School of Medicine

Division of Lifespan and Population Health

University of Nottingham

Defining the impact of paternal diet on maternal cardiometabolic ill-health in late gestation

by Afsaneh Khoshkerdar

Supervisors: Dr Adam Watkins

Supervisors: Dr Katie Woad

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Abstract

It is well-established that poor maternal nutrition prior to, or during, pregnancy can have a range of adverse consequences for the health of the mother and her offspring. For the mother, these consequences can become manifest in an increased risk of developing conditions such as preeclampsia and gestational diabetes. For her offspring, studies have shown an increased predisposition towards a series of non-communicable diseases including obesity, type-2 diabetes, and cardiovascular disease. Such connections between maternal health and offspring well-being have been defined over recent decades through the detailed examination of human epidemiological cohorts as well as a multitude of animal models.

While the association between poor maternal health and perturbed offspring development is widely acknowledged, the role that a father plays in directing the development and long-term health of his offspring has been overlooked. However, it is becoming increasingly evident that the father plays a more significant role than just contributing to his progeny's genome. Similar to the established maternal programming studies, the use of historical epidemiological data sets, supported by mechanistic animal models, reveals a complex process through which poor paternal health influences post-fertilization development and offspring well-being. Such observations have underpinned a new field within the Developmental Origins of Health and Disease (DOHaD) hypothesis, known as the Paternal Origins of Health and Disease (POHaD).

In particular, studies have focused on the role of paternal nutrition, being either increased or decreased in specific macro- and micro-nutrients including protein, fat, sugar and folate. Through these studies, a complex system involving perturbed testicular function and spermatogenesis, epididymal maturation and exosomal modifications, sperm epigenetic status and DNA integrity as well as seminal plasma composition have all been identified as mediators of paternal programming.

Therefore, this thesis aimed to investigate the impact of a paternal suboptimal diet on male physiology, fetal development, and late gestation maternal cardio-metabolic health. Furthermore, as altered sperm epigenetic status has been identified as one central programming mechanism, the impact of methyl-donor supplementation was also investigated to determine if this could negate the detrimental effects of the suboptimal diets. Additionally, as poor maternal health in pregnancy is a significant risk factor for altered fetal development, I also assessed the impact of paternal diet on maternal cardio-metabolic status in late gestation.

To achieve these aims, male C57/BL6J mice were fed one of the five diets; a control diet (CD, 18% casein, 21% sugar, 10% fat), low protein diet (LPD, 9% casein, 24% sugar, 10% fat), Western diet (WD, 19% casein, 34% sugar, 21% fat), LPD supplemented with methyl donors (MDLPD, 15% D, L-methionine, 7.5% betaine, 5% choline chloride) or WD supplemented with methyl donors (MDWD) for a minimum of 7 weeks. Males were mated with chow-fed, virgin 8–12-week-old C57BL/6J females. At embryonic day 17.5, dams were culled for the collection of a range of maternal and fetal tissues. At the end of the study, stud males were then culled for the analysis of male organ size.

I observed that male weight was not altered in response to being fed any of the experimental diets. However, I did observe differential sizing of several organs, especially within the WD and MDWD males. Of note, was an increase in gonadal fat in these males, indicating that while total body weight was not altered, adiposity appeared to increase. Following mating, there were no detrimental impacts of any diet on male fertility, as determined by late gestation litter size. While fetal growth and organ sizing appeared largely unaffected by the paternal diets, analysis of the fetal cardiac transcriptome revealed altered expression of multiple genes. In LPD and MDL fetuses, I observed differential expression of genes within central lipid, amino acid, and carbohydrate metabolic processes as well as cardiovascular disease pathways. In contrast, WD hearts demonstrated upregulation of genes involved in angiogenesis, embryonic organ development, and lipid metabolism as well as an abnormal heart morphology phenotype.

Analysis of placental morphology and gene expression revealed no difference in overall morphology or sizing of the labyrinth (Lz) or junctional (Jz) zones. Using Masson's Trichrome staining to visualize the amount of connective tissue within the placenta, revealed a significant reduction in the amount of overall staining within WD and MDWD placentas. Furthermore, I observed reduced expression of genes central within the renin-angiotensin system (RAS), apoptosis and one-carbon metabolism pathways within WD and MDWD placentas.

Finally, analysis of maternal cardiometabolic health in late gestation showed there to be minimal changes in serum and hepatic metabolites, gut microbiota or mediators of cardiovascular function in response to paternal diet.

These observations are of interest to human health as they demonstrate that in pregnancies uncomplicated by male infertility, or perturbed by maternal metabolic health, fetal cardiovascular programming still occurred. Furthermore, changes in fetal cardiac gene expression occurred in response to both a relatively moderate (LPD) and severe (WD) paternal dietary challenge. Further studies are required to define the underlying mechanisms through which poor paternal diet impacts fetal heart gene expression and the consequences of these changes for long-term offspring health.

Declaration

I hereby declare that this thesis is the result of my own work, as well as the collaboration that has been specified in the text. This work has not been submitted elsewhere for receiving any degrees or awards.

Afsaneh Khoshkerdar, DVM, MMedSci

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Abbreviations

ACE – Angiotensin-converting enzyme

ADHD – Attention-deficit hyperactivity disorder

ADHD – Attention-deficit hyperactivity disorder

AGT – Angiotensin

ART–Assisted reproduction technology

AT1 – Ang II type 1

AUD – Alcohol use disorders

BMI – Body Mass Index

C-C motif – Chemokine

CHD – coronary heart disease

CpG – 5′-Cytosine-phosphate-Guanine

CRP – C-reactive protein

CSF2 – Colony-stimulating factor 2

c-TGCs – Canal-associated TGCs

CT – Cytotrophoblast

CVD – Cardiovascular disease

DOHaD – Developmental Origins of Health and Disease

dpf – Days post fertilization

EDCs – Endocrine disruptors

EPC – Ectoplacental cone

EVT – Extra villous trophoblast

ExE – Extraembryonic ectoderm

FGF21– Fibroblast growth factor 21

GCs – Glycogen cells

GDM – Gestational diabetes mellites

GH – growth hormone

GI – Genomic imprinting

GMCSF – Granulocyte-macrophage colony-stimulating factor

GR– Glucocorticoid receptor

HBCS – Helsinki Birth Cohort Study

hCG – Human chorionic gonadotropin

HLA-G – Leukocyte Antigen-G

HRP- Horseradish peroxidase

ICM- Inner cell mass

ICR – Imprinting Control Regions

ICSI – Intra cytoplasmic sperm injection

IGF – Insulin-like growth factor

IGMR – Intergenerational Metabolic Reprogramming

IL – Interleukin

IUGR – Intra Uterine Growth Retardation

IVF – In vitro fertilization

JZ – Junctional zone

LBW – Low birth weight

LGA – Large for gestational age

LGA – Large for gestational age

LIF – Leukaemia inhibitory factor

LOPE – Late-onset preeclampsia

LZ – Labyrinth zone

MCP-1 – Monocyte chemoattractant protein 1

miRNA – Micro RNA

MMPs- Matrix metalloproteinases

MS- Multiple sclerosis

MT – Masson's Trichrome

MTHFR – Methylenetetrahydrofolate reductase

NOS- Nitric oxide synthesis

p-TGCs - Parietal-TGCs

PN – Pronuclear

PTHrP – Parathyroid hormone-related protein

RA- Rheumatoid arthritis

RAS- Renin angiotensin system

SI-IEL – Small intestine intraepithelial lymphocyte

sncRNA – Small non-coding RNA

(SpT) – Spongiotrophoblast

SSBs – Sugar-sweetened beverages (SSBs)

STB–Syncytiotrophoblast

TE–Trophectoderm

TRAIL– TNF-α -related apoptosis-inducing ligand

Tregs–T regulatory cells

uNK–Uterine natural killer cells

uPA– Urokinase-type plasminogen activator

VEGF–Vascular endothelial growth factor

vitB12 – Vitamin B12

zBMI – BMI z-score

1 Chapter 1 Introduction

1.1 Maternal metabolic and cardiovascular adaptation during pregnancy

1.1.1 General alterations in maternal physiology

In humans, pregnancy is a period of ~40 weeks in which the mother must undergo a series of remarkable, orchestrated physiological adaptations to support the development of a healthy progeny. These maternal physiological adaptations affect a range of organs including the heart, liver, adipose and wider vascular systems with the main aim of providing the growing fetus with adequate and continuous nutrients. However, these metabolic and cardiovascular alterations occur in such a manner as to align with the needs of the developing fetus. While gestation is a critical time for ensuring adequate embryonic and fetal development, it is also recognized as a time when poor maternal health can shape the long-term health of her child (Stern et al., 2021, Care et al., 2018).

The central aspect of maternal adaptation is intricately linked to the placenta, which serves as a prominent source of hormones and growth factor secretion into the maternal systemic circulation, resulting in numerous physiological consequences (Napso et al., 2018). The physiological and hormonal changes induce a large impact change on maternal body organs, including the cardiovascular, metabolic, and immune functionalities. In addition, early pregnancy is also characterized by significant hypertrophy of multiple maternal organs such as the liver, kidneys, pancreas and adipose tissues (Sferruzzi-Perri et al., 2020).

One of the first, and most significant, maternal metabolic adaptations during pregnancy is a change in the production and sensitivity to insulin during the first trimester. This results in either unchanged or increased insulin sensitivity, observed in both women and mice, which in turn modulates the availability of glucose for fetal growth (Parrettini et al., 2020, Musial et al., 2016).

This modulation of glucose availability is accompanied by a reduction in fasting blood glucose levels, aligning with the storage of a significant amount of glucose for later stages in pregnancy, when the developing fetus demands the highest level of glucose (Hadden and McLaughlin, 2009, Plows et al., 2018, Sonagra et al., 2014).

Insulin sensitivity plays a central role in the growth of the developing fetus, primarily through the adjustment of glucose metabolism and nutrient supplementation. The maternal glucose level plays a pivotal role in fetal growth, as it is transported across the placenta. The developing fetus's growth is substantially affected by insulin sensitivity, primarily through the regulation of glucose metabolism and nutrient supplementation. As maternal glucose is transported across the placenta, the maternal glucose level plays a crucial role in fetal growth. The process of insulin sensitivity modulates fetal insulin secretion and actively stimulates fetal growth. Maternal insulin sensitivity by modulating the fetal insulin secretion process, actively promotes fetal growth (Warrington et al., 2019). In both women and mice, insulin sensitivity fluctuates throughout the normal course of pregnancy, encompassing various trimesters in women and stages of pregnancy in mice. This increase in insulin levels could result from variations in insulin responses across different organs, with a more pronounced effect observed in skeletal muscles compared to adipose tissues (Di Cianni et al., 2003).

During pregnancy, insulin sensitivity decreases or insulin resistance increases, in line with an increase in insulin production of up to 50% (Stern et al., 2021). This increase in insulin level could be the result of the expansion in the number of pancreatic β-cells within the islets of Langerhans through both hyperplasia and hypertropia, as well as a reduction in apoptosis within the islets (Ernst et al., 2011, Huang et al., 2009). Additionally, at the end of the second trimester of pregnancy in women (Hufnagel et al., 2022), placental hormones such as oestrogen, progesterone, leptin, cortisol, placental lactogen and placental growth hormone modulate insulin secretion (Napso et al., 2018). An increase in the expression of prolactin receptors results in an expansion of pancreatic β-cells mass (Banerjee et al., 2016). This phenomenon promotes a rise in glucose levels

in maternal blood for maintaining euglycemia and facilitates the transportation of glucose across the placenta (Hufnagel et al., 2022).

Considering the heightened insulinotropic effects of glucose in both women and rats, along with the observation that the level of insulin in the whole body remains stable or may even increase, it is clear that glucose plays a central role in modulating insulin responses. As seen through the elevated insulinotropic effect of glucose, which stimulates insulin release from the pancreas in response to glucose, in both women and rats, and considering that whole-body insulin sensitivity remains stable or even increases, followed by the progression of insulin resistance mechanisms. This effect could occur due to the counter-regulatory effects of hormones such as estrogen and progesterone, which enhance blood glucose levels in early pregnancy (Vargas et al., 2010). Therefore, it is evident that pregnancy causes significant alterations in glucose metabolism resulting in substantial impacts on the health of both the mother and the developing fetus (Zhang et al., 2016a). In contrast, late gestation in women is characterized by an increase in insulin resistance, specifically in tissues such as skeletal muscles and adipose tissues, increasing the availability of glucose and free fatty acid for the developing fetus. In addition, the maternal skeletal muscles and adipose tissues switch to other fuel sources, such as fatty acids and ketone bodies (Ladyman and Brooks, 2021) as well as a detectable elevation in insulin resistance from the midgestational period onwards (Ladyman and Brooks, 2021, Musial et al., 2016).

In addition to changes in glucose metabolism, there are significant alterations in maternal lipid metabolism during pregnancy. Fat metabolism and storage undergo substantial alterations. Two phases of lipid metabolism have been defined during normal pregnancy, in both women and rodents. The first, or anabolic phase, occurs during the first and second trimesters in women and is characterized by adipocyte hypertrophy, a rise in de novo lipogenesis and fat storage in white adipocytes. The main driver of these changes is maternal hyperphagia throughout the pregnancy (Zeng et al., 2017). Additionally, it has been shown that enhanced levels of lipoprotein lipase (LPL) activity may promote fat deposition in both human and rat pregnancies (Herrera and Desoye,

2016). When the maternal blood triglyceride-rich chylomicrons and very low-density lipoproteins (VLDLs) are hydrolyzed, it leads to the release of glycerol and non-esterified free fatty acids that can be absorbed by adipose cells (Napso et al., 2018, McIlvride et al., 2017).

In contrast, the catabolic phase of pregnancy, which occurs in late gestation, is followed by an increase in lipolysis, lipid mobilization and a decrease in insulin sensitivity, in addition to a fall in glucose utilization (Lain and Catalano, 2007, Musial et al., 2016). Indeed, these alterations allow the mother's body to provide the growing fetus with the necessary demands of energy in late gestation.

A healthy, normal pregnancy results in significant hormonal, immunological, and metabolic changes in the mother (Newbern and Freemark, 2011). To meet the rapidly expanding fetal energy and biosynthetic requirements without endangering the mother's health and growing fetus, coordinated adjustments between mother and fetus are required during pregnancy. Disruptions in this intimate communication, caused potentially by genetic and environmental factors, can result in maladaptive metabolic responses and serious consequences for both mother and fetus (Bowman et al., 2019).

In a typical pregnancy, maternal food intake fluctuates during different stages of pregnancy. Maternal food consumption decreases in early pregnancy in some women due to nausea and sickness, which does not necessarily happen in all women. The decrease in food consumption could be a significant maternal acclimatization to the pregnancy and, enable maternal body composition to cope with this adaptation however, optimal nutrient transportation to the fetus must take place (Symonds et al., 2009b). The fluctuation in maternal food intake might be associated with hormonal effects such as insulin sensitivity, which plays a role in ensuring fetoplacental adaptation to nutrient availability. This, in turn, plays a role in determining the offspring's likelihood of developing metabolic syndrome (Symonds et al., 2009a).

1.1.2 Maternal gut microbiome

New data are showing the importance of maternal gut microbiome in placental physiology and fetal growth. Recent advances in genetic technologies have shown that billions of bacteria, collectively known as the microbiome, are present in humans and other vertebrates. These bacteria may directly, or indirectly, influence health and disease throughout life, including both maternal and infant health (Corwin et al., 2017). Indeed, the microbiome can influence pregnancy outcomes by programming and maintaining immune responses to infection during the period of gestation (Rooks and Garrett, 2016, Bessman and Sonnenberg, 2016).

The microbiome plays a versatile role in both the physical and emotional maternal responses to acute and chronic stress (Dinan and Cryan, 2017). It also plays a pivotal role in the digestion of food (Joyce and Gahan, 2014) as well as in regulating hormonal secretion and modulating metabolism such as insulin sensitivity, carbohydrate metabolism and lipid storage (Martin et al., 2019).

There is an emerging understanding of the potential link between maternal gut microbiota and fetoplacental development, which is pivotal in establishing fetal growth. The gut microbiota plays a substantial role in both the synthesis and absorption of nutrients such as carbohydrates (Rowland et al., 2018) and vitamins, including vitamin K and B groups (Rowland et al., 2018). Furthermore, the gut microbiota plays a significant role in the absorption of minerals like calcium, magnesium, and iron. It also contributes to the absorption of fatty acids including long-chain fatty acids which are essential for energy storage and signaling processes involved in physiological functions (Peng et al., 2020). Additionally, the gut microbiota plays a role in immune system functions, as well as growth and development. The protective effect of the microbiota is closely linked to the integrity of the intestinal barriers, as the gut microbiota helps prevent the colonization of pathogenic microorganisms (Karczewski et al., 2010). Intriguingly, the gut microbiota breaks down the non-digestible materials, leading to the production of short-chain fatty acids (SCFAs). These SCFAs have been well-known for their anti-inflammatory and chemo-

preventive properties (Gomaa, 2020). Furthermore, in mice, the developing weight gain and insulin resistance in late gestation have been attributed to the maternal gut microbiota (Koren et al., 2012). The microbiota has been suggested to modulate these alterations, by altering the permeability of the important cellular barriers in the intestine, resulting in the communications between the blood-born factors and local tissues (Connor et al., 2018).

The maternal gut microbiota has been reported either to remain relatively stable (DiGiulio et al., 2015), or be substantially altered during pregnancy. For example, a rise in *Proteobacteria* and *Actinobacteria* and a fall in butyrate-producing bacteria such as *Firmicutes* was observed during a period of normal pregnancy, accompanied by decreased bacterial richness (Koren et al., 2012, Ferrocino et al., 2018).

The third trimester of pregnancy is characterized by an increase in inflammation, as reduced insulin sensitivity (Barbour et al., 2007) has been linked to the alteration in immune status. This is followed by an increased level of cytokines such as TNF and IL-6 (Kirwan et al., 2002).

In a normal pregnancy, a significant decrease in the level of *Faecalibacterium*, which is involved in the production of butyrate (SCFAs), and has an anti-inflammatory role, has been observed in the last trimester of pregnancy. Of note, a decrease in the population of this bacterium has been reported in patients with metabolic syndrome (Haro et al., 2016). Indicating the existence of a pattern of bacterial shifts that takes place during the different trimesters of a naturally healthy pregnancy.

Dysbiosis in a normal population of the maternal gut microbiota has been associated with pregnancy complications such as gestational diabetes mellitus (GDM). For instance, an increased population of *firmicutes* and a reduction in the population of *actinobacteria* at the phylum level have been reported among GDM patients (Ferrocino et al., 2018).

Pregnancy complications can arise from the alteration of the maternal immune response and the secretion of inflammatory modulators, shifting from a physiological condition to a pathological one. This can result in vascular malformations observed in conditions like preeclampsia (PE), which can adversely affect fetal development and lead to growth restrictions (Edwards et al., 2017).

During pregnancy, the maternal immune system is suppressed, to prevent rejection of the allogenic fetus. In addition, the maternal immune system can play a paradoxical role at the same time by involvement of maternal innate immune system cells such as monocytes and neutrophils to provide the growing fetus with protection against pathogenic microorganisms (Luppi, 2003). Interestingly, the maternal immune system can regulate immune responses by secretion of cytokines. This regulation facilitates selective immune tolerance, which includes the involvement of cytokines such as transforming growth factor-beta (TGF-β), and interleukin-10 (IL-10), as well as the participation of the uterine Natural killer cells (uNK) and regulatory T cells. All of these mechanisms work together to support the growth and development of the fetus (Morelli et al., 2015, Luppi, 2003).

1.1.3 Maternal immune system

During pregnancy, substantial alterations in maternal immune system responses facilitate and encourage the expansion of a developing embryo (Nuriel-Ohayon et al., 2016). Pregnancy is followed by a remarkable alteration in immune responses, for example, alteration in immune responses during gestation often leads to improvement in certain autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (Jethwa et al., 2019, Confavreux et al., 1998, Hellwig et al., 2012). As pregnancy progresses, the symptoms of these diseases tend to lessen or even subside. This can be attributed to the enhanced level of oestrogen, which is a result of changes in systemic immunity in pregnancy. These changes include a reduction in the population of cytotoxic T cells, specifically CD8 markers of T cells (Pazos et al., 2012, Zoller et al., 2007). Secretion of oestrogen transcriptionally modulates the population of leukocytes

such as macrophages, neutrophils, dendritic cells and natural killer cells alongside the lymphocytes including B cells and T cells (Pierdominici et al., 2010). Various adaptations occur within the systemic immune system, including both the adaptive and innate immune systems, in addition to alterations in the population of the local immune cells in different organs, in response to pregnancy (Pazos et al., 2012). These adaptations are essential to protect both the mother and developing fetus from potential pathogens and to prevent the rejection of the semi-allogeneic fetus in early pregnancy (Mor and Cardenas, 2010). Additionally, during the period of pregnancy, elevated plasma levels of complement system factors such as C3a, C4a, C5a, C4d, C3a, C3, C9, along with increased levels of leukocytes and innate lymphoid cells, are observed, which also serves to strengthen the maternal immune system during this period (Abu-Raya et al., 2020). Furthermore, an increase in the pro-inflammatory activity of the monocytes is also evident.

A significant adaptation in maternal immune cell populations takes place during early pregnancy coincidence with uterine artery remodeling and decidualization. A subsequent decrease in the number of T cells may lead to uterine artery dysfunction due to elevated levels of the proinflammatory cytokine. The dysfunction in hemodynamics of uterine arteries, attributed to reduced T-reg cell counts, could result in increased resistance and pulsatility indices. Additionally, there may be heightened conversion of bET-1 (big endothelin-1) into the potent vasoconstrictor, ET-1 (endothelin-1)(Robertson et al., 2018, Care et al., 2018). In this phase, approximately 70% of immune cell populations are uterine natural killer (uNK) cells (Robertson et al., 2018). The remaining cells consist of dendritic cells and macrophages, along with cells related to the adaptive immune system, such as T regulatory cells (Tregs) are involved in maternal immune system adaptations during pregnancy (Liu et al., 2017). Treg cells are known for their capability to recognize seminal fluid and sperm antigens, which are necessary to be activated to dampen inflammation and prevent immune rejection (Schjenken and Robertson, 2015). Several inflammatory markers have been studied in pregnancy, such as interleukin-1, 6 and 8 (IL1α, IL1β, IL6, IL8), tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and monocyte chemoattractant protein 1 (MCP) in plasma and small intestine intraepithelial

lymphocyte (SI-IEL) in the gut (Giovannini et al., 2011). The level of these inflammatory factors was heightened in pregnant mice when compared to the non-pregnant mice (Abu-Raya et al., 2020). However, it has been reported that the level of inflammatory markers in late gestation is directly related to maternal metabolic dysfunction (Abell et al., 2015). Increased levels of TNF-α and CRP were observed and were associated with impaired glucose tolerance and insulin resistance in pregnant mice (Nguyen-Ngo et al., 2019).

1.1.4 Cardiovascular adaptation during pregnancy

Alterations in the maternal cardiovascular system are pronounced and commence in the very early stages of human pregnancy. For example, by the 8th week of pregnancy, cardiac output has increased by 20% which, in part, is a consequence of peripheral vasodilation (Soma-Pillay et al., 2016a) in response to the upregulation of endothelium-dependent agents such as nitric oxide synthesis (NOS), which is itself regulated by the secretion of oestrogen and prostaglandins such as PGI_2 (Bai et al., 2020, Lorigo and Cairrao, 2022).

The aforementioned changes are coupled with an extensive alteration in the cardiovascular system and hemodynamic adaptations during pregnancy, both systemically and within the uterus. Within a normal pregnancy, there is a significant decrease in uteroplacental vascular resistance and peripheral vasodilation which dampen responses to vasoconstrictive factors such as angiotensin II and increase the level of vascular relaxation factors such as nitric-oxide (Ramlakhan et al., 2020).

This systemic vasodilation and vascular resistance results in lowering blood pressure characterized in early pregnancy commencing at weeks 4-6 of pregnancy in women (Alexander et al., 2023). At around the second trimester of pregnancy, blood pressure decreases by 5-10 mmHg, despite a substantial increase in intravascular volume (Cheung and Lafayette, 2013, Mahendru et al., 2014). This decrease in vascular resistance serves to maintain sufficient uteroplacental perfusion while maintaining adequate blood pressure. Central to these cardiovascular changes is the secretion of progesterone. It has been linked

to the reduction in vascular resistance by promoting the relaxation and dilation of smooth muscles of blood vessels (Lorigo and Cairrao, 2022). In addition, progesterone secretion promotes the deposition of non-collagenous proteins in artery walls (Chen and Khalil, 2017).

There are various agents and hormones involved in systemic vasodilation, such as relaxin, a hormone which is released from the corpus luteum until the early second trimester and later is primarily produced by the placenta. Relaxin plays a potent vasodilatory role (Conrad, 2011). Levels of this hormone in women peak in early pregnancy, decline during the second trimester and then stay steady until the term. In contrast, relaxin concentrations in other mammals such as mice, rats, guinea pigs and hamsters peak close to term (Napso et al., 2018).

In addition to changes in vascular resistance, other alterations to the maternal cardiac physiology occur throughout pregnancy, to ensure the maintenance of the blood pressure. These changes include an increase in plasma volume (De Haas et al., 2017) as well as an increase in cardiac output, with the maximum level of cardiac output in late gestation especially close to the term (Meah et al., 2016, Soma-Pillay), and to a lesser extent, an increase in heart rate (Soma-Pillay et al., 2016b). There is a notable rise of approximately 40% in blood volume, in pregnant women, which induces morphological and functional alterations in the heart. These changes include augmented left ventricular mass, heightened left atrial volume, and a considerable decrease in the early to late diastolic transmitral flow velocity ratio (E/A) at the time of labour (Umazume et al., 2018). A gradual rise in both left ventricular end-diastolic and end-systolic diameters and volumes, as well as LV wall thickness, indicative of the progression of eccentric hypertrophy, could be mentioned as significant alterations in cardiac morphology, as will be recovered after parturition (Savu et al., 2012). Similar changes in cardiac volume during human pregnancy, leading to prolonged cardiac volume overload and hypertrophy, were observed in mouse pregnancy (Chung et al., 2012). Enhanced cardiac pressure and volume overload in normal pregnancy in mouse models alter heart morphometry, beginning with changes in chambers such as an enlarged left ventricle accompanied by a decrease in wall thickness (Chung and Leinwand, 2014).

Together, these adaptations result in a reduction in mean arterial resistance despite an increase in plasma volume and heart rate, in the first and second trimesters of pregnancy, however by the third trimester blood pressure rises to a level observed in non-pregnant women (Braunthal and Brateanu, 2019).

The reduced vascular resistance and consequent decline in blood pressure are coupled with activation of the renin-angiotensin-aldosterone system (RAS). Activation of RAS results in an increase in circulatory blood pressure and fluid retention, however, the primary goal is to maintain sodium balance (Mulder et al., 2022). RAS functions via the synchronized action of renin and the angiotensin-converting enzyme (ACE) to produce a potent vasopressor angiotensin II (Ang II). Renin catalyzes the conversion of inactive angiotensinogen to the biologically inert decapeptide, Ang I, which is further hydrolyzed by ACE to form an active octapeptide, Ang II. Being a potent vasoconstrictor, Ang II exerts physiological changes such as sodium retention, and vessel wall constriction by interacting with Ang II type 1 (AT1) receptors, leading to hypertension (Figure 1-1) (Paul et al., 2006).

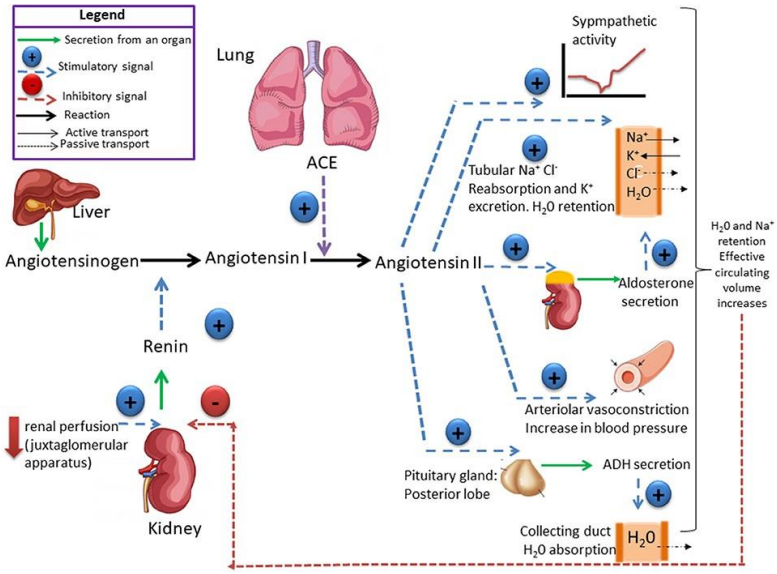


Figure 1-1 Illustrating the Renin angiotensin system (RAS). The RAS pathway. plays a central role in the retention of water and salt. Renin converts angiotensinogen into angiotensin I, which under the impact of the angiotensin-converting- enzyme (ACE), catalyses into angiotensin II. Angiotensin II regulates the secretion of aldosterone which modulates the Na^+ retention and antidiuretic hormone (ADH) which modulates the H_2O reabsorption from the collecting ducts and distal convoluted tubules of the nephron through aquaporin channels. The combination of these mechanisms regulates plasma volume and osmolarity. Adapted from (De Almeida and Coimbra, 2019).

Alongside the remarkable changes in the cardiovascular system, maternal renal blood flow and glomerular filtration (GFR) increase during pregnancy, as evidenced by the elevated creatinine clearance levels observed by the 6th week of gestation in women (Chapman et al., 1998), accompanied by an increase in renal size of up to 30% during pregnancy (Hussein and Lafayette, 2014).

In mice, an increase in GFR of nearly 34% occurs by day 12 of gestation, considering the gestational length in mice is generally between 18 and 22 days (Odutayo and Hladunewich, 2012). During a normal pregnancy upregulation of RAS components coupled with less sensitivity to Ang II, results in an increased plasma volume (Rodriguez et al., 2012). Therefore, during pregnancy, there is an increase in blood volume to cope with the increasing oxygen requirement of maternal organs and the developing fetus. There is also an increase in the volume and mass of red blood cells, driven by an increase in maternal erythropoietin hormone synthesis and secretion (De Haas et al., 2017, Chandra et al., 2012) (Figure 1-2).

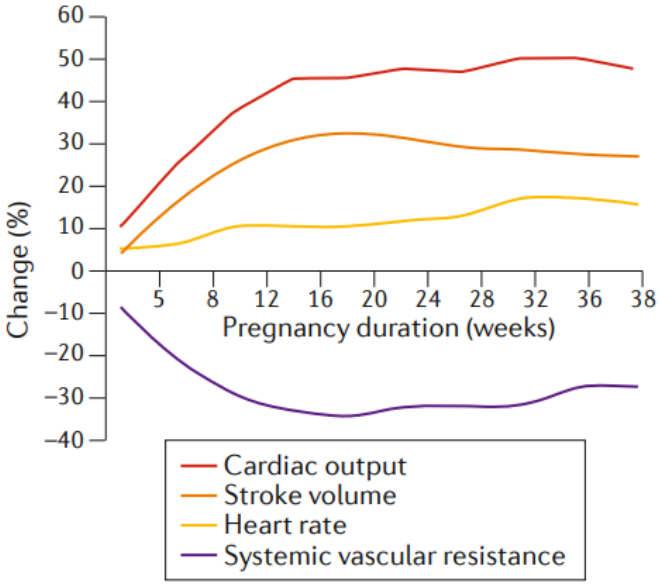


Figure 1-2 Shows the hemodynamic changes observed in women during pregnancy adapted from (Ramlakhan et al., 2020)

1.2 Placenta

The placenta is a transient, yet crucial, extraembryonic organ necessary for the survival of the fetus and acts as the interface between the mother and the fetus (Arutyunyan et al., 2023). One of the main roles of the placenta is to allow the exchange of gases, nutrients, and waste materials between both the maternal circulatory system and the developing fetus. Additionally, the placenta is an essential source of hormones (with autocrine, paracrine, and endocrine functions) and growth factors that are crucial in orchestrating a normal pregnancy. Furthermore, the placenta also plays a significant role in the immune protection of the developing fetus (Rossant and Cross, 2001). The placenta plays a significant role in determining fetal growth and weight at birth through the regulation of maternal nutrition, oxygen acquisition from the mother and minimizing immunological rejection by the maternal immune system (Desai et al., 2013, Molloy et al., 2008, Villalpando, 2008). Studies both in humans and rodents have demonstrated that a wide range of maternal factors such as over and under-nutrition, smoking, drug and alcohol intake, infection and stress can induce marked transformations in placental physiology (Ganapathy, 2011,

Zdravkovic et al., 2005, Godfrey et al., 1996). For instance, an imbalanced maternal diet characterized by elevated carbohydrate intake in early gestation and reduced consumption of dairy and meat products in late gestation may result in lower placental weight and birth weight (Godfrey et al., 1996). These transformations range from alterations to gross placental morphology such as changes in placental weight, to the more subtle changes in placental gene expression that may predict altered transport of important signals to the fetus (Nugent and Bale, 2015).

The placenta performs many functions in addition to the exchange of materials between the mother and her growing fetus. For instance, specific cells within the outer layer of the placenta in rodents, such as trophoblast giant cells and spongiotrophoblast cells, produce a range of specialized substances necessary for the developing fetus. These include hormones such as placental lactogens, angiogenic factors such as proliferin and vascular endothelial growth factor (VEGF), as well as tissue remodeling factors like matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA). Consequently, the mouse and human placenta is an intricately specialized unit that relies on various cell types for both its structure and function in facilitating the exchange between the fetus and mother (Rossant and Cross, 2001).

1.2.1 Human and mouse placental structure and development

In both women and mice, placentation is described as haemochorial, meaning that maternal blood is in direct contact with fetal trophoblast cells (Woods et al., 2018). The placenta and associated extraembryonic membranes, at the very beginning of gestation, develop from the fertilized zygote (Burton and Fowden, 2015). Blastocyst implantation occurs around 6-7 days post fertilization (*dpf*) in humans and around E4.5 in mice (Hemberger et al., 2020).

In humans at approximately 5 *dpf*, the blastocyst is comprised of two different cell lineages. These are the pluripotent inner cell mass (ICM), or embryoblast, which contains the cells that will differentiate into the fetal tissues, and the outer trophectoderm (TE). Trophectoderm differentiates into the trophoblast, and later

the trophoblast produces the outer layer of the placenta known as the epithelial covering. In addition, TE is responsible for generating a specific group of invasive extravillous trophoblast cells, which will give rise to the placenta. In humans, the placenta is made up of two main types of TE cells. First, the TE cells merge with the adjacent cells, creating a primary syncytium, and become syncytiotrophoblast (STB) cells then, these multinucleated cells rapidly invade the maternal uterine endometrium (decidua basalis) at the time of implantation which, in humans approximately occurs at 6-7 *dpf* (Turco and Moffett, 2019, Knöfler et al., 2019). Second, the remaining TE cells, which constitute the rest of the blastocyst wall, remain unfused. These precursor cells are now identified as cytotrophoblast cells (Cindrova-Davies and Sferruzzi-Perri, 2022b).

In mice, there are two syncytial layers including syncytiotrophoblast cells and sinusoidal trophoblast giant cells (TGCs) which ultimately form the transport surface of the placenta, or "interhaemal membrane" (Woods et al., 2018). However, in humans, there is just one layer of syncytial cells present. The close juxtaposition of maternal blood and fetal circulation provides the developing fetus with nutrients and gas exchange (Hemberger et al., 2020). Also, this highlights the trophoblast-associated uterine spiral arteries remodeling which is essential to occur at the early stages of placentation (Geusens et al., 2008). Extra villous trophoblast (EVT) invasion of the decidua up to the first third of the myometrium, results in spiral arteries remodeling, which alters the spiral arteries from low-flow high resistance to high-flow low-resistance arteries (Lyall et al., 2001). During a normal pregnancy, significant alterations in the uterine spiral arteries occur and are considered the first strategy for uterine adaptation. Spiral arteries become highly dilated and lose their endothelium and most of their muscle fibers. This occurs due to the fragmentation of reticulum fibres, facilitating the terminal part of the spiral arteries to remain open (Ernst, 2018). The process of uterine arteries remodeling follows by the activation of a network of uterine immunocytes such as the macrophages, dendritic cells, T regulatory cells, and the factors they secrete such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), transforming growth factor-beta (TGF-β) or granulocyte-macrophage colony-stimulating factor (CSF2) encourage spiral artery remodeling and facilitate trophoblast invasion (Lash et al., 2010,

Zhang et al., 2013, Care et al., 2018) Molecular changes in mothers support later cardiovascular system alterations. From the eighth week of gestation, cardiac output in women is modified and could grow 50 per cent by weeks 16–20 (Hunter and Robson, 1992)f by reductions in vascular peripheral resistance (Quek et al., 2016) all of which promote uteroplacental blood flow while maintaining the mother's blood pressure.

During early gestation, uterine vessels exhibit a degree of plasticity. In response to the low local oxygen concentration level and distinctly, oxidative stress arising from increasing the placental mitochondrial activities, a substantial vascular remodeling takes place (Burton and Jauniaux, 2018). Remodeling of the uterine vasculature takes place within different stages, with the first stage occurring following EVT invasion (Harris, 2010). The cytotrophoblast cells invaginate into the syncytiotrophoblast, forming structures known as primary chorionic villi. Meanwhile, the extraembryonic mesoderm differentiates into two layers, the somatic mesoderm, which contributes to the formation of the primary villi, as well as the splanchnic mesoderm, which gives rise to secondary chorionic villi (Herrick and Bordoni, 2019). At approximately 14 *dpf,* the formed blastocyst has become embedded completely in the uterine endometrium and has been covered by the epithelium, which initiates placentation (Turco and Moffett, 2019). Figures 1-3 and 1-4 represent the human embryo implantation and comparative placentation between mice and humans, respectively.

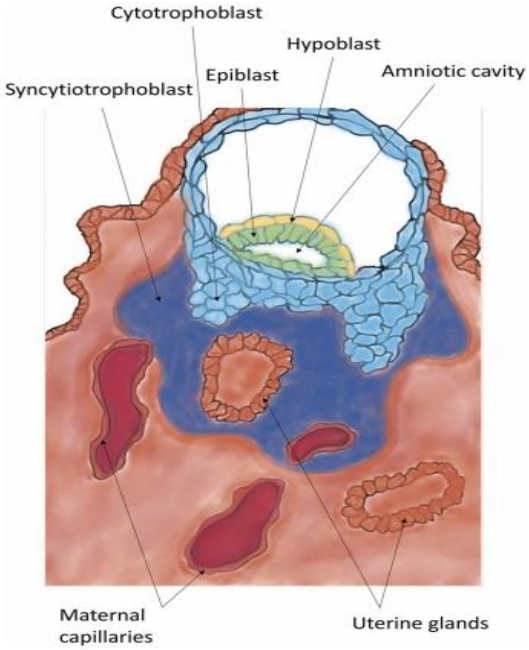


Figure 1-3 Diagram showing human embryo implantation and the initial stages of human placental development. This figure illustrates human early placentation at approximately 6–8 dpf. The formation of primary syncytium occurs following the fusion of the trophectoderm of the trophoblast with the endometrium epithelial cells. The consequence of the invasion of the trophectoderm cells into the epithelial cells of the uterus is the transformation of the maternal endometrium into a decidual tissue. Cytotrophoblast cells are derived from the remnants of the trophectoderm which forms the rest of the blastocyst wall, which remains unfused, and expansion of these cells forms the primary syncytiotrophoblast cells of the chorionic plate. These cells cover the gestational sac at the time of completion of implantation. Two distinct layers, the epiblast and hypoblast, result from the subsequent differentiation of the inner cell mass. Taken from (Cindrova-Davies and Sferruzzi-Perri, 2022a).

