweight 5-7 year old children.* Eur J Nutr, 2002. **41**(3): p. 132-8.
60.   Whitaker, R.C., *Predicting preschooler obesity at birth: the role of maternal obesity in early pregnancy.* Pediatrics, 2004. **114**(1): p. e29-36.
61.   Laitinen, J., C. Power, and M.R. Jarvelin, *Family social class, maternal body mass index, childhood body mass index, and age at menarche as predictors of adult obesity.* Am J Clin Nutr, 2001. **74**(3): p. 287-94.
62.   West-Eberhard, M.J., *Phenotypic plasticity and the origins of diversity.* Annual review of Ecology and Systematics, 1989: p. 249-278.
63.   Gluckman, P.D., et al., *Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies.* Proc Biol Sci, 2005. **272**(1564): p. 671-7.
64.   Langley-Evans, S.C., *Nutrition in early life and the programming of adult disease: a review.* J Hum Nutr Diet, 2015. **28 Suppl 1**: p. 1-14.
65.   Hales, C.N. and D.J. Barker, *The thrifty phenotype hypothesis.* Br Med Bull, 2001. **60**: p. 5-20.
66.   Desai, M., M. Beall, and M.G. Ross, *Developmental origins of obesity: programmed adipogenesis.* Curr Diab Rep, 2013. **13**(1): p. 27-33.
67.   Sinclair, K.D. and A.J. Watkins, *Parental diet, pregnancy outcomes and offspring health: metabolic determinants in developing oocytes and embryos.* Reprod Fertil Dev, 2013. **26**(1): p. 99-114.
68.   Grazul-Bilska, A.T., et al., *Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep.* Domest Anim Endocrinol, 2012. **43**(4): p. 289-98.
69.   Reynolds, R.M., et al., *Transmitting biological effects of stress in utero: implications for mother and offspring.* Psychoneuroendocrinology, 2013. **38**(9): p. 1843-9.
70.   Tarantal, A.F. and L. Berglund, *Obesity and lifespan health--importance of the fetal environment.* Nutrients, 2014. **6**(4): p. 1725-36.
71.   Champagne, F.A., *Maternal imprints and the origins of variation.* Horm Behav, 2011. **60**(1): p. 4-11.
72.   Gluckman, P.D. and M.A. Hanson, *The developmental origins of the metabolic syndrome.* Trends Endocrinol Metab, 2004. **15**(4): p. 183-7.
73.   Barker, D.J., *The origins of the developmental origins theory.* J Intern Med, 2007. **261**(5): p. 412-7.
74.   Barker, D.J., *The fetal and infant origins of adult disease.* Bmj, 1990. **301**(6761): p. 1111.
75.   Ryle, J.A. and W.T. Russell, *The natural history of coronary disease; a clinical and epidemiological study.* Br Heart J, 1949. **11**(4): p. 370-89.
76.   Morris, J.N., *Recent history of coronary disease.* Lancet, 1951. **1**(6646): p. 69-73.
77.   Gardner, M.J., M.D. Crawford, and J.N. Morris, *Patterns of mortality in middle and early old age in the county boroughs of England and Wales.* Br J Prev Soc Med, 1969. **23**(3): p. 133-40.
78.   censuses, G.-B.O.o.p. and surveys, *Occupational Mortality: The Registrar General's Decennial Supplement for England and Wales, 1970-72.* 1978: HM Stationery office.
79.   Keys, A., *Seven countries. A multivariate analysis of death and coronary heart disease.* 1980: Harvard University Press.

80.   Rose, G. and M.G. Marmot, *Social class and coronary heart disease.* Br Heart J, 1981. **45**(1): p. 13-9.

81.   Barker, D.J., *The fetal origins of coronary heart disease.* Acta Paediatr Suppl, 1997. **422**: p. 78-82.

82.   Barker, D.J., et al., *Weight in infancy and death from ischaemic heart disease.* Lancet, 1989. **2**(8663): p. 577-80.

83.   Barker, D.J. and C. Osmond, *Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales.* Lancet, 1986. **1**(8489): p. 1077-81.

84.   Gardner, M.J., P.D. Winter, and D.J.P. Barker, *Atlas of mortality from selected diseases in England and Wales 1968-1978.* 1984.

85.   General, R., *Registrar General's Statistical Review of England and Wales for the Year 1959: Supplement on Mental Health.* 1962, London: Stationery Office.

86.   Hales, C.N., et al., *Fetal and infant growth and impaired glucose tolerance at age 64.* Bmj, 1991. **303**(6809): p. 1019-22.

87.   Barker, D.J., et al., *Fetal and placental size and risk of hypertension in adult life.* Bmj, 1990. **301**(6746): p. 259-62.

88.   Barker, D.J., et al., *The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life.* Bmj, 1993. **306**(6875): p. 422-6.

89.   Barker, D.J., *Childhood causes of adult diseases.* Arch Dis Child, 1988. **63**(7): p. 867-9.

90.   Barker, D.J., et al., *Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth.* Diabetologia, 1993. **36**(1): p. 62-7.

91.   Law, C.M., et al., *Thinness at birth and glucose tolerance in seven-year-old children.* Diabet Med, 1995. **12**(1): p. 24-9.

92.   Phillips, D.I., et al., *Thinness at birth and insulin resistance in adult life.* Diabetologia, 1994. **37**(2): p. 150-4.

93.   Yajnik, C.S., et al., *Fetal growth and glucose and insulin metabolism in four-year-old Indian children.* Diabet Med, 1995. **12**(4): p. 330-6.

94.   Xiao, X., et al., *Evidence of a relationship between infant birth weight and later diabetes and impaired glucose regulation in a Chinese population.* Diabetes Care, 2008. **31**(3): p. 483-7.

95.   Frankel, S., et al., *Birthweight, body-mass index in middle age, and incident coronary heart disease.* Lancet, 1996. **348**(9040): p. 1478-80.

96.   Stein, C.E., et al., *Fetal growth and coronary heart disease in south India.* Lancet, 1996. **348**(9037): p. 1269-73.

97.   Rich-Edwards, J.W., et al., *Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976.* Bmj, 1997. **315**(7105): p. 396-400.

98.   Forsen, T., et al., *Mother's weight in pregnancy and coronary heart disease in a cohort of Finnish men: follow up study.* Bmj, 1997. **315**(7112): p. 837-40.

99.   Leon, D.A., et al., *Reduced fetal growth rate and increased risk of death from ischaemic heart disease: cohort study of 15 000 Swedish men and women born 1915-29.* Bmj, 1998. **317**(7153): p. 241-5.

100.   Forsen, T., et al., *Growth of girls who later develop coronary heart disease.* Heart, 2004. **90**(1): p. 20-4.

101.   Colditz, G.A., J.E. Manson, and S.E. Hankinson, *The Nurses' Health Study: 20-year contribution to the understanding of health among women.* J Womens Health, 1997. **6**(1): p. 49-62.

102.   Rich-Edwards, J.W., et al., *Birthweight and the risk for type 2 diabetes mellitus in adult women.* Ann Intern Med, 1999. **130**(4 Pt 1): p. 278-84.

103.   Ravelli, G.P., Z.A. Stein, and M.W. Susser, *Obesity in young men after famine exposure in utero and early infancy.* N Engl J Med, 1976. **295**(7): p. 349-53.

104.   Gluckman, P. and C. Pinal, *Glucose tolerance in adults after prenatal exposure to famine.* Lancet, 2001. **357**(9270): p. 1798.

105.   Ravelli, A.C., et al., *Obesity at the age of 50 y in men and women exposed to famine prenatally.* Am J Clin Nutr, 1999. **70**(5): p. 811-6.

106.   Schulz, L.C., *The Dutch Hunger Winter and the developmental origins of health and disease.* Proceedings of the National Academy of Sciences, 2010. **107**(39): p. 16757-16758.