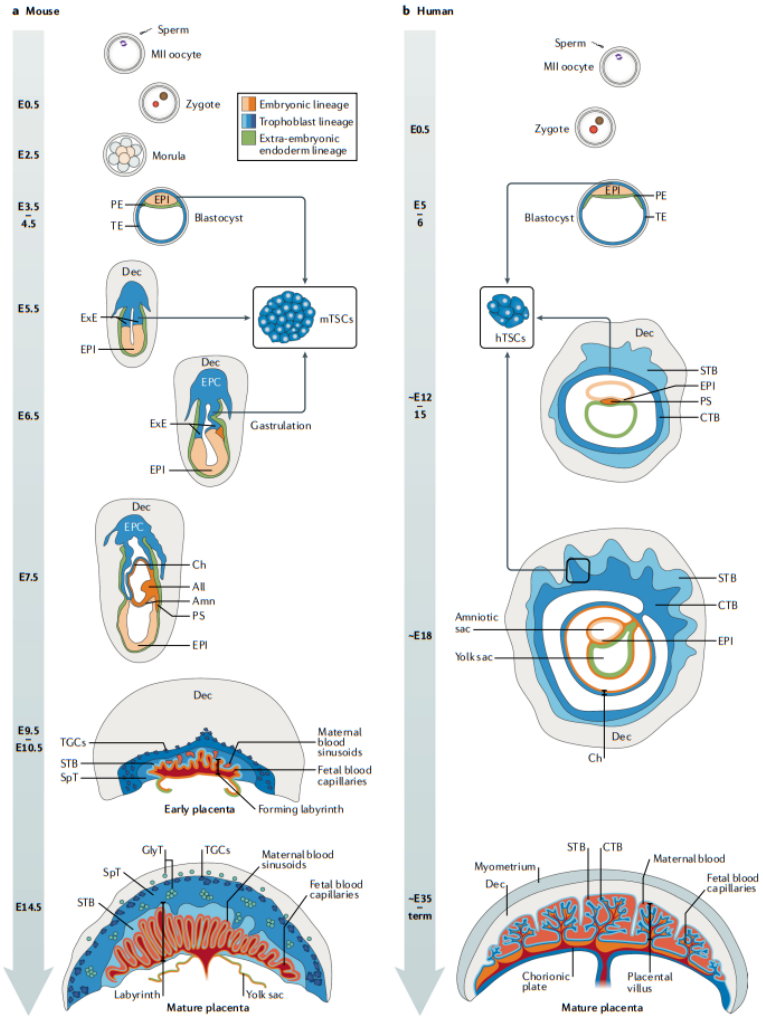


Figure 1-4 Displays a comparative schematic figure of the placentation development stages after fertilization in mice (left) and humans (right). Taken from (Hemberger et al., 2020)

Additionally, Figure 1-5 below displays the stages of human placental development during pregnancy. The process begins with the creation of the blastocyst around the fifth day of gestation (A). Approximately, around 6.5th day of gestation, while the implantation phase is occurring, the trophectoderm differentiation and syncytiotrophoblast invasion into the maternal endometrium could be observed (B).

As the pregnancy progresses to the post-implantation phase by the 12th day of gestation, the formation of primary chorionic villi derived from cytotrophoblasts initiates. In addition, the maternal vessels and endometrial glands are eroded by the syncytiotrophoblast (C). The post-implantation phase continues until the 16th day of gestation, during which the secondary chorionic villi form through the differentiation of extraembryonic mesoderm (D). Finally, the tertiary chorionic villi and a cytotrophoblastic shell develop at the 21st day of gestation, in the preplacental phase (E). Figure 1-5, section (F) illustrates, approximately the 20-week of pregnancy in women, when the placenta is fully developed.

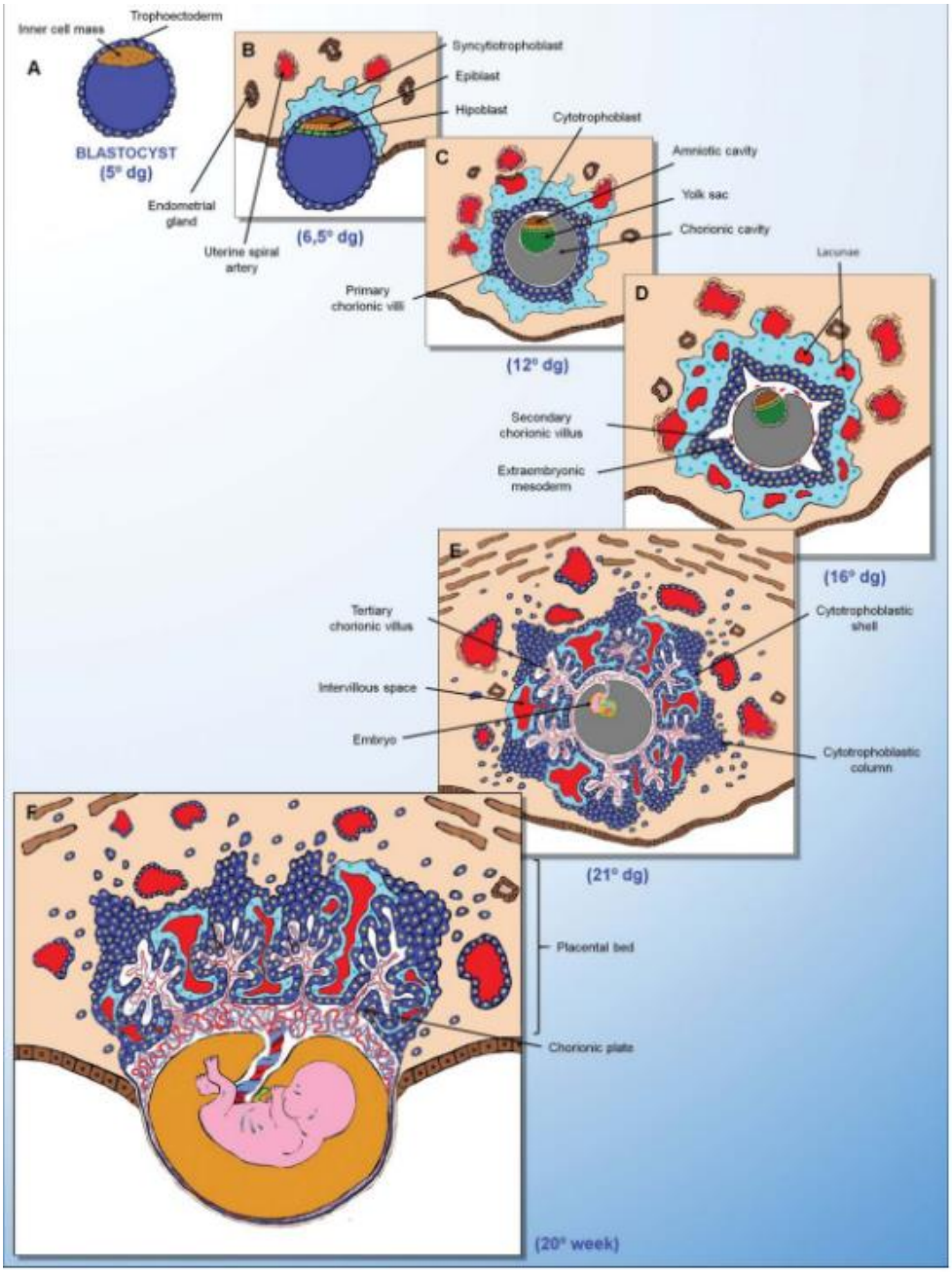


Figure 1-5 Displays the adhesion of the blastocyst to the uterine endometrium, blastocyst implantation as well as the formation of early stages of placentation (A-F) Taken from (Silva and Serakides, 2016)

Figure 1-6 below depicts the various stages of rat and mouse placental development. Following fertilization, the process of implantation initiates with the creation of the blastocyst on the 5th day of gestation (A). Between days 5 and 6.5 of gestation, which is known as the implantation phase in rodents (rat and mouse), the trophectoderm differentiates and the giant cells of primary trophoblast invade the maternal endometrium (B). Following that, the post-implantation phase initiates on day 7.5 of gestation. In this stage the differentiation of trophectoderm continues, resulting in the production of giant cells all around the embryo and the ectoplacental cone (C). In continuation of the post-implantation phase, at approximately day 8.5 of gestation, the allantois start to develop from the posterior epiblast (D). At around day 10.5 of gestation, the process of pre-placentation initiates. This involves the fusing of the chorionic ectoderm and allantois mesoderm, resulting in the formation of the placental labyrinth (E). Ultimately, the establishment of the definitive placenta on day 15 of gestation occurs. This is distinguished by the presence of the trophoblast giant cell layers and spongiotrophoblast, as well as the placental labyrinth (F).

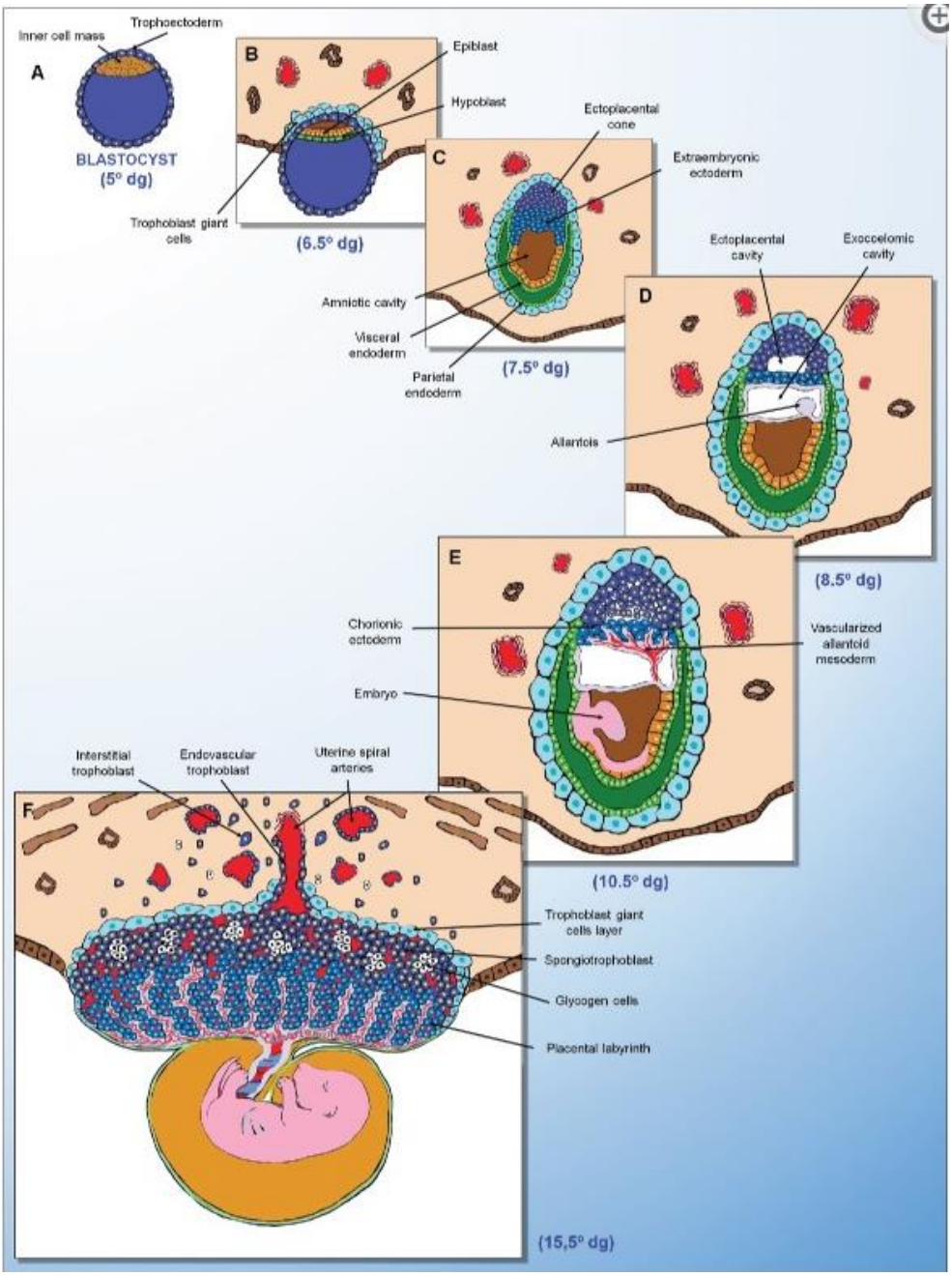


Figure 1-6 Displays the process of placentation in rodents from the blastocyst stage until the formation of the definitive placenta (A-F). Taken from (Silva and Serakides, 2016). (dg)= day of gestation

Despite the variation in the detailed anatomical structure between the human and mouse placenta, there exists a notable similarity in the overall structure and molecular mechanisms governing placental development (Watson and Cross, 2005). The process of placentation is followed by separation of the TE and ICM. Later on, at the time of implantation at embryonic day 4.5 E4.5 which is equivalent to 6.5-7 *dpf* in humans the formation of trophoblast giant cells (TGCs) from the cells that are not in touch with the ICM takes place. In mice, trophoblast cells facilitate embryo penetration into the uterine endometrium during implantation (Woods et al., 2018).

After merging the chorion and allantois, the mature placenta forms around E8.5 in mice (Hemberger et al., 2020). TGCs have a substantial role in the endocrine function of the placenta which therefore means they are considered equivalent to human extra villus cytotrophoblast cells (Rossant and Cross, 2001, Watson and Cross, 2005). Moreover, the TGCs help the yolk sac to provide nutrition and oxygen to the growing fetus by forming blood sinuses in the embryos' peripheral areas (Maris et al., 1988). Blood sinuses" refer to large, irregularly shaped blood vessels or spaces within tissue that are capable of holding blood. These sinuses are formed by the trophoblast giant cells (TGCs) at the periphery of the embryo. Their formation contributes to the process of providing nutrients and oxygen to the developing embryo by facilitating the circulation of blood (Mess and Carter, 2009). In mice, a cap-like structure is created by the proliferation of the polar trophectoderm in the blastocyst, from the extra-embryonic ectoderm cells called the ectoplacental cone (EPC), which is a temporary structure formed during early embryonic development. The EPC is a diploid derivative of the early post-implantation trophoblast, which most likely serves as a progenitor for the spongiotrophoblast formation (Rossant and Cross, 2001). Furthermore, the cells at the margins of EPC differentiate into secondary TGCs. These secondary invasive TGCs play a role in remodeling the maternal spiral arteries through a deep penetration into the maternal endometrium and create contact with maternal blood vessels between ~E7.5 and E9.5 (Tesser et al., 2010, Woods et al., 2018). Indeed, the origination of the placental junctional zone (Jz) from the cells located in the core of EPC, occurs through the expression of genes such as *Tpbpa* (Woods et al., 2018).

A remarkable feature of the TGCs is that while they cease cell division, they continue to duplicate their genome (endoreduplication) and become polyploid. In contrast, the extraembryonic ectoderm (ExE) and the EPC two diploid cell types arise from the polar trophectoderm, which are the cells next to the ICM (Watson and Cross, 2005). The chorionic epithelium is a component of the placenta that forms as a result of the invagination of the mesodermal tissues during placentation, despite the expansion of the extraembryonic ectoderm (Burton and Fowden, 2015)

1.2.2 Labyrinth zone in mice placenta

As development proceeds, at around E8.5 gestation in mice, at the posterior part of the embryo derived from the mesoderm, the allantois arises and makes contact with the chorion, which results in the formation of the chorioallantoic.

The mouse placenta is formed of different layers, including junctional, labyrinth zones and maternal decidua. Formation of labyrinth zone (Lz) is the result of the interaction among the EPC, the chorion and allantois (E9.5-10.5) (Rossant and Cross, 2001, Knott and Paul, 2014). Just before mid-gestation chorio-allantoic fusion occurs, following the invagination of the extra-embryonic mesoderm into the chorionic trophoblast layer. This interaction of the fetal blood vessels with maternal blood sinuses, which are lined by trophoblast cells, initiates the fundamental creation of the mouse placental labyrinth zone (Hemberger et al., 2020). Prior to the fusion of chorioallantoic membranes, and formation of the Lz at approximately mid-gestation, the yolk sac is the only organ responsible for providing the developing fetus with nutrients, so that chorio-allantoic fusion and the Lz formation appears to be vital for the survival of the embryo (Kuzmina, 2023).

In the mouse, the placenta is composed of three distinct regions. Labyrinth zone Lz, which is a tightly packed tissue and highly vascularized is the result of a high extent of villus branching from trophoblast and fetal blood vessels. Alongside the labyrinth development, it is supported by another ectoplacental cone (EPC)-derived layer called the spongiotrophoblast, which creates a compact layer

between the outer giant cells and the labyrinth zone at around E9.5 due to expansion and flattening of the EPC (Rossant and Cross, 2001, Watson and Cross, 2005).

1.2.3 Junctional zone in mice placenta

The other functional layer in the mouse placenta is the Jz which is located between the Lz and maternal decidua, and similar to the Lz, originates from the EPC. Overall, a few different types of trophoblast subtype cells originated from the EPC, form the Jz. The Jz includes three different subtypes, namely spongiotrophoblast (SpT), which makes up the majority of the population of cells in the middle layer of the placenta, surrounded by outer secondary TGCs and the labyrinth zone (Simmons and Cross, 2005). Alongside these are the glycogen cells (GCs), which form at E7.5, functioning through glycogen accumulations at E12.5, and secondary trophoblast giant cells (TGCs). The secondary TGCs, which are located within Jz and decidua, form three different subtypes, including maternal arterial canal-associated TGCs (c-TGCs), parietal-TGCs (p-TGCs) which line the implantation site and spiral artery associated TGCs (SpA-TGC) (Knott and Paul, 2014).

From the ICM another lineage of cells originates named extraembryonic mesoderm which forms the fetal placental vascular network (Woods et al., 2018). While the labyrinth zone modulates the maternal nutrient exchange with the growing fetus through a highly developed vascular network, at the end of pregnancy, the Jz will produce growth factors and pregnancy-related hormones such as prolactin-like hormones (Simmons et al., 2007, Woods et al., 2018).

1.2.4 Placental endocrine function and role in maternal metabolic and cardiovascular changes

By the 6th week of gestation in humans, and E11 in mice, the placenta has become established as a major endocrine organ, influencing multiple aspects of maternal and fetal physiology. The placenta releases a range of endocrine factors which target central metabolic and cardiovascular systems both in the mother and her growing fetus.

It should be noted that the human and mouse hormonal support of pregnancy various in terms of the specific endocrine factors produced and their roles. In humans, the placenta secrets an extensive range of hormones including steroids (oestrogen, progesterone), prolactin (PRL), placental lactogen (PL), growth hormone (GH), insulin-like growth factor-2 (IGF2) and Parathyroid hormone-related protein (PTHrP) (Napso et al., 2018). These factors play a substantial role in regulating maternal insulin and glucose homeostasis, lipid profile and appetite (Table 1-1) (Lin et al., 2019, Fowden et al., 2008). Additional placental products include neuroactive hormones such as melatonin, serotonin, oxytocin, kisspeptin, thyrotropin-releasing hormone, and corticotropin-releasing hormone (Napso et al., 2018). These hormones, which target the maternal brain, play a central role in maternal nursing behaviours, both during pregnancy and lactation (Bosch and Neumann, 2012). For instance, serotonin and melatonin are involved in modulating maternal mood as well as behaviours, both during gestation and the postpartum periods (Angoa-Pérez and Kuhn, 2015). Additionally, leptin, PTHrP, relaxin, activin chorionic gonadotropin and prolactin-growth hormone family are also produced by the placenta.

The placenta is also involved in the secretion of inflammatory chemokines such as TNF-α, IL6 and monocyte chemotactic protein-1 (MCP-1), which all have both local autocrine and paracrine effects (Du et al., 2014). They affect the systematic modulation of insulin signaling in hepatic and muscle cells, and potentially impact maternal metabolic adaptation (Denison et al., 2010).

Development of the fetoplacental unit is essentially dependent on both sufficient and appropriate blood supply. Fetal growth retardation which affects the health of the mother and fetus is caused by aberrant angiogenesis during early pregnancy (Egbor et al., 2006). Islami et al (2003), showed that human chorionic gonadotropin (hCG) probably plays an important role in the control of endometrial and placental vascularization by paracrine (on the endometrium) as well as juxtacrine (on the trophoblast) mechanisms.

Table 1-1 Placentally derived hormones in humans, details taken from (Costa, 2016).

Hormone	Molecular targets	Source of secretion	Functions
Progesterone	nPRs, mPRs	Syncytiotrophoblast	Decidualization. Endometrial receptivity and embryo implantation. Relaxation of myometrium; uterine quiescence. ↑Th2 cytokine production. ↓uNK cells activity. ↓Trophoblast invasion; ↑trophoblast migration. ↓HCG, Leptin and resistin placental synthesis. Preparation of breast for lactation. Hyperphagia, fat storage
HCG	LH/HCG receptor	Syncytiotrophoblast	↑Progesterone production by corpus luteum Syncytialization Angiogenesis Immunotolerance ↑Trophoblast invasion Uterine quiescence Endometrial receptivity and embryo implantation
Leptin	LepR	Syncytiotrophoblast, Extra villus trophoblast	↑CT prolifération ↓CT apoptosis. ↑Trophoblast invasion ↑HCG and ↓HPGH, progesterone and oestradiol placental production. Angiogenesis Embryo implantation Immunomodulation. Uterine quiescence ↑Pro-inflammatory cytokines and prostaglandins.
Human placental growth hormone	GHR, PRLR	Syncytiotrophoblast, Extra villus trophoblast	Synthesis of maternal IGF1. Lipolysis Insulin resistance, ↑serum glucose levels, ↑Trophoblast invasion.
Human placental lactogen	GHR, PRLR	Syncytiotrophoblast, Extra villus trophoblast	Lipolysis, ↑free fatty acids and ketone bodies. Insulin sensitivity ↑Fetal insulin and IGF1 synthesis ↓Leptin placental synthesis.
Oestradiol	nERs, mERs	Syncytiotrophoblast	Endometrial receptivity and embryo implantation. Angiogenesis Regulation of human uteroplacental blood flow, ↑myometrium contraction, labour induction Syncytialization. ↑Leptin and ↓resisting placental synthesis. Preparation of breast for lactation. Hyperlipidemia and fat storage.

nPRs = nuclear progesterone receptors, mPRs = membrane-associated progesterone receptors; Th2 = Thelper2, uNK= uterine natural killer, HCG = human chorionic gonadotropin, LH = Luteinizing hormone, CT = cytotrophoblast, LepR= leptin receptor, HPGH = human placental growth hormone, GHR = growth hormone receptor, PRLR = prolactin receptor, IGF1= insulin and insulin-like growth factor 1, nERs = nuclear oestrogen receptors, mPRs = membrane-associated progesterone receptors, CT= cytotrophoblast, HCG= human chorionic growth, Th2= T helper2, HPGH= human placental growth hormone, PLLR= prolactin receptor

1.3 Gestational complications

1.3.1 Preeclampsia

As the placenta is one of the first organs to be formed during mammalian embryogenesis, the occurrence of any problems during its formation could be critical to pregnancy success, leading to complications and potential early pregnancy loss (Rossant and Cross, 2001).

One of these life-threatening complications for both mother and baby is preeclampsia, which is defined as a pregnancy-specific hypertensive disorder (Bergman et al., 2020). After the 20^{th} week of pregnancy, approximately 10-15 percent of pregnant women experience new-onset hypertension (Duley, 2009). The initial diagnosis of preeclampsia is made when blood pressure consistently reaches 140 mm Hg or higher for systolic measurements and, as well as 90 mm Hg or higher for diastolic measurements on two separate occasions. This measurement should be taken at least with the differences of at least 4 hours apart. Alternatively, if the systolic pressure reaches 160 mm Hg or higher and the diastolic pressure reaches 110 mm Hg or higher after week 20 of pregnancy, it can be categorized as preeclampsia (Erez et al., 2022, Karrar and Hong, 2023).

Preeclampsia comprises the combination of hypertension and at least one other complication such as proteinuria or uteroplacental dysfunction. It has been classified as one of the main causes of maternal and perinatal mortality worldwide, resulting in the death of an estimated fifty thousand women and up to 1 million infants per year (Steinthorsdottir et al., 2020). Preeclampsia not only plays a predominant role in maternal mortality but is also a leading cause of iatrogenic pre-term birth and fetal growth restriction (Sasaki et al., 2007).

The chief driver of preeclampsia is abnormal and dysfunctional placentation. A strong justification for the prominent role of the placenta in this disease progression is that there is no curative treatment for it except delivering baby and placenta (Chappell and Cluver, 2021).

Abnormal remodelling of the uterine spiral arteries has been reported in women carrying a polymorphism in the angiotensinogen (*AGT*) gene, resulting in a substitution mutation of threonine for methionine and which leads to abnormal remodelling of the uterine spiral arteries (Sibai et al., 2005, Zerres, 2008). The uteroplacental mismatch (misalignments between uteroplacental blood flow), in preeclampsia is caused by decreased uteroplacental blood flow, increased fetoplacental requirements, or both mentioned conditions (Espinoza, 2012). To ensure the optimal adaptation of the uterine tissue throughout pregnancy the invasion of extravillous cytotrophoblast into the inner third of the maternal uterine myometrium, to prevent the contraction of the terminal section of the vessels (Fournier et al., 2021). This structural modulation is linked with reduced spiral artery resistance at the materno-fetal interface and, subsequently less sensitivity to vasoconstrictive substances. This provides the optimal foundation for nutrient and gas exchanges during pregnancy by enabling a higher stream of blood through these vessels(Uzan et al., 2011, Chappell and Cluver, 2021). Impaired spiral artery remodelling followed by vascular mal-perfusion (Wright et al., 2017) has been reported in women who develop preeclampsia, additionally, fetal growth restriction (Levytska et al., 2017), is one of the prominent implications of this impairment. Moreover, pre-eclampsia is associated with an impairment in the expression of nitric oxide synthesis (NOS) which in normal pregnancies provides the spiral arteries with the required vasodilation (Sutton et al., 2020).

The consequence of impaired remodeling of spiral arteries is placental ischemia (Burton et al., 2009), due to the high velocity and turbulent flow of blood and an increase in oxidative stress (Rana et al., 2019, Burton et al., 2009). Such changes result in detrimental damage to the placental villus and the secretion of unusual levels of angiogenic factors into the maternal blood (Zur et al., 2020). This leads to an imbalance in the level of the circulating levels of pro-angiogenic placental growth factor and antiangiogenic soluble fms-like tyrosine kinase 1 (sFlt-1) (Magee et al., 2022).

Preeclampsia is typically considered a maternal disease, with some levels of fetal participation (Dekker et al., 2011), so that, any underlying paternal

involvement in the disease manifestation is not well-studied. The well-defined immunogenetic association between mother and father is receiving high appreciation, as well as the distinct genetic conflict that is related to haemochorial placentation. It could be understood that the genes from different parents may have different interactions, and any potential genetic conflicts may impact the process of this type of placental development (Haig, 2015, Petroff et al., 2022, Dekker et al., 2011). Therefore, the placenta could be seen as a cornerstone for both preeclampsia and the contribution of the paternal genome. A susceptibility factor involving the father and the fetus in the development of preeclampsia is the presence of fetal versions of Human Leukocyte Antigen-G (HLA-G) from the father along with those not coming from the mother (Galaviz-Hernandez et al., 2019). HLAs could be considered as a major part of the histocompatibility complex, to recognize foreign antigens. This HLA class plays a central role in enabling the immune system to distinguish the body's cells and tissues and exogenous cells (Mosaad, 2015). The fetus's HLAs are inherently different from the mother's because it inherits them from both parents. Therefore, the genetic dissimilarities, particularly regarding the HLA genes are a substantial factor that fetus could be considered as a semi-allogenic graft in pregnancy (Aisagbonhi and Morris, 2022).

Additionally, seminal plasma and sperm, have a significant influence on maternal uterine responses resulting in the secretion of factors involved in immune response and proinflammatory cytokines (Figure 1-7) which ultimately encourages uterine spiral artery remodelling (Schjenken and Robertson, 2020).

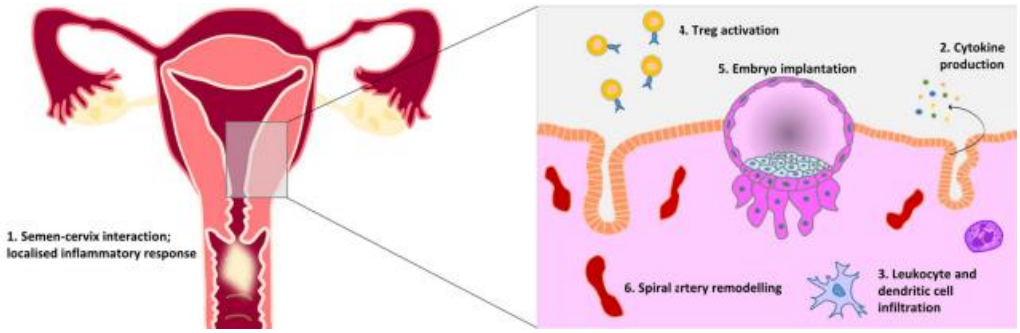


Figure 1-7 Illustrating the seminal plasma composition encourages the uterine immune system to secrete the proinflammatory cytokines. Taken from (Khoshkerdar et al., 2021)

Pathologies that affect fetal development and growth are often caused by the failure of uterine microvascular remodelling (D'Errico and Stapleton, 2018). Studies supporting the role of the seminal plasma in the occurrences of PE have shown an increased risk of PE in women who have experienced types of contraceptives which prevent the female reproductive tract (FRT) from being exposed to the seminal plasma as well as sperm (Kenny and Kell, 2018). Similarly, studies have demonstrated the period of exposure to their partner's seminal plasma is beneficial for women, as the longer exposure to the partner's semen (sexual cohabitation) has a substantial role in attenuating the maternal uterine immunological responses against the familiar paternal antigens present in the placenta (Bromfield, 2014).

Seminal plasma contains several components such as cytokines and TGF-β (Ahmadi et al., 2022) which can have profound and substantial implications for uterine adaptation in the early stages of pregnancy. However, the main paternally derived antigenic stimulatory agents, after the deposition of the semen in the FRT, are most likely delivered by sperm. The risk of gestational hypertension and preeclampsia is three times higher in women conceiving via intra cytoplasmic sperm injection (ICSI), using surgically obtained sperm from men with complete azoospermia (a complete absence of sperm in the semen), in the cases of never being exposed to their partner's sperm than in women conceiving via standard in vitro fertilization (IVF) and ICSI using sperm obtained thorough masturbations (Dekker et al., 2011, Wang et al., 2002).

Studies have also shown that maternal preeclampsia can also affect the long-term health of her children. Offspring born from preeclamptic mothers are lighter at birth and display increased blood pressure in childhood when compared with babies born from non-preeclamptic pregnancies (Hoodbhoy et al., 2021). According to clinical studies, PE is associated with long-term increased blood pressure in these offspring (Davis et al., 2012). Although the precise underlying mechanism is still not fully understood, RAS dysregulation may play a critical role in the long-term programming of hypertension in these offspring (Alexander et al., 2023).

1.3.2 Gestational Diabetes Mellitus (GDM)

Gestational diabetes mellitus (GDM) is defined as the development of hyperglycemia or glucose intolerance, which commences with pregnancy and usually subsides after giving birth (Brown et al., 2017). Currently, GDM is the most common metabolic complication in pregnancy, impacting 1% to >30% of all pregnancies (McIntyre et al., 2019). However, one of the most recent meta-analyses reported a global incidence of GDM at 14.7%, according to the criteria established by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) (Saeedi et al., 2021). GDM is associated with an increased risk of maternal and neonatal complications, which include fetal overweight or increased adiposity, large for gestational age (LGA) weight at birth, an increased risk of future obesity, cardiovascular disease (CVD) and type II diabetes mellitus (T2DM), and even neurodevelopmental issues such as attention-deficit hyperactivity disorder (ADHD) (McIntyre et al., 2019). In GDM pregnancies, the heightened levels of pro-inflammatory cytokines, including TNF-α and IL-6, in the maternal circulatory system have been associated with reduced nutrient transport through the placenta, as well as reduced insulin and insulin-like growth factor (IGF) signalling and cell survival pathways (Musa et al., 2023). Additionally, TNF-α and leptin are potential mediators of insulin- resistance (Kirwan et al., 2002). Increased leptin has also been associated with fetal macrosomia, where it mediates increased expression of the glycerol transporter aquaporin-9, resulting in enhanced nutrient transport to the fetus through the placenta (Pérez-Pérez et al., 2020). Placental secretion of TNF-α has been

recognized as a prominent factor contributing to insulin insensitivity throughout gestation. Moreover, disruptions in the placental production of TNF-α have been associated with impaired insulin signalling transduction, which is responsible for skeletal muscle and white adipose tissue glucose uptake (Musa et al., 2023). Such as in the case of GDM, placental dysfunctions such as increased levels of inflammatory mediators, oxidative stress and increased lipid accumulation have been recognized (Khoshkerdar et al., 2021). As the placenta is derived from both parents, paternally imprinted genes are a player in early lineage and placental development (Plasschaert and Bartolomei, 2014). Two of the most well-studied imprinted genes are insulin-like growth factor-2 (*Igf2)* and *H19.* So, the contribution of the father to the mother's health during pregnancy could be justified through his contribution to the placental formation.

1.4 DOHaD hypothesis

1.4.1 Background and epidemiological data

It is well-established that germ cells carry information inherited from their ancestors, destined for their descendants. Primarily, this 'memory' is stored within the nucleic acid sequence constituting the DNA of the genome, or the genotype.

Around 60 years ago, the understanding of the importance of the intrauterine environment and its influence on long-term life health was established. The pioneering research of Rose (1964), defined the existence of a familial pattern in the occurrence of coronary heart disease (CHD), stillbirth and newborn mortalities (Waterland and Michels, 2007, Rose, 1964). The first geographical correlation between new-born mortality and CVD were described by Forsdahl (Laurén et al., 2003, Forsdahl, 1977), which found a notable positive correlation between mortality risk from arteriosclerotic heart disease among people aged 40-69 years and the rate of new-born mortality in the same cohort. This suggested that poverty in early life was a significant risk factor for later-life disease, irrespective of later-life living standards (Forsdahl, 1977). These initial human observations revealed a direct link between the disproportionate intrauterine growth and the occurrence of non-communicable diseases (NCDs),

including cardiovascular disease, hypertension, obesity, as well as conditions including allergies and cancers, in addition to the neurodevelopmental imperfections later in life. The findings are a strong manifestation of the theory of the DOHaD concept.

Perhaps, one of the most influential epidemiological case studies in the field of developmental origin of health and diseases is derived from the Dutch Hunger Winter cohort study (Schulz, 2010, Paneth and Susser, 1995). The study's examination of the effects of food scarcity on various stages of pregnancy has significantly contributed to the understanding of the DOHaD hypothesis. The Dutch Hunger Winter close to the end of World War II (1944–1945) cohort study documented the impacts of maternal food restriction before and during pregnancy and compared the long-term physical and mental consequences of maternal food deprivation in their offspring at birth and later in life. It appears that the timing of famine exposure plays a central role in the occurrence of offspring ill-health consequences. Early gestation appears to be the most vulnerable time frame for exposure to food scarcity, resulting in an increased rate of psychological problems such as schizophrenia and depression, and cognitive problems, as well as severe physical outcomes in offspring, later in life. Any individual who had been exposed to the Dutch famine in utero at any stage of pregnancy, displayed enhanced levels of glucose in their adulthood, which could be a result of damage to the pancreas beta cells responsible for insulin secretion (De Rooij et al., 2006). In contrast, when pregnant mothers were exposed to the famine during early pregnancy, their babies were born heavier compared to the normal birth weight of the population, they showed enhanced levels of atherogenic lipid profiles, impaired blood coagulation and increased risk of coronary heart diseases as well as hypertension and obesity (Roseboom et al., 2000, Lumey et al., 2009). Altogether, being exposed to famine at the time of early gestation doubled an individual's CHD risk. It is well established that perturbed gestational and postnatal nutrition availability is associated with altered fetal growth, metabolism, and placental efficiency, which could contribute to the development of future diseases (Govoni et al., 2019, Olsen, 2014). Individuals who were exposed to the famine during mid-gestation demonstrated nephrological issues such as enhanced risk of the

occurrence of micro-albuminuria in adulthood (Painter et al., 2005, De Rooij et al., 2022). Exposure to famine in late gestation has been attributed to being born shorter and lighter weight babies and glucose intolerance and diabetes type 2 were observed in adulthood of individuals being exposed to famine in late gestation (De Rooij et al., 2022, Hoffman et al., 2017).

Other human studies have also investigated the incidence of heart disease related to birthweight, A retrospective self-reported study of birth weight among American females in 1976, observed a negative association between birth weight and the incidence of non-fatal cardiovascular diseases and stroke in later life (Rich-Edwards et al., 1997). Similar to the analysis of Barker and colleagues, this study accounted for variables that could potentially influence the outcomes, including socioeconomic status, ethnicity and lifestyle including cigarette smoking in adulthood.

Barker's hypothesis was formulated following a retrospective study which involved recovering historical birth data and death records in Hertfordshire (UK). The study found a high geographic correlation between rates of infant mortality from 1921 to 1925 and specific classes of later adult death. Additionally, it revealed an association between birthweight and rates of adult death from coronary and ischemic heart disease in 1968-78 with infant mortality in 1921-25 (Barker and Osmond, 1986). The interpretation of this hypothesis was influenced by several factors, including the strong correlation observed in 1920 between low birth weight and fetal mortality rates. This correlation was initially based on suboptimal intrauterine environment and conditions rather than postnatal factors. Additionally, a paradoxical relationship was observed between the occurrence of cardiovascular disease and the affluence rate, with the occurrence of the highest rate of CVD among non-prosperous individuals. These observations led Barker to hypothesize that undernutrition during gestation was an important early origin of adult cardiometabolic disorders due to fetal programming that permanently shaped the body's structure, function, and metabolism and contributed to adult disease (Barker, 1995). While Barker suggested all these adverse clinical outcomes and fatality rates were results of the fetus's adaptation responses to the intrauterine environment conditions which

makes a clear link between the periconceptional period as well as intrauterine conditions and future health. Therefore, the hypothesis of DOHaD was introduced. The DOHaD concept proposes that a mother's physiological condition, diet and lifestyle in the periconceptional period and throughout pregnancy have fundamental and long-lasting impacts on her offspring's health in adulthood, which magnify the sensitivity of early embryo to the environmental conditions which can build pathways in programming and characteristics of future diseases risk (Fleming et al., 2018, Barker and Thornburg, 2013, Fleming et al., 2015).

Another substantial birth cohort study with high relevance to reproductive medicine was the Southampton Women's Survey (SWS) which ran between 1998 and 2002 and considered many areas of women's health and nutrition, especially during preconception, throughout pregnancy and early childhood. The SWS enrolled pregnant women aged 20–34 years, in their first trimester and gathered thorough data on a variety of characteristics, to measure the pre-pregnant characteristics including nutrition, lifestyle, medical history, and socioeconomic position. The survey's major goal was to investigate how these factors influence pregnancy outcomes, baby development, and long-term health outcomes in both mothers and children. The SWS has produced vital insights into the links between maternal diet, lifestyle, and health and their impact on the health and development of the next generation by tracking participants over time (Wadhwa et al., 2009, Inskip et al., 2006). The SWS has yielded a broad range of findings regarding the risk of cardiometabolic ill-health in offspring's later life due to maternal diet, stress, physical activity and body composition during pregnancy. Additionally, it has provided valuable insight into nutritional intervention for women during pregnancy. The study also explored how maternal diet influences the modification of epigenetic factors, specifically DNA methylation. Furthermore, the phenotypic data obtained in late childhood enabled the identification of key periods for programming adiposity and comorbidities both before and after birth (Nutrition).

The Helsinki Birth Cohort Study (HBCS) is an ongoing socio-economic study, comprised of 6544 men and 6050 women born between 1934 and 1944 in

Helsinki, Finland. The major goal was to investigate how prenatal and early-life characteristics, such as birth weight, gestational age, and childhood growth, may influence the chance of metabolic disease later in life, and to explore the association between maternal, placental, and neonatal characteristics and growth trajectories across the life-span (Lahti et al., 2014). One such finding identified an association between shorter length at birth, shortened height up to two years of age, and lower weight at two years, with an increased risk of alcohol use disorders later in life (Lahti et al., 2014).

One of the major challenges in epidemiological research is our limited ability to comprehend the underlying mechanisms. Therefore, animal models have played a critically significant role in demonstrating how alterations in the fetal environment can result in metabolic programming later in life through alterations in the epigenetic mechanism. Animal evidence studies according to the gestational intervention, such as iron deficiency and maternal under-feeding, confirm the occurrence of offspring phenotypical traits similar to those seen from human epidemiological data (Langley-Evans, 2015). The confirmation of the effects of the epigenetic factors could be established by conducting animal experiments on microinjections. A diverse range of animal models has been developed to test aspects of the DOHaD hypothesis, including rats, mice, guinea pigs, pigs, sheep, and more recently baboons (Langley-Evans, 2013, Hsu and Tain, 2021). (Hsu and Tain, 2021) DNA methylation status is largely established in utero and then persists, hence providing a potential link between early life environment and phenotypic changes observed in later life. In a maternal low protein diet rat model study, a significant decline in the level of DNA methylation of the glucocorticoid receptor (GR) and peroxisomal proliferator-activated receptor (*Ppara)* was observed, coupled with an enhanced level of corresponding mRNA in the progeny. Resultant changes in the activity of GR and *Ppara* might contribute to the risk of increased disease, such as hypertension and induction of dyslipidemia (Lillycrop et al., 2005). The role of maternal environmental conditions and lifestyle in the occurrence of epigenetic inheritance both during oogenesis and pregnancy has been well-established and studied extensively (Gluckman et al., 2008, Jimenez-Chillaron et al., 2009, Radford et al., 2014). Perhaps one of the first animal model studies investigating

in the field the heritable epigenetic effects of environmental intervention and their implications on the phenotypic manifestations in the offspring has originated from the studies conducted on pregnant rats treated with endocrine disruptor vinclozolin. The consequences of this exposure including infertility and behavioural alterations have been passed on through multiple generations of these rats' offspring (Anway et al., 2005, Crews et al., 2007).

In addition, changes in histone modifications, in response to environmental status, have also been confirmed using animal models. A rat model of IUGR resulting from placental insufficiency has been used to study the development of adult-onset diabetes (Park et al., 2008). It demonstrated that IUGR resulted in a reduction in acetylation of histones H3 and H4 associated with the critical pancreatic regulator *Pdx1*, increased *Pdx1* methylation and silenced *Pdx1* expression, leading to diabetes (Park et al., 2008, Bianco-Miotto et al., 2017).

Suggesting alterations in the expression of the miRNA in female gametes have been investigated (Carletti and Christenson, 2009). By microinjecting the specific RNAs into a zygote that has not been exposed to the specific environmental conditions, as a causation the offspring exhibits the phenotypes related to the specific environmental exposure (Lee and Conine, 2022). A rodent model of maternal protein restriction demonstrated an enhanced level of the imprinted miRNA, miR-483-3p in the adipose tissue of offspring with low birth weight (Ferland-McCollough et al., 2012). Enhanced miR-483-3p expression early in life leads to restriction in the storage of lipids in adipocytes, resulting in lipotoxicity as well as insulin resistance, therefore, making the offspring more prone to metabolic disease later in adulthood.

Fetal programming occurs during critical periods of development when changes become permanent and lead to later pathophysiological events (Bertram and Hanson, 2001). A rat study conducted by Langley et al. (1994) revealed that a maternal low-protein diet during pregnancy was associated with alterations in placental growth and fetal growth, in addition to organ development such as liver and lung. Late gestation growth retardation was one of the main manifestations of this experiment, as well as the differences in growth rate. Both sexes of male

and female grew quickly at the time after birth until week 4, however after puberty, they were smaller than the offspring born from control dams, which showed that intrauterine low protein exposure tends to alter the offspring growth drastically. This was one of the first studies to show experimentally that fetal exposure to maternal undernutrition increased systolic blood pressure in later life, mirroring data from human epidemiological data sets.

Short and long-term adverse effects of pregnancy outcomes including low birthweight (LBW), premature birth and infant mortality are influenced by the maternal diet, both before conception and mainly during the period of pregnancy. Insufficient nutritional intake during these vital periods leads to differential adaptation in the development of essential tissues and subsequently downregulation of fetal growth (Woldeamanuel et al., 2019). Therefore, emerging evidence from human and animal model studies suggests there is a direct link between poor maternal metabolic health and aberrant fetal development and programming long-term effects on offspring health and adult diseases (Lin et al., 2019, Okubo et al., 2012, Wroblewska-Seniuk et al., 2009, Godfrey and Barker, 2001, Barker, 1997a).

1.4.2 Critical windows in fetal programming

As discussed earlier during the Dutch Hunger Winter different phenotypical traits were documented in the individuals who were born in the different trimesters of pregnancy being exposed to the famine, the results were heterogeneous. The differences could be discussed through the understanding of experimental and epidemiological data from the DOHaD hypothesis indicating that different stages in development and gestation show differential sensitivities to environmental perturbations These windows are critical points in DOHaD as they can create a possibility to intervene and prevent programmed adverse phenotypes in the offspring. One of these critical stages is during the process of gametogenesis which could be influenced by epigenetic mechanisms. Epigenetics can be defined as any heritable alters that happen in gene functions followed by mitotically or meiotically changes, these changes cannot be elucidated by alters in DNA sections (Gluckman et al., 2016, Huang et al., 2013).

Not only the period of pregnancy could imply a significant alteration in the growing fetus, but also periconception period influences on development are believed to occur through environment-induced modification of the embryo's epigenome (Gluckman et al., 2010, Lane et al., 2014). During gametogenesis, the sperm and oocyte genome undergo remarkable epigenetic reprogramming such as DNA methylation. Histon modifications as well as miRNA alterations (Hieronimus and Ensenauer, 2021). Post fertilization, substantial reprogramming occurs in the parental genome, as most epigenetic marks are deleted and allow de novo methylation to take place. Allowing programming of the embryo from the beginning, however, certain epigenetic marks avoid reprogramming in turn this means these epigenetic marks can carry forward environmental information affecting the programming of phenotypes through to the next generation (Barbosa et al., 2016). The results of this epigenetic reprogramming can impact embryo metabolism (Sookoian et al., 2013), and partially sustained over life. Intriguingly, during the period of embryogenesis, the second wave of genome-wide epigenetic remodelling occurs in the primordial germ cells (PGCs) (Zeng and Chen, 2019).

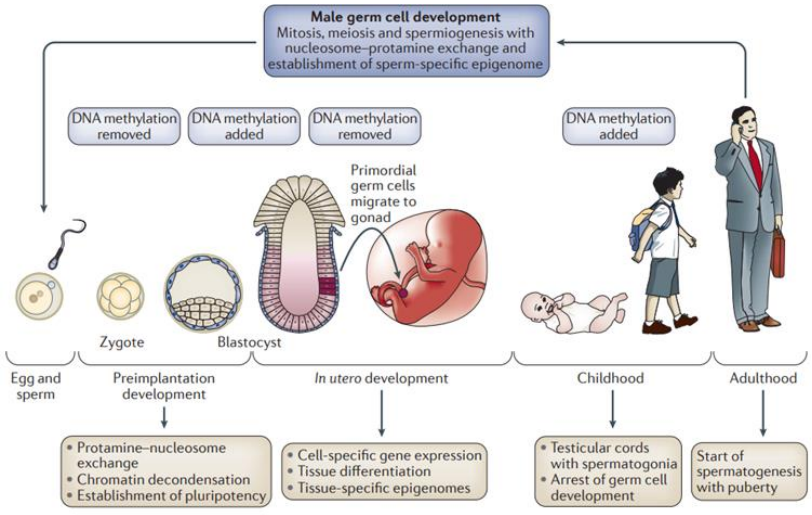


Figure 1-8 Shows the central epigenetic events in human sperm development. In early embryo development stages, the replacement of protamine with nucleosomes initiates these alterations, which leads to the relaxation of chromatin. After fertilization, a re-programming phase occurs during the preimplantation stage, establishing totipotency and allowing for specialized cell differentiation. The reprogramming of the inherited epigenetic profiles begins following the migrations of the primordial germ cells (PGCs), during fetal development and this will proceed in spermatogonia cells during childhood. During adulthood, the epigenetic makeup of sperm is established by DNA hypermethylation and rearrangements of epigenetic factors related to retaining histones. At the time of fertilization both the sperm's epigenome and genome are transmitted to the oocyte (Schagdarsurengin and Steger, 2016).

The next vulnerable window could be referred to the in-utero stages including the preimplantation and fetal development periods. The importance of the preimplantation period plays a substantial role in human ART, including the molecular and morphological changes, as well as differentiation lineage in ICM and TE. This vulnerable window in ART is accompanied by various clinical conditions such as SGA, LGA, placentation deficits and imprinting disorders (Minucci and Alessi, 2022). The importance of imprinted genes in fetal growth is considerably essential, and any dysregulation of imprinting gene expression in the placenta may lead to developmental dysfunction (Fowden et al., 2011).

Imprinted genes have a substantial role in the placenta and expressed imprinted genes such as *PHLDA2* and *IGF2R* have a primary role in providing nutrients for the fetus (Sibley et al., 2004, Moore et al., 2015). The significant aspect of the imprinting gene process is monoallelic expression determined by parental origin. This entails that imprinted genes are expressed solely from one parent, while the copies inherited from the other parent remain silent. When the paternal allele is expressed, the maternal copy stays unexpressed/silenced and vice versa. This expression of either paternal or maternal allele is typically driven by differential levels of DNA methylation and histone modifications (Elhamamsy, 2017). Genomic imprinting plays a key role in maintaining normal embryogenesis, and prenatal and postnatal growth. In genomic imprinting, several epigenetic processes are involved to result in a unique epigenetic signature observed at a subset of loci in the genome (Elhamamsy, 2017). Histone modification and non-coding RNA and DNA methylation, of cytosines in areas of DNA that are rich in CpG dinucleotides known as imprinting control regions (ICR) are taking part in the gene imprinting process (Fowden et al., 2011).

In addition to the importance of preimplantation environment in human ART, maternal environmental conditions at this stage have a crucial role in programming the long-term ill-health of the individuals, *in vivo*. A mouse model of maternal LPD-fed (9% of casein in Emb-LPD compared to 18% of casein in normal diet), which studied the specific stage of E0-E3.5 of pregnancy. Exposure to the perturbed intrauterine environment though for a very short period, leads to substantial adverse effects on the cardiometabolic health of their

offspring later in life (Fleming et al., 2017, Safi-Stibler and Gabory, 2020). The fetal period has been associated with tissue differentiation as well as organogenesis. Organogenesis in these stages is extremely vulnerable to any alteration in uterine conditions, as well as maternal metabolic diseases, especially diabetes. Maternal gestational diabetes is a pathological condition during pregnancy, which puts the fetus at risk of macrosomia, and it could leave substantial impacts on the organogenesis and overall growth of the fetus. Occurrence of GDM in early gestation has been associated with congenital anomalies and malformation, through increased levels of oxidative stress, and activation of cellular stress signals which leads to enhanced levels of apoptosis in humans (Zawiejska et al., 2020, Wu et al., 2020). Different animal model experiments such as maternal caloric restriction, LPD or uterine artery ligation report the role of epigenetic alterations in the occurrence of phenotypic traits such as insulin deficiency and dyslipidemia in adult offspring, with epigenetic alterations such as a reduction in H3K14ac and enhanced level of H3K9me2, associated with increased Hdac1/4, Kmt1a and HP1α binding (Raychaudhuri et al., 2008).

Post-natal development represents another critical window within DOHaD, making the post-natal period vulnerable to any deviation from optimal environmental conditions. Developments of some specific organs such as the intestine, liver and adipose tissues, as well as the maturation of the nervous system, have been linked with parental care and social environments. The importance of this period could be associated with the nutritional transition from umbilical cord feeding to oral feeding (Hue-Beauvais et al., 2017, Symonds et al., 2009a). Experimental studies in rodents and rabbits documented the association with lactation nutritional status to the susceptibility of long-term metabolic diseases.

1.4.3 Paternal origins of health and disease (POHaD)

Extensive experimental and epidemiological evidence exists connecting poor maternal health and offspring programming, however, there is now a growing body of data recognizing the link between paternal factors at the time of conception and the health of his offspring (Eberle et al., 2020, Sharp et al., 2018). By expanding the studies in the field of DOHaD, animal model experiments have investigated the substantial impacts of environmental factors on male germ cells as well as a non-genetic inheritance of pre-conceptional traits (Soubry, 2018). However, the impacts of parental lifestyle in the vulnerable periods of pre- or periconception have been overlooked.

Experimental animal studies indicated the impacts of paternal transgenerational epigenetic influences through the manipulation of the male germ line. Unfortunately, the data derived from human studies regarding paternal influences and epigenetic effects are scarce. These environmental factors include stress, imbalanced (over/under nutrition) diets and exposure to substances like bisphenol and heavy metals. (Soubry, 2018). According to animal studies and a limited number of epidemiological studies, the concept of paternal origin of health and disease (POHaD) as an extension of the DOHaD paradigm has been introduced (Soubry et al., 2013, Soubry, 2021, Soubry, 2018).

The POHaD paradigm concentrates on the importance of further investigations to find out how the father could pass on the effects of the environmental factors to his offspring (Kim and Lee, 2017). For the first time, the theory of POHaD was introduced by (Houfflyn et al., 2017), emphasized how the father's health status, both at the time of conception and even prior to it, has the potential to significantly impact the long-term health of his offspring. The influence extends not only to the transmission of environmental information to subsequent generations but also to the transfer of NCDs. Furthermore, it can have significant implications on their offspring's reproductive ill-health, as well as extensive cardio-metabolic outcomes later in life (Billah et al., 2022).

A study conducted by Swedish researchers in Överkalix, a small, geographically isolated pastoral parish of northeast Sweden, demonstrated that a father's limited access to dietary resources during pre-adolescence (between the ages of 9-12), which was defined as a "slow growth period" was associated with decreasing chance of diabetes and cardiovascular mortality in his offspring, as well as impacting on their grand-children life spans. This data strongly supports the idea that there are some paternal factors which can be transmitted to multiple generations, via epigenetic inheritance (Kaati et al., 2002, Pembrey et al., 2006b, Bygren et al., 2001). In contrast, exposure of the fathers or grand-fathers at a similar period had enhanced the risk of the mortality rates related to diabetes (Pembrey et al., 2006b, Kaati et al., 2002).

Other epidemiological studies indicate the importance of paternal metabolic health at the reproductive age associated with the metabolic health of their children. A large cohort study using the UK Biobank studying a huge number of individuals including 230,000, demonstrated that paternal diabetes at the time of conception resulted in low birth weight and enhanced the chance of diabetes in their children (Tyrrell et al., 2013, Hur et al., 2017). Investigating the role of paternal lifestyle and factors such as age, smoking, exercise, and diet on his offspring's metabolic ill-health is receiving more attention. Human studies have shown a strong association between advanced paternal age (APA), and pregnancy complications such as placental abruption, miscarriage, premature birth, and low birth weight (Alio et al., 2012, Khandwala et al., 2018).

In vivo and *in vitro* studies in mice showed that APA has a significant negative reproductive outcome (Katz-Jaffe et al., 2013). In mice, APA is related to long-term impacts on postnatal development and behavioural features in the offspring, as well as reproductive fitness and longevity (Denomme et al., 2020). Yet, little is known about the direct impact of APA on the molecular processes regulating embryonic growth in utero. Recent research has demonstrated a correlation between APA and increased levels of reactive oxygen species (ROS) and DNA damage in sperm and testis tissue (Vaughan et al., 2020, Dutta and Sengupta, 2016, WHO, 2010). Oxidative stress is identified by an excess of reactive oxygen species (ROS), such as superoxide anions, peroxides, and

hydroxyl radicals. These harmful molecules are generated in more quantities than the cells' antioxidant systems can keep up with (Bakos et al., 2011a). So, one of the serious consequences of the increased level of oxidative stress in sperm, is increased lipid peroxidation, loss of sperm motility and eventually reduction of sperm-oocyte binding. So increased sperm ROS and lower rates of pregnancy and fertilization are linked to older paternal ages. There is a significant link between higher paternal age at conception to lower infant birthweight and a higher chance of premature birth (Halvaei et al., 2020). A population-based cohort study showed that increased paternal age was negatively associated with maternal health as identified through an increased risk of gestational diabetes (Khandwala et al., 2018). Disruptions in epigenetic modifications, such as DNA methylation, which control gene expression without changing the DNA sequence, are considered a potential underlying mechanism linking alterations in fetal growth and development observed with increased paternal age. For the creation and upkeep of epigenetic markers, gametogenesis and embryogenesis are critical stages. It is theorized that as men age, epigenetic mistakes can develop during spermatogenesis (Yatsenko and Turek, 2018). Errors in DNA methylation associated with ageing have already been found in mouse and human sperm (Kobayashi et al., 2016, Milekic et al., 2015). The paternal epigenome has been shown to play a crucial role in embryo development; hence, temporal changes in the sperm epigenetic landscape with ageing the fathers and age-related DNA methylation errors may be transmitted to the offspring and subsequently affect offspring development (Denomme et al., 2020).

While the precise underlying mechanism of paternal programming mechanisms has yet to be defined, human and animal model studies have identified inter and trans-generational paternal programming, highlighting epigenetic mechanisms as a central mechanism in this transmission. While the role of maternal environmental exposure and the transmission of the implications across several generations have been well-defined, more work needs to be done to understand the mechanisms underlying paternal programming.

Indeed, experimental studies on rodent models have shown a wide range of potentially detrimental effects of paternal programming on male offspring and grand-offspring reproductive outcomes, including anatomical changes, reduced testosterone concentrations, poor sperm morphology, reduced sperm quality, reduced sperm-oocyte binding and increased oxidative DNA damage (Fullston et al., 2015, McPherson et al., 2014, Aitken and Curry, 2011). Similarly in female offspring, oocyte quality, embryo development and quality have all been reported in response to paternal obesity (Billah et al., 2022). In addition, animal experiments on studying the impacts of paternal diet result in the understanding of intergenerational paternal programming.

The first report of the mammalian, non-genetic, transmission of intergenerational metabolic diseases has been conducted in an HFD-fed male mouse model (Ng et al., 2010b). This study emphasized the impacts of paternal obesity on the occurrence of NCDs in their offspring in a sex-specific manner through the non-genetic mechanism which these alterations resulted in changes to the sperm DNA sequence that affected offspring health. The significant reduction in the secretion of insulin with increased impairment in glucose tolerance by increasing the age of these female offspring was observed in F1 female offspring., in response to paternal HFD (Ng et al., 2010b). Ng et al. added, that hypomethylation of the *Il13ra2* gene, which is a part of Jak-Stat signaling pathways considered one of the central pancreatic genes, was observed. Other animal studies in rodent models, showed the adverse effects of paternal obesity and HFD on male semen parameters, and negative impacts on sperm quality, as well as deleterious implications on the early developmental stages resulting in a reduction in embryo quality (embryo development rate) in addition to the number of embryos recovered (McPherson et al., 2013).

Obesity and paternal HFD-fed models in rodents have been interlinked with sperm epigenetic alterations in different levels of the microRNA and/or DNA methylation as well as histone modification (de Castro Barbosa et al., 2016, Fullston et al., 2013, McPherson et al., 2013). The other types of paternal diet interventions such as paternal low-protein diets (Carone et al., 2010, Watkins and Sinclair, 2014a, Watkins et al., 2018) and methyl donor manipulated models

have documented significant changes in offspring metabolic status (Lambrot et al., 2013b), following by NCDs in these offspring in adulthood with considerable changes in sperm epigenetic status of the fathers.

Noteworthy, a significant finding from a paternal LPD-fed demonstrated that the offspring derived from these fathers exhibited an elevation in the level of cytosine methylation in the promoter regions of the genes such as *Pparα* involved in governing the central pathways in the synthesis of the lipid and cholesterol (Ly et al., 2019). However, no significant alteration in the level of the cytosine methylation in the loci of imprinted genes was reported in the offspring derived from LPD-fed fathers, as well as no alterations in cytosine methylation profiles of paternal sperm were reported (Carone et al., 2010). The obtained results from the animal studies suggest that the reason for these phenotypical manifestations in the offspring of LPD-fed fathers is associated with the molecular mechanism in male spermatogonial cells. Epidemiological studies in humans have demonstrated a strong association between transgenerational paternal nutrition status and the occurrence of NCDs such as diabetes, obesity and cardiovascular dysfunctions in their children and grandchildren (Power et al., 2003, Kasturi et al., 2008).

As well as sperm DNA methylation, chromatin structure and histone modifications, sperm sncRNAs have the main role in paternally induced intergenerational metabolic programming effects (Nilsson and Skinner, 2015). Sperm miRNAs are transferred into the oocyte during fertilization, which has the potential to alter gene expression within the embryo. It has been reported sperm born microRNA-34c is essential for first cleavage in mice (Fullston et al., 2016). Studies, involving the microinjection of excessive quantities of individual microRNAs into pronuclear (PN) stage embryos exhibited solid proof that changes in the expression of microRNA levels during the early stages of embryo development can trigger characteristics and phenotypes in adult offspring. The phenotypes influenced by the paternal miRNAs include cardiac hypertrophy (microRNA-1(Wagner et al., 2008)), coat color changes (microRNA-221/222 (Rassoulzadegan et al., 2006)), embryo and offspring overgrowth (microRNA-

124 (Grandjean et al., 2009)), and an obesity phenotype microRNA-19b (Grandjean et al., 2015) (Fullston et al., 2016)).

As the importance of the different stages of exposure to impaired environmental conditions was discussed in the concept of the DOHaD framework, the importance of the pre-conceptional window in response to paternal environments and its role in fetal programming has been receiving attention. The pre-conception body mass index (BMI) of fathers revealed an association with the child BMI z-score (zBMI) in their children as well as growth rate and weight status, particularly in boys. These effects were more pronounced in children with an overweight or obese biological mother during the periconceptional period. However, more research is required to define precisely how father weight impacts children's BMI, particularly regarding the potential differences considering the child's gender (Deveci et al., 2023).

The precise mechanisms underlying how sperm epigenetic alterations such as DNA methylation in exposure to environmental factors can escape from being reset during the early embryo developmental stages remains yet elusive. Therefore, various mechanisms have been recommended to transduce these non-DNA sequence-based inheritance in mammals. Including the chemical modification of the DNA and histones, or it could be related to the transfer of small regulatory RNAs that complement the genomic sequences (Perez and Lehner, 2019, Zheng et al., 2021).

The term transgenerational epigenetic inheritance refers to the transmission, which involves the transferring of programming effects, and physiological traits, as well as the transmission of pathologies including metabolic outcomes, being passed down via germ cells, across generations excluding the primary exposure to the original triggers, either exposure of the germ cells or developing fetus (Champroux et al., 2018). In mice, injection of sperm transfer RNAs (tRNAs) from males fed a high-fat diet into normal zygotes results in metabolic disorders and alterations in gene expression in the first-generation (F1) offspring (intergenerational). This suggests that paternal tRNA may be a further epigenetic mechanism by which paternal diet influences the sperm epigenetics status

followed by offspring health (Chen et al., 2016). As well as, a paternal HFD study reported, induction of the obesity in father by 21%, as well as the observation of obesity and insulin resistance in the next two generations (F2), by influencing the DNA methylation status of spermatogonia germ cells (F0), as well as alterations in the expression and abundance of different mi-RNAs such as miR-205, miR-340, in testis and sperm (Fullston et al., 2013). Morgan et al. (2020b), reported a fascinating mice model of paternal LPD, which directed perturbation in RAS pathway dysfunction CVD in the offspring of LPD-fed fathers. These impairments were also programmed into the second generation (F2).

1.4.4 Paternal programming

These long-term risks for the offspring include cardiovascular, metabolic, immune, and neurological morbidities, often termed developmental programming (Fleming et al., 2018). An offspring's healthy development is highly dependent on the quality of the parental gametes at the time of conception. Parental lifestyle factors, such as lack of exercise, smoking, and poor diet typically may pose risks to the development of a healthy embryo and offspring's ill health later in life.

To prevent undesirable offspring outcomes the human and animal model studies, reported that treatment intervention in the preconception window should not be limited to the women but also it should also be shifted to the father as well (Hieronimus and Ensenauer, 2021).

Perhaps one of the first studies that led to the understanding of paternal metabolic programming, originated from studying the offspring of male rodents who chemically induced diabetes. In this study Okamoto (1965), demonstrated the offspring of rats and rabbits exposed to drug-induced diabetes, their offspring showed the development of diabetes up to multigeneration without being exposed to the diabetogenic drug.

In rodents, a paternal low-protein diet, has been shown to affect offspring cardio-metabolic health, such as increased body weight and adiposity at the time of

birth, hypotension and increased heartbeats, and liver gene expression mimicking non-alcoholic fatty liver disease (NAFLD) (Watkins and Sinclair, 2014a). Analysis of sperm from LPD-fed male mice displayed global hypomethylation in line with the disruption of testicular one-carbon metabolism (1-C) genes including the folate cycle genes *Dhfr*, *Mthfr* and *Mtr* and the DNA methyltransferases *Dnmt1*, *Dnmt3L* (Watkins et al., 2018). 1-C metabolism, mediated by the folate cofactors, plays a central role in multiple physiological functions including the biosynthesis of purines and thymidine. The other roles of the 1-carbon metabolism pathway have been associated with maintaining the balance of amino acids including glycine, serine, and methionine, as well as supporting the epigenetic regulations, in addition to playing a protective role against oxidative stress (Ducker and Rabinowitz, 2017, Lyon et al., 2020). Therefore, there is an association between paternal diet resulting in sperm epigenome alterations and associations with fetal development.

In rats, a paternal high-fat diet resulted in impaired metabolic health in female offspring, through hyper methylation of DNA and transcriptome changes in the pancreas and adipose tissues (Lane et al., 2014). Alteration in the expression of the miRNA let-7c in the sperm of F0 rats and their F1 offspring was observed in the male mice fed with a high-fat diet. Moreover, the differential expression of miRNA let-7c in metabolic tissues of the offspring, by changing the expression of anticipated target genes located in adipose tissue (de Castro Barbosa et al., 2016).

Epidemiological studies conducted in the area of paternal programming suggest the existence of traits or characteristics that are inherited from the father to his offspring through genetic and epigenetic transmission (Pembrey et al., 2006b). Epidemiological studies in humans have proven the role of paternal programming as a transgenerational factor. Such studies have shown direct relationships between food intake in grandparents or paternal smoking exposure with elevated body mass index in male grandchildren (Pembrey et al., 2006a).

It is well-established that risk factors such as paternal advanced age, smoking and exposure to chemicals such as phthalates and bisphenol A (BPA) are

correlated with elevated risks of cancer and neurological disorders in children, through paternal programming (Lee et al., 2009, Van Balkom et al., 2012). For instance, paternal smoking is associated with an increased risk of leukaemia in children (Lee et al., 2009). There is mounting evidence for pathways of paternal transgenerational epigenetic effects that can be attributed to sperm and seminal fluid with mechanisms that go beyond sperm DNA damage (Lane et al., 2014).

An extensive body of data firmly confirmed the inverse impact of maternal BMI on oocyte quality, plus embryo and offspring development (Metwally et al., 2007, Bartolacci et al., 2019, van Duijn et al., 2021). Intriguingly, independent of the mother's weight, children born from pre-conceptionally obese fathers are more prone to developing obesity and metabolic impairments (Billah et al., 2022, Campbell and McPherson, 2019, Shi and Qi, 2023).

1.4.5 Epigenetic mechanisms

Epigenetics can be defined as any heritable alters that happen in gene functions These changes cannot be elucidated by alteration in the DNA sequence. During gametogenesis, the sperm and oocyte DNA undergo remarkable epigenetic reprogramming. Following fertilization, substantial reprogramming occurs in the parental genome, as most epigenetic marks are deleted and allow de novo methylation to take place. Allowing programming of the embryo from the beginning, certain epigenetic marks avoid reprogramming in turn this means these epigenetic marks can carry forward environmental information affecting the programming of phenotypes through to the next generation (Barbosa et al., 2016).

Research has focused on how environmental conditions impact germ cell mutations within the coding and promoter sections of genes (Lambrot et al., 2013a, Yauk et al., 2008, Ge et al., 2013, Trapphoff et al., 2013). Alternatively, cells can inherit and pass on information that lies outside the genomic sequence. These 'epigenetic' modifications go beyond traditional genetics and involve the hereditary transfer of elements regulating patterns of gene expression, which are passed to offspring cells during cell division.

Epigenetics is today usually defined as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA section” (Gluckman et al., 2016). Epigenetics encompasses multiple chemical reactions and processes that modify and regulate gene activity without affecting the underlying DNA sequence (Rhon-Calderon et al., 2019). The three main mechanisms governing epigenetic gene transcription are DNA methylation, histone modifications, and regulation by noncoding RNA-associated gene activation (expression) or silencing (repression) (Goyal et al., 2019). Epigenetic modifications are predisposing alterations that do not result from changes in the DNA sequence, which are affected by genetic variability and environmental influences in disease. They alter DNA accessibility and chromatin structure, thereby regulating patterns of gene expression (Pisarska et al., 2018). One of the most well-studied epigenetic marks is DNA methylation, the addition of a methyl group directly on a cytosine base. This epigenetic mark plays a role in genomic imprinting, a phenomenon in mammals that regulates gene expression in a parent-of-origin-specific manner (Rhon-Calderon et al., 2019). The role of DNA methylation in epigenetics is well-established, cytosine methylation is one of the most prominent epigenetic alterations in mammalians, which has frequently been attributed as a mechanism of transgenerational inheritance (Cedar and Bergman, 2012, Daxinger and Whitelaw, 2012).

Shea et al. (2015) demonstrated that a paternal low-protein diet, high-fat diet, and caloric restriction, in mice influence the landscape of cytosine methylation in sperm and cause the transgenerational transfer of metabolic phenotypes in offspring. impacts of affecting. Transfer of epigenetic information such as changes in the DNA methylation profile (Ng et al., 2010a, Fullston et al., 2013), histone modification (Carone et al., 2010) and miRNA expression (Fullston et al., 2013) caused by dietary factors and carried by gametes may influence the metabolic phenotype of the offspring.

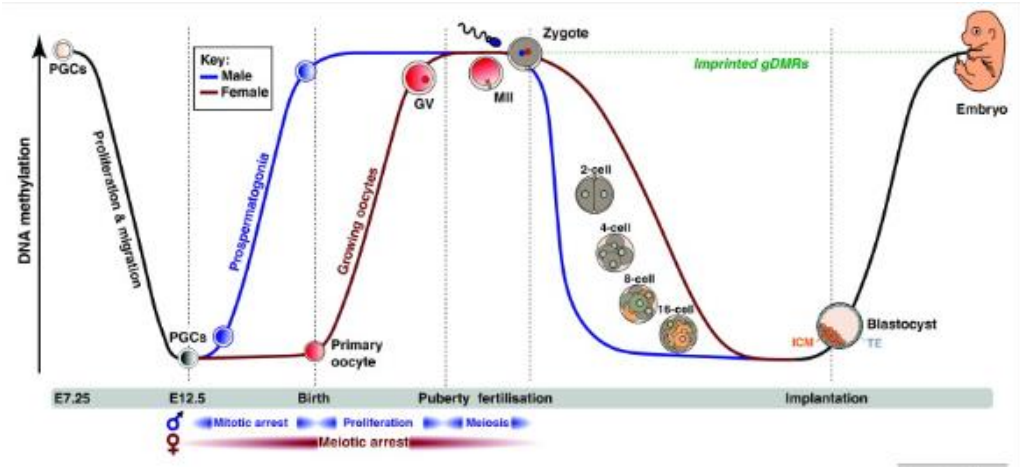


Figure 1-9 The above schematic figure demonstrates the two waves of DNA methylation alterations in mammalian development, representing the two main phases of DNA demethylation and remethylating during the lifetime. The DNA of the primordial germ cells (PGC) are highly methylated primarily. During the migration of the PGCs global demethylation takes place. The process of De novo methylation plays a central role in creating the sex-specific germ cell methylation pattern, including methylation marks imprinted loci in germ cells based on sex, which happens at the next phase of germ cell development, prior to the birth in males and after in females (Smallwood and Kelsey, 2012).

Therefore, it is clear that disrupting epigenetic reprogramming during the early stages of embryogenesis can have significant impacts on the programming of the developing embryo (Kundakovic and Jaric, 2017). For instance, experimental studies have documented the impacts of the paternal insulted diet on the embryos derived from exposed sperm and future offspring. Studies in rodents reported the impacts of paternal diets on alterations of epigenetic patterns in the embryos derived from exposed sperms (Barakat et al., 2020, Sharma et al., 2016b)..

Methylation of cytosines is correlated with inactivation or silencing of the associated promoter, whereas hypomethylation usually leads to activation of gene expression. Silencing of gene expression is either due to inhibition of transcription factor binding to methylated cytosines or repression mediated by methyl-CpG-binding proteins (Gunes et al., 2016). Histones are basic proteins rich in lysine and arginine located within the nucleus and are subject to post-translational modifications on their N- and C-terminal tails via acetylation, methylation, phosphorylation, and ubiquitination (Gunes et al., 2016). Epigenetic modifications of histones will be applied to proteins (histones),

which control the chromatin compaction and the accessibility of DNA. The two central types of histone modifications encompass acetylation and methylation and their consequence on the gene expressions ultimate (expression or repression), according to the numbers and positioning of these markers within the histone complex (Lapehn and Paquette, 2022).

One of the most recent aspects of epigenetic modifications is the epigenetic modification of non-coding RNAs (ncRNAs), which could result in long-term/persistent changes in offspring. Recent transcriptomic investigations have disclosed that in the eukaryotic cells, the genomes transcribe almost 90% of the eukaryotic cell DNA. Although just a small section of these transcriptions are responsible for protein production, which these transcription sequences make up the non-coding RNAs (Kaikkonen et al., 2011). Based on the informative research, several non-coding RNAs have been classified into different groups, this categorization is determined based on their length and functions, such as transfer RNA (tRNA), ribosomal RNA (rRNA), micro RNA (miRNA), and long non-coding RNA (lncRNA) among others (Washietl et al., 2007, Zhang et al., 2017c). Of note, two types of coding and non-coding RNAs have been detected within the sperm. As well as small noncoding RNAs (sncRNA) species as such, microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), piwi-interacting RNAs (piRNAs), and tRNA-derived small RNAs have also been found in sperm. They are synthesized in the nucleus and the process of maturation takes place within the cytoplasm, the biogenesis of these miRNAs requires the presence of two main RNase III enzymes, known as Drosha and Dicer also known as Dicer1, which contributes to the process of the RNA-induced silencing complex (RISC), plays an important role in the process of the gene silencing. The role of these small RNAs in the transgenerational effects of paternal lifestyle is indeed significant. Discoveries regarding their influence on offspring development have been crucial, offering fresh insights into the realm of paternal fertility (Yuan et al., 2016). Short RNA molecules which are called (miRNAs) bind onto mRNAs and can influence gene expression, by preventing the expression of the mRNAs or signaling the destabilizing of the mRNA resulting in translational silencing (Cannell et al., 2008). Mature miRNAs exert their impact via binding to the 3’ untranslated region of a specific mRNA and

either cause degradation or constrain protein translation. Additionally, miRNAs target genes involved in epigenetic modulations, such as DNA methyltransferases or histone acetylase enzymes (Pajares et al., 2021). Epigenetic inheritance remains the likeliest candidate to carry the impacts of environmental alteration on paternal information to offspring (Rando, 2012), instead of any alterations in genetic status. There is evidence that paternally derived RNAs play an important role in the development of obesity, metabolic disorders, and stress responses in animals (Braun et al., 2017). The influence of paternal diet on the gene expression and metabolism in the progeny, also designated as paternal-diet-induced intergenerational metabolic reprogramming (IGMR), was demonstrated in rodents, strongly suggesting the non-genetic intergenerational transmission of metabolic sequelae of paternal diet through sperm (Okada and Yamaguchi, 2017, Colaco and Sakkas, 2018). IGMR was originally observed in rodents, this robustly indicates the non-genetic intergenerational transmission of metabolic sequelae of paternal diet through sperm. Genomic imprinting (GI) is a unique phenomenon that occurs in placental mammals, marsupials, and a subset of flowering plants (Colaco and Sakkas, 2018b).

Solid inverse correlation between the increased BMI and semen parameters quality including ejaculate volume (Eisenberg et al., 2014, Bieniek et al., 2016), sperm concentration (Tsao et al., 2015), sperm motility (Martini et al., 2010) and sperm morphology (Martini et al., 2010, Bieniek et al., 2016), in men, has been reported. On the other hand, there are conflicting debates regarding increased BMI and adverse effects on semen quality, (MacDonald et al., 2010). There are large datasets derived from animal models which support an association between impaired male fertility and obesity-inducing diets (Rato et al., 2013), mice (Bakos et al., 2011a) and rabbits (Morgan et al., 2014). Impaired embryo development and fetal growth in the offspring of high-fat-fed male mice have been reported (McPherson et al., 2013). These impairments have been related to the elevated level of the reactive oxygen species (ROS) both in sperm and/or seminal plasma, also negative offspring health outcomes have been linked to the increased sperm ROS (Eberle et al., 2020). The paternal high-fat diet has been associated with impaired cardiometabolic outcomes in offspring particularly in

a sex-specific manner (Chen et al., 2012). The underlying mechanism related to impacts of over/under nutrition paternal diets in rodents has been attributed to the elevated rates of apoptosis, sperm DNA damage and perturbed testicular steroidogenesis (Vendramini et al., 2014).

Research on a HFD-fed male mice have shown specific alterations in adiponectin (involved in the modulation of the metabolism of the glucose and fatty acids) and leptin (encompassed in food intake modulation) levels in their offspring in a sex-specific manner, which males affected mostly (Fullston et al., 2013). These alterations are associated with changes in the acetylation of the H3K9 and methylation of H4K20 in the leptin and adiponectin promotors in the adipose tissues of the offspring (Masuyama et al., 2016).

Folate serves as the initial building block in the 1-C metabolic pathway. In the liver, it is metabolized into 5-methyltetrahydrofolate before being distributed to various tissues. In the next step, 5-methyltetrahydrofolate, aided by vitamin B12 as a cofactor, is used by methionine synthase to transfer a methyl group to homocysteine, resulting in the production of methionine and S-adenosyl-methionine (SAM). SAM acts as the universal methyl donor necessary for methylation processes involving DNA, RNA, proteins, and lipids, and it plays a crucial role in maintaining these methylations. Additionally, 5-methyltetrahydrofolate, with the help of methylenetetrahydrofolate reductase (MTHFR), is converted into tetrahydrofolate and 5,10-methylenetetrahydrofolate. These substances are essential for the biosynthesis of purines and thymidylate, respectively. While folate's critical role in biosynthesis and methylation is well-established, most research has focused on its impact on the reproductive health of women. Surprisingly, there has been limited investigation into the role of folate in male reproduction, particularly concerning spermatogenesis and the expression of genes related to spermatogenesis.

However, the function of the level of paternal essential minerals and vitamins has not been extensively investigated. Interestingly, paternal serum and seminal plasma folate concentration can influence sperm DNA integrity (Aarabi et al.,

2015, Boxmeer et al., 2009). Micronutrients such as folate, methionine, and vitamin B12 (vitB12) are essential substrates implicated in methyl group transportation which vitally impacts epigenetic mechanisms and gene expression through the implication of 1-C metabolism. 1-C metabolism is effectively involved in DNA, lipid, and protein synthesis, in addition to histone and DNA methylation (Saber Cherif et al., 2019, Hoek et al., 2020). Vitamin B12 has an essential role in homeostasis and growth as it is a cofactor for the synthesis of methionine, a forerunner of S-adenosyl methionine which provides the methyl groups for the methylation processes of macromolecules such as DNA, lipids, proteins, and neurotransmitters acting in the 1-C metabolism (Samavat et al., 2019). Suboptimal levels of these micronutrients involved in the 1-C cycle can increase the levels of homocysteine and correlate with pregnancy complexities including growth retardation (Kasture et al., 2018). Apoptosis is vital in maintaining the homeostasis of various cells including placental trophoblastic cells (Sharp et al., 2010). Additionally, it has been well-established that there is a close association between paternal over/undernutrition and the modification of imprinted genes such as *Igf2, Peg3, Cdkn1c,* and *Gnas* in spermatozoa, and intriguingly the positive correlation with the DNA methylation and histone modification of these genes in the placenta has been reported (Deshpande et al., 2022, Mitchell et al., 2017). As already discussed, changes in the physiology of the placenta contribute to the health of the offspring and maternal health during and after pregnancy. Additionally, it has been shown that paternal diet-induced obesity potentially results in promoting anomalies in placental development (Mitchell et al., 2017).

The molecular/epigenetic mechanism through which this phenomenon occurs is not yet fully known. Fully understanding the mechanism is further complicated by differential effects of disease risk depending on which grandparent is exposed to the stressor and the sex of the grandchild being assessed. Although it is impossible to collect tissue from retrospective studies focusing on historical famines to assess the inheritance of epigenetic factors between each generation, we might rely on another undernutrition-style insult in human populations caused by religious fasting to explore an epigenetic mechanism.

The influence of paternal fasting during Ramadan on the health of his offspring has not been studied extensively. However paternal food deprivation before conception in mice, led to a notable decrease in the serum glucose level of his offspring, regardless of their sex (Anderson et al., 2006).

1.5 Seminal plasma

Seminal plasma is composed of the secretion of several accessory sex glands which vary by species, including the prostate, seminal vesicles bulbourethral glands and secretions from the epididymis (Cheng and Ko, 2019). A large percentage of seminal fluid is made up of the seminal vesicle glands secretions both in mice and men (Barbagallo et al., 2022). The full composition of seminal plasma in men has been detailed in (Figure 1-10).