107.   de Rooij, S.R., et al., *Glucose tolerance at age 58 and the decline of glucose tolerance in comparison with age 50 in people prenatally exposed to the Dutch famine.* Diabetologia, 2006. **49**(4): p. 637-43.

108.   Roseboom, T.J., et al., *Adult survival after prenatal exposure to the Dutch famine 1944--45.* Paediatr Perinat Epidemiol, 2001. **15**(3): p. 220-5.

109.   Langley-Evans, S.C., *Developmental programming of health and disease.* Proc Nutr Soc, 2006. **65**(1): p. 97-105.

110.   Ravelli, A.C., et al., *Glucose tolerance in adults after prenatal exposure to famine.* Lancet, 1998. **351**(9097): p. 173-7.

111.   Roseboom, T.J., et al., *Coronary heart disease after prenatal exposure to the Dutch famine, 1944-45.* Heart, 2000. **84**(6): p. 595-8.

112.   Painter, R.C., et al., *Microalbuminuria in adults after prenatal exposure to the Dutch famine.* J Am Soc Nephrol, 2005. **16**(1): p. 189-94.

113.   Rickard, I.J. and V. Lummaa, *The predictive adaptive response and metabolic syndrome: challenges for the hypothesis.* Trends Endocrinol Metab, 2007. **18**(3): p. 94-9.

114.   Hales, C.N. and D.J. Barker, *Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis.* Diabetologia, 1992. **35**(7): p. 595-601.

115.   Eriksson, J.G., et al., *Catch-up growth in childhood and death from coronary heart disease: longitudinal study.* Bmj, 1999. **318**(7181): p. 427-31.

116.   Stanner, S.A., et al., *Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study.* Bmj, 1997. **315**(7119): p. 1342-8.

117.   Vaiserman, A., *Early-life origin of adult disease: evidence from natural experiments.* Exp Gerontol, 2011. **46**(2-3): p. 189-92.

118.   Stanner, S.A. and J.S. Yudkin, *Fetal programming and the Leningrad Siege study.* Twin Res, 2001. **4**(5): p. 287-92.

119.   Primatesta, P., E. Falaschetti, and N.R. Poulter, *P-493: Birthweight and blood pressure in children: Does the association exists?* American Journal of Hypertension, 2003. **16**(S1): p. 216A-217A.

120.   Levitt, N.S., et al., *An inverse relation between blood pressure and birth weight among 5 year old children from Soweto, South Africa.* J Epidemiol Community Health, 1999. **53**(5): p. 264-8.

121. Thame, M., et al., *Blood pressure is related to placental volume and birth weight.* Hypertension, 2000. **35**(2): p. 662-7.
122. Fall, C.H., et al., *Size at birth, maternal weight, and type 2 diabetes in South India.* Diabet Med, 1998. **15**(3): p. 220-7.
123. Hoy, W.E., et al., *A new dimension to the Barker hypothesis: low birthweight and susceptibility to renal disease.* Kidney Int, 1999. **56**(3): p. 1072-7.
124. Oken, E. and M.W. Gillman, *Fetal origins of obesity.* Obes Res, 2003. **11**(4): p. 496-506.
125. Garn, S.M. and D.C. Clark, *Trends in fatness and the origins of obesity Ad Hoc Committee to Review the Ten-State Nutrition Survey.* Pediatrics, 1976. **57**(4): p. 443-56.
126. Curhan, G.C., et al., *Birth weight and adult hypertension, diabetes mellitus, and obesity in US men.* Circulation, 1996. **94**(12): p. 3246-50.
127. Curhan, G.C., et al., *Birth weight and adult hypertension and obesity in women.* Circulation, 1996. **94**(6): p. 1310-5.
128. Wang, X., et al., *Metabolic syndrome in obese children born large for gestational age.* Indian J Pediatr, 2007. **74**(6): p. 561-5.
129. Armitage, J.A., et al., *Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals?* J Physiol, 2004. **561**(Pt 2): p. 355-77.
130. Gnanalingham, M.G., et al., *Ontogeny and nutritional programming of adiposity in sheep: potential role of glucocorticoid action and uncoupling protein-2.* Am J Physiol Regul Integr Comp Physiol, 2005. **289**(5): p. R1407-15.
131. Zhu, M.J., et al., *Maternal nutrient restriction affects properties of skeletal muscle in offspring.* J Physiol, 2006. **575**(Pt 1): p. 241-50.
132. Shankar, K., et al., *Maternal obesity at conception programs obesity in the offspring.* Am J Physiol Regul Integr Comp Physiol, 2008. **294**(2): p. R528-38.
133. Page, K.C., et al., *Maternal and postweaning diet interaction alters hypothalamic gene expression and modulates response to a high-fat diet in male offspring.* Am J Physiol Regul Integr Comp Physiol, 2009. **297**(4): p. R1049-57.
134. Howie, G.J., et al., *Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet.* J Physiol, 2009. **587**(Pt 4): p. 905-15.
135. Zheng, J., et al., *Maternal high-fat diet modulates hepatic glucose, lipid homeostasis and gene expression in the PPAR pathway in the early life of offspring.* Int J Mol Sci, 2014. **15**(9): p. 14967-83.
136. Samuelsson, A.M., et al., *Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming.* Hypertension, 2008. **51**(2): p. 383-92.
137. Nivoit, P., et al., *Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance.* Diabetologia, 2009. **52**(6): p. 1133-42.
138. Howie, G.J., et al., *Timing of maternal exposure to a high fat diet and development of obesity and hyperinsulinemia in male rat offspring: same metabolic phenotype, different developmental pathways?* J Nutr Metab, 2013. **2013**: p. 517384.
139. Taylor, P.D., et al., *Impaired glucose homeostasis and mitochondrial abnormalities in offspring of rats fed a fat-rich diet in pregnancy.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(1): p. R134-9.

140.   Borengasser, S.J., et al., *Maternal obesity during gestation impairs fatty acid oxidation and mitochondrial SIRT3 expression in rat offspring at weaning.* PLoS One, 2011. **6**(8): p. e24068.

141.   Oben, J.A., et al., *Maternal obesity during pregnancy and lactation programs the development of offspring non-alcoholic fatty liver disease in mice.* J Hepatol, 2010. **52**(6): p. 913-20.

142.   Bruce, K.D., et al., *Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression.* Hepatology, 2009. **50**(6): p. 1796-808.

143.   Gabory, A., et al., *Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics.* Biol Sex Differ, 2013. **4**(1): p. 5.

144.   Dahlhoff, M., et al., *Peri-conceptional obesogenic exposure induces sex-specific programming of disease susceptibilities in adult mouse offspring.* Biochim Biophys Acta, 2014. **1842**(2): p. 304-17.

145.   Gabory, A., et al., *Maternal diets trigger sex-specific divergent trajectories of gene expression and epigenetic systems in mouse placenta.* PLoS One, 2012. **7**(11): p. e47986.

146.   Walker, S.P., et al., *Inverse relationship between gestational weight gain and glucose uptake in human placenta from female foetuses.* Pediatr Obes, 2014. **9**(3): p. e73-6.

147.   Zhang, L., et al., *Maternal obesity in ewes results in reduced fetal pancreatic beta-cell numbers in late gestation and decreased circulating insulin concentration at term.* Domest Anim Endocrinol, 2011. **40**(1): p. 30-9.

148.   Yan, X., et al., *Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring.* Biol Reprod, 2011. **85**(1): p. 172-8.

149.   Zhu, M.J., et al., *Maternal obesity markedly increases placental fatty acid transporter expression and fetal blood triglycerides at midgestation in the ewe.* Am J Physiol Regul Integr Comp Physiol, 2010. **299**(5): p. R1224-31.

150.   Kruse, M., et al., *High-fat intake during pregnancy and lactation exacerbates high-fat diet-induced complications in male offspring in mice.* Endocrinology, 2013. **154**(10): p. 3565-76.