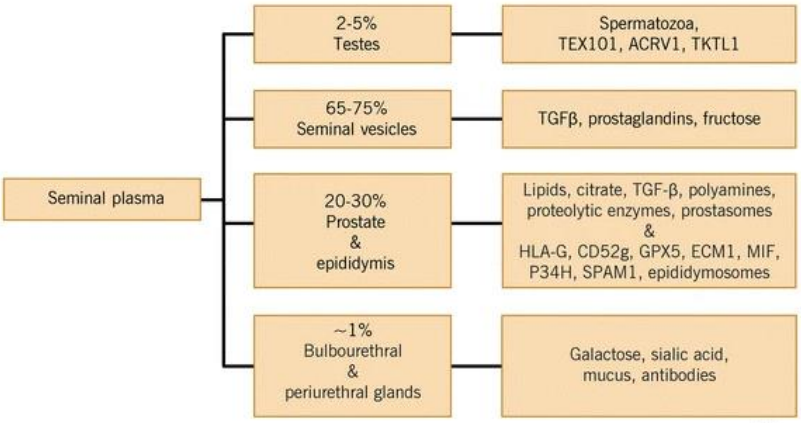


Figure 1-10 Chart showing the full composition of seminal plasma with the different origins and components in humans. Taken from (Samanta et al., 2018).

The activation of cellular and molecular pathways can influence reproductive functions and outcomes, through the interaction of seminal fluid and FRT tissues (Schjenken and Robertson, 2015). Post-coitus, seminal fluid serves two main functions; facilitating oocyte fertilization by inducing molecular and cellular alterations to increase the likelihood of fertilization, and modulation of the female genital tract molecular responses, which assist in conception and promote the development of pregnancy. The response of the FRT including the cervix, fallopian tubes and uterine endometrium results in the secretion of signaling agents such as pro-inflammatory cytokines and their molecular effects promote

the likelihood of conception and pregnancy by the production of the embryotropic factors. The composition of seminal fluid includes hormones including LH and FSH in addition to prolactin (Vitku et al., 2017) as well as cytokines, and plays a particular role in signalling within the maternal reproductive tract, which can have implications for the early embryo implantation and affect the long-term health of offspring consequently (Bromfield et al., 2014). Therefore, seminal fluid has substantial implications during implantation and proceeding pregnancy in addition to its primary impact which is delivering the sperm for fertilization (Robertson, 2005).

Seminal fluid interactions with the FRT, about secretion of the pro-inflammatory cytokines and immune system agents, act on both a local and systematic level. Components of seminal fluid trigger the activation of systemic immune responses which is the second wave of infiltration, including the infiltration of neutrophils, macrophages and dendritic cells from the peripheral circulation into the subepithelial stroma in the maternal reproductive tract (Sharkey et al., 2012b). Signaling functions of seminal plasma, are including the activation of maternal inflammatory responses at the time of the peri-conception, removing the microorganisms and clearance of surplus of sperm by modulating the activation and recruiting of maternal uterine immune system cells, including leukocytes and neutrophils, activation of the endometrial receptivity prior to embryo implantation and modulation of the maternal immune tolerance, in addition of programming the health of offspring (Schjenken and Robertson, 2015). There are numerous agents such as the isoforms of TGF-β, TNF-α, and cytokines including chemokine (C-C motif) ligand 3 (CCL3), in seminal plasma. These factors in the composition of the seminal plasma actively induce the immune responses in FRT following coitus (Schjenken and Robertson, 2020).

Studies in humans reported that deposition of seminal fluid in the upper vagina or the ectocervix promotes the secretion of pro-inflammatory cytokines and recruitment of leukocytes, which increase the receptivity of the endometrium to facilitate the process of embryo implantation (Schjenken and Robertson, 2020). These agents include granulocyte-macrophage colony-stimulating factor (GM-CSF) which activates the production of granulocytes, macrophage and platelets

(Tanhay. Kalat. Sabz et al., 2021), as well as infiltration of leukocytes into the FRT, including macrophages, dendritic cells, and memory T cells (Schjenken and Robertson, 2020). In addition to the secretion of pro-inflammatory cytokines, there are central modulatory cytokines at play. These include TNF-α interleukin (IL)-1A, IL-6, colony-stimulating factor 2 (CSF2), and CSF1. Notably, IL-1B which has a significant role in extravillous trophoblast (EVT) migration and invasion, as well as in promoting the remodeling of uterine spiral arteries (Hayder et al., 2021). Eliciting the pro-inflammatory cytokine's secretion in response to seminal fluid occurs, through the activation of the gene expression and synthesis of the chemokines and cytokines synthesized locally. IL6 is one of the critical cytokines which plays an important role in immune tolerance of pregnancy in addition to modulatory roles in embryo implantation and placental development (Prins et al., 2012, Meuleman et al., 2015). Leukaemia inhibitory factor (LIF), which is necessary for embryo implantation as well as TNF-α from the epithelial cells of the cervix and endometrium in women has been reported (Sharkey et al., 2012a). LIF and CSF, which are secreted from the oviduct in response to seminal fluid, are embryo trophic factors such as CSF1, CSF2, CSF3, IL-6, LIF, and VEGF. These factors assist in facilitating the process of embryo implantation by inducing maternal immune tolerance against the paternal antigens (Fleming, 2018). Seminal fluid factors directly can induce the reproductive tract immune cells responses, including the secretion of the IL-10 and TGF-β as well as the induction of formation of immune suppressive regulatory T cells (Treg cells) (Remes Lenicov et al., 2012, Meuleman et al., 2015).

It is well-established that seminal fluid plays a role in inducing endometrial receptivity, through the modulation of angiogenesis, embryo attachment and implantation (Schjenken and Robertson, 2020). This effect is attributed to the activity of the various lineages of immunological cells particularly, uNK cells, dendritic cells, and T cells which make up about 20-25% of the CD45 leukocytes (Schjenken and Robertson, 2020). The secretions of uNK cells have critical roles in placental development, as they are necessary for the occurrence of uterine spiral artery remodeling, and facilitate trophoblast invasion with full access to maternal blood flow (Rätsep et al., 2015). One of the most essential factors in

maternal immunological adaptation is the modulation of secretion of the Treg cells, which has a critical role in the regulation of adaptive tolerance to proceed with the development of the pregnancy (Robertson et al., 2018).

Notably, factors such as LIF and GM-CSF present in seminal plasma as one of the most important embryo trophic factors promoting embryo survival by setting off the embryo programming and offspring phenotype characteristics, and susceptibility to diseases later in life have been identified as active role players in these developmental pathways (Bromfield, 2014). In addition, seminal plasma by suppressing the TNF-α -related apoptosis-inducing ligand (TRAIL) from the oviduct, which promotes the blastomere apoptosis, (Bromfield et al., 2014).

1.6 Aim and objectives.

The role of maternal diet and uterine environment on embryo development, fetal growth and long-term offspring health has been well established. However, the role of paternal diet and its mechanism on fetal development has not been fully defined yet. Nevertheless, the growing body of research emphasizing the role of paternal health and reproductive fitness at the time of conception affects sperm quality, seminal plasma factors and embryogenesis but also may affect the long-term health of his progeny (Janny and Menezo, 1994, Borini et al., 2006, Colaco and Sakkas, 2018a, Campbell et al., 2015, Bromfield, 2014, Schjenken et al., 2021a, Watkins and Sinclair, 2014a, Batra et al., 2022, Fullston et al., 2015). Interestingly, an aberration in testicular gene expression, hormonal receptors, and testicular inflammations in response to an imbalanced diet has been reported (Fullston et al., 2013).

Traditionally, the focus of studies is focused on sperm quality, and sperm genome and epigenome alteration in response to paternal health and wellbeing. Indeed, the substantial role of seminal plasma has been receiving attention recently. Therefore, this thesis research aimed to investigate the impact of paternal suboptimal diet both undernutrition (low protein diet-LPD) and overnutrition (western diet-WD) on male physiology, fetal development, and late gestation maternal cardio-metabolic ill-health. Maternal cardiovascular health significantly influences uterine vascular growth and remodeling, crucial

factors in ensuring a healthy placenta. Any dysfunction in maternal cardiac function may lead to impaired fetal growth and abnormal development, potentially impacting the offspring's long-term health and well-being (Blackmore and Ozanne, 2015). Thus, ensuring maternal cardiac health is essential for optimal fetal development. In light of this, the observed alterations in fetal heart transcriptomes and development may indeed be linked to the effects of the paternal diet. Maternal metabolic impairment in cases like GDM has been associated with negative affects the fetal cardiac health for example in GDM cardiometabolic genes indicate the presence of fuel-induced epigenetic reprogramming of cardiac tissue during prenatal development (Depla et al., 2021). Furthermore, as altered sperm epigenetic status has been identified as one central programming mechanism, the impact of methyl-donor supplementation was also investigated to define whether the addition of methyl-donor groups could negate the detrimental effects of the suboptimal diets. Additionally, as poor maternal health in pregnancy is a significant risk factor for altered fetal development, therefore, the impact of paternal diet on maternal cardio-metabolic status in late gestation was examined. Therefore, it’s well worth finding out if there are any paternal influences on maternal cardiovascular health at late gestation or not. The impact of the paternal sperm and seminal fluid composition at the time of conception on maternal uterine cardiovascular and systemic adaptations have been discussed earlier, however, it’s still not clear if paternal diet could impose any alteration regarding these adaptions in a normal pregnancy.

2 Chapter 2 Materials and Methods

2.1 Dietary regimens

All experimental procedures were conducted under the UK Home Office Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012, which transposed Directive 2010/63/EU into UK law, and with the approval of the local ethics committee at the University of Nottingham.

Eight-week-old male C57BL/6 mice (Harlan Ltd, Belton, Leicestershire, UK) were maintained at the Bio Support Unit at the University of Nottingham. Animals were housed in controlled 12/12 hours light/dark conditions with a constant temperature (21 °C ± 3 °C) and ad-libitum access to water. After a period of acclimatization, males were allocated to one of five diets including; a control diet (CD: 18% casein, 21% sugar, 10% fat) an isocaloric low protein diet (LPD: 9% casein, 24% sugar, 10% fat,) or LPD supplemented with 1-carbon methyl donors (MDL; 5 g/kg diet choline chloride, 15 g/kg diet betaine, 7.5 g/kg diet methionine, 15 mg/kg diet folic acid, 1.5 mg/kg diet vitamin B12), a Western diet (WD: 19% casein, 34% sugar, 21% fat) or a Western diet supplemented with methyl donors (MD-WD; 5 g/kg diet choline chloride, 15 g/kg diet betaine, 7.5 g/kg diet methionine, 15, 15 mg/kg diet folic acid, 1.5 mg/kg diet vitamin B12). Additionally, a mixture of vitamins AIN76 (0.5 g/kg) was added to the diets of CD, LPD, and MDL groups, while a higher dosage (1 g/kg) was added to the diets of WD and MDWD groups. Dietary composition is provided in Appendix 1. The rationale for the addition of these supplements could be associated with observed the administration of folic acid supplementation alongside a protein-restricted diet during pregnancy serves to mitigate the alterations in phenotype and epigenotype observed in the offspring induced by the protein-restricted diet (Burdge et al., 2009).

Males were fed diets for a minimum of 7 and a maximum of 33 weeks to allow sufficient time for dietary influence on sperm, as a full cycle of spermatogenesis in mice takes approximately 35 days (Perrard et al., 2016). Subsequently, males were mated with virgin 8-12-week-old female C57BL/6J mice, which were maintained on a standard Rat and Mouse No.1 Maintenance chow diet (Special

Dietary Services Ltd, UK). Females were housed overnight in males' mating cages, and copulatory plugs were examined the following morning. The presence of copulatory plugs indicated successful mating, while if absent, a maximum of three mating attempts with the same females was permitted. Pregnancy in females was confirmed by the presence of a vaginal plug the day after successful mating. Pregnancy was allowed to progress to embryonic day 17.5 (term being day 19) before the dam was culled via cervical dislocation and the fetal tissues including, heart, liver, head, kidneys, placenta, and yolk sac were collected from the middle fetuses within each uterine horn (where possible). A total number of 87 litters was used in this study. The fetal and placental sex were not determined in this study, due to lack of relevant data.

All tissues were weighed prior to being snap-frozen in liquid nitrogen and stored at -80^{o} C. Maternal kidneys, hearts, liver and faecal samples collected from the lower gut, were snap-frozen using liquid nitrogen and stored at -80°C. Placentas were either snap-frozen in liquid nitrogen or fixed in 4% formalin (Sigma-Aldrich, UK) at 4°C overnight prior to storage in 70% ethanol. Maternal blood samples, taken via heart puncture, were allowed to clot on ice before centrifugation at 10,000 ×g, 4°C for 10 minutes, after which serum was aliquoted and stored at −80°C. Maternal faecal pellets were collected from the descending colon and stored at −20 °C in DNase/RNase-free tubes.

The stud males were kept on these dietary regimens for a period of 26-28 weeks, starting from the initiation of mating until they were euthanized and culled . At the end of the study, the males were euthanized and culled at 33 weeks of age using cervical dislocation. Various tissues including the heart, liver, kidneys, testes, seminal vesicles, gonadal fat, and interscapular fat were collected. These tissues were either snap-frozen and stored at -80°C or, in the case of the testes, fixed in formalin (4%). (Sigma-Aldrich, UK, at 4°C overnight). Blood serum samples taken via heart puncture were allowed to clot on ice before centrifugation at 10,000 ×g, 4°C for 10 minutes, after which serum was aliquoted and stored at −80°C.

2.2 Maternal tissue and serum angiotensin converting enzyme assay.

In this assay, modifications were made to the protocol of Santos, Krieger et al., (1985). Eight maternal sets of tissues were used, from each of the five treatment groups. Maternal placental and kidney samples were cut into a desired piece, a quarter of the placenta (20-25mg) and approximately a quarter of the maternal kidney (approximately 5-6 mg) were cut using a disposable scalpel and ground to a powder using a pestle and mortar placed on dry ice to prevent the sample thawing.

Initially, the optimization of the converting angiotensin-converting enzyme (ACE) assay, including the time of incubation and volume of sample used, was conducted for each of the biological samples, including the maternal serum. To achieve this, a time course and a volume course experiment were designed to find an optimal sample volume and incubation time within their respective linear ranges, to the data points of the standard curve, which represents the association of ACE concentration and the measured signals. As such, the incubation time and concentration of the biological samples were gradually increased until consistent and accurate results were obtained for the serum samples. Several trials of time and volume optimizations were conducted on the biological replicates including the placenta and kidney however no measurable activity signals were observed, because the obtained results were off the standard curve response, and no measurable enzymatic activity was detected. However, no reliable and consistent results were observed for the placental and kidney lysates *ACE* activity assay due to a high degree of background noise such as inaccurate or inconsistent readings across different volumes and time ranges an increase in signal was observed when a larger volume of maternal serum was used or when the same volume was incubated for an extended period. However, no such changes were observed for the maternal placenta or kidney. Consequently, the decision was made not to proceed further with the analysis of ACE activity within these tissues. For serum, a volume of 20 µL and an incubation time of 10 minutes were chosen. The ACE function of the serum was examined (triplicate)

using a colourimetric technique to determine the amount of hippurate released from hippuryl-l-histidyl-l-leucine by ACE in the presence of chloride ion.

For the assessment of tissue and serum ACE activity, 200µL of sodium borate buffer solution (Boric acid 0.5m and 1.125 m NaCl, pH 8.3) was added to the tissue and serum samples. The biological tissue samples were disrupted using the Tissue Lyser II (Qiagen, UK) with the addition of stainless-steel beads for the placenta and kidney. Samples were disrupted for 1 minute at 25 Hz/s (two runs, each one for 30 seconds). The tubes containing the disrupted tissue samples were centrifuged (Fisher Scientific), at 10,000 ×g at 4ºC for 10 minutes. Following that, 50µL aliquots of each lysate supernatant were transferred to the 0.5 ml collecting tubes. 20 µL of lysate supernatant or serum, 55µL of sodium borate buffer (0.5 M) and 25µL N- Hippuryl-histidine-leucine hydrate (2mM in 5 ml sodium borate buffer; Sigma-Aldrich, UK) to a final volume of 100µL were vortexed and incubated using a heating block at 37ºC for 30 minutes. Afterwards, 150µL of 1M, NaOH was added to each sample, and mixed and 400 µL of O-phthaldialdehyde (0.4 g per 20 ml of methanol, Merck- UK) was added, vortexed and incubated at room temperature for 10 minutes. Eventually, 60µL of 3M hydrochloric acid was added to each sample. Samples were vortexed and centrifuged at 10,000 ×g at room temperature for 3 minutes and the clear supernatants were transferred to new collection tubes. Aliquots of 100µL of each sample were pipetted into a 96-well clear plate (Thermo Fisher Scientific), and three technical replicate readings for each sample were taken. The optical density of each sample was measured at 405 nm using an iMark Microplate Reader BIO-RAD. Serum ACE activity was expressed as a concentration of His-Leu (mM) per µL of serum per minute. The solution of His-Leu generated using maternal serum samples containing only 80µL of sodium borate buffer and 20µL of *N*-Hippuryl-His-Leu were considered as blanks or negative controls which were free of lysate or serum, as well as standard curve solution of His-Leu (0 to 2mM). Initially, a series of optimization experiments was conducted to determine the optimal sample volume and incubation time. Various sample volumes were tested while maintaining a consistent incubation time. Similarly, different incubation times were tested while keeping the sample volume constant. The objective was to observe a linear increase in signal with both

increasing time and sample volume. The initial optimization experiments revealed a linear increase in optical density with increasing volumes of kidney and placenta lysates (R^2=0.99 and 0.95 for kidney and placenta, respectively). However, when a fixed volume (10 µL) of kidney and placenta lysates was incubated over different time periods, there was no significant increase in optical density (R^2 = 0.08 and 0.74 for kidney and placenta, respectively). Subsequently, it was observed that any increase in optical density signal for kidney and placenta samples could be attributed solely to absorbance by the increasing sample volumes, rather than an increase in ACE activity. Initial test experiments revealed a proportional increase in signal intensity with increasing volumes of serum (**Error! Reference source not found.** B) and incubation time ***Error! Reference source not found.*** C).

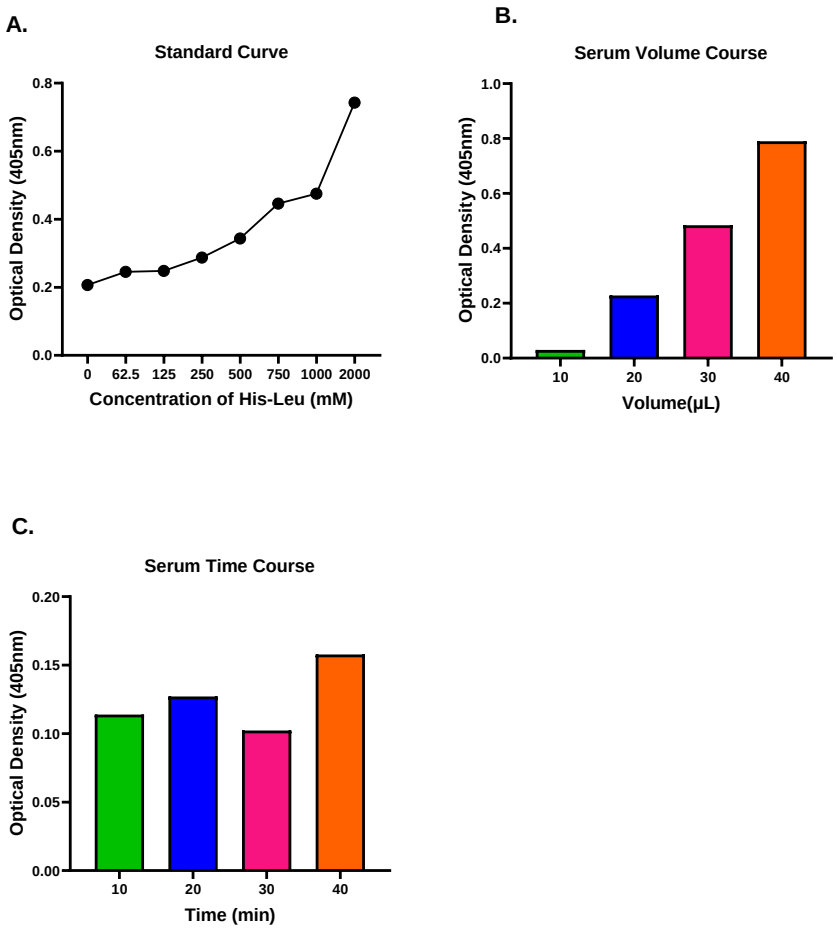


Figure 2-1 (A-C) demonstrates the serum ACE activity optimization.

2.3 General RNA extraction

2.3.1 Placental RNA extraction

Placental RNA was extracted from frozen placental tissues using the RNeasy mini kit (Qiagen), in accordance with the manufacturer's instructions. The following process was performed on dry ice using a disposable scalpel blade pestle and mortar. Approximately 20-25mg (1/4 placenta), was cut off and placed in a 2ml RNase-free tube (Qiagen) with a 5 mm RNase-free-stainless steel bead (Qiagen) and 700µL Qiazol lysis reagent. The samples were then disrupted using the TissueLyser II, with the tubes pulsed at a frequency of 25Hz/s for 30 seconds. Then the tubes were placed on ice for one minute, and then the pulsing of the tubes was repeated for a total of four times, to ensure the entire samples had been disrupted thoroughly. The disrupted samples were incubated at room temperature for 5 minutes for placental tissues, and then 140µL of chloroform (Thermo-Fisher) was added to the tubes containing the placental samples in the fume hood, tightly sealed, and shaken vigorously for 15 seconds. The tubes were then centrifuged (Fisher Scientific) for 15 minutes at 4°C, 13,000 ×g. After centrifugation, the samples were separated into three phases: an upper colourless, aqueous phase containing RNA; an intermediate white phase; and a lower, red, organic phase. The upper aqueous phase was transferred to a new collection tube and 550 µL of 70% (Sigma-Aldrich, UK) was for the placental samples. The ethanol was added to the samples and thoroughly mixed by pipetting up and down several times. Samples were transferred into a RNeasy Mini spin column and centrifuged at 8,500 ×g for 30 seconds at room temperature (15–25°C), and the flow-through was discarded. Afterwards, 350µL of buffer RW1 were added to the RNeasy Mini spin column and centrifuged at 8,500 ×g to wash the column for 30 seconds. The flow-through was discarded. On-column DNA digestion was performed by adding one aliquot (10µL) of DNase solution mixed with 70µL RDD buffer (Qiagen) onto each column per sample, then the samples were incubated at room temperature for 15 min. Next, 350µL buffer RW1 was added to the RNeasy Mini Spin column, centrifuged for 30s at 8,500 x g to wash the column, flow-through

was discarded. Then, 500µL Buffer RPE was pipetted onto the RNeasy Mini spin column. The columns were centrifuged at 8,500 ×g for two minutes and the flow-through was discarded.

The membrane of the RNeasy Mini spin column was dried by centrifuging for 1 minute at maximum speed (~16,000 ×g). The RNeasy Mini spin columns were transferred to a new RNase/DNase-free 1.5 ml collection tube (Qiagen), and 30µL RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane and incubated for 5 minutes at room temperature. To elute the RNA, centrifugation was performed for 1 minute at 8,500 ×g. The flow-through was collected and 1µL of RNA was used to measure total RNA concentration and purity ratios using the NanoDrop 1000 Spectrophotometer V3.7 with three readings taken per sample. The isolated RNA was stored at -80°C.

2.3.2 Maternal liver RNA extraction

The procedure for extracting maternal liver RNA closely resembled that of placental RNA extraction, albeit with minor variations. RNA extraction from frozen maternal liver tissue, approximately 20-25mg in weight, was performed using the RNeasy Mini plus kit (Qiagen). In contrast to the placental RNA extraction method which employed Qiazol, 600µL of Buffer RTL Plus lysis reagent was utilized for liver tissue samples. After centrifugation at 13,000 ×g for 3 minutes at room temperature, liver tissue samples were treated with 600 µL of 50% ethanol (Sigma-Aldrich, UK) and thoroughly mixed. Another distinction between the placental and liver RNA extraction protocols was that, following the addition of 700µL of buffer RW1 to the RNeasy Mini spin column, centrifugation at 8,500 ×g for 30 seconds was conducted once instead of being repeated twice.

2.3.3 Fetal heart RNA extraction

The procedure for extracting fetal heart RNA closely resembled that of placental RNA extraction, with one minor difference. Fetal heart RNA was extracted from frozen heart tissue (approximately 4 mg) using the RNeasy Mini Kit, miRNeasy

Micro Kit (Qiagen). RNA isolation was carried out according to the manufacturer's instructions for the RNeasy kit.

2.3.4 Placental cDNA conversion

Maternal placental RNA was used as the template for cDNA synthesis, using the Precision nanoScript2 Reverse Transcription Kit (Primerdesign, UK) method, in accordance with the manufacturer's instructions. Specifically, 1µg of RNA was added to each reaction in a 0.2 ml PCR tube (Eppendorf).

To prepare the master mix, the following components were combined: RT primer, dNTP mix primer (1µL per reaction) and RNase/DNase free water (Qiagen) to a final volume of 10µL per reaction. Subsequently, another master mix (10µL per reaction) was provided consisting of nanoscript2 4x buffer (5.0µL per reaction); dNTP mix 10mM (1.0µL per reaction), RNase/DNase free water (3.0µL per reaction), and nanoscript2 enzyme (1.0µL per reaction), respectively. Each reaction was set at a total of 20µL, with 12.3µLof the Mastermix added to each 7.7µL of RNA sample.

In addition, to the experimental samples, two controls were also prepared. One control containing all components except the nanoScribe™ enzyme (-RT) to evaluate any contaminations in the cDNA samples. The other control, a no template control contained no RNA, with the volume of RNA being substituted with RNase-free water.

All reactions were mixed and placed in the Techne® Touchgene Gradient Thermocycler. Cycling conditions were as follows: 20 minutes at 42^{o}C; 10 minutes at 75°C; and 4 °C indefinitely. A volume of 180µL of RNase/DNase-free water was added to each tube to dilute the cDNA samples (1:10) before storage at -20°C.

2.3.5 Placental and liver Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

For all analyses of gene expression by RT-qPCR, a 19µL reaction was used. The reaction mixture comprised 2× Precision PLUS SYBR Green Mastermix (PrimerDesign, UK); 175nM forward and reverse primer mix (Eurofins), and 1µL of cDNA (5ng/µL) product. The volume made up to 19µL using 8.3µL RNase-free water (Qiagen). The plate was sealed with an optical adhesive lid (PrimerDesign) and spun using a benchtop plate spinner (Axygen) to ensure all samples were at the bottom of each well. The Applied Biosystems® 7500 Real-Time PCR machine was run for 40 cycles, with a single cycle consisting of: a pre-cycle step at 95°C for 20 seconds for enzyme activation; denaturation at 95°C for 5 seconds, and 60°C for 30 seconds. To confirm the presence of specific products for each primer set a melting-curve step was included, comprising 15 seconds at 95°C, 60 seconds at 60°C, 15 seconds at 95°C, and finally 15 seconds at 60°C. Each sample was analysed in triplicate. An average cycle threshold for each sample was taken from the triplicate reactions. The melt curve was produced on 7500™-computer software. A post-amplification melting curve confirmed the presence of specific products for each primer set.

These two reference genes were *Sdha* (succinate dehydrogenase) and *Tuba* (tubulin A). The two reference genes remained stable and were amplified to normalise data and determined to exhibit similar expression across the different treatment groups following analysis using NormFinder software (Andersen et al., 2004).

Genes of interest were chosen for their roles in the renin-angiotensin system, apoptosis, and one-carbon metabolism pathways (see Appendix 2 for a list of primers and their details). Ct values were converted to relative expression values using the delta-delta Ct method with gene-of-interest expression normalised to *Pgk1* and *Tbp* for liver tissue and *Sdha* and *Tbp* for placental tissue.

To control for technical variation, all samples were normalised to one of the control diet samples. The expression of genes of interest was presented relative to the control diet sample.

2.4 Placental histology

2.4.1 Placental fixation, embedding and sectioning.

Placentas (n=40; 8 from each treatment group and all taken from separate litters) were fixed in 4% formalin (Sigma, UK) prior to being processed into paraffin wax on a Thermo-Scientific Excelsior tissue processor, ahead of morphological and histological assessment. Formalin-fixed, paraffin-embedded whole placental tissues were sectioned at 5μm through the mid-line using a Leitz 1512 rotary microtome (Leica). The paraffin-embedded placental samples were all unused, ensuring consistency in sectioning by employing an equal number of cuts to maintain uniform plane sectioning. It is noteworthy that the assessment of placental sex was not conducted for this experiment. The cut sections were floated out on distilled water at 37º C before being mounted on SuperFrost Plus adhesion slides (Menzel-Glaser, Brauncshweig, Germany). Sections were placed on a hot plate for approximately an hour prior to being dried overnight at 37º C in a laboratory oven (Leader, GP/30/CLAD). From each placenta, 5 series of serial sections (8 slides) were taken.

2.4.2 Periodic Acid Schiff (PAS) staining

For the analysis of placental glycogen content, one series of placental sections were stained using PAS stain. A total of 40 placental tissue slides (8 slides per diet group) were stained according to the following protocol. The placental sections were deparaffinized and dehydrated in Histo-clear I, Histo-clear II (National Diagnostic), Industrial Methylated Spirit (IMS, 100% and 70%) and distilled water for 3 minutes each. Afterwards, placental tissue sections were incubated in Periodic Acid reagent (0.5%) (Sigma-Aldrich, UK), for 5 minutes at room temperature. Next, slides were washed in distilled water four times over a period of 10 minutes (2 minutes and 30 seconds per wash). Following this, Schiff's reagent (Sigma-Aldrich, UK), was applied to each section and then incubated in the dark (tray incubator) for 10 minutes at room temperature. Subsequently, slides were rinsed in running, lukewarm water for 10 minutes. After that, slides were placed in Haris Haematoxylin (Sigma-Aldrich, UK) for 30 seconds to obtain the desired contrast. Slides were rinsed in tap water for 3 minutes. Next, slides were dipped in Acid Alcohol (1% HCl in Ethanol) (Sigma-Aldrich, UK) four times before being rinsed in tap water for 1 minute.

Afterwards, slides were dehydrated in IMS (70% then 100%), Histo-clear II and Histo-clear I (National Diagnostic) for 3 minutes each. Finally, one or two drops of DPX mounting medium (Sigma-Aldrich, UK), were added to each slide and covered by a coverslip.

2.4.3 Masson's Trichrome staining

For the analysis of the accumulation of extracellular matrix (ECM) in placental tissues, specifically for highlighting the collagen fibre content, one series of placental sections were stained using Masson's Trichrome staining. A total of 40 placental tissue slides (8 slides per diet group) were stained according to the following protocol. The placental sections were deparaffinized and dehydrated in Histo-clear I, Histo-clear II (National Diagnostic), Industrial Methylated Spirit (IMS, 100% and 70%) and distilled water for 3 minutes each. Then, placental sections were incubated in Bouin's Fluid provided by the manufacturer, overnight at room temperature in a fume hood. Afterwards, placental tissue sections were rinsed in running tap water until sections were completely clear and then were rinsed with distilled water. Equal parts of Weigert's (A) and Weigert's (B), each 1000µL were prepared and placental sections were stained with working Weigert's Iron Haematoxylin for 5 minutes and then rinsed with running tap water for 2 minutes, and subsequently rinsed in distilled water. In the next step, Biebrich Scarlet / Acid Fuchsin Solution were applied to slides for 5 minutes and then sections were rinsed in distilled water. The staining was followed by applying Phosphomolybdic/Pahosphotungstic Acid Solution (1%) (ScyTek) for 10 minutes, afterwards, without rinsing Aniline Blue Solution was applied to sections for 10 minutes, and then rinsed in distilled water. In the following step, Acetic Acid (Sigma-Aldrich, UK), solution (1%) was applied to the slides for 5 minutes. The next sections were dehydrated very quickly in 2 changes of 95% IMS alcohol, followed by 2 changes of absolute alcohol. Finally, placental sections were cleared in IMS III, IMS IV, Histo-clear III and Histo-clear IV, each for 2 minutes and each section was mounted in synthetic resin.

2.5.4 Image analysis

To investigate and analyse the structure of placental morphology including the different layers of the placenta, slides stained with PAS and MT were viewed at a magnification of 20× for PAS staining and 40× for MT, and the entire placenta as a single field of view was captured using a Leica DMRB, SP multichannel microscope, by using the tile scanning technic using Glide scanner, to capture ans stich the different sections of the placental images together. The magnified sections were taken using a glide scanner (Oasis). Images were imported into ImageJ (National Institutes of Health (NIH), https://imagej.net/software/fiji/) for the analysis of placental cross-section morphology. Measurements of the whole placental area, as well as areas of the Junctional and Labyrinth zones, as well as the chorionic plate, were collected.

2.5 Maternal hepatic assays

2.5.1 Maternal hepatic cholesterol assay

Kit components

Maternal hepatic cholesterol levels were quantified using a Cholesterol Quantitation Kit (MAK043; Sigma-Aldrich, UK) according to the manufacturer's instructions. Approximately 10 mg of frozen maternal liver tissue was cut, and ground using a pestle and mortar on dry ice and used for the assay.

As part of the preparation steps, all vials containing the kit reagents were briefly centrifuged before opening. The kit's cholesterol assay buffer was brought to room temperature before use. The cholesterol probe was warmed up to room temperature to thaw the solution prior to using it. Cholesterol Esterase and Enzyme Mix were then each reconstituted in cholesterol assay buffer and mixed thoroughly by pipetting up and down and stored in a cool place (on ice), protected from light while were utilized.

Cholesterol standard preparation

To generate the cholesterol standard solution, 20µL of the 2 µg/µL cholesterol standard solution was diluted with 140µL of the cholesterol assay buffer to produce a 0.25 µg/µL cholesterol solution. To generate 0 (blank), 1, 2, 3, 4, and 5 µg/well standards, 0, 4, 8, 12, 16, and 20µL of the 0.25 µg/µL cholesterol standard solution was added as a duplicate to a 96-well plate (Thermo Fisher Scientific). Subsequently, 50µL, 46µL, 42µL, 38µL, 34µL and 30µL of the cholesterol assay buffer were added to each well to bring the volume to 50µL.

Sample preparation

From each sample, approximately 10mg of hepatic tissue was disrupted in 200µL of chloroform: isopropanol: IGEPAL (IGEPAL CA-630 is a nonionic, non-denaturing detergent suitable for solubilization, isolation and purification of membrane protein complexes. It is chemically indistinguishable from Nonidet P-40) with the ratio of (7:11:0.1) solution, by adding stainless steel beads using the Qiagen TissueLyser II machine. This process was repeated twice, with each cycle running for 10 minutes at a frequency of 25 Hz. Subsequently, to remove any non-homogenized tissue, tubes containing the lysates were centrifuged at 13,000×g for 10 minutes (Fisher Scientific) to remove any insoluble material and cellular debris. The supernatants were transferred to a new tube (Eppendorf, Qiagen) and were air-dried in a fume hood under the vacuum at room temperature to remove chloroform (Sigma-Aldrich, UK). The pellets were discarded. Then the samples were placed under a vacuum for 30 minutes to remove any residual organic solvents. Then, the dried lipids were dissolved in 200µL of the cholesterol assay buffer, and the tubes were vortexed until the mixtures became cloudy and homogenous.

Assay Reaction

50µL of Assay Reaction was mixed which contained Cholesterol Assay Buffer 44µL, Cholesterol Probe 2µL, Cholesterol Enzyme Mix 2µL, and Cholesterol Esterase 2µL, were added and mixed well in each well of the 96-well plate

(Thermo Fisher Scientific) using pipetting carefully to prevent making any air bubbles. Then the plate was wrapped in aluminium foil to protect it from light and incubated for 60 minutes at 37 °C at room temperature. After 60 minutes of incubation, the absorbance was read at 585 nm using a Bio-Rad iMark Microplate Reader with Microplate Manager Software (MPM 6. exe).

2.5.2 Maternal hepatic free fatty acid assay

Kit components

Maternal hepatic free fatty acid levels were quantified using the Free Fatty Acid Quantitation Kit (MAK044; (Sigma-Aldrich, UK), in accordance with the manufacturer's instructions. Approximately 10 mg of frozen maternal liver tissue using a pestle and mortar on dry ice, was used for the assay.

Prior to running the assay in the preparation step, all the vials containing the kit reagents were briefly centrifuged before the vials were opened to perform the assay. Fatty acid assay buffer was brought to room temperature before use. The fatty acid probe was warmed up to room temperature to thaw the solution prior to using it. Acyl-CoA Synthetase (ACS) reagent and Enzyme Mix were then each reconstituted with 220µL of fatty acid assay buffer and were mixed thoroughly by pipetting up and down.

To redissolve the Palmitic Acid Standard, after freezing the aqueous phase was placed in a digital heating block (AccuBlock (Labnet)) at 95°C for 1 minute and spun for 30 seconds. Once the standard had a clear colour it was placed in a heating block for another 1 minute and vortexed for another 30 seconds.

All samples and standards were run as duplicates; 10µL of the 1 nmol/µL standard solution was diluted with 90 µL of the fatty acid assay buffer to prepare a 0.1 nmol/µL standard solution. Subsequently, 0, 2, 4, 6, 8, and 10 µL of the 0.1 nmol/µL standard solution were added to a 96-well plate (Thermo Fisher Scientific), to generate 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1 nmol/well standard. Then, 50µL, 48µL, 46µL, 44µL, 42µL, and 0µL of the fatty acid assay buffer were added to each well to bring the volume to a total of 50µL.

Sample preparation

For sample preparation, 10mg of hepatic tissue was disrupted by adding stainless steel beads (Qiagen) and using the Qiagen TissueLyser II machine. This process was repeated twice, with each cycle running for 10 minutes, each at a frequency of 25 Hz. Subsequently, to remove any non-lyzed tissues, the samples were centrifuged at 13,000 ×g for 10 minutes (Fisher Scientific). Any insoluble material was then carefully removed. Subsequently, the lower phase was collected and air dried at 50°C using a fume hood to remove chloroform. The samples were then dried under a vacuum (fume hood) for 30 minutes to remove the trace amount of chloroform. And then, the dried lipids at the bottom of the tubes were dissolved in 200µL of fatty acid assay buffer by vortexing extensively for 5 minutes, until the solution became cloudy. Finally, after removing the remnant of chloroform the samples were brought to a final volume of 50µL by adding fatty acid assay buffer.

Assay Reaction

Prior to assay reaction preparation, 2 µL of the ACS reagent was added to all wells including the standards and samples, then incubated at 37°C for 30 minutes. For each well, a master reaction mixture was prepared, by adding 44 µL of the fatty acid assay buffer, 2µL of the fatty acid probe, 2 µL of the enzyme mix, and 2µL of the enhancer, equivalent per 50µL. Then, 50µL of the master reaction mix was added to each well, mixed well with the pipetting, and then the plate was incubated at room temperature for another 30 minutes. During the incubation time, the plate was light protected being covered with a thin layer of aluminium foil.

After 30 minutes of the incubation period, the colorimetric absorbance of hepatic free fatty acid was measured using BIO-RAD iMark Microplate Redear, Microplate Manager Software (MPM 6. exe), at 595 nm.

2.5.3 Maternal hepatic triglyceride assay

Maternal hepatic triglyceride levels were measured using the Triglyceride Quantification Kit (MAK266; (Sigma-Aldrich, UK). For this assay, 100 mg of the frozen maternal hepatic tissue samples were used. Prior to performing the assay in the preparation step the vials of the kit were briefly centrifuged before opening. The triglyceride assay buffer was brought to room temperature before being used. The triglyceride probe was melted in a digital heating block (37°C) for 4 minutes before being utilized. The triglyceride enzyme and lipase were each reconstituted by adding 220µL of triglyceride assay buffer and thoroughly mixed by pipetting up and down. Since the triglyceride Standard was stored in the freezer, it may have caused it to separate from the aqueous phase was placed in a digital heating block (98°C) for 1 minute and redissolved before use. After removing the vial from the heating block, it was vortexed for 30 seconds until the solution became clear from a cloudy colour. The heating and vortexing steps were repeated one more time.

Triglyceride standard preparation for colorimetric detection

All samples and standards were run as duplicates. Then, 40µL of the 1 mM triglyceride standard was diluted with 160 µL of triglyceride assay buffer to prepare a 0.2 mM standard solution. Subsequently, 0, 10, 20, 30, 40, and 50µL of the 0.2 mM standard solution were added into a 96-well plate to generate 0 (blank), 2, 4, 6, 8, and 10 nmol/well standards, respectively. Triglyceride assay buffer was added to each well to bring the final volume to 50µL, 48µL, 40µL,30µL, 20µL, and 10µL, respectively.

Sample preparation

100 mg of hepatic tissue per sample (n=40) was mixed in 1 ml solution of 5% Nonidet P40 (Sigma-Aldrich, UK). Substrates were disrupted using stainless steel beads (Qiagen), using a TissueLyser II machine (Qiagen) at 25 Hz, in 2 sets of 10 minutes each. Samples were then heated in a heating block at 98°C

for 4 minutes, until the samples became cloudy, and then cooled down to room temperature for 20 minutes. To ensure that all the triglyceride content was dissolved the heating step was repeated one more time. Samples were then centrifuged (Fisher Scientific) at 13000 ×g for 2 minutes to remove insoluble materials and separate the supernatants. Removed supernatants, were diluted 10 times by adding pure water. Finally, 50µL of diluted samples were added to a 96-well plate (Thermo Fisher Scientific) as duplicates.

In the next step, 2µL of lipase was added to the standard and the wells containing the samples. To control the sample background, 2µL of lipase was substituted with 2µL of triglyceride assay buffer in blank wells. The solution was mixed well and incubated for 20 minutes at room temperature to convert triglyceride to glycerol and fatty acid.

Assay Reaction

For each well, a master reaction was set up, containing 46µL of triglyceride assay buffer, 2µL of triglyceride probe, and 2µL of triglyceride enzyme mix. Then, 50µL of the master reaction mix was added to each well including the background control, standards, and wells including samples, then were mixed thoroughly using the pipetting. The plate was then incubated at room temperature for 60 minutes. To protect the plate against the light, it was covered with a thin layer of aluminium foil. Colorimetric absorbance of hepatic triglyceride was measured after 60 minutes of incubation at room temperature, at 595 nm, using a BioRad iMark Microplate Reader, with Microplate Manager Software (MPM 6. exe).

2.5.4 Maternal serum glucose assay

A Glucose Colorimetric Detection Kit (EIAGLUC; Thermo Fisher Scientific) was used to measure maternal serum glucose levels (collected as described in section 2.1). All serum samples were thawed on ice, prior to performing the assay. Initially, 5µL of thawed serum per female was diluted 1:20 in assay buffer.

To prepare the Glucose standard, the vial was thoroughly mixed, and then 15 µL of glucose standard was added to another tube containing 135µL of glucose assay buffer to prepare a 32 mg/dL glucose standard.

Meanwhile, seven tubes were labelled with the following concentrations, 16, 8, 4, 2, 1, 0.5, and 0 mg/dL glucose. To prepare 150µL of solution with a concentration of 32mg/ml glucose, 15µL of glucose standard solution was added to 135µL of assay buffer. Subsequently, stepwise dilutions were carried out to, create final concentrations of 16, 8, 4, 2, 1, 0.5, and 0 mg/dL glucose respectively. In the next step, a one-fold horseradish peroxidase (HRP) solution was prepared with the following concentration, 30µL of HRP concentrate 100-fold was mixed with 2.97 ml of assay buffer to prepare a total volume of 3 ml. Then, glucose oxidase was diluted with assay buffer at a ratio of 1:10. Finally, 275µL of glucose oxidase concentrate (10-fold) was mixed with 2.475 ml of the assay buffer to obtain a total volume of 2.75 ml.

Assay procedure

All reagents were brought to room temperature, and thoroughly mixed before use. Subsequently, 20µL of both the standards and samples were added to their respective wells in duplicates. Following this, 25µL one-fold HRP was added to each well. Afterwards, 25µL of substrate and 25µL 1 × glucose oxidase were added to each well accordingly. The plate was then incubated at room temperature for 30 minutes.

After the incubation period, the colourimetric absorbance of serum glucose was measured at 595 nm, using BIO-RAD iMark Microplate Redear, Microplate Manager Software (MPM 6. exe).

2.5.5 Maternal serum insulin assay

Maternal serum insulin was determined by enzyme-linked immunosorbent assay using the rat/mouse insulin ELISA kit (EMD Millipore Corporation, USA). All kit components were supplied and stored at 4°C. The 96-well plate (Thermo Fisher Scientific) used was sufficient for background, 6 rat/mouse insulin

standards, 2 quality controls (QC1 and QC2), and 39 unknown samples in duplicate. Prior to assay preparation, all kit reagents were brought to room temperature.

The two bottles of 10-fold concentrated wash buffer were mixed by adding 900 ml of distilled water. The required number of strips was assembled in an empty plate holder, and the strips were washed three times with 300 µL of diluted wash buffer per wash using the automated strip washer (BioTek ELx50). Any residual wash buffer from all wells was removed by inverting the plate and gently tapping it several times on absorbent wipes. The next step proceeded before drying the wells and adding 10µL of the assay buffer to each of the blank and sample wells using 0.2, 0.5, 1, 2, 5, and 10 ng/ml of assay buffer, respectively. 10µL of the matrix solution was added to each of the blank, standard and control wells. The assay procedure was continued by adding 10µL of the rat insulin standards to the appropriate wells in order of ascending concentration (indicated on the bottles). 10µL of QC1 and 10µL of QC2 were added to the corresponding wells. In the next step, 10µLof each sample was added to the remaining wells in duplicate. Then, 80µL of the detection antibody was added to all wells. The plate was then covered with a plate sealer and incubated at room temperature for 2 hours using an orbital shaker for microtiter plates set at a moderate speed of approximately 400 to 500 ×g. At the end of the incubation period, the plate seal was removed, and the solutions were decanted from the plate. The plate was carefully drained on the absorbent surface to ensure that all solution residue was removed from the wells. Then, the wells were washed three times with 300µL of diluted wash buffer per wash using the automated strip washer (BioTek ELx50). Subsequently, 100µL of the enzyme solution was added to each well. The plate was then covered with a self-adhesive plate cover and incubated for 30 minutes with moderate shaking at room temperature on the shaker for microtiter plates. After 30 minutes of incubation, the plate adhesive was removed, the solutions were decanted from the plate, and the plate was tapped to remove any residual liquid. The wells were then washed six times with 300µL of diluted wash buffer per wash using the (BioTek ELx50) automated strip washer. After the wash step, 100µL of substrate solution was added to each well, the plate was covered with a sealer and placed in the plate shaker for 20 minutes.

After the incubation period, the self-adhesive plate sealer was removed and 100µL of the stop solution was added to each well. The plate was shaken manually, taking care to avoid air bubbles, and then the absorbance was measured at 450 nm with BIO-RAD iMark Microplate Redear using Microplate Manager software (MPM 6. exe); after 5 minutes, the absorbance was read at 595 nm.

2.6 Maternal faecal DNA extraction

Frozen faecal samples were weighed, (80-120 mg), using the QIAamp Fast DNA Stool Mini Kit (catalog. no. 51604, Qiagen, UK) and frozen stool samples were placed in 2ml microcentrifuge tubes provided by the supplier, on ice. To create a homogeneous consistency, 1 ml of inhibitEX was added to each stool sample and was kept at room temperature for 5 minutes before the heating up step. Samples were vortexed continuously for one minute. The tubes containing the suspensions were heated using the digital heating block at 95°C, for 10 minutes, followed by vortexing for 15 seconds. In the next step, the samples were centrifuged (Fisher Scientific) at 13,000×g at room temperature for 60 seconds to precipitate any non-lyzed particles. Meanwhile, after centrifugation, 600µL of each supernatant was pipetted to a new 1.5 ml microcentrifuge tube, mixed thoroughly with 600µL of buffer AL and vortexed for 15 seconds. Afterwards, 45µL of Proteinase K was added mixed completely and vortexed for 15 seconds. 600µL of the lysate was transferred into the QIAmp spin column and centrifuged at 13000 ×g for 1 minute. The QIAmp spin column was placed into a new 2 ml collection tube and the flow-through was discarded. This step was repeated until the entire volume of lysate was passed through the spin column. 500µL of buffer AW1 was added to the spin column and was centrifuged at maximum speed for 1 min. Afterwards, the spin column was placed into a new 2 ml collection tube and the filtrate tube was discarded. Following the previous step, 500µL of buffer AW2 was added into the spin column and centrifuged at maximum speed for 3 minutes. After discarding the filtrate tube, the spin column was placed into a new 2 ml collection tube and was centrifuged at 13000 ×g for 3 minutes, to further drying of the spin column membranes. The spin columns were placed in new 1.5 ml collection tubes. In the next step, 50µL of buffer ATE was added

directly onto the QIAamp membrane, and incubated at room temperature for 1 minute, followed by centrifugation for 1 minute at 13000 x g to elute the DNA. The flow-through was collected and 1µL of DNA was used to measure total DNA concentration and purity ratios using the NanoDrop 1000 Spectrophotometer V3.7. DNA samples were diluted to 5ng/µl in 10mM Tris pH8 (10 mM Tris-HCl -HCl containing 1mM EDTA•Na2) (Sigma-Aldrich, UK), in a volume of 20µL. The labelled DNA tubes were then stored at -20°C to be transferred to the DeepSeq following the Illumina 16 S Metagenomic Sequencing Library Preparation protocol.

To generate 16 S rRNA amplicons, specific forward and reverse primers were used, forward 5' (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG) and Reverse 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGT ATCT AATCC) primers, which included Illumina adapter-overhang sequences. Illumina dual index barcodes from the Illumina XT Index Kit v2 (Set A: FC-131-2001) were attached to each amplicon. PCR clean-up steps were performed using AMpure XP beads (Beckman; A63882). The libraries were quantified using the Qubit Fluorometer and the Qubit dsDNA HS Kit (Thermo-Fisher Scientific). Fragment-length distribution of the library was analyzed using the Agilent TapeStation 4200 and the Agilent D1000 ScreenTape Assay (Agilent; 5067–5582 and 5067–5583). Equimolar amounts of the libraries were pooled, and the library pool was size selected using the Blue Pippin (Sage Science) and a 1.5% Pippin Gel Cassette (Sage Science; BDF2010). The sequencing was performed on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600 cycles) (Illumina; MS-102-3003) to generate 300 bp paired-end reads. The raw reads were processed by the Qiime2 pipeline and trimmed. The classification was performed using Greengenes version 13.8.

2.7 Maternal heart RNA extraction

Total RNA was extracted from frozen maternal heart tissues using the RNeasy Plus Mini Kit (Qiagen). To achieve this, per each sample, a frozen full maternal

heart was crushed manually to create a homogenous sample using pestle mortar on dry ice, the pestle mortar was wiped using 70% IMS, and RNase Zap (Sigma-Aldrich, UK), prior to grinding the samples, to remove any contamination. After grinding, a maximum of 25 mg of the tissue powder was weighed and transferred into a 2 ml RNase/DNase-free tube (Eppendorf), and the rest of the samples were transferred into separate collection tubes and were kept in the freezer at -80°C. One 5 mm stainless steel ball was added to each tube containing 25 mg of samples, afterward 600µL of the provided Buffer RLT Plus, and anti-foaming Reagent DX (0.5% v/v) were added to each tube. The tubes were pulsed for 60 seconds at a frequency of 30 Hz using the Qiagen TissueLyser. The tubes were then centrifuged (Fisher Scientific) for 3 minutes at full speed (13000 ×g) to precipitate any non-lyzed tissue. Each homogenised lysate was transferred to a gDNA Eliminator spin column in a 2 ml collection tube, and subsequently centrifuged for 30 seconds at 8,000 ×g. After centrifugation, the column was discarded, and 600 µl of 70% ethanol was added to the flow-through and mixed by pipetting thoroughly. In the next step, 700µL of each sample was transferred to RNeasy spin columns in supplied 2 ml collection tubes. The tubes were centrifuged for 15 seconds at 8000 ×g. The flow-through was discarded and 700µL of Buffer RW1 was added into each spin column to be centrifuged for 15 seconds at 8000 ×g. The flow-through was again discarded and 500µL of Buffer RPE was added to the spin columns followed by centrifugation for 15 seconds at 8000 ×g. This step was repeated after discarding the flow-through and the tubes were centrifuged for 2 minutes at 8000 ×g. After discarding the flow-through, the RNeasy spin columns were placed in new 2 ml collection tubes and were centrifuged at full speed for 1 minute to further dry the spin column membranes. The RNeasy spin columns were placed in new 1.5 ml collection tubes and 30µL of RNase-free water was added directly to the spin column membrane of each tube, followed by centrifugation for 1 minute at 8000 ×g to elute the RNA. The flow-through was collected and 1µL of RNA was used to measure total RNA concentration and purity ratios using the NanoDrop 1000 Spectrophotometer V3.7 three times per sample. The labelled RNA tubes were then stored at -80°C to be used for cDNA synthesis.

2.7.1 cDNA synthesis using the Rt^2 First Strand Kit

cDNA was prepared from the extracted RNA using a Rt^2first strand kit (Qiagen, UK) per the manufacturer's instructions.

The provided reagents of the Rt^2profiler first strand kit were thawed on the ice and were brought to room temperature before use. The reagents were briefly spun without vortexing to ensure bringing the contents to the bottom of the tubes. To prepare a genomic DNA elimination mix, 50 ng/µL of RNA from each sample was added to the appropriate volume of RNase-free water, and 2µL of buffer GE, in 0.2 ml PCR tubes, and the total volume of each tube was brought to 10µL.

The genomic DNA elimination mix was incubated for 5 minutes at 42°C and was placed on ice immediately for at least one minute. To prepare the reverse-transcription mix, 4µL of 5 × buffer BC3, µL of Control P2, and 2µL of RE3 Reverse Transcriptase Mix water were mixed accordingly and the total volume was brought to 10µL by adding 3µL of RNase-free water? to each tube.

10µL of the Reverse-Transcription mix was added to the genomic DNA elimination mix, then mixed gently by pipetting up and down. The reaction tubes were then placed in a thermocycler with the following cycling conditions: 42°C for 15 minutes, heat inactivation at 95°C for 5 minutes. A volume of 91µL of RNase-free water was added to each reaction, the reaction was mixed well via pipetting up and down several times. The samples were then stored at -20°C for subsequent real-time PCR.

2.7.2 Real-Time PCR for Rt^2-Profiler PCR Array

To perform gene expression analysis of maternal hearts, for real-time PCR reactions, 96-well plate Rt^2 Profiler PCR Arrays (PAMM-174ZC, mouse cardiovascular diseases; Qiagen) in combination with Rt^2 SYBR Green Mastermixes (Qiagen) were used in accordance with the manufacturer's instructions.

To start the procedure, the Rt^2 SYBR Green Mastermix was centrifuged briefly (10–15 seconds) to bring the contents to the bottom of the tube. The PCR components mix was prepared in a 5 ml RNase-free collecting tube well by pipetting up and down. Then the PCR components were transferred to a loading reservoir, and 25 µl of the PCR components mix was added to each well of the Rt^2 Profiler PCR Array plate, using an 8-channel pipettor carefully to avoid any air bubble generation. After expelling the PCR components into each well, the Rt^2 Profiler PCR Array plate was sealed tightly using the optical adhesive film, the plate was centrifuged at room temperature for one minute to ensure any air bubbles were removed. The real-time cycler Applied Biosystems® 7500 Real-Time PCR machine (Applied Biosystems) was programmed. The qPCR reaction was followed by the generation of a post-PCR run melt curve to ensure the specificity of the primers and to check for any non-specific amplification. The analysis of generated data was conducted using Qiagen's GeneGlobe Data Analysis Centre (www.qiagen.com/shop/pcr/primersets/rt2-profiler-pcr-arrays/#resources), to be able to normalize the obtained array data, 2 housekeeping genes including phosphoglycerate kinase 1 (*Pgk1*) and tubulin, alpha 1a (*Tuba1a*), using GeneGlobe Data Analysis web, (www.qiagen.com/shop/genes-and-pathways/dataanalysis-center-overview-page).

2.8 Fetal heart microarray analysis

RNA was isolated from whole snap frozen and stored fetal hearts (4-5 mg) as collected under section 2.1. In total, RNA was isolated from 8 fetal hearts per treatment group (4 females and 4 males per group, with each pair coming from a separate litter). The extracted RNA samples were diluted in 700 µl of Qiazol reagent to 100 ng/µL and made into 20µL aliquots for microarray preparation. The samples were taken to the Nottingham Arabidopsis Stock Centre (NASC; School of Biosciences, Sutton Bonington Campus), where the RNA was quality control checked. RNA integrity was assessed using microfluidic analysis (Agilent), and only samples with an RNA Integrity Number (RIN) ≥8 was used GeneChip WT PLUS Reagent Kit - Manual Target Preparation for GeneChip™ Whole Transcript (WT) 28 Expression Arrays (ThermoFisher Scientific), a

reverse transcription priming method, was used to generate a complete transcriptome coverage. First-strand cDNA was synthesised from the extracted RNA using Reverse Transcriptase (RT), followed by a second-strand cDNA synthesis using DNA Polymerase and RNase H. Complementary RNA (cRNA) was synthesised by in vitro transcription (IVT) of the second-strand cDNA using T7 RNA polymerase. This method is based on the RT-IVT or Eberwine method (Van Gelder et al., 1990). cDNA was then purified in preparation for the second cycle of single-strand cDNA synthesis by reverse transcription of cRNA. The cRNA template was then hydrolysed using RNase H leaving only a single-strand cDNA. In preparation for fragmentation and labelling, the cDNA strand was purified to remove salts, enzymes and unincorporated dNTPs. The cDNA was labelled with biotin and hybridised to DNA probes on Clariom™ S Assay Mouse GeneChip™ (ThermoFisher Scientific).

The obtained results were analysed using Partek® Genomics Suite® analysis software WebGestalt. The gene list for each dietary group was restricted to those with a false detection rate (FDR) < 0.01, $p < 0.05$. Then, the gene lists showing significant expression alterations for each dietary comparison were analysed using the WEBbased GEneSeTAnaLysis Toolkit (WebGestalt) for Gene Set Enrichment Analysis (GSEA) (http://www.webgestalt.org/) with an FDR≤ 0.05. For further gene enrichment analysis, ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/) was used (see Appendix Figure 0-1).

2.9 Data and statistical analyses

Maternal, paternal, and fetal data analyses were performed using a linear mixed model using SPSS (IBM SPSS Statistics 27.0.1.0.). The normality of the data was assessed using the D'Agostino & Pearson normality test. If the data were normally distributed, a one-way ANOVA with Tukey's post-hoc test was conducted. If the data were not normally distributed, a Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons was performed. Area Under the Curve (AUC) analysis was carried out for animal weight progression data. Outliers in data sets were identified using PRISM's Robust Regression and Outlier Removal (ROUT) method and data was statistically analysed with the

outliers excluded from the data sets. Statistical analysis and graphical representation were completed using Prism GraphPad software (7-10). Statistical significance was accepted if a p-value was less than 0.05 and confidence intervals of 95% were used. Further information on the specific statistical tests used is detailed in the results chapters.

Global gene lists of microarray data sets were generated through the Partek Genomics Suite (Partek®). Fetal heart microarrays were analyzed using Partek Genomics Suite analysis software. False detection rate (FDR) and fold change thresholding were set to 0.05 and 1.1, respectively. The gene list for each dietary groups was generated using an ($FDR < 0.05, p < 0.05$) Lists of differentially expressed genes(DEGs) were analyzed using WEBbased GEneSeTAnaLysis Toolkit (Web Gestalt) for Gene Set Enrichment Analysis (GSEA) (http://www.webgestalt.org/). For further gene enrichment analysis (ShinyGO 0.77), (http://bioinformatics.sdstate.edu/go/) was used (https://geneglobe.qiagen.com/gb/).

For q-PCR, the Ct values were transformed into relative expression values by utilizing the CT method, and the stability of the housekeeper genes was determined using the NormFinder. The two genes, out of a panel of reference genes, are the most stable were indicated. Then a normalization factor in EXCEL based on the geometric mean of the expression of the two most stable reference genes output was calculated and provided a normalization factor, which was applied to normalize the expression levels of each gene compared to CD1.

3 Chapter 3 Parental Physiological Characteristics and Maternal Cardio-metabolic Adaptation in Response to Paternal Diet in Late Gestation

3.1 Introduction

The sub-optimal maternal environment can impair fetal growth and development which can then impact negatively on the health of the offspring. The uterine environment during early life can have a significant impact on fetal growth and development, which in turn can affect the risk of developing chronic diseases such as diabetes, cardiovascular disease, and obesity later in life (Watkins et al., 2020). Accumulating research shows various factors such as maternal sub-optimal diets, over or undernutrition (Wu et al., 2004), stress (Van den Bergh et al., 2020), and exposure to heavy metals and toxins such as alcohol and tobacco (Sabra et al., 2018) can all have detrimental impacts on fetal development.

Pregnancy is a unique physiological state as the mother's metabolic system adjusts to ensure that sufficient nutrients are transferred to the fetus to maintain appropriate growth and development. The mother's nutritional and metabolic health before and during gestation, as well as the placenta's synthesis of endocrine substances, such as chorionic gonadotropin, placental lactogen, estrogen and progesterone which effectively have a significant role in fetal growth and maternal metabolic efficiency, all affect how the mother's metabolism adapts during pregnancy (Parrettini et al., 2020).

During a normal pregnancy, the mother's body undergoes significant hemodynamic alterations to provide the optimum level of nutrient availability for fetal development, as well as enable the mother to manage the additional physical and metabolic requirements during gestation (Tan and Tan, 2013). Maternal metabolic adaptations during pregnancy depend on different factors such, as maternal metabolic and nutrient status as well as the endocrine secretion of the placenta. During the first stage of pregnancy, the mother's body actively increases fat storage in early pregnancy. This is known as the “anabolic phase” and functions to provide a source of energy for fetal growth later in gestation. Enhanced levels of the lipid could be associated with increased insulin resistance

in the anabolic phase (Sferruzzi-Perri et al., 2020, Sivan and Boden, 2003). The last trimester of pregnancy in humans and late gestation in mice is considered a ‘’catabolic stage’’, accompanied by reduced insulin sensitivity, or enhanced insulin resistance (Meo and Hassain, 2016). overall, these modifications to maternal metabolism, in both humans and mice, during pregnancy are crucial to ensure that the developing fetus receives the essential nutrients required for proper growth and development (Parrettini et al., 2020, Zeng et al., 2017).

The significance of appropriate maternal adaptation during pregnancy is highlighted in conditions where these coordinated responses become maladaptive. One of the most significant maternal metabolic adaptations during late pregnancy is that the mother’s body becomes more resistant to insulin, which increases glucose availability to the fetus (Catalano et al., 2003). This adaptation is essential as the fetus requires a constant supply of glucose for energy.

A healthy, normal pregnancy results in significant hormonal, immunological, and cardio-metabolic changes in the body (Newbern and Freemark, 2011). To meet the rapidly expanding fetal energy and biosynthetic requirements without endangering the mother's health and fertility, coordinated adjustments between mother and fetus are required during pregnancy. As the mother’s different body organs are going through massive physiological challenges and adaptations during pregnancy, the heart is one of the most crucial organs which faces a significant challenge. There are several cardiovascular changes which occur during pregnancy such as substantial elevation in blood volume, cardiac hypertrophy and significant vascular re-structuring specifically around the decidualized endometrium (Aasa et al., 2015). Adaption in maternal cardiac function happens through an increase in blood volume, plasma volume and red blood cell mass, through the increase in the secretion of maternal erythropoietin (Sanghavi and Rutherford, 2014). The effects of erythropoietin are initially released by interstitial cells in the kidney, which is essential to protect the red blood cells from destruction. The secretion of this hormone could be enhanced under the impact of placental lactogen (Ervasti et al., 2008). An increase in the concentration of sodium ions and water reabsorption under the impact of the

anti-diuretic hormone (ADH) at nephron levels leads to an increase in the plasma volume or plasma expansion (Vricella, 2017). There is also evidence of a link between maternal plasma volume and fetal growth (Goodlin et al., 1983, Vricella, 2017).

The renin-angiotensin system (RAS) has a substantial function in controlling and modulating blood pressure, electrolyte balance and fluid homeostasis (Tamanna et al., 2020). Meanwhile, pregnancy has been associated with increases in the different components of RAS (Irani and Xia, 2008), specifically with the enhanced level of angiotensin-converting enzyme (ACE) activity which is the central component of the RAS. Perturbed RAS activity has been documented in pregnancy difficulties such as pre-eclampsia (Rodriguez et al., 2012).

Another important factor in maternal gestational health is the gut microbiota. Similar to other maternal systems, the microbiota undergoes significant adaptation in gestation. However, the gut microbiota is shaped based on diet and genetics (Morrison and Regnault, 2016). Shifts, including the type and abundance of bacteria, in maternal intestinal microbiota could be an indication of improper maternal metabolic adaptation and may have subsequent pregnancy consequences (Gohir et al., 2019). The importance of the gut microbiota in various physiological aspects including immune system development (Mor et al., 2017), hormonal (Lin et al., 2020) and energy metabolism during pregnancy has been studied (Xue et al., 2020, Guo et al., 2022). Gut microbiota bacterial composition and abundance during early pregnancy are similar to the gut microbiota composition in non-pregnant women (Vavreckova et al., 2022). However, later in pregnancy, the abundance of bacteria associated with inflammatory status will increase significantly in 70% of pregnant women. As pregnancy progresses, there is an alteration in the ratio of *Firmicutes* and *Bacteroidetes,* with the higher level of *Firmicutes* seen in obesity (Santacruz et al., 2010). The gut microbiota plays a substantial role in the synthesis and absorption of nutrients such as carbohydrates (Rowland et al., 2018), vitamins such as K and B vitamin groups (Rowland et al., 2018), minerals such as calcium, magnesium and iron and fatty acids including long-chain fatty acids

which are crucial for energy storage and signaling process involved in physiological functions (Peng et al., 2020), immune system functions, as well as growth and development. Finally, it is well-studied that gut microorganisms have recently been linked to metabolic alterations brought on by pregnancy, which can lead to weight gain and insulin resistance during late gestation (Koren et al., 2012).

Disruptions in this intimate communication caused by genetic and environmental factors can result in maladaptive metabolic responses and serious consequences for both mother and fetus (Bowman et al., 2019). Pregnancy complications such as GDM, IUGR and preeclampsia result from inadequate or improper adaptations in maternal cardio-metabolic status and can have significant short-and long-term health implications for both the fetus and the mother (Yang and Wu, 2022). In GDM, increased levels of serum insulin, hyperglycemia and insulin resistance have been reported (He et al., 2020, Feng et al., 2022, Liang et al., 2010), which affects 15.8% of pregnancies worldwide (Association, 2019).

Underlying such impaired adaptation could be changes in placental function and/or endocrine status (Fowden et al., 2009). For example, imbalanced maternal nutrition is one of the factors that can lead to intrauterine growth restriction (IUGR), which is associated with an increased risk of chronic diseases in adulthood (Sharma et al., 2016a). As well as maternal obesity and cases of gestational diabetes mellitus (GDM) have been associated with fetal macrosomia and NCDs in adulthood (Catalano, 2010). The impact of maternal health during pregnancy could leave a long-term consequence on herself and her offspring. The association between a mother's ill health and her offspring's health has been the subject of substantial research. However, the role a father plays in directing the health of his offspring has been largely overlooked. The impact of paternal reproductive fitness and paternal environmental conditions on sperm quality, post-fertilization and offspring development has not received a large notice (Morgan and Watkins, 2020). For instance, an imbalanced diet can have a significantly negative impact on paternal reproductive physiology, particularly in the areas of fertility (Soubry, 2018) and the health of his offspring

(Watkins et al., 2018). Studies in human and mouse models show that the quality of a father's diet can affect the quality of his sperm, and subsequently in his fertility potential, such as a reduction in sperm fertility markers, fertilized embryos and litter size, such as high-fat diet in men (Showell et al., 2014) and mouse model (Schagdarsurengin and Steger, 2016, Marcho et al., 2020, Binder et al., 2012).

These influences on male reproductive fitness encompass factors such as sperm count, as demonstrated by Sermondade et al. (2013), who showed a J-shaped curve. Hence, being underweight also affected fertility negatively (Sermondade et al., 2013), sperm motility negatively associated with obesity (Nätt et al., 2019), and morphology varies depends on diet (Kusuyama et al., 2020). For instance, a systematic review showed that a diet high in processed foods, saturated fats and sugar has been linked to reduced semen quality, lower sperm counts and reduced sperm motility in men (Salas-Huetos et al., 2017, Morgan and Watkins, 2020). In addition to the importance of macronutrients (protein, carbohydrates, fats) the role of how a poor diet led to a reduced intake of micronutrients such as folate, Vit B12, and methionine is substantial. This is particularly significant given that most methyl donors necessary for DNA methylation in mammals primarily come from components found in food (Finer et al., 2014).

Still, a challenging question remains to be answered; how can the paternal diet impact maternal metabolic health? we are beginning to understand that paternal diet affects fetal and offspring health and that this could be mediated through modifications to maternal health during pregnancy.

3.2 Hypothesis

This chapter of the thesis hypothesises that the dietary status of the father at the time of conception can influence fetal development and long-term offspring health by altering sperm epigenetic status. Additionally, it is hypothesized that changes in fetal development may be correlated with disruptions induced by the father to late gestation maternal cardio-metabolic health, subsequently affecting fetal growth. This study aims to investigate whether direct programming effects

driven by alterations in sperm epigenetic status or paternal-induced disruptions to late gestation maternal cardio-metabolic health mediate changes in fetal development. Specifically, we will examine the impact of paternal low-protein or high-fat diet with or without methyl-donor groups supplementation on maternal metabolic and cardiovascular health to elucidate the mechanisms underlying paternal programming of offspring health.

3.3 Aims

- To examine paternal physiological characteristics in response to different types of over/undernutrition with and without methyl-supplemented diets.

- To investigate the impact of these paternal diets on maternal physiological characteristics in addition to metabolic profiles and gut microbiome in late gestation.

- To investigate the maternal cardiovascular responses to paternal diet in late gestation

3.4 Method and Materials

Animals

All experimental procedures were conducted under the UK Home Office Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012, which transposed Directive 2010/63/EU into UK law, and with the approval of the local ethics committee. 7-week-old C57BL/6 mice males were housed in controlled 12/12-h light/dark conditions with a constant temperature (21 °C ± 3 °C) and *ad libitum* access to water (food/water intake was not recorded), animals were housed in filtered or conventional (IVC) cages. Male mice were fed one of five diets including, an isocaloric control diet CD;18% casein; isocaloric low protein diet (LPD; 9% casein;) or LPD supplemented with 1-carbon methyl donors MDL; a western diet (WD, 24% fat) or WD supplemented with methyl donors MDWD as described in detail in chapter 2 section 1 with (full dietary composition provided in appendix 1). After 7 weeks on the respective diets, each

male mated one single virgin 8-12-week-old female C57BL/6J mouse. Pregnancy in females was confirmed by the presence of a vaginal plug the day after successful mating. No appearance of false pregnancy was observed in females. Pregnancy was allowed to progress to embryonic day 17.5 (term being day 19). Stud males and pregnant females were culled via cervical dislocation. Blood samples were taken via heart puncture, centrifuged at 10,000 × $\boldsymbol{g}$ at 4°C, for 10 min and the serum aliquoted and stored at −80°C. Stud males and dam body organs in addition to fecal samples collected from the lower gut were weighed prior to being snap frozen and stored at −80°C.

3.4.1 Maternal hepatic cholesterol assay

A sub-set of frozen maternal liver samples (E17.5) was used to measure cholesterol content levels using a Cholesterol Quantitation Kit (MAK043; Sigma-Aldrich) in accordance with the manufacturer's instructions. The Hepatic Cholesterol Assay is described in Chapter 2 (section 2.6.1).

3.4.2 Maternal hepatic free fatty acid assay

A sub-set of frozen maternal liver samples (E17.5) was used to measure free fatty acid content levels quantified using the Free Fatty Acid Quantitation Kit (MAK044 from Sigma-Aldrich) in accordance with the manufacturer's instructions. The Hepatic Free Fatty Acid Assay is described in Chapter 2 (section 2.6.2)

3.4.3 Maternal hepatic triglyceride assay

A sub-set of frozen maternal liver samples (E17.5) was used to measure triglyceride content levels quantified using a Triglyceride Quantitation Kit (MAK266 from Sigma-Aldrich) in accordance with the manufacturer's instructions. The Hepatic Free Fatty Acid Assay is described in Chapter 2 (section 2.6.3).

3.4.4 Maternal serum glucose assay

A sub-set of frozen maternal serum samples (E17.5) were used to measure and analyzed the serum glucose level quantified using Thermo Fisher Scientific Glucose Colorimetric Detection Kit Quantification Kit (Catalog Number EIAGLUC) in accordance with the manufacturer's instructions. The serum glucose assay is described in Chapter 2 (section 2.6.4).

3.4.5 Maternal Serum Insulin Assay

A sub-set of frozen maternal serum samples (E17.5) was used to measure the serum insulin level using a rat/mouse insulin ELISA kit (EMD Millipore Corporation, USA), in accordance with the manufacturer's instructions. The serum insulin assay is described in Chapter 2 (section 2.6.5).

3.4.6 Maternal faecal DNA extraction

Total DNA was isolated from frozen maternal faecal samples, using the QIAamp DNA stool mini kit (Qiagen, UK). Microbiome sequencing was conducted on the V3-V4 region of the 16 S rRNA gene following the Illumina 16 S Metagenomic Sequencing Library Preparation protocol as described in Chapter 2 (section 2.7).

3.4.7 Serum and placental Angiotensin Converting Enzyme assay

Prior to the analysis of my samples of interest, the ACE assay was optimized to ensure that all sample volumes and incubation times fell within the linear phase of the assay. Initially, a series of factorial analyses was set up, varying the volume of maternal serum or tissue (kidney and placental) as well as the duration of incubation (see section 2.2 for full details on the assay). An increase in signal was observed when a larger volume of maternal serum was used or when the same volume was incubated for an extended period. However, no such changes were observed for the maternal placenta or kidney. Consequently, the decision was made not to proceed further with the analysis of ACE activity within these tissues. For serum, a volume of 20 µL and an incubation time of 10 minutes were chosen.

3.4.8 Maternal heart Real-Time PCR for RT2-Profiler PCR Arrays

Whole, frozen maternal E17.5 heart samples were used for this experiment using the commercial kits (QIAGEN, PAMM-174ZC, mouse cardiovascular diseases), in accordance with the manufacturer's instructions, which are described in Chapter 2 (section 2.8).

3.5 Statistics

All data were assessed for normality using the D'Agostino-Pearson normality test. Normally distributed data were analyzed by an ordinary one-way ANOVA test, while data that did not pass the D'Agostino-Pearson normality (non-normally distributed data) test was analyzed using a nonparametric test (Kruskal-Wallis). Appropriate post-hoc tests were conducted to determine significant differences between treatment groups. Statistical differences for correlation analyses were measured by Spearman's correlation. Data were graphed with Prism, version 8 (GraphPad Software Inc., San Diego, CA, USA). All data are presented as mean +/- SEM. P values of < 0.05 were considered statistically significant. Additionally, PRISM's Robust Regression and Outlier Removal (ROUT) method was employed to detect outliers within the datasets, and statistical analysis was conducted after excluding these outliers from the data sets.

3.6 Results

3.6.1 Assessing the impact of diet on the paternal body and organ weights throughout the experiment.

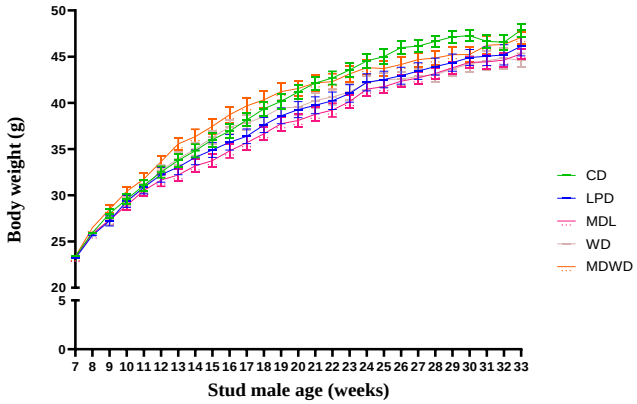
In the designed experiments, the impacts of the over and under-nutrition diets with and without methyl supplements were assessed regarding the paternal physiological characteristics including body and organ weights. Male C57/BL6J mice were fed one of five diets; control diet (CD; 18% casein), low protein (LPD; 9% casein), Western diet (WD; 19% casein), LPD or WD supplemented with methyl-donors termed MDL and MDWD respectively), for at least 7 weeks before mating, (see appendix 1 for diet composition). The stud males were

maintained on these diets for a duration of 26-28 weeks, spanning from the time of mating until they were culled.

Males from all groups demonstrated broadly similar growth profile patterns (Figure 3-1) throughout 26 weeks of study, and no significant difference was observed in stud male growth across different groups. Both the MDL and LPD had the lowest growth rate while WD and MDWD exhibited the highest growth compared to the CD males. At weeks 12 and 13, the rate of growth remained similar across all groups.

No significant differences were observed between any of the stud male groups in terms of overall growth trajectory over the period of the experimental study. To analyze data further, the Area Under the Curve (AUC), analysis was conducted to assess the animal weight progression data, for every individual was calculated and followed by a one-way ANOVA test to see if any significant differences in terms of weight gain exist across the different treatment groups. None of the groups exhibited significant differences when compared to each other and the control group.

A.



B.

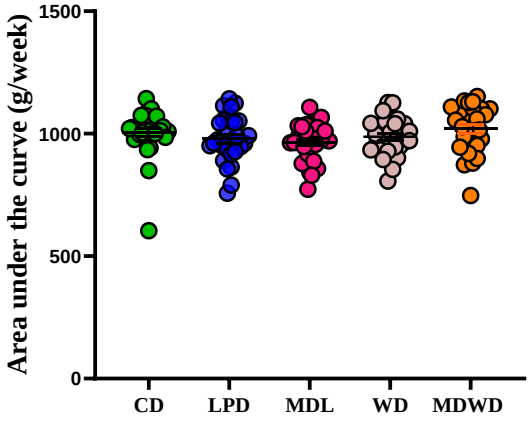


Figure 3-1 Changing the weight of stud males in response to diet. (A) Weekly body weight of stud males fed either a control diet (CD), low protein diet (LPD), methyl donor low protein diet (MDL), western diet (WD) or methyl donor western diet (MDWD). Males were acclimatized for one week prior to being assigned to their respective diet from 7 weeks of age. Males were maintained on their diet until cull at 33 weeks of age. (B) Area under the growth curve for each treatment group. Individual points represent a mean weight measurement at a given week ± SEM (n=30/group). Data were analyzed by D'Agostino & Pearson normality test was utilized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.

At 33 weeks of age, males were culled for analysis of organ sizing. Their final organ weights were expressed as shown in (Figure 3-2 A-F). The WD group showed significantly higher liver weight when compared to the CD, LPD and MDL groups. No significant difference was observed between CD, LPD and MDL (Figure 3-2 A). A significant decrease was found in heart weight in LPD, MDL and MDWD groups when compared to CD (Figure 3-2 B). The WD group exhibited a significant increase in mean kidney weight when compared to the LPD and MDL groups (Figure 3-2 C). There were no observed differences in

mean testicular weight in any of the examined groups (Figure 3-2 D). A significant decrease was observed in seminal vesicle weight in the MDL and LPD groups compared to the CD group, and no significant differences were observed between LPD and MDL compared to each other (Figure 3-2 E). Lastly, an increase in gonadal fat pad weight was observed in the MDL, WD, and MDWD groups compared to the CD group. Additionally, a significant increase in MDL when compared to CD was noticed. This increase coincided with both the WD and MDWD groups compared to the LPD group as well (Figure 3-2 F).