151.   King, V., et al., *Post-weaning diet determines metabolic risk in mice exposed to overnutrition in early life.* Reprod Biol Endocrinol, 2014. **12**: p. 73.

152.   Connor, K.L., et al., *Nature, nurture or nutrition? Impact of maternal nutrition on maternal care, offspring development and reproductive function.* J Physiol, 2012. **590**(Pt 9): p. 2167-80.

153.   Ozaki, T., et al., *Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring.* J Physiol, 2001. **530**(Pt 1): p. 141-52.

154.   Holemans, K., et al., *Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring.* Br J Nutr, 1999. **81**(1): p. 73-9.

155.   Woodall, S.M., et al., *A model of intrauterine growth retardation caused by chronic maternal undernutrition in the rat: effects on the somatotrophic axis and postnatal growth.* J Endocrinol, 1996. **150**(2): p. 231-42.

156.   Vickers, M.H., et al., *Dysregulation of the adipoinsular axis -- a mechanism for the pathogenesis of hyperleptinemia and adipogenic diabetes induced by fetal programming.* J Endocrinol, 2001. **170**(2): p. 323-32.

157. Wallace, J., *Adaptive maternal, placental and fetal responses to nutritional extremes in the pregnant adolescent: lessons from sheep.* Cambridge Studies in Biological and Evolutionary Anthropology, 2011. **1**(59): p. 112-127.

158. Hawkins, P., et al., *Effect of maternal nutrient restriction in early gestation on development of the hypothalamic-pituitary-adrenal axis in fetal sheep at 0.8-0.9 of gestation.* J Endocrinol, 1999. **163**(3): p. 553-61.

159. Hawkins, P., et al., *Effect of maternal undernutrition in early gestation on ovine fetal blood pressure and cardiovascular reflexes.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(1): p. R340-8.

160. Ozaki, T., et al., *Effects of undernutrition in early pregnancy on systemic small artery function in late-gestation fetal sheep.* Am J Obstet Gynecol, 2000. **183**(5): p. 1301-7.

161. Edwards, L.J. and I.C. McMillen, *Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation.* J Physiol, 2001. **533**(Pt 2): p. 561-70.

162. Vonnahme, K.A., et al., *Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep.* Biol Reprod, 2003. **69**(1): p. 133-40.

163. Gardner, D.S., et al., *Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep.* Hypertension, 2004. **43**(6): p. 1290-6.

164. Hellmuth, C., et al., *Influence of moderate maternal nutrition restriction on the fetal baboon metabolome at 0.5 and 0.9 gestation.* Nutr Metab Cardiovasc Dis, 2016.

165. Li, C., et al., *Effects of maternal global nutrient restriction on fetal baboon hepatic insulin-like growth factor system genes and gene products.* Endocrinology, 2009. **150**(10): p. 4634-42.

166. Schlabritz-Loutsevitch, N., et al., *Moderate maternal nutrient restriction, but not glucocorticoid administration, leads to placental morphological changes in the baboon (Papio sp.).* Placenta, 2007. **28**(8-9): p. 783-93.

167. Colman, R.J., et al., *Caloric restriction delays disease onset and mortality in rhesus monkeys.* Science, 2009. **325**(5937): p. 201-4.

168. Schlabritz-Loutsevitch, N.E., et al., *Metabolic adjustments to moderate maternal nutrient restriction.* Br J Nutr, 2007. **98**(2): p. 276-84.

169. Franco Mdo, C., et al., *Severe nutritional restriction in pregnant rats aggravates hypertension, altered vascular reactivity, and renal development in spontaneously hypertensive rats offspring.* J Cardiovasc Pharmacol, 2002. **39**(3): p. 369-77.

170. Kind, K.L., et al., *Restricted fetal growth and the response to dietary cholesterol in the guinea pig.* Am J Physiol, 1999. **277**(6 Pt 2): p. R1675-82.

171. Simmons, R.A., L.J. Templeton, and S.J. Gertz, *Intrauterine growth retardation leads to the development of type 2 diabetes in the rat.* Diabetes, 2001. **50**(10): p. 2279-86.

172. Garofano, A., P. Czernichow, and B. Breant, *In utero undernutrition impairs rat beta-cell development.* Diabetologia, 1997. **40**(10): p. 1231-4.

173. Kind, K.L., et al., *Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig.* Am J Physiol Regul Integr Comp Physiol, 2003. **284**(1): p. R140-52.

174. Hyatt, M.A., et al., *Maternal nutrient restriction in early pregnancy programs hepatic mRNA expression of growth-related genes and liver size in adult male sheep.* J Endocrinol, 2007. **192**(1): p. 87-97.

175. Brameld, J.M., et al., *Maternal nutrition alters the expression of insulin-like growth factors in fetal sheep liver and skeletal muscle.* J Endocrinol, 2000. **167**(3): p. 429-37.

176. Heasman, L., et al., *Maternal nutrient restriction during early to mid gestation alters the relationship between insulin-like growth factor I and bodyweight at term in fetal sheep.* Reprod Fertil Dev, 2000. **12**(7-8): p. 345-50.

177. Jones, H.N., et al., *High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice.* Faseb j, 2009. **23**(1): p. 271-8.

178. Ashino, N.G., et al., *Maternal high-fat feeding through pregnancy and lactation predisposes mouse offspring to molecular insulin resistance and fatty liver.* J Nutr Biochem, 2012. **23**(4): p. 341-8.

179. Winzell, M.S. and B. Ahren, *The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes.* Diabetes, 2004. **53 Suppl 3**: p. S215-9.

180. Srinivasan, M., et al., *Maternal high-fat diet consumption results in fetal malprogramming predisposing to the onset of metabolic syndrome-like phenotype in adulthood.* Am J Physiol Endocrinol Metab, 2006. **291**(4): p. E792-9.

181. Woods, S.C., et al., *A controlled high-fat diet induces an obese syndrome in rats.* J Nutr, 2003. **133**(4): p. 1081-7.

182. Cerf, M.E., et al., *Islet cell response in the neonatal rat after exposure to a high-fat diet during pregnancy.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(5): p. R1122-8.

183. Ghebremeskel, K., et al., *Maternal diet high in fat reduces docosahexaenoic acid in liver lipids of newborn and sucking rat pups.* Br J Nutr, 1999. **81**(5): p. 395-404.

184. Khan, I.Y., et al., *Gender-linked hypertension in offspring of lard-fed pregnant rats.* Hypertension, 2003. **41**(1): p. 168-75.

185. Khan, I.Y., et al., *A high-fat diet during rat pregnancy or suckling induces cardiovascular dysfunction in adult offspring.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(1): p. R127-33.

186. Frias, A.E., et al., *Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition.* Endocrinology, 2011. **152**(6): p. 2456-64.

187. McCurdy, C.E., et al., *Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates.* J Clin Invest, 2009. **119**(2): p. 323-35.

188. Fan, L., et al., *Maternal high-fat diet impacts endothelial function in nonhuman primate offspring.* Int J Obes (Lond), 2013. **37**(2): p. 254-62.

189. Gohir, W., et al., *Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet.* Gut Microbes, 2015: p. 0.

190. Bayol, S.A., B.H. Simbi, and N.C. Stickland, *A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning.* J Physiol, 2005. **567**(Pt 3): p. 951-61.

191. Buettner, R., J. Scholmerich, and L.C. Bollheimer, *High-fat diets: modeling the metabolic disorders of human obesity in rodents.* Obesity (Silver Spring), 2007. **15**(4): p. 798-808.

192. Buettner, R., et al., *Defining high-fat-diet rat models: metabolic and molecular effects of different fat types.* J Mol Endocrinol, 2006. **36**(3): p. 485-501.

193.   Farley, D., et al., *Feto-placental adaptations to maternal obesity in the baboon.* Placenta, 2009. **30**(9): p. 752-60.