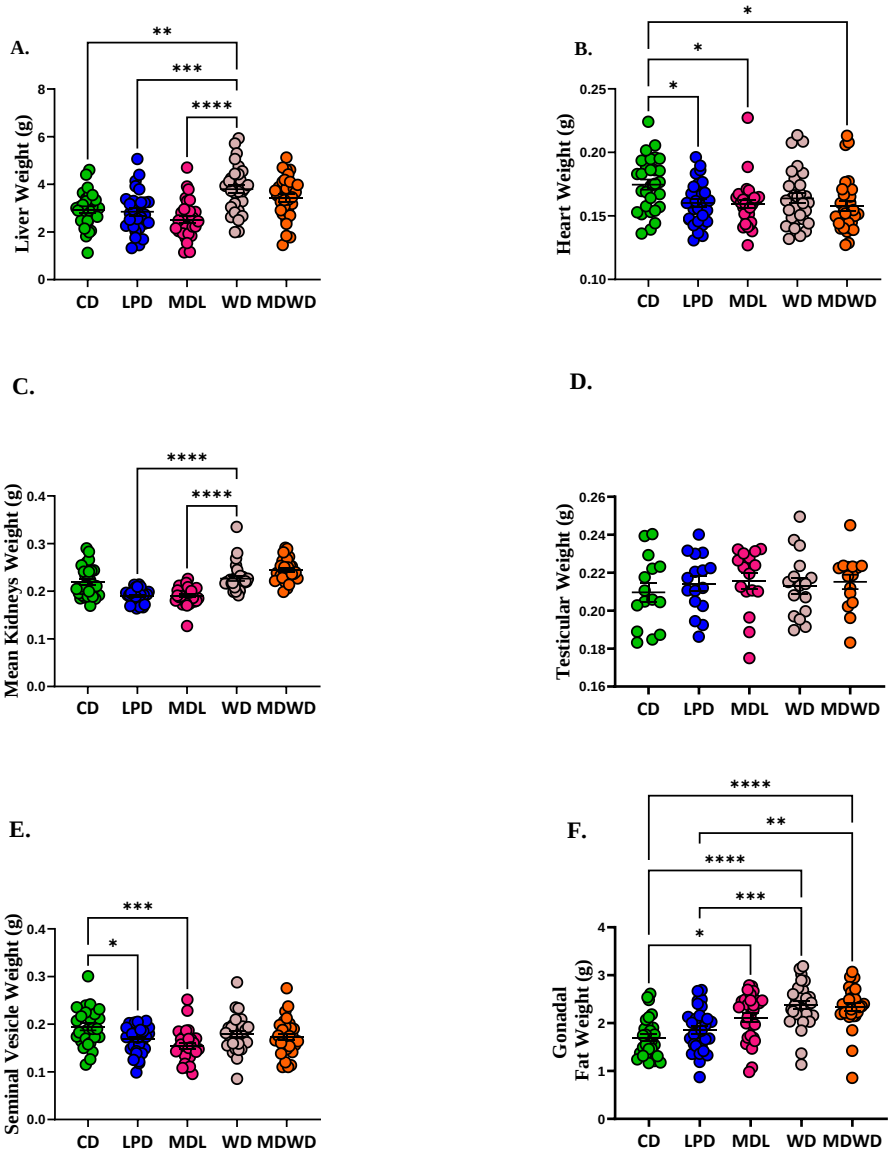


Figure 3-2 Impact of paternal diet on stud male organs weight. Stud males were fed a control diet (CD), low protein diet (LPD), methyl donor low protein diet (MDL), western diet (WD) or methyl donor western diet (MDWD). The total male body weight at the time of cull (A), liver (B), heart (C), mean kidneys (D) testicular (E), seminal vesicles and (F), and gonadal fat weighs respectively. Data are represented as mean ± SEM. n = *30 males per dietary group. Data were analyzed by D'Agostino & Pearson normality test was utilized followed by one-way ANOVA followed by Bonferroni post hoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.* *P < *0.05,* **P < *0.01,* ***P < *0.001,* ****P < *0.0001.*

At 33 weeks of age, males were culled for analysis of organ size, along with the determination of the relative organ-to-body weight ratio. Their final organ-to-body weight ratio is represented, (Figure 3-3 A-F). The WD and MDWD groups

showed significantly higher liver over body weight ratio when compared to the MDL and LPD groups, in addition to a significant increase in the WD group when compared to CD (Figure 3-3 A). No difference was observed between CD, LPD and MDL (Figure 3-3 A). No significant difference was found in heart weight relative to total body weight among any of the groups (Figure 3-3 B). The MDWD group exhibited a significant increase in mean kidney weight relative to total body weight compared to the CD and MDL groups (Figure 3-3 C). There were no observed differences in mean testicular weight relative to body weight in any of the examined groups (Figure 3-3 D). A significant decrease was observed in seminal vesicle weight relative to total body weight in the MDL group compared to the CD group (Figure 3-3 E). Lastly, an increase in gonadal fat pad weight relative to total body weight was observed in the MDL, WD, and MDWD groups compared to the CD and LPD groups, as well as no significant differences between LPD and MDL were observed. This increase coincided with both the WD and MDWD groups compared to the LPD group as well (Figure 3-3 F).

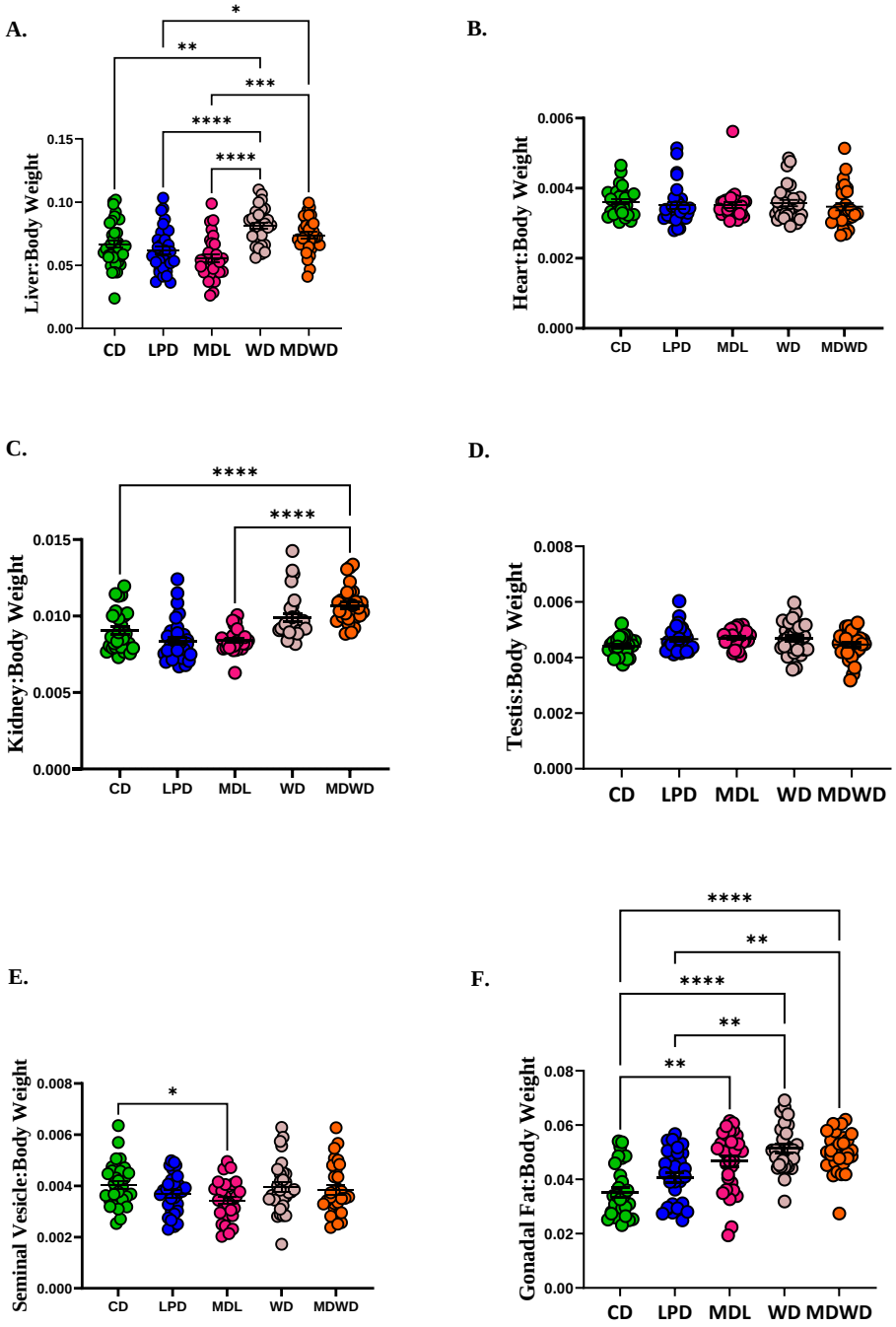


*Figure 3-3 Impact of paternal diet on organ weight relative to body weight. stud males were fed a control diet (CD), low protein diet (LPD), methyl donor low protein diet (MDL), western diet (WD) or methyl donor western diet (MDWD). the ratio of male (A) liver, heart (B), mean kidneys (C), testicular (D) seminal vesicle glands (E), and gonadal fat pad weights (F) expressed relative to body weight. data are mean ± sem. n = 30 males per dietary group. Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

3.6.2 Paternal diet has minimal influence on maternal physiological characteristics.

In this experiment, virgin females aged 8-12 weeks were mated with males who had been fed each of the 5 different diets. Males were left in, with females until the appearance of a sign of pregnancy which was the formation of a vaginal plug. At embryonic day 17.5 these females were weighed before termination of pregnancy and being culled at a stage of carrying E17.5 fetuses. No significant changes in maternal weight were observed across the different treatment groups in response to the paternal diet (Figure 3-4 A). For further analyses, maternal weight immediately after mating E.05 was analyzed and there was no significant difference in any of the groups (Figure 3-4 B). Additionally, the difference in weight gain from E0.5 to E17.5 was assessed, as shown in (Figure 3-4 C). The assessment was performed subsequent to the deduction of the total conceptus mass at E17.5 (Figure 3-4 D). The resultant graph illustrates the incidence of fetal resorptions in response to the paternal diet (Figure 3-4E). Upon comparison to the CD, no significant differences were evident across the various treatment ggroups in response to the paternal diet.

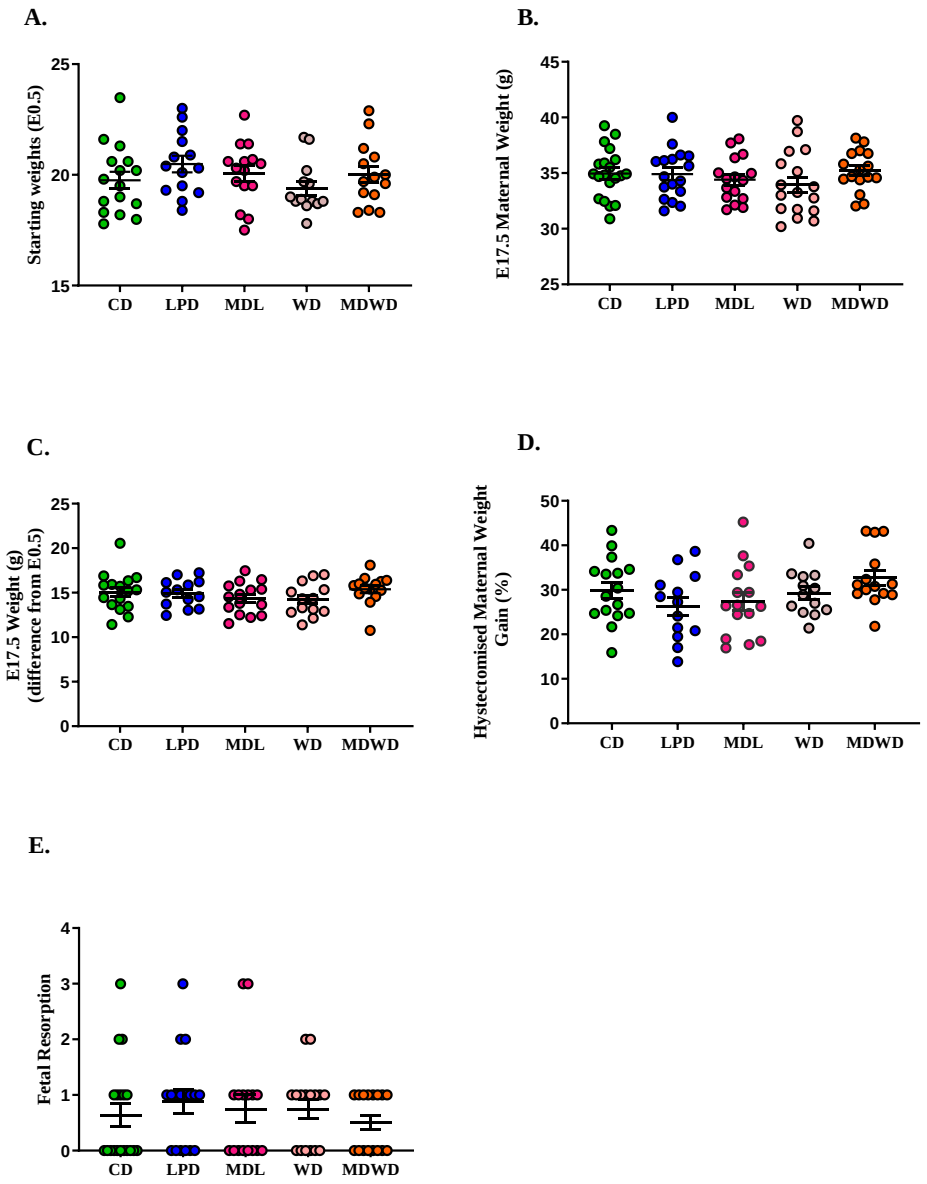


Figure 3-4(A-D) Displays maternal pregnancy-associated weight parameters (E17.5). There are no significant differences in maternal total body weight (A) and body weight at the start of gestation. E0.5 (B), as well as weight gain during pregnancy until E 17.5, (C), the weight gain percentage when conceptus weight excluded (n=14-16) (D), and the number of fetal resorption (n=17-19) . Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.

As well as the maternal weight characteristics, the organs were subsequently weighed post-partum, as represented in (Figure 3-5 A-E). This assessment was conducted after subtracting the total conceptus mass at E17.5. No significant alteration between groups, in terms of maternal different organ weight was observed. Dam's different organ weights including heart (A), liver (B), left kidney (C), right kidney (D), as well as whole uterus weight (E) were not significantly different between the treatment groups. However, slight variations in LPD heart samples could be observed in late gestation, although this variation is not statistically significant.

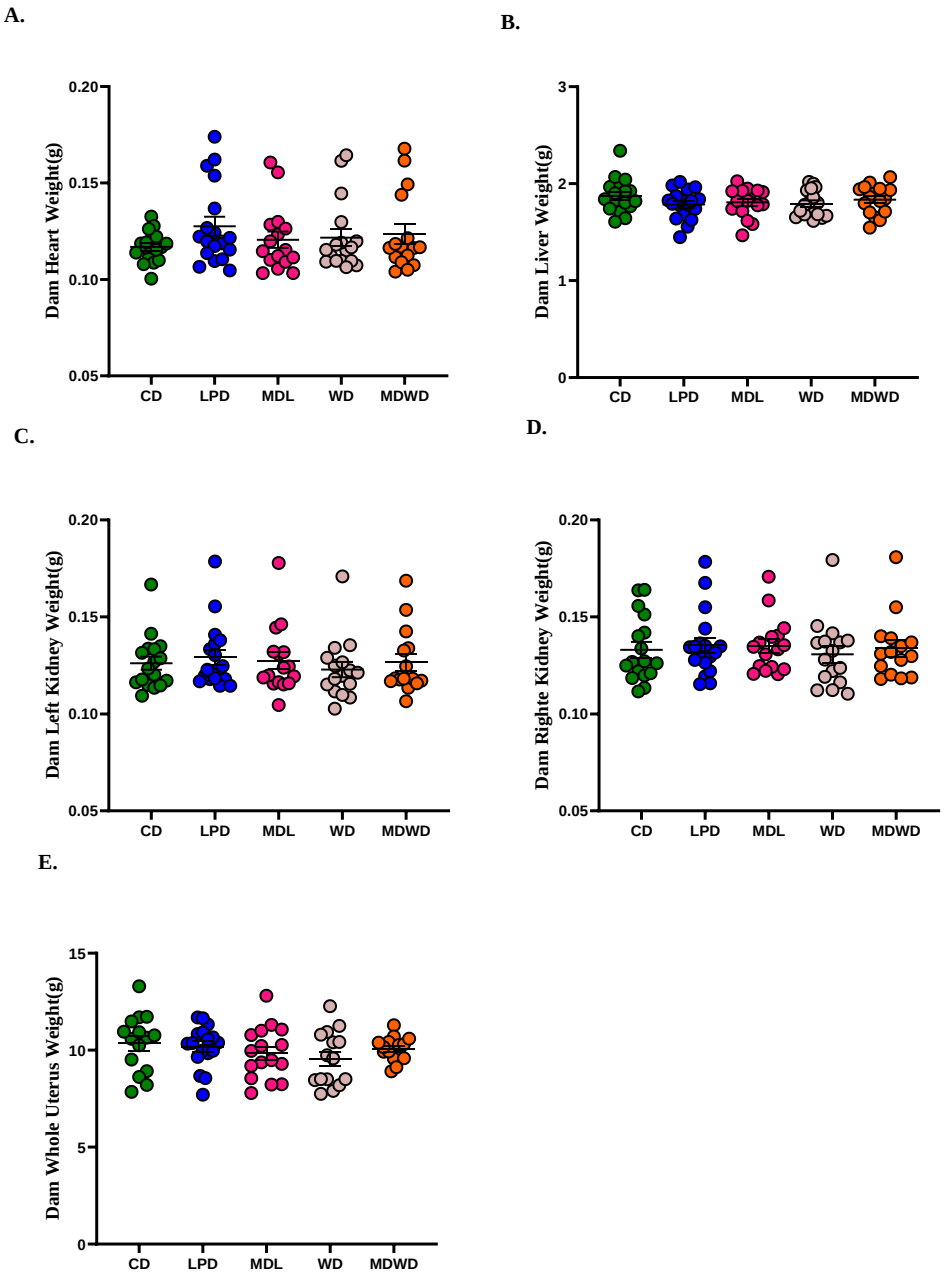


Figure 3-5 (A-E) displays maternal organ weight characteristics in late gestation (E17.5). There are no significant differences in maternal heart weight (A), liver weight (B), left (C), right (D) kidneys and whole uterus (E) weight, across different treatment groups when compared to CD. Data are the mean ± SEM (n=18 liver), (n = 19 heart) and (n=18 kidney). Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.

3.6.3 Maternal organ weight over body weight ratio at late gestation in response to paternal diets

No effects of paternal diet on maternal organ/body ratio at late gestation were observed. No statistically significant alterations were observed in any of the groups when compared to the CD or each other. However, interestingly there is more variability in heart weight relative to maternal body weight in the LPD group when compared to the other treatment groups. Their final organ-to-body weight ratio is represented in, (Figure 3-6 A-E). No significant changes in the liver, heart, left and right kidneys respectively as well as the whole uterus over body weight ratio, were observed when compared to the when compared to CD and each group.

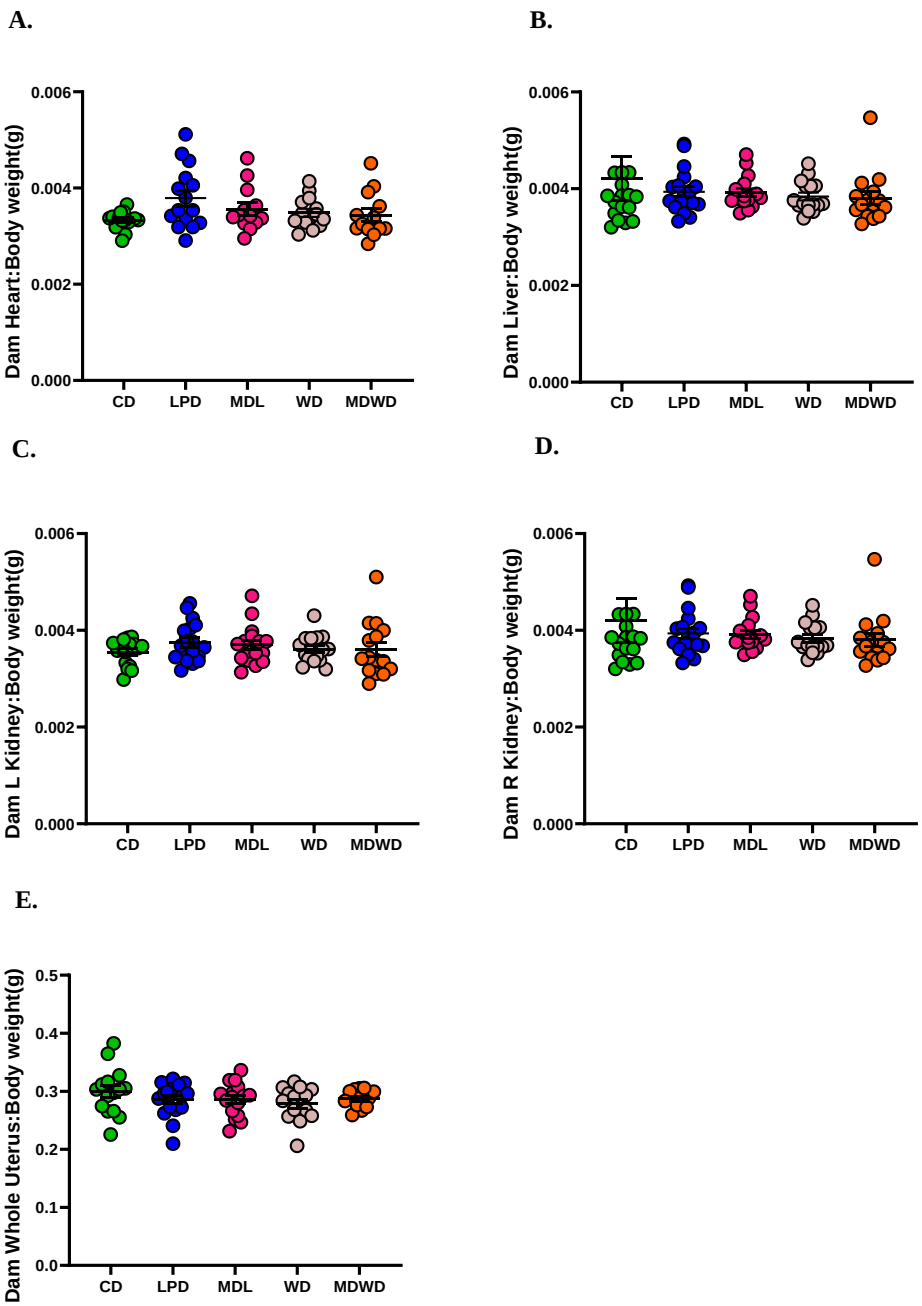


Figure 3-6 (A-E) represents the impacts of paternal diet on the Maternal organ-to-body ratio compared to CD and each group. Data points represent individual dams. (n=18 liver), (n = 19 heart), (n=18 kidneys) and (n=18 uteri). Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.

3.6.4 Paternal diet has minimal influence on maternal hepatic and serum biochemistry profiles.

In this experiment, virgin females aged 8-12 weeks were mated with males who had been fed each of the 5 different diets. These females were weighed before culling at a stage of carrying E17.5 fetuses. To determine whether paternal diet may have any potential influence on maternal metabolic health during late gestation, maternal metabolic status was analyzed. To achieve this, maternal liver triglycerides, cholesterol, free fatty acid serum insulin and glucose at late gestation (E17.5) were analyzed represented in (Figure 3-7 A-E).

No significant alteration between groups, in terms of maternal liver triglycerides was observed (Figure 3-7. A), liver cholesterol (Figure 3-7. B), liver free fatty acid (Figure 3-7. C), serum insulin (Figure 3-7. D), as well as serum glucose (Figure 3-7. E) were not significantly different between the treatment groups.

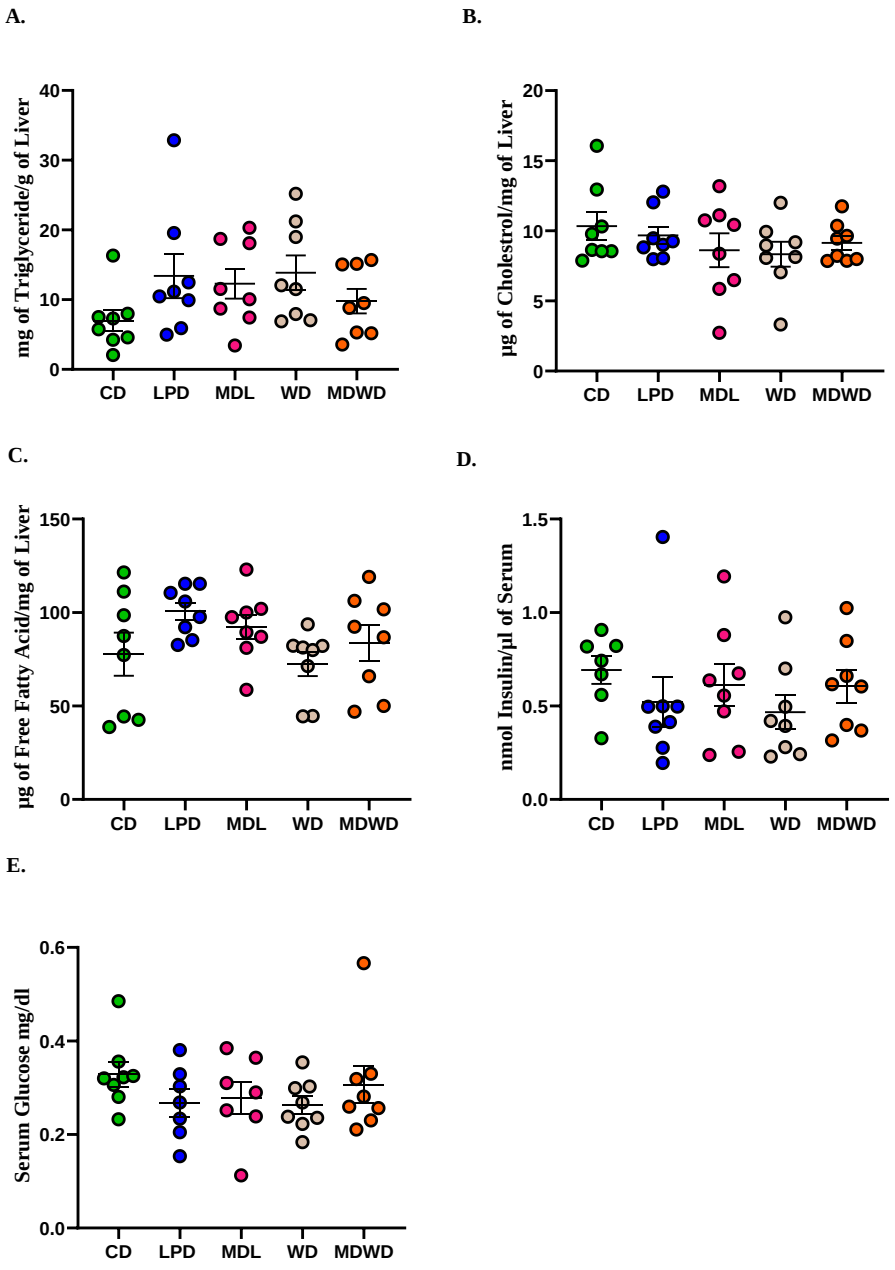


Figure 3-7 (A-E) Represents the impacts of paternal diet on maternal liver and serum metabolic status among different groups compared to CD and each group. Data points represent individual dams(n=8 for liver and serum samples). D'Agostino & Pearson normality test analysed data was utilised followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM.

For further assessment of maternal metabolic status in response to paternal diet, the ratio of the maternal liver and serum metabolic profile was assessed (Figure 3-8). To achieve this, the ratio of maternal liver triglycerides over serum insulin represents a significant increase in the WD group when compared to CD (Figure 3-8 A). No significant differences in maternal liver free fatty acid over serum

insulin (Figure 3-8 B.), serum glucose over serum insulin (Figure 3-8 C.), and liver triglycerides over liver cholesterol (Figure 3-8 D.), were observed in response to paternal diet.

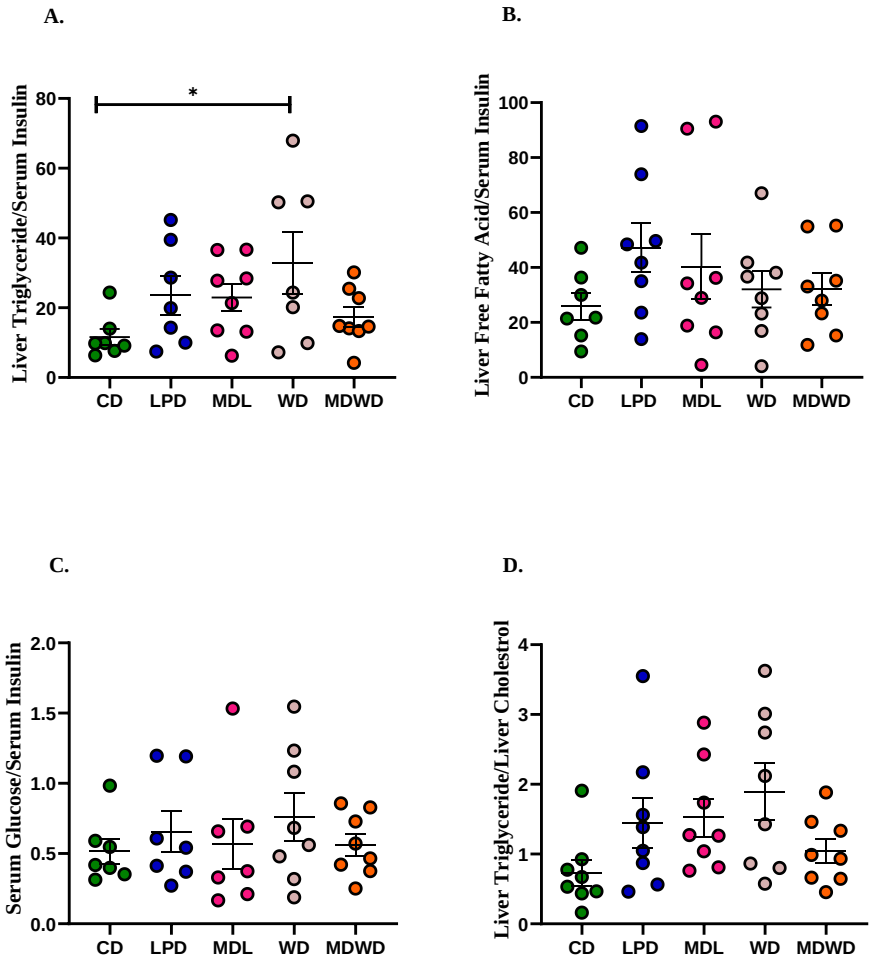


Figure 3-8 (A-D) Represent the maternal hepatic and serum metabolic ratio profiling including liver triglycerides over serum insulin ratio (Figure A), liver free fatty acid over serum insulin (Figure B), serum glucose/serum insulin (Figure C.), and the ratio of the liver triglycerides and cholesterol (Figure D.). D'Agostino & Pearson normality test analysed data was utilised followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM., P <0.05.*

3.6.5 Paternal diet has minimal influence on maternal liver central metabolism gene expression.

The maternal expression of several genes involved in hepatic central metabolism, in response to the paternal diet, was investigated. (Figure 3-9 A-F) displays the expression of the two housekeeping genes *Pgk1* and *Ppib* and hepatic *Cyp7a1, G6pc, Akt2,* and *Adipor1*. Interestingly both housekeeping genes behave similarly between the different treatment groups. However, there was no significant difference in the expression of any of the genes across any of the different groups when compared to CD or each other. The stability of these housekeeping genes was assessed by considering the inter and intra-group variation in gene expression.

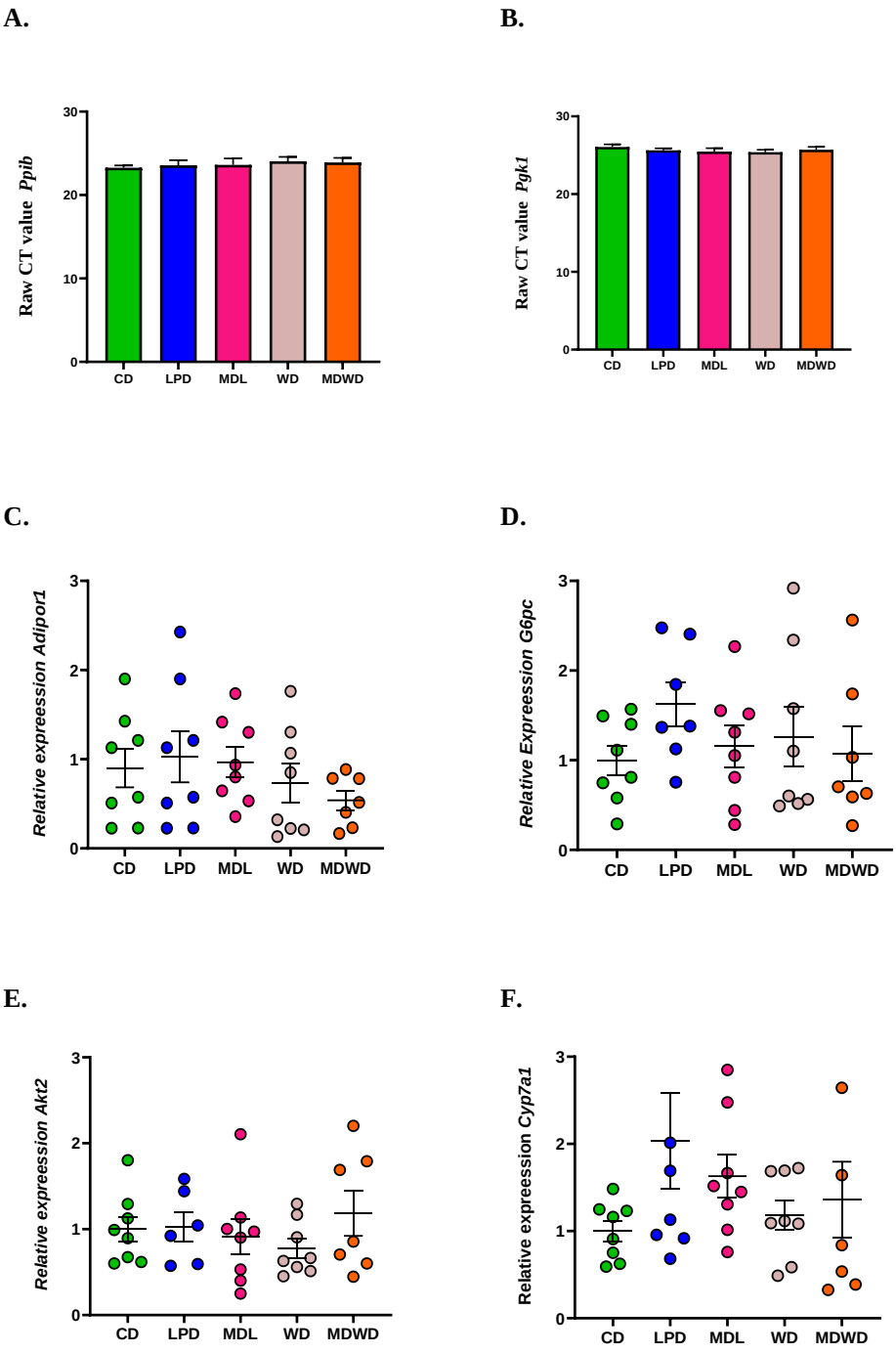


Figure 3-9 (A-F). T maternal hepatic expression of genes involved in central metabolism. All gene expression profile data demonstrated relative to CD expression normalized to Pgk1 and Ppib housekeeping gene expression. n = 6–8 liver samples (A, B). The quantification of maternal hepatic CT values of housekeeping genes of Pgk1 and Ppib Placental expression of Adipor1 involved in fatty acid oxidation and glucose uptake (C), G6pc involved in glycogenolysis and gluconeogenesis (D), Akt2 involved in glycogen synthesis and lipid metabolism in the liver (E), and Cyp7a1 involved in cholesterol metabolism in the liver (F). Statistical significance was determined using either one-way ANOVA with Tukey post-hoc test or Kruskal-Wallis with Dunns post-test, comparing the five diet groups for each gene. $p < 0.05$ is considered as a significant difference.

3.6.6 Maternal faecal microbiota displays minimal alterations in response to paternal diet.

To assess whether maternal gut bacterial diversity was influenced by paternal diet, Faith's phylogenetic diversity index was used to measure species evenness (a measure of relative abundance of the different species of bacteria. Box plots indicate a measure of the phylogenetic diversity of species among 5 different treatment groups, (n = 6) that were included in the analyses (Figure 3-10 A-E). No, significant differences in bacterial species diversity were observed, between the groups. Moreover, the other components of the maternal gut microbiota analysis of bacterial profiles at the phylum, order, and family level were represented (Figure 3-10 B-D). Analysis of bacterial profiles at these levels showed no significant differences between treatment groups, compared to CD and each other.

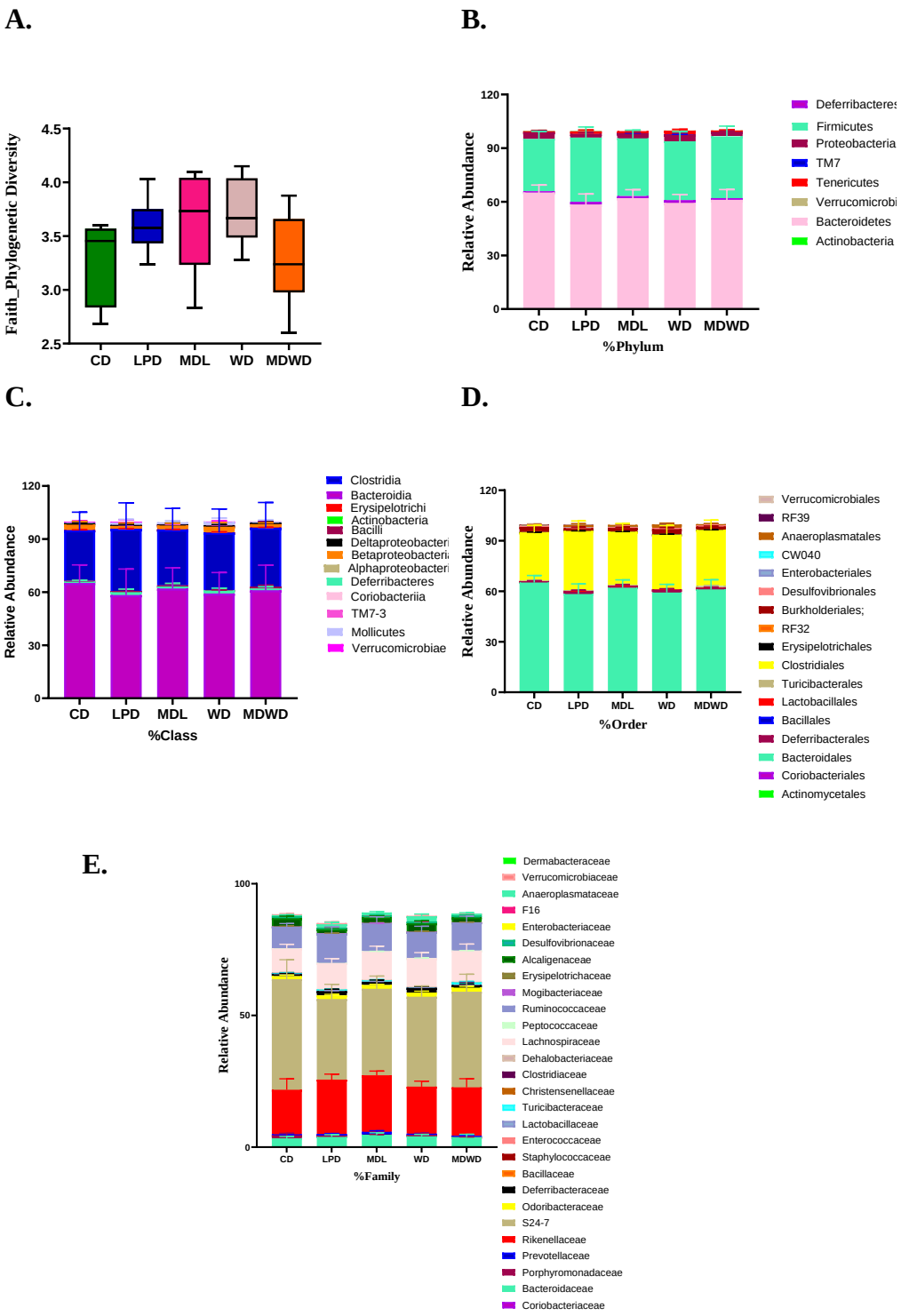


Figure 3-10 (A-E) Displays no significant difference in bacterial diversity across different treatment groups was observed in maternal lower-gut microbiota, in late gestation. Figures (B-E), represent maternal gut microbiota in different levels including (B)%Phylum, (C) %class (D), %Order, and (E) % Family were analyzed in response to paternal diet. No statistically significant alterations were observed in the diversity or relative abundance of different bacterial species in the maternal gut in response to the paternal diet. Statistical analyses were conducted using one-way ANOVA for normally distributed data & Kruskal-Wallis for non-parametric analyses (n=6 per group, each mated with separate stud males).

3.6.7 Assessment of maternal relative bacterial phylum correlation in response to paternal diet.

To understand whether poor paternal diet may influence the broader maternal bacterial profiles, each bacterial component at the family level were correlated with each other. All experimental groups showed a significant positive correlation between the prevalence of *Actinobacteria*, and *Bacteroidetes, Deferribacteres, Firmicutes, Proteobacteria and TM7*. e. However, this positive correlation between the *Actinobacteria*, and *Proteobacteria* disappears in MDL and MDWD groups (Figure 3-11, A-E). In the maternal CD group, a significant positive correlation between the levels of *Bacteroidetes, Deferribacteres* (r=0.939, p=0.005), *Firmicutes* (r=0.960, p=0.002), *Proteobacteria* (r=0.811, p=0.05), and *TM7* (r=0.971, p=0.001) was observed. Interestingly, MDL, WD and MDWD groups represent a very similar pattern of correlation across the phyla.

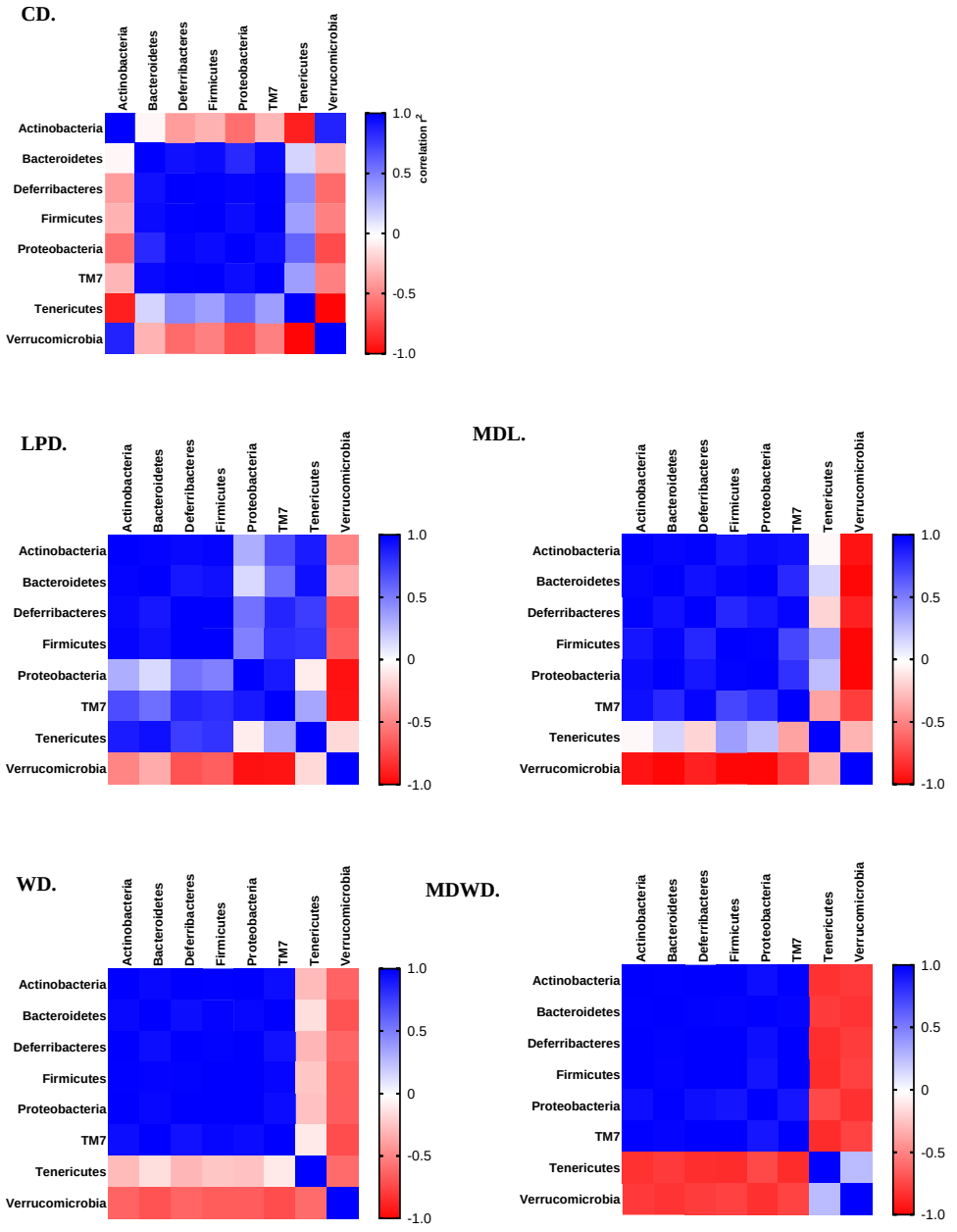


Figure 3-11 (A-E) *Represent heatmap graphs which correlate different bacterial prevalence in phylum taxonomy in maternal lower-gut, late gestation (E17.5), in response to different paternal diets. (n=6). Statistical differences were analyzed using Spearman's correlation.*

3.6.8 Maternal serum ACE activity assay and gene expression relating to cardiac function

Maternal placenta, kidney lysates as well and serum were analyzed for the determination of ACE activity. Initially, a series of optimization experiments to determine the most appropriate volume of sample as well as the appropriate

incubation time were conducted. The ACE Assay optimization is described in Chapter 2 (section 2.2).

Samples of maternal serum were measured against the standard of His-Leu (the dipeptide product of ACE). Maternal serum ACE assay showed no significant difference among different groups in response to paternal diet (**Error! Reference source not found.**).

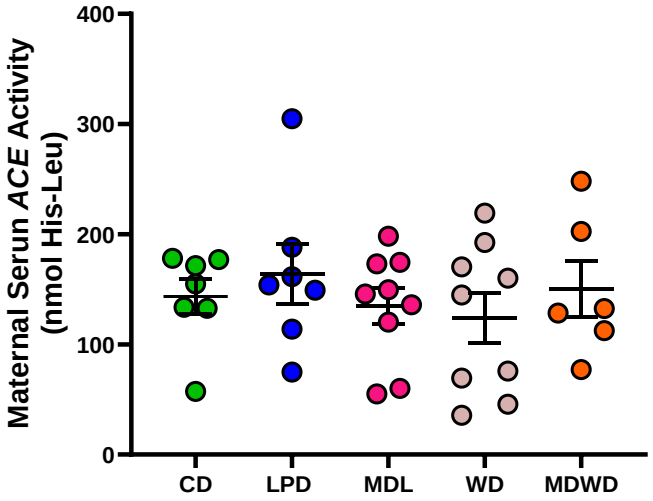


Figure 3-12 demonstrates the serum activity analysis showed as nmol of His-Leu formed. Statistical analyses of the data exhibited no significant difference in ACE *activity enzyme across the different five treatment groups. (*n=8*). Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM*

3.6.9 Paternal diet has no statistically substantial effect on maternal cardiovascular gene expression.

To investegate whetehr maternal cardiac function may be alterd in response to poor paternal diet , the expression of central genes in the regulation of cardiac function were examined. Maternal cardiovascular gene expression was measured using the RT^2-Profiler cardiovascular diseases array. There was no significant difference in the expression levels of any of the 5 housekeeping genes including *Actb*, *B2m*, *Gapdh*, *Gusb* and *Hsp90ab1* between dietary groups ($P < 0.1$). The expression of all 5 housekeeping genes exhibited some variability across the different samples. Therefore, to account for any potential variability regarding the amount of synthesized cDNA, data using genes of interest were normalized, by using two of the housekeeping genes including *Actb* and *Hsp90ab1*. In addition, there were no significant differences in gene expression when comparing each of the treatment groups with CD and each other, (n=5, per each treatment group).

Gene function analysis of the differentially expressed genes (genes of interest) characterised them into the nine different functional gene families, of which those involved in including apoptosis (Table 3-1 A), cardiac remodeling (Table 3-1 B), cell cycle (Table 3-1 C), Cell Growth (Table 3-1 D), Sarcomere Structural Protein (Table 3-1 E)**,** Signal Transduction (Table 3-1 F), Stress and Immune Response (Table 3-1 G), Transcriptional Regulation and Transporter genes (Table 3-1 H and I respectively). For analysis, the fold expression differences and individual P values ≤0.05 were considered significant.

Table 3-1. A

Apoptosis	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Ccl2	1.41	0.392606	0.72	0.522381	1.06	0.40047	1.02	0.312707
Maoa	1.54	0.333634	1.62	0.394912	1.68	0.15502	2.81	0.346311
Ndufb5	0.04	0.150163	7.14	0.477626	1.16	0.400741	0.12	0.625896
Nppa	0.47	0.304933	0.02	0.185459	0.06	0.446394	0.17	0.887847
Nppb	2.27	0.347882	2.85	0.389768	1.06	0.40047	20.89	0.338604
Npr1	0.48	0.365972	0.95	0.486617	0.29	0.554466	1.64	0.396456
Pde3a	0.63	0.532268	2.11	0.987669	3.01	0.334938	2.45	0.325743
Snca	3.54	0.548981	1.58	0.55931	6.91	0.235384	8.28	0.18265
Thbs2	2.58	0.89445	2.95	0.40151	6.36	0.349354	3.75	0.313707
Ubb	0.48	0.177333	0.82	0.622626	1.06	0.40047	1.02	0.312707
Zyx	0.78	0.429918	0.34	0.389078	1.22	0.586087	0.3	0.588682

Table 3-1.B

Cardiac Remodelling	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Aebp1	0.73	0.586098	0.86	0.53187	1.5	0.367203	1.77	0.314182
Anxa4	0.98	0.675871	1.59	0.787362	2.07	0.338142	1.06	0.269141
Col1a1	0.09	0.218046	0.02	0.166226	0.01	0.250535	0.01	0.193335
Col3a1	0.2	0.499722	0.28	0.616265	0	0.027927	2.54	0.155465
Dcn	3.26	0.76535	0.98	0.595349	0.11	0.407196	0.77	0.400652
Dmd	0.17	0.357846	1.35	0.371219	0.3	0.485843	0.7	0.82099
F2r	1	0.779712	2.01	0.50623	4.04	0.287891	0.74	0.24004
Fn1	0.19	0.327668	0.39	0.529978	1.12	0.547561	1.71	0.398604
Gja1	1.02	0.886544	2	0.914007	2.94	0.330292	2.21	0.323255
Mmp13	0.48	0.177333	2.44	0.222251	1.06	0.40047	1.02	0.312707
Ren1	1.1	0.787381	1.74	0.595392	0.52	0.467845	0.67	0.523073
Rtn4	1.77	0.180494	4.44	0.455166	0.3	0.652306	43.62	0.237757
Tnni3	0.05	0.674115	0	0.356851	0.03	0.412581	0.13	0.594065
Tnnt2	0	0.334956	0	0.263226	0.04	0.490402	0.14	0.508299

Table 3-1.C

Cell Cycle	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Ccnd1	0	0.341185	0.01	0.294033	0.05	0.413551	0.03	0.344188
Cdkn1b	0.87	0.616614	0.25	0.216881	0.23	0.364358	0.04	0.75185
G0s2	1.04	0.489973	0.75	0.950882	12.99	0.276291	3.44	0.347235
Rarres1	2.12	0.246327	4.78	0.100703	3.59	0.296013	1.47	0.404656

Table 3-1.D

Cell Growth	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Ccn2	0.88	0.776414	2.06	0.421433	7.22	0.357412	2.17	0.277203
Ptn	4.69	0.327584	11.17	0.130128	2.22	0.312537	1.32	0.447574
Sfrp4	0.39	0.941829	0.34	0.157409	0.51	0.911339	0.49	0.757603
Spock1	0.48	0.177333	5.26	0.389296	12.87	0.292227	1.02	0.312707

Table 3-1.E

Sarcomere Structural Protein	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Cryab	0	0.126318	1.81	0.722675	2.72	0.368742	0.13	0.467626
Crym	0.23	0.132772	0.74	0.454012	2.84	0.150735	0.48	0.584196
Myh10	2.37	0.387309	6.59	0.484783	3.5	0.901361	2.2	0.595034
Myh6	0.01	0.333202	0.08	0.718669	0.31	0.415007	0.14	0.817761
Nebl	0.03	0.312886	0.41	0.320933	0.04	0.414832	2.86	0.404712
Postn	0.12	0.226451	1.57	0.816297	0.58	0.783315	0.65	0.856407

Table 3-1.F

Signal Transduction	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Adra1b	4.87	0.602275	0.76	0.437122	19.12	0.246047	3.28	0.209272
Adra1d	1.42	0.61334	2.51	0.721265	2.37	0.652617	7.74	0.32782
Adrb1	0.14	0.279556	1.8	0.527076	1.46	0.644997	0.35	0.768475
Adrb2	0.24	0.267714	0.15	0.506314	0.1	0.257466	1.09	0.554376
Adrb3	9.43	0.346543	0.83	0.717269	6.68	0.314726	1.18	0.378525
Agtr1a	1.09	0.602696	2.44	0.447268	2.11	0.337826	2.42	0.287676
Ar	1.29	0.541309	1.03	0.474169	3.85	0.319112	2.87	0.269433
Dusp6	2.42	0.275563	6.12	0.119841	0.65	0.363427	2.05	0.227225
Epor	2.02	0.386539	1.39	0.475961	6.64	0.642783	0.85	0.856637
Frzb	1.06	0.514918	0.91	0.831565	4.05	0.292018	4.56	0.29779
Hmgcl	1.04	0.477631	0.58	0.776062	0.38	0.398376	4.15	0.776306
Hmgcr	3.71	0.30109	14.45	0.267065	1.06	0.40047	1.02	0.312707
Map2k5	1.85	0.361597	0.52	0.3047	0.36	0.823343	0.15	0.248992
Mapk1	0.44	0.705568	0.15	0.36079	4.58	0.287202	6.13	0.35553
Pde7a	0.48	0.177333	25.75	0.311582	1.06	0.40047	1.02	0.312707
Rassf1	0.31	0.873252	0.1	0.587574	1.03	0.308363	0.35	0.358441
Slc12a1	0.38	0.174682	0.57	0.270446	1.21	0.329553	0.81	0.427611

Table 3-1.G

Stress and Immune Response	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
C6	0.48	0.177333	0.94	0.910405	1.06	0.40047	2.19	0.28773
Ccl11	4.65	0.243366	17.35	0.161052	7.6	0.172516	26.24	0.067903
Ccl2	1.41	0.392606	0.72	0.522381	1.06	0.40047	1.02	0.312707
Cxcl12	4.72	0.694684	1.55	0.695057	1.34	0.330702	1.51	0.663647
S100a1	45.28	0.790291	0.42	0.335229	6.09	0.591938	1.32	0.476781
S100a8	3.55	0.663579	2.63	0.470361	7.43	0.323255	1.25	0.480715

Table 3-1.H

Transcriptional Regulation	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Creb5	1.22	0.645514	0.16	0.537115	0.95	0.396171	0.31	0.557678
Crem	7.78	0.42992	11.06	0.283736	13.44	0.282586	5.58	0.311456
Enah	0.13	0.304503	0.32	0.635541	0.02	0.374944	0.22	0.411923
Hmgn2	2.77	0.347065	0.72	0.522381	1.06	0.40047	1.02	0.312707
Klhl3	2.81	0.347959	8.54	0.354344	1.06	0.40047	1.02	0.312707
Msi2	2.08	0.865744	25.48	0.312959	1.04	0.617614	4.86	0.270658
Nfia	2.43	0.218197	2.9	0.373134	2.37	0.430282	25.61	0.154368
Nkx2-5	4.59	0.290605	51.39	0.020649	15.13	0.221699	16.3	0.231326
Stat1	2.98	0.34676	2.45	0.389755	1.06	0.40047	4.43	0.345015
Tcf4	15.31	0.454426	0.57	0.437431	7.21	0.960983	2.38	0.631217

Table 3-1.I

Transporters	**LPD vs CD**		**MDL vs CD**		**WD vs CD**		**MDWD vs CD**	
Gene Symbol	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
Atp2a2	0.01	0.145847	2.76	0.897429	0.02	0.48402	0.01	0.53997
Atp5a1	0.05	0.346588	0.59	0.894128	0.11	0.407114	1.07	0.401399

Table 3-1 A-I represents the maternal cardiovascular disease gene expression results. For presented array genes, fold expression changes and individual P values have been listed. There were no differences in the expression of any of the housekeeping genes, and the test genes were observed across different groups in response to the paternal diet. Gene function analysis of the expressed genes characterized them into the following cardiovascular process, listed in Table *1. A-I.*

3.7 Discussion

The main findings of this current Chapter show that this study has demonstrated that any impact of paternal diet on offspring outcome is not dependent on maternal physiological response to paternal diet. The different sub-optimal paternal diets had minimal and subtle impacts on the growth profiles of the males. However, by the end of the study, a significant change in the gross weight of several organs, as well as their relative organ: body weight ratios, were observed. In addition to studying the impact of the diets on male health, the late gestation (E17.5) well-being of females mated with these males, was also assessed. Here, while the male diet had a very subtle impact on overall maternal cardio-metabolic health, the obtained data suggest there may be some alterations in the interconnectedness of specific bacterial species within the maternal gut, in response to different paternal diets.

There were no alterations in the weight gain and growth trajectory of stud males across the different dietary treatment groups, throughout the study. This is not completely in agreement with similar studies, as male mice fed low-protein diets had reduced weight gain, especially during the pre-mating phase of feeding (Watkins and Sinclair, 2014a). However, some studies reported no noticeable changes in body weight observed in response to LPD-fed males when compared to the CD-fed males (Morgan et al., 2020a, da Cruz et al., 2018b). It should be noted that both experiments utilized the same LPD in both experiments. No information regarding the scale of weight changes in the LPD-fed males has been reported. The consumption of lower amounts of protein is associated with an increased risk of weight gain in humans (Apolzan et al., 2007). This weight gain in response to a low level of protein consumption has been associated with hyperphagia, both in men (Apolzan et al., 2007), and mice (Blais et al., 2018). Consumption of LPD elevates the expression of the *Ucp-1* gene in both brown and white adipose tissue respectively BAT and WAT, which the expression of this gene has been associated with the contribution to energy expenditure (Hill et al., 2017). Increasing the expression of *Ucp-1* in BAT and WAT as well as, hepatic amino acid sensing and signaling, plays a central role in hyperphagia via the activation of fibroblast growth factor 21 (FGF21), secreted from hepatocytes

which has a central role in the activation of β-klotho receptors in the brain to promote hyperphagia (Laeger et al., 2014, Hill et al., 2017).

Increased total body weight in males fed a WD, has been reported in animal model studies (Hasegawa et al., 2020). These studies used similar fat percentages (22%, and 21% respectively) and different types of fats (SF00-219 diet which contains 0.15% Cholesterol to mimic the WD), to our experiment. However, the period of exposure to these diets was less than the period of our experiments, 10 and 14 weeks respectively (Binder et al., 2015a, Schjenken et al., 2021a). Interestingly, Binder, (2015) utilized the same strain of the mice, which suggests the differences observed in male weight gain may not be attributed to the strain of mice. The role of choline and methionine levels in animal weight gain after feeding them a(Binder et al., 2015a, Schjenken et al., 2021a).(Binder et al., 2015a, Schjenken et al., 2021a). Interestingly, Binder, (2015) utilized the same strain of the mice, which suggests the differences observed in male weight gain may not be attributed to the strain of mice. The role of choline and methionine levels in animal weight gain after feeding them HFD, or WD show choline and methionine deficiency has a substantial role in weight gain of animals feeding HFD, WD, and cafeteria diets (Bortolin et al., 2018). One possible reason for the absence of weight gain in the WD-fed males in our experiment could be due to the differences in the profile of the dietary fatty acids, such as different compositions of saturated/mono-unsaturated/polyunsaturated, as well as trans, by implying various effects on glucose-insulin homeostasis. Saturated fatty acid has the most deleterious impact on enhancing the HbA1c. The composition of the diet fatty acids has an essential role in the induction and promotion of obesity, through modulation of the lipogenic enzymes by influencing SREBP-1 proteolytic cascade alongside peroxisome proliferator-activated receptor α (PPARα), in mice liver (Nakatani et al., 2003, Speakman, 2019).

Supplementation of the LPD with methyl donors has been shown to increase weight gain in male mice when compared to the LPD alone (Morgan et al., 2020a). It is important to consider, that diets low in methionine and cysteine have been associated with reduced food intake in both animals and humans

(Plaisance et al., 2010, Ables and Johnson, 2017). This could be due to a difference in the period that they kept animals on MD-LPD, which was at least 7 weeks (Morgan et al., 2020a).

No significant alterations in organ weight ratios, such as those of the testes and heart, relative to the total body weight, were observed in relation to different male diets. However, significant associations were found between male diets and the weight ratios of organs such as the liver, kidneys, seminal vesicles, and gonadal fat pads, with overall body weight.

Observation of a significant increase in liver weight in response to WD when compared to CD, is in accordance with the other HFD studies (Bodden et al., 2021, Fan et al., 2015b, Ng et al., 2010b). An increase in liver weight could be possibly due to the increase in lipid droplet accumulation in hepatocytes indicating serious steatosis (Fan et al., 2015b, Bodden et al., 2021, Simões et al., 2021). In animal HFD studies, liver steatosis has been reported accompanied by perturbed glucose levels, which could be an indication of non-alcoholic fatty liver diseases (NAFLD). Which is characterized by the storage of lipid droplets in the liver, and the potential development into non-alcoholic steatohepatitis (NASH), which is a kind of proinflammatory liver condition, that results in liver fibrosis and cirrhosis in its end stages (Vancells Lujan et al., 2021). Additionally, lipid accumulation in hepatocytes could result in perturbed beta-oxidation as a result of impaired liver mitochondrial functions (Li et al., 2008). NAFLD is a common component of the metabolic disease (Wu et al., 2013), which has been related to insulin resistance, followed by consumption of a poor diet. Hence, it could be comprehended that the pre-diabetic and metabolic syndrome conditions could be predicted in the WD-fed males. However, confirmation would require the examination of glucose tolerance as well as TG or other lipid metabolite marker profiles.

Heart weight measurement showed a significant decrease in the weight of hearts derived from LPD, MDL, and MDWD groups when compared to CD. A study on LPD-fed male mice reported a significant decrease in heart: body weight ratio in the LPD group, however the heart: body weight ratio in our experiment does

not mirror this, despite a significant decrease in heart tissues derived from LPD-fed males (Watkins and Sinclair, 2014a).

There could be various mechanisms directing heart weight loss in these groups when compared to CD. It could be related to diminishing sources of amino acids, which could lead to cardiomyocytes atrophy, and the similar diminish in heart weights derived from males fed MDL, could suggest that supplementation of methyl donor group, did not mitigate the negative effects of LPD on heart weight. The reduction in dietary protein content significantly impacts caloric intake and energy expenditure, therefore, it may cause depletion in the availability of micronutrients such as vitamins and minerals. The decrease in heart weight of the MDWD group suggests that the supplementation of the WD group could have prevented the uptake of essential proteins for the growth of the cardiomyocytes, and interestingly the supplementation of this suboptimal diet with methyl donor was not effectively enough to mitigate the negative effects of WD. Additionally, the negative association between the secretion of leptin and food intake could be another potential reason for preventing the absorption of essential amino acids for cardiomyocyte development (Poetsch et al., 2020). However, whether leptin directly affects cardiac function or acts through a leptin-regulated neurohumoral pathway remains unclear.

It is worth noting that heart weight alone cannot provide a comprehensive picture of cardiac function. Additional assessments, such as blood pressure monitoring and analysis of gene expression involved in cardiac calcium signaling (*Adcy, Plcb, Prkcb*), as indicated by (Watkins and Sinclair, 2014a, Li et al., 2016a) are essential for a thorough investigation. These logical investigations are necessary for a better understanding of the role of dietary factors in the health and function of the heart.

Interestingly, despite the absence of a significant alteration in kidneys weight, relative to total body weight, between the LPD and CD treatment groups observed in this experiment, other studies conducted on mice have reported a significant reduction in kidney weight in the LPD when compared with the CD group (Watkins and Sinclair, 2014a, Morgan et al., 2020b). In this study, a

significant increase in kidney size (mean weight of right and left kidneys) was observed in the WD group. One possible reason for this could be fat deposition in kidneys known as peri-renal fat. As it is evident, abnormal deposition of visceral fat has been linked with cardiometabolic diseases (Shuster et al., 2012). Accumulation of peri-renal fat has been attributed to an increased risk of metabolic diseases. Additionally, studies have identified an increased risk of chronic kidney disease (CKD) with peri-renal fat deposition (Huang et al., 2020). A significant reduction in kidney weight in the LPD group compared to WD was observed, while no significant difference between LPD kidneys compared to CD was observed.

A significant increase in gonadal fat pads derived from WD-and MDWD-fed males was observed. Gonadal fat mass: body mass has been associated with an increase in white adipose tissue (WAT), in mice (Kerley-Hamilton et al., 2012). Indeed, excessive levels of WAT have been associated with increased secretion of the pro-inflammatory adipokines, including adiponectin, TNF-α, leptin, and interleukin 6, which have been associated with an increased level of inflammation, perturbed modulation of the glucose/lipid metabolism (Lin et al., 2011, Yan et al., 2007). Alterations in the level of adipokines and inflammatory factors, as well as an imbalance of glucose/lipid metabolism, have been associated with the perturbed hypothalamic–pituitary–gonadal axis (HPGA). It could potentially suggest, this increased WAT around the testes could disturb the process of spermatogenesis, by disturbing the optimum temperature required for spermatogenesis, as spermatogenesis is highly sensitive to the increased heat. Indeed, an increased level of adipocytokines, would potentially result in negative influences on modulating the pituitary-testicular axis, suggesting results in conditions such as hypogonadotropic hypoestrogenic hyperandrogenemia, which may negatively impact spermatogenesis and male fertility (Reis and Dias, 2012, Davidson et al., 2015). It could be a result of differences in the time of being on the diet, and the percentage of the fat and sugar in the diets consumed in these experiments conducted by Reis and Dias, (2012) and Davidson et al, (2015) when compared to our experiment.

Additionally, the lack of epididymal measurement in this study needs to be pointed out, as a decrease in epididymal weight and increase in gWAT alongside the normal testes weight has been reported in HFD-fed male mice, with a lower sperm count despite the normal sperm viability, morphology, motility and acrosomal reactions (Gómez-Elías et al., 2019). Both increase and decrease (Yang et al., 2022), epididymis adipose tissue has been associated with (HPGA), and consequent influences on male fertility.

However, a slight increase in seminal vesicles weight in both WD and MDWD groups was observed. However, it is important to note that the absence of alterations in seminal vesicle weight does not necessarily imply changes in seminal vesicle secretions volume and agents, encompasses fructose, proteins such as SPMI which are essential for sperm motility, citric acid, potassium, and prostaglandins (Noda and Ikawa, 2019). A study on high-fat-fed male mice (Schjenken et al., 2021b) showed a non-significant increase in seminal vesicle weight, which aligns with the data obtained in this experiment.

In general, no significant differences in organ weights were observed between the under/over-nutrition diets when compared to methyl-donor supplemented diets, across all the diet groups. It could be suggested, the methyl-donor supplementation may have mitigated the adverse effects of suboptimal groups, in terms of the organ weights.

In continuation, this chapter discusses the results obtained from studying the association between paternal diet and maternal physiological and metabolic characteristics, or any potential gut dysbiosis in late gestation (E17.5).

Collectively, maternal metabolic profiling including glucose, insulin, lipid profile and their ratios demonstrates a minimal impact on maternal responses to the paternal diet in late gestation. Potentially, this could suggest that an inappropriate stage of pregnancy has been chosen for looking into the impact of paternal diet on maternal metabolic status, as the majority of the maternal metabolic adaptations occur in the first and second stages of pregnancy (Hadden

and McLaughlin, 2009, Symonds and Ramsay, 2010). Therefore, any changes in response to paternal factors have been normalized by this stage of pregnancy.

The impact of parental under/overnutrition has been associated with a reduction in uterine pro-inflammatory cytokines essential for implantation and embryo trophic functions. In a mouse model study, females mated with males fed an LPD, exhibited a reduction in the uterine proinflammatory cytokines and chemokines at 3.5 days post-coitus. This was coupled with reduced expression of prostaglandin synthesis genes as well as reduced uterine blood vessel development (Watkins et al., 2018). Hence, the suppression of uterine inflammatory responses could potentially impair the secretion of the embryotropic factors and modulation of the maternal immune cytokines such as regulatory T cell expansion and leukocyte infiltration. These processes are essential to prevent the rejection of the allograft embryo by the maternal immune system, as well as embryo development and blastocyst implantation (Orsi et al., 2007, Bromfield et al., 2014).

As such, the obtained results from our study are in line with the other studies. A paternal HFD study on rats has reported no alteration in maternal metabolic status during gestation in response to paternal diet. However, this study has considered the assessment of the placental lactogen coding-gene (*Prl3d1*) at two different points of gestation including E14.5 and E18.5which secretion of this hormone plays a substantial role in the maternal glucose metabolism and metabolic status (Jazwiec et al., 2022). The placental lactogen is a marker of maternal metabolic status and fetal growth, through modulating the secretion of insulin from pancreatic beta cells, throughout pregnancy (Sibiak et al., 2020). Therefore, it is worth highlighting that our study did not include an examination of the placental lactogen in this set of experiments which could be valuable in understanding the potential impact of paternal diet on maternal metabolic status. While the underlying mechanisms linking paternal diet to maternal metabolic status have not been fully defined, assessing placental lactogen, a key marker of maternal metabolic status, could provide valuable insights, as changes in this hormone have been associated with pregnancy complications.

Moreover, data derived from both human studies (Chen et al., 2012, McCowan et al., 2011), and research on animal models (McPherson et al., 2017, Lambrot et al., 2013a, Watkins and Sinclair, 2014a) have shown that the perturbed paternal diet can impact his offspring's health through various mechanisms. Given that the underlying mechanism of paternal programming is not fully defined it has become of interest to investigate whether the paternal diet may influence maternal health status. As well as considering the prominent role of maternal cardio-metabolic health in fetal development.

Any impairment in maternal adaptation in terms of cardiovascular, metabolic and immunological system aspects potentially would be an underlying aetiology of pregnancy complications such as gestational diabetes. The results of maternal metabolic biomarkers assessment in this chapter represent a minimal impact of paternal diet on maternal metabolic biomarkers alterations during late gestation.

Investigating the influence of paternal factors on maternal health during pregnancy is an ongoing research subject. For instance, factors such as paternal advanced age have been associated with maternal health during gestation, including the risk of gestational diabetes in births, as well as remarkable adverse effects on their babies such as premature birth and low birth weight. However, the overall risk of occurrence stays low and steady (Khandwala et al., 2018). Thus, the placenta emerges as a remarkable organ linking paternal factors to maternal metabolic status.

The exact underlying mechanism that may lead to maternal gestational diabetes at the time of birth is not fully clarified yet. However, these mechanisms may be connected with the shortened telomers of sperm, the same as the relations between advanced paternal age and the occurrence of preeclampsia (García-Ortíz et al., 2011). Shortened sperm telomeres have been associated with cellular ageing as well as decreased chromosomal integrity, as telomers play a central role in chromosomal stability coupled with protection of genomic resistance (Sharqawi et al., 2022).

It is well established that any alterations in placental development are likely to be associated with fetal maldevelopment. Binder et al. (2015a), observed significant changes in gene expression and DNA methylation in the placentas of the mice offspring derived from paternal HFD, in a sex-specific manner. It was demonstrated that comparison of placentas from sons born to obese fathers, with those born to non-obese males, showed a significant increase in the expression of *Ppara* and *Casp12* (Binder et al., 2015a). Fascinatingly, a strong association between the hyper-expression of *Ppara* and *Casp12* in the placenta has been related to some pregnancy complications such as late-onset preeclampsia (LOPE), which manifests around week 34 of pregnancy onwards (Permadi et al., 2020). As a result of the key role of *Ppar* in transducing the nutritional signals to influence the gene expression of pathways mainly targeted the lipid and energy metabolism (Wahli and Michalik, 2012), involved in placental development (Matsuda et al., 2013). Paternal HFD studies in animal models have been associated with gestational complications such as GDM and preeclampsia (Binder et al., 2012). For instance, a high-fat diet in male mice reduced fetal growth and increased the risk of perturbation of uterine vascularization in female mice mated with the sperm of the male mice fed the high-fat diet (Jazwiec et al., 2022).

Pparα, β and *ϒ* are involved in the regulation of lipid metabolism, glucose and amino transportation as well as insulin-sensitising activity. Their functions have the potential to impact the uptake of fatty acid in the trophoblast, directly influencing the regulation and distribution of these metabolites within the placenta (Matsuda et al., 2013).

Additionally, a decrease in the expression of *Pparα*, *ϒ* has been associated with reduced insulin sensitivity. This reduction in expression can lead to increased inflammation, resulting in enhanced expression of pro-inflammatory cytokines such as IL-6 and TNF-α (Choi et al., 2015).

Dysregulation in the expression of this gene has been related to disrupted pregnancies such as intrauterine growth restriction IUGR, gestational diabetes mellitus GDM and preeclampsia PE. Therefore, it is very plausible to conclude

that misexpression of these genes which are the master regulators of glucose, fat and amino acid transporters in the trophoblasts, could be a substantial factor in the development of GDM and PE (Peng et al., 2021, Olivo-Vidal et al., 2016). However, studies have demonstrated that GDM occurs earlier in pregnancy and then can become normalized in later gestation, as the initiation and progression of GDM could be varied in individuals. Therefore, the maternal blood insulin and glucose tolerance test should have been conducted earlier in gestation, as by progressing the pregnancy maternal body adaptations in regulating glucose uptake and insulin secretion will improve to provide the optimum level of availability glucose for the consumption of the developing fetus (Bochkur Dratver et al., 2022, Parveen et al., 2022).

Hormones such as placental lactogen may interfere with the rate of metabolism throughout gestation including in late gestation (Sibiak et al., 2020). As, impairments in the level of these hormones would be potentially linked to the occurrence of maternal metabolic dysfunctions, encompassing insulin resistance, and impaired lipid metabolism. In this chapter the emphasis is not on the late gestation hormonal profile and their alterations in response to the paternal environment, however, their assessment could be very insightful which has been missed from my experiment, due to the time restrictions.

However, the precise underlying mechanism of how the paternal diet affects maternal metabolic responses remains unclear. Nonetheless, it is worth mentioning a few epigenetic mechanisms through which a father's diet could impact maternal health. The paternal high-fat diet causes alterations in sperm miRNA expression (de Castro Barbosa et al., 2016, Claycombe-Larson et al., 2020) and there is an association between the paternal high-fat diet and the placental epigenetic aberrations, and glucose metabolism disruption significant in response to the paternal high-fat diet. A paternal high-fat diet resulting in obesity alters placental gene expression and dysregulated the expression of genes such as *Peg3, Peg9, Peg10*, and the nutrient transporter gene *Slc38a2*. Furthermore, paternal high-fat diet and aberrant DNA methylation in the promoter of *Peg9* gene in the placentas of the first generation. These alterations in gene expression consequently result in the dysregulation of reproductive

fitness and DNA methylation ultimately results in the disruption of reproductive fitness and elevated risk of long-term diseases in offspring (Claycombe-Larson et al., 2020). The other factor that manifests the contribution of the father to the development of the placenta is imprinted genes. Alteration in the expression of placental imprinted genes in the placentas derived from paternal LPD-fed diet has been reported, and the consequences of this alteration in these imprinted genes are making the offspring more susceptible to the cardio-metabolic dysfunctions later in life. Paternal LPD altered both paternally *Mest* and *Snrpn* and maternally imprinted genes *Cdkn1c*, *H19*, and *Grb10* in these placentas (Watkins et al., 2017). In mammals, offspring inherit two copies of each allele from their parents. However, for a small minority of genes, one of the parental alleles is silenced, while the other remains expressed, these are termed imprinted genes (Bajrami and Spiroski, 2016). The significance of imprinted genes in mammals is that many of them are expressed within the placenta and play a significant role in the regulation of placental function and fetal growth (Georgiades et al., 2002). Therefore, perturbed expression of the imprinted genes may result in pregnancy complications, such as IUGR and macrosomia, which results in health implications for both mother and baby.

Over-expression of a few paternally imprinted genes such as *PEG10* and *MEST* has been expressed in the IUGR term placenta (Caniçais et al., 2021). As well as paternally imprinted genes *IGF2* has been overexpressed in placentas derived from macrosomia babies, followed by increased levels of maternal glucose in term (Petry et al., 2011).

Intriguingly, in late gestation, a shift occurs whereby metabolism switches from the anabolic status to the catabolic one, which is accompanied by less fat mass deposition in the mother. Additionally, lipoprotein lipase (LPL) activity, lipolysis of the adipose tissues, and elevated FFA levels are expected, which makes the lipid sources be used up as the major sources of energy for mother and fetus, despite glucose and amino acids being kept for the fetus (Parrettini et al., 2020).

One significant metabolic biomarker in late gestation metabolic profile assessment is LPL, due to its crucial role in lipid metabolism. LPL is responsible for breaking down fats into forms such as triglycerides (TG) and free fatty acids (FFA). Any deficiency in LPL levels or function could result in gestational complications, such as intrauterine growth restriction (IUGR) and an increase in placental LPL levels, potentially leading to the development of IUGR. (Gagné-Ouellet et al., 2017).

Literature suggests that the level of the adipokine leptin measurement in late gestation could be considered as a valuable clinical indicator for the assessment of maternal health, as a disturbance in the level of these metabolites has been associated with maternal pregnancy complications as a pathophysiological and prognostic factor (Miehle et al., 2012). In addition, studies in humans show it seems leptin and adipokines play a fundamental role in early pregnancy, such as contributing to vital processes including trophoblast invasion, proliferation and finally apoptosis in placental cells (Thagaard et al., 2019, D'Anna et al., 2006). Intriguingly, the importance of leptin level in late gestation is directly related to the healthy maintenance of lipid level circulation such as TG, cholesterol, and FFA, and overall energy homeostasis (Catalano et al., 2006, Ramos-Lobo and Donato Jr, 2017, Masuyama and Hiramatsu, 2012). Thus, measuring this biomarker could be a very insightful evaluation, however no significant difference in maternal hepatic lipid profile was observed in this experiment, so it would not be anticipated to see a significant difference in the leptin and adiponectin levels in dams across the different treatment groups.

Another component of the maternal metabolic status analyzed within this Chapter was the maternal gut microbiota. As the microbiome plays a significant role in various healthy states, it is assumed to offer essential aid for the growth of the fetus (Charbonneau et al., 2016). The gut microbiota including shifts in bacterial relative abundance and populations, undergoes alterations which are normal but, similar to those seen in metabolic syndrome, and these changes are significant during pregnancy (Pitlik and Koren, 2017). However, during a healthy pregnancy, there is a noticeable rise in the number of bacteria present

and significant changes occur in the makeup of the gut microbiota (Nuriel-Ohayon et al., 2016).

Studies on human samples during the initial three months of pregnancy, show that the gut microbiota of the mother is comparable to that of healthy females who are not pregnant. However, from the start of pregnancy until the third trimester, there is a substantial shift in the gut microbiota composition. This transformation is identified by a rise in the presence of *Actinobacteria* and *Proteobacteria* phyla, and the decrease in individual diversity which is referred to as alpha diversity (Koren et al., 2012). In contrast, other studies, demonstrate no significant differences between the pre-conception period and the first trimester of pregnancy (Schoenmakers et al., 2019). However, as the pregnancy progresses the overall number of bacterial populations increases. Separately, other studies have observed no alteration regarding the gut microbiota over the pregnancy (DiGiulio et al., 2015).

As it has been mentioned earlier, studies have emphasized how mothers directly can influence the development of the fetal microbiome, and clearly, there is an essential requirement to know how a father's influences can impact the offspring's microbiome development as well as his impact on maternal investment during pregnancy (Korgan et al., 2018).

Pregnancy manipulates the intestinal microbiota diversity and composition in mice, in a strain-dependent way (Rodríguez et al., 2015). The results of this experiment at the phylum level represented the presence of phyla *Deferribacteres, Firmicutes, Proteobacteria, TM7, Tenericutes, Verrucomicrobia, Bacteroidetes,* and *Actinobacteria,* however *Bacteroidetes* and *Firmicutes* substantially more prevalent at the phylum level in the obtained results.