194.   Gorski, J.N., et al., *Postnatal environment overrides genetic and prenatal factors influencing offspring obesity and insulin resistance.* Am J Physiol Regul Integr Comp Physiol, 2006. **291**(3): p. R768-78.

195.   Caluwaerts, S., et al., *Diet-induced obesity in gravid rats engenders early hyperadiposity in the offspring.* Metabolism, 2007. **56**(10): p. 1431-8.

196.   Morris, M.J. and H. Chen, *Established maternal obesity in the rat reprograms hypothalamic appetite regulators and leptin signaling at birth.* Int J Obes (Lond), 2009. **33**(1): p. 115-22.

197.   Hausman, D.B., H.M. McCloskey, and R.J. Martin, *Maternal dietary fat type influences the growth and fatty acid composition of newborn and weanling rats.* J Nutr, 1991. **121**(12): p. 1917-23.

198.   Rasmussen, K.M., *Effects of under- and overnutrition on lactation in laboratory rats.* J Nutr, 1998. **128**(2 Suppl): p. 390s-393s.

199.   Ferezou-Viala, J., et al., *Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(3): p. R1056-62.

200.   Liang, C., M.E. Oest, and M.R. Prater, *Intrauterine exposure to high saturated fat diet elevates risk of adult-onset chronic diseases in C57BL/6 mice.* Birth Defects Res B Dev Reprod Toxicol, 2009. **86**(5): p. 377-84.

201.   Taylor, P.D. and L. Poston, *Developmental programming of obesity in mammals.* Exp Physiol, 2007. **92**(2): p. 287-98.

202.   Bouanane, S., et al., *Hepatic and very low-density lipoprotein fatty acids in obese offspring of overfed dams.* Metabolism, 2010. **59**(12): p. 1701-9.

203.   White, C.L., M.N. Purpera, and C.D. Morrison, *Maternal obesity is necessary for programming effect of high-fat diet on offspring.* Am J Physiol Regul Integr Comp Physiol, 2009. **296**(5): p. R1464-72.

204.   Kim, K.A., et al., *High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway.* PLoS One, 2012. **7**(10): p. e47713.

205.   Vuguin, P.M., et al., *Shared effects of genetic and intrauterine and perinatal environment on the development of metabolic syndrome.* PLoS One, 2013. **8**(5): p. e63021.

206.   Mark, P., et al., *A maternal high-fat diet in rat pregnancy reduces growth of the fetus and the placental junctional zone, but not placental labyrinth zone growth.* Journal of Developmental Origins of Health and Disease, 2011. **2**(01): p. 63-70.

207.   Aagaard-Tillery, K.M., et al., *Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome.* J Mol Endocrinol, 2008. **41**(2): p. 91-102.

208.   Adams Waldorf, K.M., et al., *Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys.* Reprod Sci, 2008. **15**(2): p. 121-7.

209.   Bayol, S.A., S.J. Farrington, and N.C. Stickland, *A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring.* Br J Nutr, 2007. **98**(4): p. 843-51.

210. Plagemann, A., et al., *Increased number of galanin-neurons in the paraventricular hypothalamic nucleus of neonatally overfed weanling rats.* Brain Res, 1999. **818**(1): p. 160-3.
211. Schmidt, I., et al., *The effect of leptin treatment on the development of obesity in overfed suckling Wistar rats.* Int J Obes Relat Metab Disord, 2001. **25**(8): p. 1168-74.
212. Muhlhausler, B.S., et al., *Impact of glucose infusion on the structural and functional characteristics of adipose tissue and on hypothalamic gene expression for appetite regulatory neuropeptides in the sheep fetus during late gestation.* J Physiol, 2005. **565**(Pt 1): p. 185-95.
213. Kirk, S.L., et al., *Maternal obesity induced by diet in rats permanently influences central processes regulating food intake in offspring.* PLoS One, 2009. **4**(6): p. e5870.
214. Bouret, S.G., S.J. Draper, and R.B. Simerly, *Trophic action of leptin on hypothalamic neurons that regulate feeding.* Science, 2004. **304**(5667): p. 108-10.
215. Gupta, A., et al., *Hypothalamic alterations in fetuses of high fat diet-fed obese female rats.* J Endocrinol, 2009. **200**(3): p. 293-300.
216. Kim, S., et al., *Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model.* Gene, 2004. **340**(1): p. 99-109.
217. Strakovsky, R.S., et al., *Gestational high fat diet programs hepatic phosphoenolpyruvate carboxykinase gene expression and histone modification in neonatal offspring rats.* J Physiol, 2011. **589**(Pt 11): p. 2707-17.
218. Turnbaugh, P.J., et al., *An obesity-associated gut microbiome with increased capacity for energy harvest.* Nature, 2006. **444**(7122): p. 1027-31.
219. Schneeberger, M., et al., *Akkermansia muciniphila inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice.* Sci Rep, 2015. **5**: p. 16643.
220. Whitman, W.B., D.C. Coleman, and W.J. Wiebe, *Prokaryotes: the unseen majority.* Proc Natl Acad Sci U S A, 1998. **95**(12): p. 6578-83.
221. Chow, J., et al., *Host-bacterial symbiosis in health and disease.* Adv Immunol, 2010. **107**: p. 243-74.
222. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
223. Nieuwdorp, M., et al., *Role of the microbiome in energy regulation and metabolism.* Gastroenterology, 2014. **146**(6): p. 1525-33.
224. Samuel, B.S., et al., *Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41.* Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16767-72.
225. Round, J.L. and S.K. Mazmanian, *The gut microbiota shapes intestinal immune responses during health and disease.* Nat Rev Immunol, 2009. **9**(5): p. 313-23.
226. Cebra, J.J., *Influences of microbiota on intestinal immune system development.* Am J Clin Nutr, 1999. **69**(5): p. 1046s-1051s.
227. Isolauri, E., et al., *Lactobacillus casei strain GG reverses increased intestinal permeability induced by cow milk in suckling rats.* Gastroenterology, 1993. **105**(6): p. 1643-50.
228. Ewaschuk, J.B., et al., *Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function.* Am J Physiol Gastrointest Liver Physiol, 2008. **295**(5): p. G1025-34.

229. Thompson, G.R. and P.C. Trexler, *Gastrointestinal structure and function in germ-free or gnotobiotic animals.* Gut, 1971. **12**(3): p. 230-5.
230. De Filippo, C., et al., *Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14691-6.
231. Turnbaugh, P.J., et al., *Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome.* Cell Host Microbe, 2008. **3**(4): p. 213-23.
232. de La Serre, C.B., et al., *Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut microbiota and gut inflammation.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(2): p. G440-8.
233. Claesson, M.J., et al., *Gut microbiota composition correlates with diet and health in the elderly.* Nature, 2012. **488**(7410): p. 178-84.
234. Yatsunenko, T., et al., *Human gut microbiome viewed across age and geography.* Nature, 2012. **486**(7402): p. 222-7.
235. Ley, R.E., et al., *Obesity alters gut microbial ecology.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.
236. Everard, A., et al., *Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.* Proc Natl Acad Sci U S A, 2013. **110**(22): p. 9066-71.
237. Louis, S., et al., *Characterization of the Gut Microbial Community of Obese Patients Following a Weight-Loss Intervention Using Whole Metagenome Shotgun Sequencing.* PLoS One, 2016. **11**(2): p. e0149564.
238. Dao, M.C., et al., *Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology.* Gut, 2016. **65**(3): p. 426-36.
239. Remely, M., et al., *Gut microbiota composition correlates with changes in body fat content due to weight loss.* Benef Microbes, 2015. **6**(4): p. 431-9.
240. Rajilic-Stojanovic, M., et al., *Phylogenetic analysis of dysbiosis in ulcerative colitis during remission.* Inflamm Bowel Dis, 2013. **19**(3): p. 481-8.
241. Png, C.W., et al., *Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria.* Am J Gastroenterol, 2010. **105**(11): p. 2420-8.
242. Collado, M.C., et al., *Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly.* Appl Environ Microbiol, 2007. **73**(23): p. 7767-70.
243. Hansen, C.H., et al., *Early life treatment with vancomycin propagates Akkermansia muciniphila and reduces diabetes incidence in the NOD mouse.* Diabetologia, 2012. **55**(8): p. 2285-94.
244. Karlsson, C.L., et al., *The microbiota of the gut in preschool children with normal and excessive body weight.* Obesity (Silver Spring), 2012. **20**(11): p. 2257-61.
245. Le Chatelier, E., et al., *Richness of human gut microbiome correlates with metabolic markers.* Nature, 2013. **500**(7464): p. 541-6.
246. Johansson, M.E., J.M. Larsson, and G.C. Hansson, *The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions.* Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4659-65.
247. Derrien, M., et al., *Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium.* Int J Syst Evol Microbiol, 2004. **54**(Pt 5): p. 1469-76.

248.   Reunanen, J., et al., *Akkermansia muciniphila Adheres to Enterocytes and Strengthens the Integrity of the Epithelial Cell Layer.* Appl Environ Microbiol, 2015. **81**(11): p. 3655-62.

249.   Koren, O., et al., *Host remodeling of the gut microbiome and metabolic changes during pregnancy.* Cell, 2012. **150**(3): p. 470-80.

250.   Perez, P.F., et al., *Bacterial imprinting of the neonatal immune system: lessons from maternal cells?* Pediatrics, 2007. **119**(3): p. e724-32.

251.   Collado, M.C., et al., *Distinct composition of gut microbiota during pregnancy in overweight and normal-weight women.* Am J Clin Nutr, 2008. **88**(4): p. 894-9.

252.   Everard, A., et al., *Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice.* Diabetes, 2011. **60**(11): p. 2775-86.

253.   Cani, P.D., et al., *Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia.* Diabetologia, 2007. **50**(11): p. 2374-83.

254.   Nadal, I., et al., *Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents.* Int J Obes (Lond), 2009. **33**(7): p. 758-67.

255.   Yoshinaga, K., R.A. Hawkins, and J.F. Stocker, *Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy.* Endocrinology, 1969. **85**(1): p. 103-12.

256.   Gibori, G., E. Antczak, and I. Rothchild, *The role of estrogen in the regulation of luteal progesterone secretion in the rat after day 12 of pregnancy.* Endocrinology, 1977. **100**(6): p. 1483-95.

257.   Albrecht, E.D., G.W. Aberdeen, and G.J. Pepe, *The role of estrogen in the maintenance of primate pregnancy.* Am J Obstet Gynecol, 2000. **182**(2): p. 432-8.

258.   Henricks, D.M., et al., *Plasma estrogen and progesterone levels after mating, and during late pregnancy and postpartum in cows.* Endocrinology, 1972. **90**(5): p. 1336-42.

259.   Tulchinsky, D., et al., *Plasma estrone, estradiol, estriol, progesterone, and 17-hydroxyprogesterone in human pregnancy. I. Normal pregnancy.* Am J Obstet Gynecol, 1972. **112**(8): p. 1095-100.

260.   SIITERI, P.K. and P.C. MacDonald, *Placental Estrogen Biosynthesis During Human Pregnancy 1.* The Journal of Clinical Endocrinology & Metabolism, 1966. **26**(7): p. 751-761.

261.   Raftogianis, R., et al., *Estrogen metabolism by conjugation.* J Natl Cancer Inst Monogr, 2000(27): p. 113-24.

262.   Goldin, B.R., et al., *Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women.* N Engl J Med, 1982. **307**(25): p. 1542-7.

263.   Flores, R., et al., *Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: a cross-sectional study.* J Transl Med, 2012. **10**: p. 253.

264.   Heimer, G.M. and D.E. Englund, *Enterohepatic recirculation of oestriol studied in cholecystectomized and non-cholecystectomized menopausal women.* Ups J Med Sci, 1984. **89**(2): p. 107-15.

265.   Levitz, M. and J. Katz, *Enterohepatic metabolism of estriol-3-sulfate-16-glucosiduronate in women.* J Clin Endocrinol Metab, 1968. **28**(6): p. 862-8.

266.   Winter, J. and V.D. Bokkenheuser, *Bacterial metabolism of natural and synthetic sex hormones undergoing enterohepatic circulation.* J Steroid Biochem, 1987. **27**(4-6): p. 1145-9.

267.   Van Eldere, J., et al., *Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans.* Appl Environ Microbiol, 1988. **54**(8): p. 2112-7.

268.   Aries, V., et al., *Degradation of bile salts by human intestinal bacteria.* Gut, 1969. **10**(7): p. 575-6.

269.   Shimizu, K., et al., *Normalization of reproductive function in germfree mice following bacterial contamination.* Exp Anim, 1998. **47**(3): p. 151-8.

270.   Syukuda, Y., *[Establishment of a new breeding colony of germfree CF no. 1 mice (author's transl)].* Jikken Dobutsu, 1978. **27**(3): p. 271-81.

271.   Reyniers, J.A., *Design and operation of apparatus for rearing germfree animals.* Annals of the New York Academy of Sciences, 1959. **78**(1): p. 47-79.

272.   Jarvenpaa, P., et al., *In vitro metabolism of estrogens by isolated intestinal micro-organisms and by human faecal microflora.* J Steroid Biochem, 1980. **13**(3): p. 345-9.

273.   Fuhrman, B.J., et al., *Associations of the fecal microbiome with urinary estrogens and estrogen metabolites in postmenopausal women.* J Clin Endocrinol Metab, 2014. **99**(12): p. 4632-40.

274.   Wada-Hiraike, O., et al., *Role of estrogen receptor beta in colonic epithelium.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2959-64.

275.   Menon, R., et al., *Diet complexity and estrogen receptor beta status affect the composition of the murine intestinal microbiota.* Appl Environ Microbiol, 2013. **79**(18): p. 5763-73.

276.   Uchihashi, M., et al., *Influence of age, reproductive cycling status, and menstruation on the vaginal microbiome in baboons (Papio anubis).* Am J Primatol, 2015. **77**(5): p. 563-78.

277.   Lorenzen, E., et al., *The vaginal microbiome is stable in prepubertal and sexually mature Ellegaard Gottingen Minipigs throughout an estrous cycle.* Vet Res, 2015. **46**: p. 125.

278.   Chaban, B., et al., *Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle.* Microbiome, 2014. **2**: p. 23.

279.   Ley, R.E., et al., *Microbial ecology: human gut microbes associated with obesity.* Nature, 2006. **444**(7122): p. 1022-3.

280.   Turnbaugh, P.J., et al., *A core gut microbiome in obese and lean twins.* Nature, 2009. **457**(7228): p. 480-4.

281.   Furet, J.P., et al., *Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss: links with metabolic and low-grade inflammation markers.* Diabetes, 2010. **59**(12): p. 3049-57.

282.   Santacruz, A., et al., *Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women.* Br J Nutr, 2010. **104**(1): p. 83-92.

283.   Turnbaugh, P.J., et al., *The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice.* Sci Transl Med, 2009. **1**(6): p. 6ra14.

284.   Paul, H.A., et al., *Diet-induced changes in maternal gut microbiota and metabolomic profiles influence programming of offspring obesity risk in rats.* Sci Rep, 2016. **6**: p. 20683.

285.   Hallam, M.C., et al., *Maternal high-protein or high-prebiotic-fiber diets affect maternal milk composition and gut microbiota in rat dams and their offspring.* Obesity (Silver Spring), 2014. **22**(11): p. 2344-51.

286.   Fujiwara, R., J. Watanabe, and K. Sonoyama, *Assessing changes in composition of intestinal microbiota in neonatal BALB/c mice through cluster analysis of molecular markers.* Br J Nutr, 2008. **99**(6): p. 1174-7.

287.   Bomhof, M.R., et al., *Combined effects of oligofructose and Bifidobacterium animalis on gut microbiota and glycemia in obese rats.* Obesity (Silver Spring), 2014. **22**(3): p. 763-71.

288.   Cluny, N.L., et al., *Interactive effects of oligofructose and obesity predisposition on gut hormones and microbiota in diet-induced obese rats.* Obesity (Silver Spring), 2015. **23**(4): p. 769-78.

289.   Cani, P.D., et al., *Oligofructose promotes satiety in rats fed a high-fat diet: involvement of glucagon-like Peptide-1.* Obes Res, 2005. **13**(6): p. 1000-7.

290.   Pettker, C.M., et al., *Value of placental microbial evaluation in diagnosing intra-amniotic infection.* Obstet Gynecol, 2007. **109**(3): p. 739-49.

291.   Rautava, S., et al., *Probiotics modulate host-microbe interaction in the placenta and fetal gut: a randomized, double-blind, placebo-controlled trial.* Neonatology, 2012. **102**(3): p. 178-84.

292.   Steel, J.H., et al., *Bacteria and inflammatory cells in fetal membranes do not always cause preterm labor.* Pediatr Res, 2005. **57**(3): p. 404-11.

293.   Jimenez, E., et al., *Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section.* Curr Microbiol, 2005. **51**(4): p. 270-4.

294.   Jimenez, E., et al., *Is meconium from healthy newborns actually sterile?* Res Microbiol, 2008. **159**(3): p. 187-93.

295.   Dandona, P., et al., *Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss.* J Clin Endocrinol Metab, 1998. **83**(8): p. 2907-10.

296.   Vozarova, B., et al., *Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion.* Obes Res, 2001. **9**(7): p. 414-7.

297.   Pradhan, A.D., et al., *C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus.* Jama, 2001. **286**(3): p. 327-34.

298.   Cai, D., et al., *Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB.* Nat Med, 2005. **11**(2): p. 183-90.

299.   Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes.* Diabetes, 2007. **56**(9): p. 2356-70.

300.   De Souza, C.T., et al., *Consumption of a fat-rich diet activates a proinflammatory response and induces insulin resistance in the hypothalamus.* Endocrinology, 2005. **146**(10): p. 4192-9.

301.   Saghizadeh, M., et al., *The expression of TNF alpha by human muscle. Relationship to insulin resistance.* J Clin Invest, 1996. **97**(4): p. 1111-6.

302.   Challier, J.C., et al., *Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta.* Placenta, 2008. **29**(3): p. 274-81.

303.   Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

304. Gay, N.J. and F.J. Keith, *Drosophila Toll and IL-1 receptor.* Nature, 1991. **351**(6325): p. 355-6.

305. Kirschning, C.J., et al., *Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide.* J Exp Med, 1998. **188**(11): p. 2091-7.

306. Yang, R.B., et al., *Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling.* Nature, 1998. **395**(6699): p. 284-8.

307. Kawai, T., et al., *Unresponsiveness of MyD88-deficient mice to endotoxin.* Immunity, 1999. **11**(1): p. 115-22.

308. Aderem, A. and R.J. Ulevitch, *Toll-like receptors in the induction of the innate immune response.* Nature, 2000. **406**(6797): p. 782-7.

309. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

310. Chow, J.C., et al., *Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction.* J Biol Chem, 1999. **274**(16): p. 10689-92.

311. Yang, H., et al., *Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1.* J Biol Chem, 2000. **275**(27): p. 20861-6.

312. Lee, J.Y., et al., *Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4.* J Biol Chem, 2001. **276**(20): p. 16683-9.

313. Shi, H., et al., *TLR4 links innate immunity and fatty acid-induced insulin resistance.* J Clin Invest, 2006. **116**(11): p. 3015-25.

314. Medzhitov, R., et al., *MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways.* Mol Cell, 1998. **2**(2): p. 253-8.

315. Zhang, F.X., et al., *Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes.* J Biol Chem, 1999. **274**(12): p. 7611-4.

316. Molteni, R.A., S.J. Stys, and F.C. Battaglia, *Relationship of fetal and placental weight in human beings: fetal/placental weight ratios at various gestational ages and birth weight distributions.* J Reprod Med, 1978. **21**(5): p. 327-34.

317. Silasi, M., et al., *Abnormal placentation, angiogenic factors, and the pathogenesis of preeclampsia.* Obstet Gynecol Clin North Am, 2010. **37**(2): p. 239-53.

318. Fowden, A.L., et al., *Placental efficiency and adaptation: endocrine regulation.* J Physiol, 2009. **587**(Pt 14): p. 3459-72.

319. McNamara, H., et al., *Feto-placental ratio: useful predictor of neonatal outcome in preterm infants.* Am J Obstet Gynecol, 1998. **178**: p. S123.

320. Reynolds, C.M., et al., *Maternal high fat and/or salt consumption induces sex-specific inflammatory and nutrient transport in the rat placenta.* Physiol Rep, 2015. **3**(5).

321. Martino, J., et al., *Maternal body weight and gestational diabetes differentially influence placental and pregnancy outcomes.* J Clin Endocrinol Metab, 2015: p. jc20152590.

322. Henao-Mejia, J., et al., *Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity.* Nature, 2012. **482**(7384): p. 179-85.

323. Hildebrand, F., et al., *Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice.* Genome Biol, 2013. **14**(1): p. R4.

324. Caligioni, C., *Assessing Reproductive Status/Stages in Mice.* Curr Protoc Neurosci, 2009. **Appendix**: p. Appendix-4I.

325. Whelan, F.J., et al., *The loss of topography in the microbial communities of the upper respiratory tract in the elderly.* Ann Am Thorac Soc, 2014. **11**(4): p. 513-21.

326. Martin, M., *Cutadapt removes adapter sequences from high-throughput sequencing reads.* EMBnet. journal, 2011. **17**(1): p. pp. 10-12.

327. Masella, A.P., et al., *PANDAseq: paired-end assembler for illumina sequences.* BMC Bioinformatics, 2012. **13**: p. 31.

328. Ye, Y., *Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment.* Proceedings (IEEE Int Conf Bioinformatics Biomed), 2011. **2010**: p. 153-157.

329. Wang, Q., et al., *Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.* Appl Environ Microbiol, 2007. **73**(16): p. 5261-7.

330. Caporaso, J.G., et al., *QIIME allows analysis of high-throughput community sequencing data.* Nat Methods, 2010. **7**(5): p. 335-6.

331. DeSantis, T.Z., et al., *Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB.* Appl Environ Microbiol, 2006. **72**(7): p. 5069-72.

332. McMurdie, P.J. and S. Holmes, *phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.* PLoS One, 2013. **8**(4): p. e61217.

333. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.

334. Wickham, H., *ggplot2: elegant graphics for data analysis*. 2009: Springer Science & Business Media.

335. Byers, S.L., et al., *Mouse estrous cycle identification tool and images.* PLoS One, 2012. **7**(4): p. e35538.

336. Liang, C., K. DeCourcy, and M.R. Prater, *High-saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice.* Metabolism, 2010. **59**(7): p. 943-50.

337. Mistry, A.M. and D.R. Romsos, *Intracerebroventricular leptin administration reduces food intake in pregnant and lactating mice.* Exp Biol Med (Maywood), 2002. **227**(8): p. 616-9.

338. Mistry, A.M., A.G. Swick, and D.R. Romsos, *Leptin rapidly lowers food intake and elevates metabolic rates in lean and ob/ob mice.* J Nutr, 1997. **127**(10): p. 2065-72.

339. Mukherjea, R., et al., *Elevated leptin concentrations in pregnancy and lactation: possible role as a modulator of substrate utilization.* Life Sci, 1999. **65**(11): p. 1183-93.

340. Henson, M.C., et al., *Serum leptin concentrations and expression of leptin transcripts in placental trophoblast with advancing baboon pregnancy.* J Clin Endocrinol Metab, 1999. **84**(7): p. 2543-9.

341. Tomimatsu, T., et al., *Increase of mouse leptin production by adipose tissue after midpregnancy: gestational profile of serum leptin concentration.* Biochem Biophys Res Commun, 1997. **240**(1): p. 213-5.

342. Chien, E.K., et al., *Increase in serum leptin and uterine leptin receptor messenger RNA levels during pregnancy in rats.* Biochem Biophys Res Commun, 1997. **237**(2): p. 476-80.

343. Garcia, M.D., et al., *Gestational profile of leptin messenger ribonucleic acid (mRNA) content in the placenta and adipose tissue in the rat, and regulation of the mRNA levels*

of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation. Biol Reprod, 2000. **62**(3): p. 698-703.

344.   Helland, I.B., et al., *Leptin levels in pregnant women and newborn infants: gender differences and reduction during the neonatal period.* Pediatrics, 1998. **101**(3): p. E12.

345.   Hardie, L., et al., *Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy.* Clin Endocrinol (Oxf), 1997. **47**(1): p. 101-6.

346.   Highman, T.J., et al., *Longitudinal changes in maternal serum leptin concentrations, body composition, and resting metabolic rate in pregnancy.* Am J Obstet Gynecol, 1998. **178**(5): p. 1010-5.

347.   Schubring, C., et al., *Levels of leptin in maternal serum, amniotic fluid, and arterial and venous cord blood: relation to neonatal and placental weight.* J Clin Endocrinol Metab, 1997. **82**(5): p. 1480-3.

348.   Schubring, C., et al., *Longitudinal analysis of maternal serum leptin levels during pregnancy, at birth and up to six weeks after birth: relation to body mass index, skinfolds, sex steroids and umbilical cord blood leptin levels.* Horm Res, 1998. **50**(5): p. 276-83.

349.   Sivan, E., et al., *Leptin in human pregnancy: the relationship with gestational hormones.* Am J Obstet Gynecol, 1998. **179**(5): p. 1128-32.

350.   Tamura, T., et al., *Serum leptin concentrations during pregnancy and their relationship to fetal growth.* Obstet Gynecol, 1998. **91**(3): p. 389-95.

351.   Butte, N.F., *Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus.* The American journal of clinical nutrition, 2000. **71**(5): p. 1256s-1261s.

352.   Henson, M.C. and V.D. Castracane, *Leptin in pregnancy.* Biology of Reproduction, 2000. **63**(5): p. 1219-1228.

353.   Kiess, W., et al., *Leptin as a metabolic regulator during fetal and neonatal life and in childhood and adolescence.* J Pediatr Endocrinol Metab, 1998. **11**(4): p. 483-96.

354.   Mantzoros, C.S. and S.J. Moschos, *Leptin: in search of role(s) in human physiology and pathophysiology.* Clin Endocrinol (Oxf), 1998. **49**(5): p. 551-67.

355.   Harigaya, A., et al., *Relationship between concentration of serum leptin and fetal growth.* The journal of clinical endocrinology & metabolism, 1997. **82**(10): p. 3281-3284.

356.   Koistinen, H.A., et al., *Leptin concentration in cord blood correlates with intrauterine growth.* J Clin Endocrinol Metab, 1997. **82**(10): p. 3328-30.

357.   Sierra-Honigmann, M.R., et al., *Biological action of leptin as an angiogenic factor.* Science, 1998. **281**(5383): p. 1683-6.

358.   Bouloumie, A., et al., *Leptin, the product of Ob gene, promotes angiogenesis.* Circ Res, 1998. **83**(10): p. 1059-66.

359.   Bennett, B.D., et al., *A role for leptin and its cognate receptor in hematopoiesis.* Curr Biol, 1996. **6**(9): p. 1170-80.

360.   Cioffi, J.A., et al., *Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction.* Nat Med, 1996. **2**(5): p. 585-9.

361.   Hirose, H., et al., *Serum leptin level: possible association with haematopoiesis in adolescents, independent of body mass index and serum insulin.* Clin Sci (Lond), 1998. **94**(6): p. 633-6.

362.   Yuen, B.S., et al., *Abundance of leptin mRNA in fetal adipose tissue is related to fetal body weight.* J Endocrinol, 1999. **163**(3): p. R11-4.

363.   Clapp, J.F. and W. Kiess, *Cord blood leptin reflects fetal fat mass.* Journal of the Society for Gynecologic Investigation, 1998. **5**(6): p. 300-303.

364.   Hassink, S.G., et al., *Placental leptin: an important new growth factor in intrauterine and neonatal development?* Pediatrics, 1997. **100**(1): p. E1.

365.   Matsuda, J., et al., *Serum leptin concentration in cord blood: relationship to birth weight and gender.* J Clin Endocrinol Metab, 1997. **82**(5): p. 1642-4.

366.   Varvarigou, A., C.S. Mantzoros, and N.G. Beratis, *Cord blood leptin concentrations in relation to intrauterine growth.* Clinical endocrinology, 1999. **50**(2): p. 177-183.

367.   Geary, M., et al., *Leptin concentrations in maternal serum and cord blood: relationship to maternal anthropometry and fetal growth.* Br J Obstet Gynaecol, 1999. **106**(10): p. 1054-60.

368.   Butte, N.F., J.M. Hopkinson, and M.A. Nicolson, *Leptin in human reproduction: serum leptin levels in pregnant and lactating women.* J Clin Endocrinol Metab, 1997. **82**(2): p. 585-9.

369.   Lin, K.C., et al., *Difference of plasma leptin levels in venous and arterial cord blood: relation to neonatal and placental weight.* Kaohsiung J Med Sci, 1999. **15**(12): p. 679-85.

370.   Yura, S., et al., *A positive umbilical venous-arterial difference of leptin level and its rapid decline after birth.* Am J Obstet Gynecol, 1998. **178**(5): p. 926-30.

371.   Senaris, R., et al., *Synthesis of leptin in human placenta.* Endocrinology, 1997. **138**(10): p. 4501-4.

372.   Sattar, N., et al., *Leptin levels in pregnancy: marker for fat accumulation and mobilization?* Acta Obstet Gynecol Scand, 1998. **77**(3): p. 278-83.

373.   Grinspoon, S., et al., *Serum leptin levels in women with anorexia nervosa.* J Clin Endocrinol Metab, 1996. **81**(11): p. 3861-3.

374.   Casanueva, F.F., et al., *Serum immunoreactive leptin concentrations in patients with anorexia nervosa before and after partial weight recovery.* Biochem Mol Med, 1997. **60**(2): p. 116-20.

375.   Kim, D.W., et al., *Obesity during pregnancy disrupts placental morphology, cell proliferation, and inflammation in a sex-specific manner across gestation in the mouse.* Biology of reproduction, 2014. **90**(6): p. 130.

376.   Stewart, F.M., et al., *Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers.* J Clin Endocrinol Metab, 2007. **92**(3): p. 969-75.

377.   Basu, S., et al., *Pregravid obesity associates with increased maternal endotoxemia and metabolic inflammation.* Obesity (Silver Spring), 2011. **19**(3): p. 476-82.

378.   Roberts, K.A., et al., *Placental structure and inflammation in pregnancies associated with obesity.* Placenta, 2011. **32**(3): p. 247-54.

379.   Aye, I.L., et al., *Increasing maternal body mass index is associated with systemic inflammation in the mother and the activation of distinct placental inflammatory pathways.* Biology of reproduction, 2014. **90**(6): p. 129.

380.   Madan, J.C., et al., *Maternal obesity and markers of inflammation in pregnancy.* Cytokine, 2009. **47**(1): p. 61-64.

381.   Founds, S.A., et al., *A comparison of circulating TNF-alpha in obese and lean women with and without preeclampsia.* Hypertens Pregnancy, 2008. **27**(1): p. 39-48.

382.   Winkler, G., et al., *Tumor necrosis factor system in insulin resistance in gestational diabetes.* Diabetes Res Clin Pract, 2002. **56**(2): p. 93-9.

383. Linnemann, K., et al., *Leptin production and release in the dually in vitro perfused human placenta.* J Clin Endocrinol Metab, 2000. **85**(11): p. 4298-301.

384. Hunt, J.S., *Macrophages in human uteroplacental tissues: a review.* Am J Reprod Immunol, 1989. **21**(3-4): p. 119-22.

385. Minami, M., et al., *Estrous cycle and vaginal flora in conventionalized and gnotobiotic BALB/c mice.* BIFIDUS-FLORES ET FRUCTUS., 1987. **1**(1): p. 25-32.

386. Santiago, G.L., et al., *Longitudinal study of the dynamics of vaginal microflora during two consecutive menstrual cycles.* PLoS One, 2011. **6**.

387. Fischer, C.C., R.E. Persson, and G.R. Persson, *Influence of the menstrual cycle on the oral microbial flora in women: a case-control study including men as control subjects.* J Periodontol, 2008. **79**(10): p. 1966-73.

388. McCafferty, J., et al., *Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model.* Isme j, 2013. **7**(11): p. 2116-25.

389. Arthur, J.C., et al., *Intestinal inflammation targets cancer-inducing activity of the microbiota.* Science, 2012. **338**(6103): p. 120-3.

390. Deloris Alexander, A., et al., *Quantitative PCR assays for mouse enteric flora reveal strain-dependent differences in composition that are influenced by the microenvironment.* Mamm Genome, 2006. **17**(11): p. 1093-104.

391. Campbell, J.H., et al., *Host genetic and environmental effects on mouse intestinal microbiota.* Isme j, 2012. **6**(11): p. 2033-44.

392. McCormack, J.T. and G.S. Greenwald, *Progesterone and oestradiol-17beta concentrations in the peripheral plasma during pregnancy in the mouse.* J Endocrinol, 1974. **62**(1): p. 101-7.

393. Wood, G.A., et al., *Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus.* Reproduction, 2007. **133**(5): p. 1035-44.

394. Walmer, D.K., et al., *Lactoferrin expression in the mouse reproductive tract during the natural estrous cycle: correlation with circulating estradiol and progesterone.* Endocrinology, 1992. **131**(3): p. 1458-66.

395. Fata, J.E., V. Chaudhary, and R. Khokha, *Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle.* Biol Reprod, 2001. **65**(3): p. 680-8.

396. Murr, S.M., et al., *Plasma progesterone during pregnancy in the mouse.* Endocrinology, 1974. **94**(4): p. 1209-11.

397. Choudary, J.B. and G.S. Greenwald, *Ovarian activity in the intact or hypophysectomized pregnant mouse.* Anat Rec, 1969. **163**(3): p. 359-72.

398. Kosaka, T., T.R. Saito, and K.W. Takahashi, *Changes in plasma progesterone levels during the estrous cycle and pregnancy in 4-day cyclic mice.* Jikken Dobutsu, 1988. **37**(3): p. 351-3.

399. Michael, S.D., *Plasma prolactin and progesterone during the estrous cycle in the mouse.* Proc Soc Exp Biol Med, 1976. **153**(2): p. 254-7.

400. Ramsay, J.E., et al., *Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways.* J Clin Endocrinol Metab, 2002. **87**(9): p. 4231-7.

401. Kameyama, K. and K. Itoh, *Intestinal colonization by a Lachnospiraceae bacterium contributes to the development of diabetes in obese mice.* Microbes Environ, 2014. **29**(4): p. 427-30.

402. Larsen, N., et al., *Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults.* PLoS One, 2010. **5**(2): p. e9085.

403. Karlsson, F.H., et al., *Gut metagenome in European women with normal, impaired and diabetic glucose control.* Nature, 2013. **498**(7452): p. 99-103.

404. Qin, J., et al., *A metagenome-wide association study of gut microbiota in type 2 diabetes.* Nature, 2012. **490**(7418): p. 55-60.

405. Wu, X., et al., *Molecular characterisation of the faecal microbiota in patients with type II diabetes.* Curr Microbiol, 2010. **61**(1): p. 69-78.

406. Marlene, R., et al., *Abundance and diversity of microbiota in type 2 diabetes and obesity.* Journal of Diabetes & Metabolism, 2013. **2013**.

407. Zhang, X., et al., *Human gut microbiota changes reveal the progression of glucose intolerance.* PLoS One, 2013. **8**(8): p. e71108.

408. Ciubotaru, I., et al., *Significant differences in fecal microbiota are associated with various stages of glucose tolerance in African American male veterans.* Transl Res, 2015. **166**(5): p. 401-11.

409. Salyers, A.A., et al., *Fermentation of mucins and plant polysaccharides by anaerobic bacteria from the human colon.* Appl Environ Microbiol, 1977. **34**(5): p. 529-33.

410. Ding, S., et al., *High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse.* PLoS One, 2010. **5**(8): p. e12191.

411. Biswas, S.K. and A. Sodhi, *In vitro activation of murine peritoneal macrophages by monocyte chemoattractant protein-1: upregulation of CD11b, production of proinflammatory cytokines, and the signal transduction pathway.* J Interferon Cytokine Res, 2002. **22**(5): p. 527-38.

412. Fan, S.T. and T.S. Edgington, *Coupling of the adhesive receptor CD11b/CD18 to functional enhancement of effector macrophage tissue factor response.* J Clin Invest, 1991. **87**(1): p. 50-7.

413. Ding, A., S.D. Wright, and C. Nathan, *Activation of mouse peritoneal macrophages by monoclonal antibodies to Mac-1 (complement receptor type 3).* J Exp Med, 1987. **165**(3): p. 733-49.

414. Zhu, M., et al., *Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta.* Placenta, 2010. **31**(5): p. 387-391.

415. Nguyen, T., et al., *IL-10 produced by trophoblast cells inhibits phagosome maturation leading to profound intracellular proliferation of Salmonella enterica Typhimurium.* Placenta, 2013. **34**(9): p. 765-74.

416. Stephens, J.M., et al., *Tumor necrosis factor alpha-induced glucose transporter (GLUT-1) mRNA stabilization in 3T3-L1 preadipocytes. Regulation by the adenosine-uridine binding factor.* J Biol Chem, 1992. **267**(12): p. 8336-41.

417. Cornelius, P., et al., *Regulation of lipoprotein lipase mRNA content in 3T3-L1 cells by tumour necrosis factor.* Biochem J, 1988. **249**(3): p. 765-9.

418. Cornelius, P., et al., *The growth factor-like effects of tumor necrosis factor-alpha. Stimulation of glucose transport activity and induction of glucose transporter and immediate early gene expression in 3T3-L1 preadipocytes.* J Biol Chem, 1990. **265**(33): p. 20506-16.

419.   Aye, I.L., T. Jansson, and T.L. Powell, *TNF-alpha stimulates System A amino acid transport in primary human trophoblast cells mediated by p38 MAPK signaling.* Physiol Rep, 2015. **3**(10).

420.   Gordon, M.C., et al., *Insulin and glucose modulate glucose transporter messenger ribonucleic acid expression and glucose uptake in trophoblasts isolated from first-trimester chorionic villi.* Am J Obstet Gynecol, 1995. **173**(4): p. 1089-97.

421.   Karl, P.I., K.L. Alpy, and S.E. Fisher, *Amino acid transport by the cultured human placental trophoblast: effect of insulin on AIB transport.* Am J Physiol, 1992. **262**(4 Pt 1): p. C834-9.

422.   Jansson, N., et al., *Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments.* J Clin Endocrinol Metab, 2003. **88**(3): p. 1205-11.