There are several studies in agreement with the results of this experiment, which have reported very similar phyla extracted from the gut of the same strain of pregnant mice at late gestation (Greenberg et al., 2022, Safari et al., 2020). Bacteria such as *Proteobacteria, Actinobacteria, Enterobacteriaceae* and

Bifidobacterium have been reported as the dominant gut microbiota during late gestation. As an illustration, a significant increase in the population of *Proteobacteria* and *Actinobacteria* has been reported (Amir et al., 2020) as pregnancy progresses, some changes happen including an increase in the population of *Proteobacteria*, *Actinobacteria*, and opportunistic pathogens. Additionally, there is a decrease in the number of short-chain fatty acid producers and a reduction in species richness (Koren et al., 2012).

The *Firmicutes/Bacteroidetes* ratio was analyzed as any disturbance in this ratio has been related to pregnancy complications, for instance, a higher ratio of *Firmicutes/Bacteroidetes* has been associated with GDM in late gestation (Hou et al., 2022, Mills et al., 2019). Interestingly these two phyla are the two main dominant phyla in the intestine, as they have a substantial role in the metabolism of fat and bile acid, and subsequently a significant role in the energy balance of the host (Li et al., 2021). An increased abundance of the *firmicutes* has been associated with an increase in the metabolism of carbohydrates, followed by hyperglycemia and obesity. However, the role of the *Bacteroides* has been reported with weight loss due to a smaller number of genes for the expression of enzymes involved in the hydrolyses of lipid and carbohydrate metabolism when compared to *firmicutes* (Li et al., 2021, Hou et al., 2022).

The assessment of maternal vaginal microbiome was not included and could be considered as a study limitation. As it could be a potential indirect association between paternal seminal plasma content intervention the maternal metabolic health during gestation. As, we know the human maternal vaginal microbiome could be altered in response to the paternal seminal plasma microbiome (Mändar et al., 2015). Not only seminal fluid microbiome could influence the maternal vaginal microbiota but also it can impact the offspring's metabolic phenotypes consequently. A study in rats reported that offspring born to males fed a high-protein diet exhibited lower adiposity and higher insulin sensitivity compared to those born to males fed a high-fat diet. This suggests a potential indirect link between paternal gut microbiota and the gut microbiota of offspring via the mothers, possibly due to alterations in maternal reproductive tract physiology as well as the vaginal microbiome (Chleilat et al., 2021a).

Maternal late gestation serum ACE activity and cardiovascular diseases gene expression assessment was the other component of the maternal metabolic status analyzed within this Chapter. No significant differences in maternal serum ACE activity across the different treatment groups in response to paternal diet were observed when compared to CD. To our knowledge, no data has documented the impact of paternal diet on maternal RAS activity. However, in mice low protein paternal diet influences his offspring's cardiovascular programming and health in later life. Paternal low-protein diet influences offspring's ACE activity and vascular malfunction in a transgenerational manner, fascinatingly, the second generation of low-protein-fed diet fathers showed a decrease in fetal weight and alteration in tissue ACE activity (Morgan et al., 2020b). Whilst minimal findings exist regarding the impact of paternal diet on maternal cardiovascular and ACE activity levels during the late gestation, the significance of the RAS function has been documented in other maternal tissues, such as placenta and kidney (Pringle et al., 2011).

3.8 Conclusion

This chapter revealed that minimal and subtle alterations in maternal cardio-metabolic health in response to paternal diet in late gestation were observed. However, significant alterations in the fetal heart transcriptomic profile were observed in response to the different paternal diet treatments. Whether the changes observed in the current study are due to paternal influences on maternal hormonal status in pregnancy are currently unknown as no assessment of maternal endocrine assessments were conducted in these sets of experiments. Further understanding of maternal endocrinology and immune systems could provide insight into maternal responses to paternal diet, thus indicating the need to examine earlier stages of pregnancy.

3.9 Experimental limitations and strengths

The strength of this study revealed that, this study has demonstrated, any impact of paternal diet on offspring outcome is not dependent on maternal physiological response to paternal diet. Inflammatory factors are key regulators, which directly impact maternal metabolic dysfunctions during pregnancy. These factors

include tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β) and MCP-1 and were not analyzed in this experiment. The full data related to maternal weight characteristics (weight at conception period and near term (E17.5). Full maternal metabolic biomarkers measurement was not included in our experiment as we did not measure the leptin or adiponectin levels. Furthermore, no glucose tolerance tests were carried out in our experiments, as it is considered a very reliable screening test for the development of GDM (Rani and Begum, 2016).

The impacts of the maternal uterine immune system reactions both to the seminal plasma and sperm antigens could imply significant alterations in the metabolic system of the mother. Alterations in the maternal immune system could be manifested in her metabolic status changes such as being prone to gestational complications including IUGR, PE and gestational diabetes. The presence of immunomodulatory factors in seminal plasma and their interactions with FRT has been attributed to the secretion of hormones such as estradiol and progesterone (MacDonald et al., 2010). The assessment of maternal immunological modulation by components of seminal plasma could be offered as future studies.

4 Chapter 4 Impact of paternal diet on fetal and placental development and fetal heart gene expression in late gestation

4.1 Introduction

It is well-established that maternal over/undernutrition during, and even before, pregnancy increases the risk of her offspring developing an extended range of non-communicable diseases, such as type 2 diabetes, obesity and cardiovascular disease, as a consequence of the impaired intra-uterine environment (Marshall et al., 2022). Barker (1997b), has shown that offspring cardiovascular and metabolic diseases, such as hypertension, dyslipidemia and diabetes, are associated with intrauterine growth complications of both under and overgrowth such as small for gestational age (SGA) or large for gestational age (LGA). A significant body of research has investigated how maternal over and undernutrition impacts fetal cardiometabolic ill-health.

One of the most crucial organs in the fetus is the heart, which develops in parallel with the placenta. Furthermore, it has been suggested, that these organs can regulate one another reciprocally during pregnancy (Ward et al., 2023). As the fetal heart and placenta appear to be the first two organs to differentiate, it is believed that their development is intertwined (Burton and Jauniaux, 2018) Therefore, any dysfunction in early uteroplacental vascularization could be interlinked with cardiovascular-ill health in later life (Burton et al., 2016).

Epidemiological data suggested that small placental and increased surface areas is a predictor of later coronary disease in men (Eriksson et al., 2011). Surface growth of placenta indicates the recruitment of maternal spiral arteries, whereas thickness indicates the extent of endometrial invasion. According to research from Finland, placental morphological traits, such as smaller placentas and irregular vascular networks predict the risk of coronary artery disease, heart failure, hypertension and various cardiovascular malignancies (Thornburg et al., 2010). This suggests that the size of the placenta might play a role in fetal programming for coronary disease (Eriksson et al., 2011, Forsen et al., 1997). This implies that the function of the heart in the future is influenced by factors such as hemodynamics, growth factor, and oxygen/nutrient signals during

prenatal development, which are influenced or modulated by the placenta (Thornburg et al., 2010). It is worth noting that changes in placental shape and size could result from an adverse maternal environment (Camm et al., 2018).. As well as a positive correlation between the incidence of the hypertension and reduced placental weight and surface area (Barker et al., 2010)

The role of maternal diet at the critical time of preconception and its link with offspring health has been well-defined. Studies in rodents document compromised health of the offspring in response to poor maternal diets (Rodford et al., 2008, Watkins et al., 2010, Watkins et al., 2008a). For example, in mice, maternal suboptimal low protein diet during the time of oocyte maturation, resulted in phenotypical changes such as increased weight, hypertension and abnormal anxiety-related behavior in offspring and also increased susceptibility to cardiovascular disease (Watkins et al., 2008b). However, the role of the father has remained ill-defined. According to the fetal programming hypothesis programming factors associated with paternal genes could impact fetal phenotypes irrespective of the fetal genome (Hocher, 2014). Perturbed paternal nutrition at the time of conception has also been associated with impaired offspring cardio-metabolic health. Underlying the paternal programming are suggested changes in sperm epigenetic status and seminal plasma composition mediated through specific semen-associated pathways (Morgan et al., 2022).

In a study reported by Watkins and Sinclair, (2014), offspring born from male mice fed a low-protein diet displayed cardiovascular and metabolic dysfunction (Watkins and Sinclair, 2014a). These included a decrease in the activity of signaling mechanisms known to regulate vascular smooth muscle function. As a result, the offspring showed relative hypotension, decreased by 9.2 mmHg, elevated heart rate and impaired levels of glucose/insulin later in life (Watkins and Sinclair, 2014b).

The mechanisms of paternal cardiac programming have not been studied as well as maternal programming. There are two potential pathways through which the father could influence fetal heart programming. The first is through the alteration of the sperm epigenome, involving elements of DNA methylation, histone

modifications and different types of RNAs (including short and long non-coding RNAs). Under this mechanism, paternal over/undernutrition could exert their impacts through altering the sperm epigenome, which could potentially impact on gene expression patterns in embryo and fetal development, s predisposing them to the adulthood metabolic disease (Pascoal et al., 2022). In a mouse model study, parental undernutrition, such as a low-protein diet, targeted spermatogenesis and induced a state of sperm DNA hypomethylation. This was linked to a decrease in the expression of testicular folate cycle enzymes (Watkins et al., 2018).

A second potential mechanism by which paternal nutrition might impact fetal programming is through the seminal plasma. Seminal plasma constituents, such as pro-inflammatory cytokines, could manipulate the early uterine spiral artery remodeling, placentation and fetal heart programming and development, due to the intimate association between placental and cardiac development (Schjenken and Robertson, 2020). However, the precise underlying mechanism remains poorly defined. Paternal LPD led to significant alterations in serum *ACE* activity and kidney *ACE* activity in juvenile F2 mice offspring, suggesting that a poor paternal diet could affect offspring phenotype across more than one generation (Morgan et al., 2020b), similar to the way a father's high-fat diet can affect fetal programming (Aguila et al., 2021). Paternal undernourishment has a significant impact on the metabolic processes of the embryo, the growth of the fetus, and the cardiometabolic health of the offspring (Watkins et al., 2018). Placentas derived from LPD-fed fathers have been shown to exhibit reduced weight when compared to the control group, as well as the reduced proportion of the junctional zone of the placenta (Morgan et al., 2021).

Paternal overnutrition, in both human populations and animal models, has been linked to subsequent cardiovascular risks in their offspring. For example, in a cross-sectional study of Chinese children, a positive association between paternal body mass index (BMI) and increased risk of CVD in children has been documented (McCarthy et al., 2015).

4.2 Hypothesis

Paternal sub-optimal diets, with or without methyl donor supplements, will adversely affect fetal and placental development. These dietary influences are likely to induce alterations in global gene expression. Therefore, this study aims to investigate the impact of various paternal diets on placental morphological changes and fetal heart transcriptomic alterations during late gestation.

4.3 Aims

- To assess the morphological characteristics of the fetus and placenta during late gestation.
- To explore the influence of paternal diet on placental gene expression during late gestation.
- To quantify histo-morphological changes in the placenta during late gestation in response to paternal diet
- To analyze the global transcriptomic changes in the fetal heart during late gestation in response to paternal diet.

4.4 Method and Materials

4.4.1 Animal treatments

All experimental procedures were conducted under the UK Home Office Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012, which transposed Directive 2010/63/EU into UK law, and with the approval of the local ethics committee at the University of Nottingham. Eight-week-old C57BL/6 male mice (Harlan Ltd, Belton, Leicestershire, UK) were maintained at the Bio Support Unit at the University of Nottingham. Animals were housed in controlled 12/12 hours light/dark conditions with a constant temperature (21 °C ± 3 °C) and *ad libitum* access to water. After a period of acclimatization of 3-5 days, males were allocated to one of five diets including; a control diet (CD: 18% casein, 10% fat, 21% sugar), an isocaloric low protein diet (LPD: 9% casein, 24% sugar, 10% fat) or LPD supplemented with 1-carbon methyl donors (MDL; 5 g/kg diet choline chloride, 15 g/kg diet betaine, 7.5 g/kg diet methionine, 15 mg/kg diet folic acid, 1.5 mg/kg diet vitamin B12), a Western

diet (WD: 19% casein, 34% sugar, 21% fat) or a Western diet supplemented with methyl donors (MDWD; 5 g/kg diet choline chloride, 15 g/kg diet betaine, 7.5 g/kg diet methionine, 15, 15 mg/kg diet folic acid, 1.5 mg/kg diet vitamin B12). Dietary composition is provided in Appendix 1. Males were fed diets for a minimum of 8 weeks then, mated with virgin 8-12-week-old female C57BL/6J mice, which were maintained on standard Rat and Mouse No.1 Maintenance chow diet (Special Dietary Services Ltd, UK). Pregnancy in females was confirmed by the presence of a copulatory plug the day after successful mating. Pregnancy was allowed to progress to embryonic day 17.5 (term being day 19) before the dam was culled via cervical dislocation and the fetal tissues including, heart, liver, head, kidneys, placenta, and yolk sac were collected from the middle fetuses within each uterine horn (where possible). All tissues were weighed before being snap frozen in liquid nitrogen and stored at -80ºC. Prior to RNA extraction the fetal hearts were snap frozen in liquid nitrogen and stored at -80°C.

4.4.2 Fetal heart microarray

RNA samples were extracted from the whole heart as described previously (see section 2.8.1). After extraction, RNA concentration and quality were determined using a Nanodrop (Eppendorf). All samples were then diluted to 100 ng/ μL using nuclease-free water. The Nanodrop machine was utilized to evaluate the level of organic, proteins, nucleotides and contaminants in the samples.

For each dietary treatment group, a total of 8 biological replicate samples were employed, including 4 female and 4 male samples, each taken from different litters and the RNA was submitted for microarray analysis (see the full description in section 2.9).

4.4.3 Placental RNA extraction and gene expression analysis

Frozen placentas were used for RNA extraction. RNA was isolated from approximately 20-25 mg of placental tissue using the RNeasy mini kit (Qiagen, UK). The full RNA extraction procedure is detailed in section 2.3.1. The cDNA templates were created using the nanoScript2 RT kit (PrimerDesign, UK), in

accordance with the manufacturer's instructions (see the full description in section 2.4.1). Below is the list of placental genes which were analyzed and comprised the RAS pathway including *Ace, Ace2, Agtr1a, Agtr1b, Agtr2, Atp6AP2, Ren1*, apoptosis component pathway including *(Bad, Bax, Bcl2, Casp1, Fas)*and 1-C metabolism *Mthfr, Mtr, Mat2a, Mat2b, Achy, Dhfr*.

4.4.4 Placental Periodic Acid Schiff (PAS) staining

For the analysis of placental glycogen content, one series of placental sections was stained using PAS staining. A total of forty placental tissue slides (8 slides per diet group) were stained according to manufacturer protocol (See the full description in section 2.5.2).

4.4.5 Masson's Trichrome staining

For the analysis of the deposition of extracellular matrix (ECM), including collagen, in placental tissues, one series of placental sections was stained using Masson's Trichrome. A total of 40 placental slides (8 slides per diet group) were stained according to the manufacturer's protocol (See the full description in section 2.5.3).

4.4.6 Data analysis

Fetal heart microarrays were analyzed using Partek Genomics Suite analysis software. The gene list for each dietary group was generated using an ($FDR < 0.05$, $p < 0.05$) Lists of differentially expressed genes(DEGs) were analyzed using WEBbased GEneSeTAnaLysis Toolkit (Web Gestalt) for Gene Set Enrichment Analysis (GSEA) (http://www.webgestalt.org/). For further gene enrichment analysis (ShinyGO 0.77), (http://bioinformatics.sdstate.edu/go/) was used (https://geneglobe.qiagen.com/gb/).

4.4.7 Statistical analysis

All fetal data were analyzed using a multilevel random effects regression model (SPSS, version 28; IBM Corp., Armonk, NY, USA), followed by a Bonferroni

post hoc test. Correlations between parameters were conducted using Pearson correlation. Data were assessed for normality (Shapiro–Wilk test) using SPSS (Version 28). Data were analyzed for interactions between treatment group and offspring data by Kruskal–Wallis test and reported where appropriate. $P < 0.01$ was considered statistically highly significant, and $P < 0.05$ considered statistically significant.

4.5 Results

4.5.1 Fetal physiological characteristics in response to paternal diet

There was no significant effect of diet on litter size (Figure 4-1.1 A), or mean conceptus weight (Figure 4-1.1 B) across the different groups, either when compared to CD or each other group. Following dissection, fetal weight was significantly decreased in fetuses sired by LPD vs CD (P=0.0464), MDL vs CD (P=0.0071) and WD vs CD fathers (P=0.0429) and MDL vs WD (P=0.0076). Furthermore, both LPD (P=0.0429) and MLD fetuses were significantly lighter than WD fetuses (P=0.0079,)(Figure 4-1.1 C). Placental weight was found to be significantly increased in MDWD (P=0.0466) when compared to MDL (Figure 4-1.1 D). A significant increase in the fetal: placental weight ratio was observed in the WD group when compared to CD (P=0.0151), a highly significant increase in WD when compared to LPD (P=0.0009), as well as MDL (P=0.00086) and a very highly significant increase when compared to MDWD (P<0.0001 (Figure 4-1.1 E). A significant decrease in yolk sac weight derived from MDL fathers when compared to LPD (P=0.0012) and CD (P=0.0252) fathers, as well as a significant decrease in yolk sacs derived from MDL males when compared to MDWD (P=0.0392), was observed (Figure 4-1.1 F).

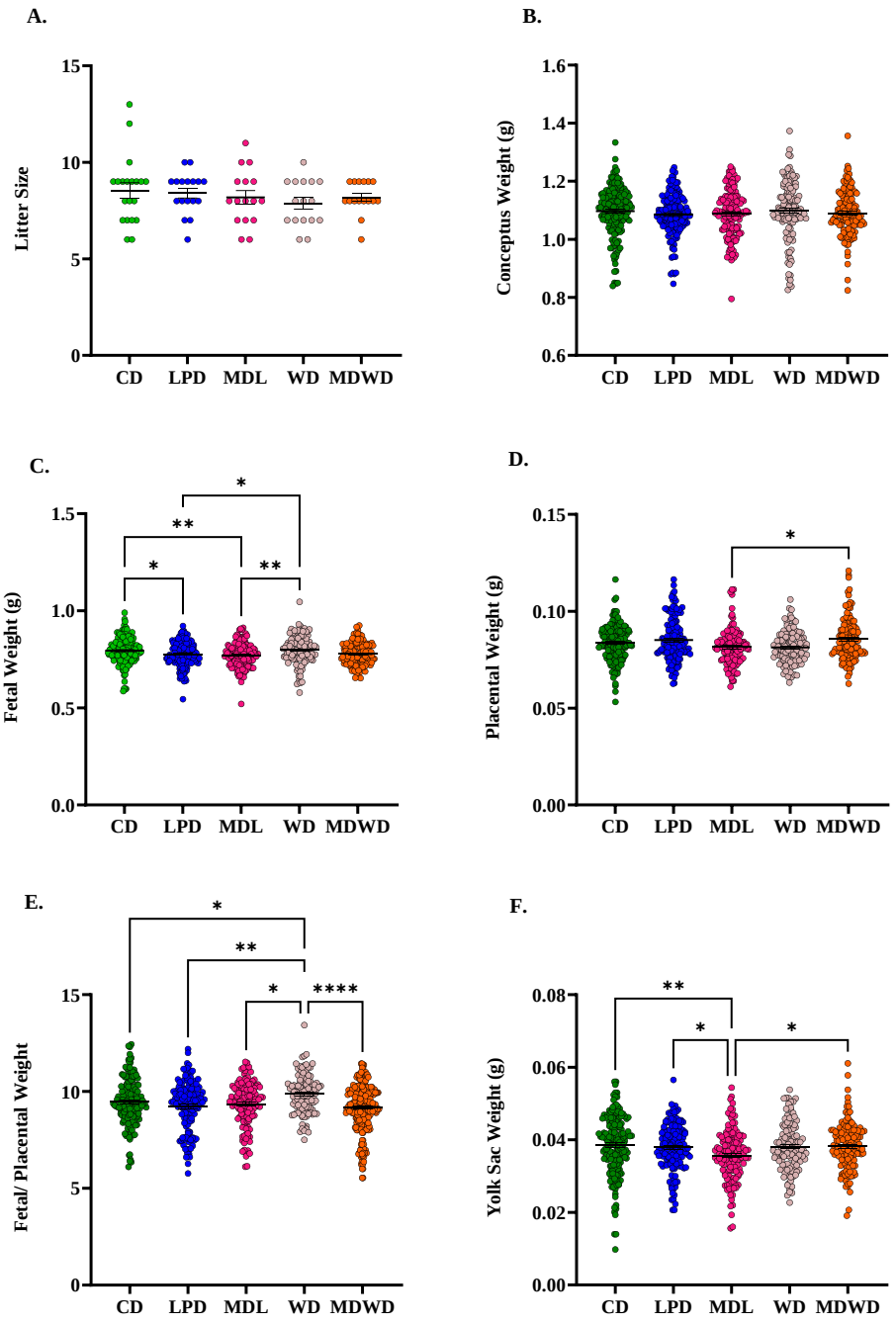


*Figure 4-1.1 The impact of paternal diet on fetal physiological characteristics in late gestation (E17.5). Litter size (A), conceptus weight (B), fetal weight (C), placental weight (D), fetal/placental ratio (E), and yolk sac weight (F). To calculate the fetal organ weights, fetal organs were dissected. Data is shown as a dot plot, each data point being the individual value, and the line representing the mean ± SEM, (n=20 liters, n=128-171), statistical significance determined using a generalized linear mixed model analysis. Shapiro-Wilk normality test (IBM SPSS Statistics 27.0.1.0) was used to assess the normality of all data. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

The distribution of fetal weights studied is shown in Figure 4-2. A total of 122 fetuses were considered SGA (below the 10th percentile) and they were obtained from 5 different treatment groups. On average, fetal weight was significantly lower by 10.17 % in SGA fetuses when compared to AGA fetuses (a total of 535 fetuses). A total of 45 LGA fetuses (above the 90th percentile) were obtained from 5 different groups including CD and these were on average, 10.57% heavier than the AGA group. All groups had a lower number of AGA fetuses, compared to CD (17). Fetal weight distributions revealed the fetuses derived from MDL and WD-fed fathers produced a higher proportion of SGA fetuses; 22.9%, and 23.4 % respectively, fall below the 10th centile when compared to CD fetal weights, when compared to CD both MDL and WD revealed significantly Chi^2 test accordingly equal by 0.0006, 0.0004.

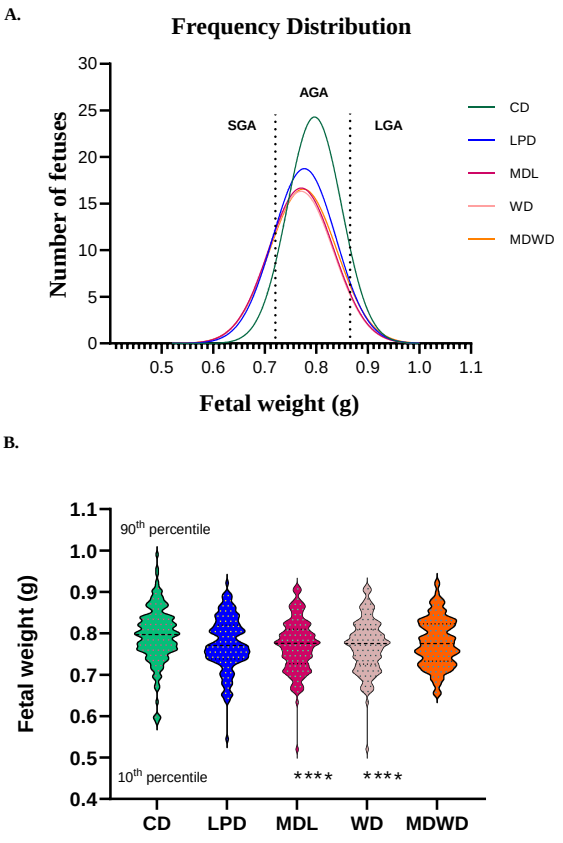


*Figure 4-2 The impact of paternal diet on fetal weight distribution in late gestation (E17.5). Frequency distribution curve for fetal weights (A), fetal weight distribution (B). Data is shown as a dot plot, each data point being the individual value, and the line representing the mean ± SEM, (n=20 liters, n=128-171), statistical significance determined violin plot of all fetuses with the 10th and 90th centiles, significant differences in 90th centile proportions and 10th centile proportions calculated using Chi^2 test, *p < 0.05, **p < 0.01 vs CD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.*

heart weight across the different groups when compared to CD or each other group (Figure 4-3 A). Similarly, there was no difference in the fetal weight/heart weight ratio across the different treatment groups (Figure 4-3 B). Analysis of mean fetal liver weight revealed no significant alterations in all the treatment groups when compared to CD (Figure 4-3 C). However, fetal/liver weight ratio was significantly elevated in LPD (P=0.005) and MDWD groups when compared to CD P=0.0451) (Figure 4-3 D).

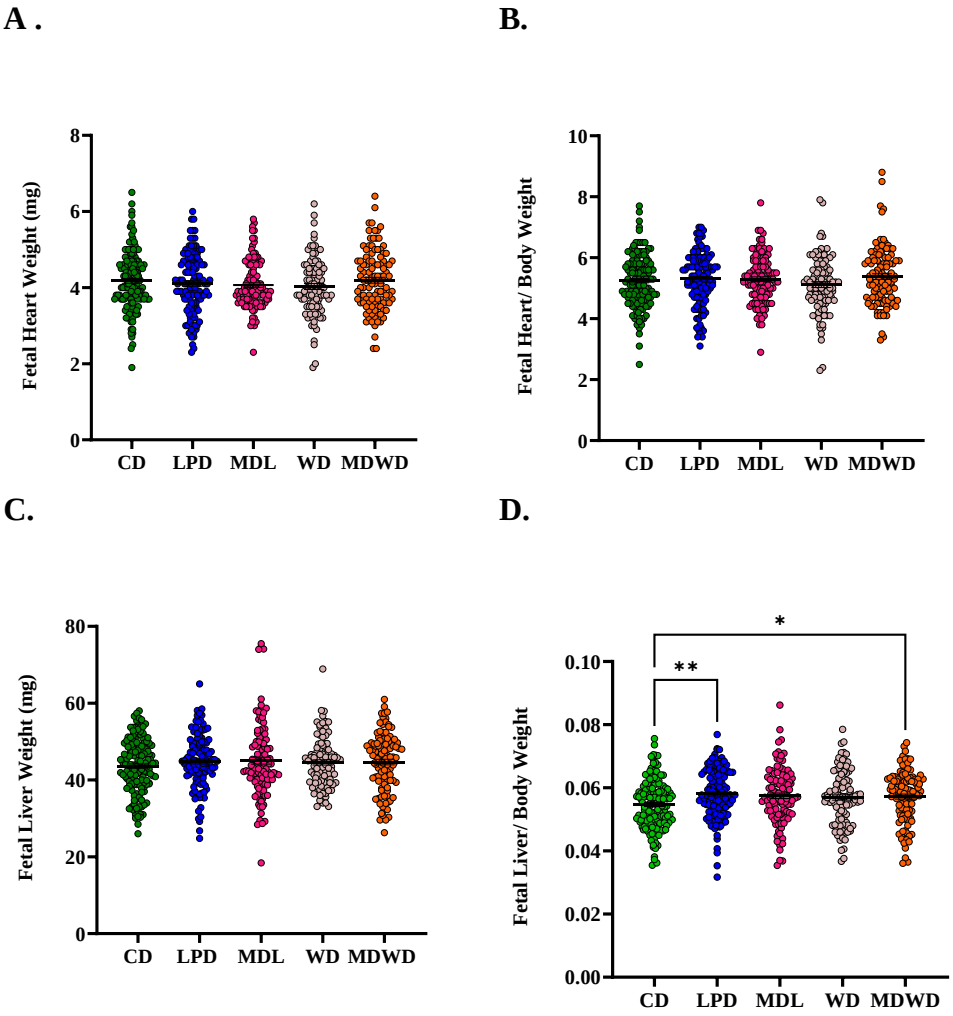


Figure 4-3 The impact of paternal diet on fetal physiological characteristics in late gestation (E17.5). *Fetal heart weight (A), fetal/heart ratio (B), fetal liver weight (C), fetal/liver ratio, (D). Data is shown as a dot plot, each data point being the individual value, and the line representing the ± SEM, (n=20 litters with 128-171 fetuses in each). Statistical significance is determined using a generalized linear mixed model analysis. Shapiro-Wilk normality test, (IBM SPSS Statistics 27.0.1.0) was used to assess the normality of all data. All fetal offspring organ weight data were analyzed using a multilevel random effect regression model using GLM.*

4.5.2 Fetal heart global gene expression in response to paternal diet

4.5.2.1 Gene Set Enrichment analysis

To verify the consequences of the transcriptional alterations in these fetal hearts the gene ontology and pathways analysis were conducted.

Microarray results were first visually examined using a principal components analysis (PCA) plot. A PCA plot containing 8 biological replicates from 5 different treatment groups including CD, LPD, MDL, WD and MDWD was generated, enabling heterogeneity of expression profiles within and between the groups to be defined. The two groups of WD and MDWD are clearly separated CD, LPD, and MDL groups, meaning there is a prominent difference in gene expression between the WD and MDWD and the rest of the groups. The dots within WD and MDWD in samples are tightly concentrated which means that these samples have similar expression profiles to each other.

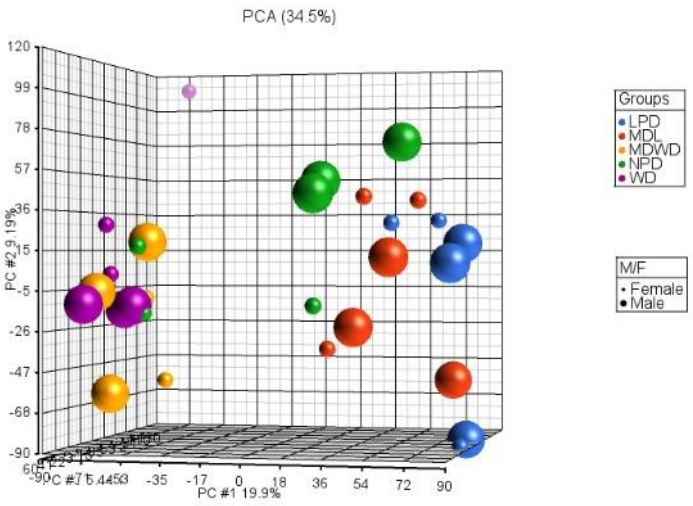


Figure 4-4 Principal component analysis (PCA) plot of the gene expression of fetal heart samples analyzed by microarray. Samples are clustered according to their similarities in gene expression. Each dot represents one sample, and each color represents one dietary treatment group (n=8, 4 males and 4 females).

Gene Set Enrichment Analysis (GSEA)

To determine the impact of paternal over/under nutrition, with and without methyl donor supplementation, on the fetal heart transcriptomic profiles, a microarray approach was employed. The analyzed data displays the overall number of DEGs in different treatment groups.

Analysis of DEGs in all treatment groups relative to control diet group, with false detective rate (FDR < 0.05, P ≤ 0.05), revealed the greatest number of DEGs between CD vs LPD (4282 genes), but the smallest difference shown to be between MDL and CD (2318 genes). CD vs WD and CD vs MDWD displayed a similar number of DEGs to the CD vs LPD group respectively. Additionally, LPD vs MDL and WD vs MDWD revealed a similar number of differentially expressed genes, 1456 and 1177 respectively. Table 4-1 and Figure 4-5 (A-E), display the total number of DEGs and the number of up/down regulated genes.

Table 4-1 represents the numbers of total and up/down regulated DEGs across treatment diet groups when compared to CD FDR ≤0.05 and P-value <0.05, n=8.

Table 4-1 Indicates the number of fetal cardiac differentially expressed genes in each treatment group when compared to CD and each other in response to paternal diet.

Diets	Total Genes	Up-regulated Genes	Down-regulated Genes
LPD vs CD	4,282	1,720	2,562
MDL vs CD	2,318	979	1,339
LPD vs MDL	1456	870	586
WD vs CD	4,148	2,044	2,104
MDWD vs CD	4,094	1,934	2,160
WD vs MDWD	1177	590	580

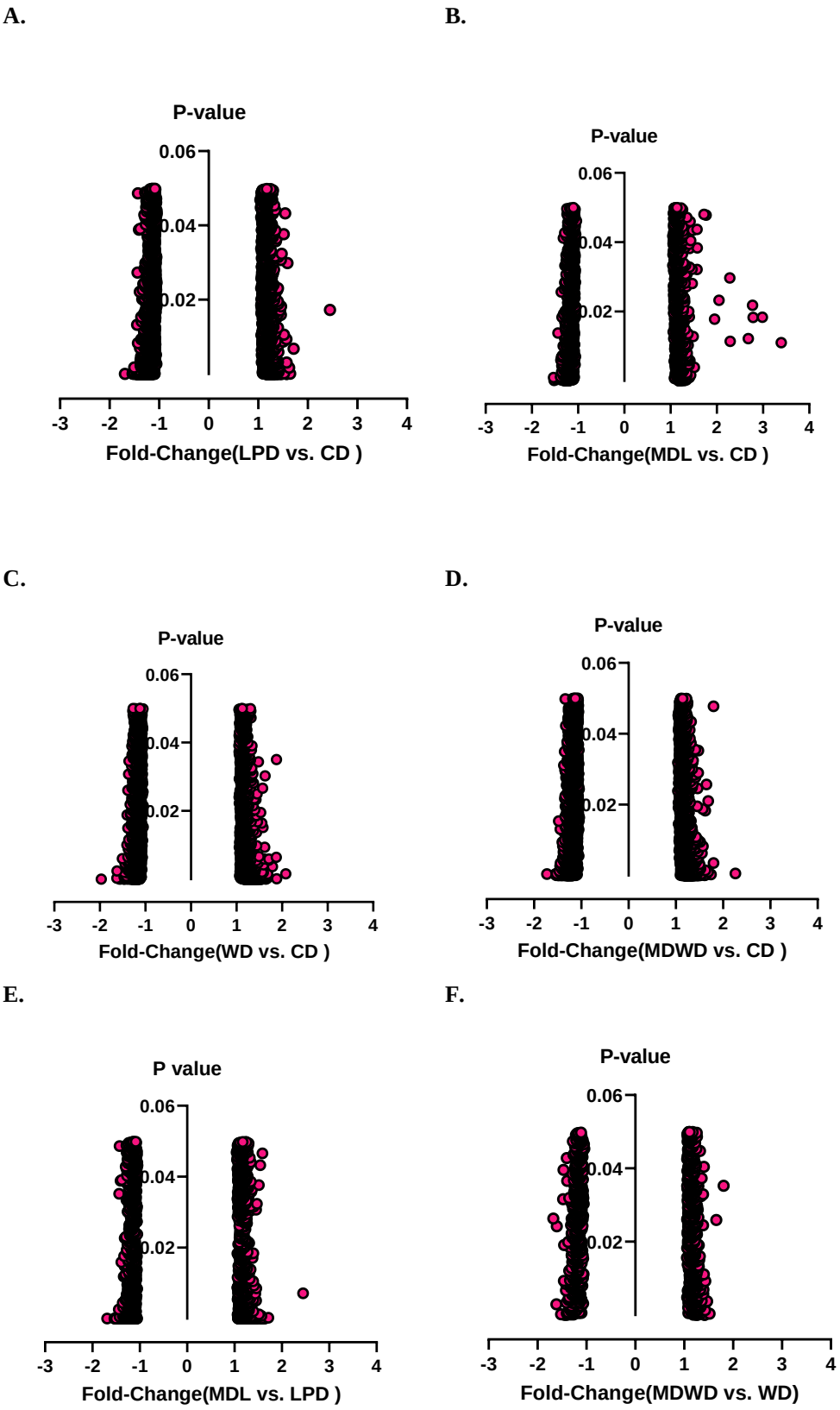


Figure 4-5 (A-F), represent analyses of fetal cardiac transcriptomic profiles using volcano plots, displays up/down regulated differentially expressed genes of fetal heart tissues at E17.5 derived from males-fed different treatment diets when compared to CD. (FDR ≤0.05 and P-value <0.05, n=4 males and 4 females).

Comparative analyses of the DEGs were performed and the overlapping and exclusive genes displayed by Venn diagram (Figure 4-6). The most genes changed in response to paternal diet when compared to CD was observed following WD with 1289 (27.8%) up-regulated transcripts. Additionally, Figure 4-6 B illustrates the most genes downregulated in response to paternal diet belonged to LPD group when compared to CD, with 1470 (24.8%), transcripts downregulated. Figure 4-6 A and B illustrate the full set of genes altered exclusively to a specific group or common across two or more than two groups.

Overview of all the genes up and downregulated (Figure 4-6 A, Figure 4-6 B) in E17.5 fetal cardiac microarray tissue. Figure 4-6 A displays all the upregulated common genes, Figure 4-6 B displays the down-regulated genes in LPD, MDL, WD and MDWD when compared to CD.

Common upregulated genes between different treatment groups compared to CD represent, 581 common upregulated genes in "LPD vs CD" and "MDL vs CD" are mainly involved in transcriptomic gene pathways, such as Ubl conjugation, transcription, transcription regulation, DNA binding, RNA-binding, nucleic acid binding, zinc finger and metal ion binding.

1289 common genes between “WD vs CD" and "MDWD vs CD" were upregulated. Common genes between these two groups are involved in endoplasmic reticulum and membrane with enrichment score of 16.24, lysosome and lysosomal membrane with the enrichment score of 8.47, TATA box binding proteins, CENP-A containing nucleosome, Histone H4 with enrichment score of 7.11. protein kinase, ATP binding sites, serin/threonine-protein kinase with enrichment factor of 3.4.

A. Up-regulated genes **B.** Down-regulated genes

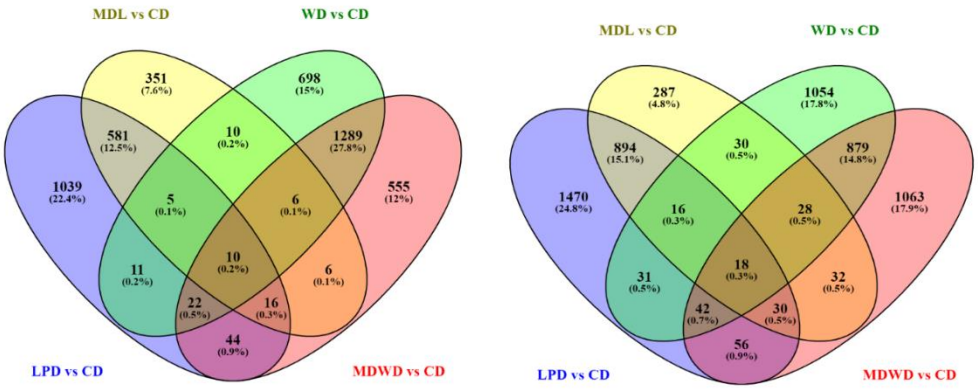


Figure 4-6 (A, B) Analysis of cardiac fetal samples at E17.5, using Venn diagrams to display the number and percentage of differentially expressed up regulated genes (A) exclusively or common across different treatment groups , and downregulated genes (B) .The displayed numbers in each of the circle represents genes number that have been altered in a certain group, whilst the overlapping areas shows the number of altered genes that are shared between two or more than two groups.

4.5.2.2 Analysis of the Fetal Heart Global Gene Expression in Response to Paternal Diet

4.5.2.2.1 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal LPD vs CD

Using data for CD vs LPD group, down-regulation of genes associated with carbohydrate transport, ribose phosphate metabolic process, nucleoside triphosphate metabolic process, cell junction organizations, epithelial tube morphogenesis, ERK1 and ERK2 cascade, small molecule catabolic process, organophosphate biosynthesis process, cofactor metabolic process and anion transmembrane transport was observed, when compared to fetal heart samples derived from CD-fed fathers (Table 4-2 and Figure 4-7 A). DEGs with a fold change of at least 1.5, whether regulated positively or negatively, were analyzed considering the distribution of gene ontology across all expressed pathways. This analysis revealed a significant downregulation of genes involved in these pathways (A). This analysis illustrates the distribution of gene ontology categories across all the expressed pathways and indicates a substantially

expressed which means a significant downregulation of genes associated with expressed pathways (Figure 4-7 A).

In addition, preliminary pathways analysis of DEGs in LPD vs CD revealed a significant downregulation in a series of pathways including metabolism and metabolic pathways, carbon metabolism and biosynthesis of amino acids (Figure 4-7 B). Additionally, pathways including abnormal cardio-vascular system physiology, abnormal heart ventricle physiology, metabolic pathways and anion transmembrane transport were also down regulated in LPD hearts when compared to CD hearts (Figure 4-7 C).

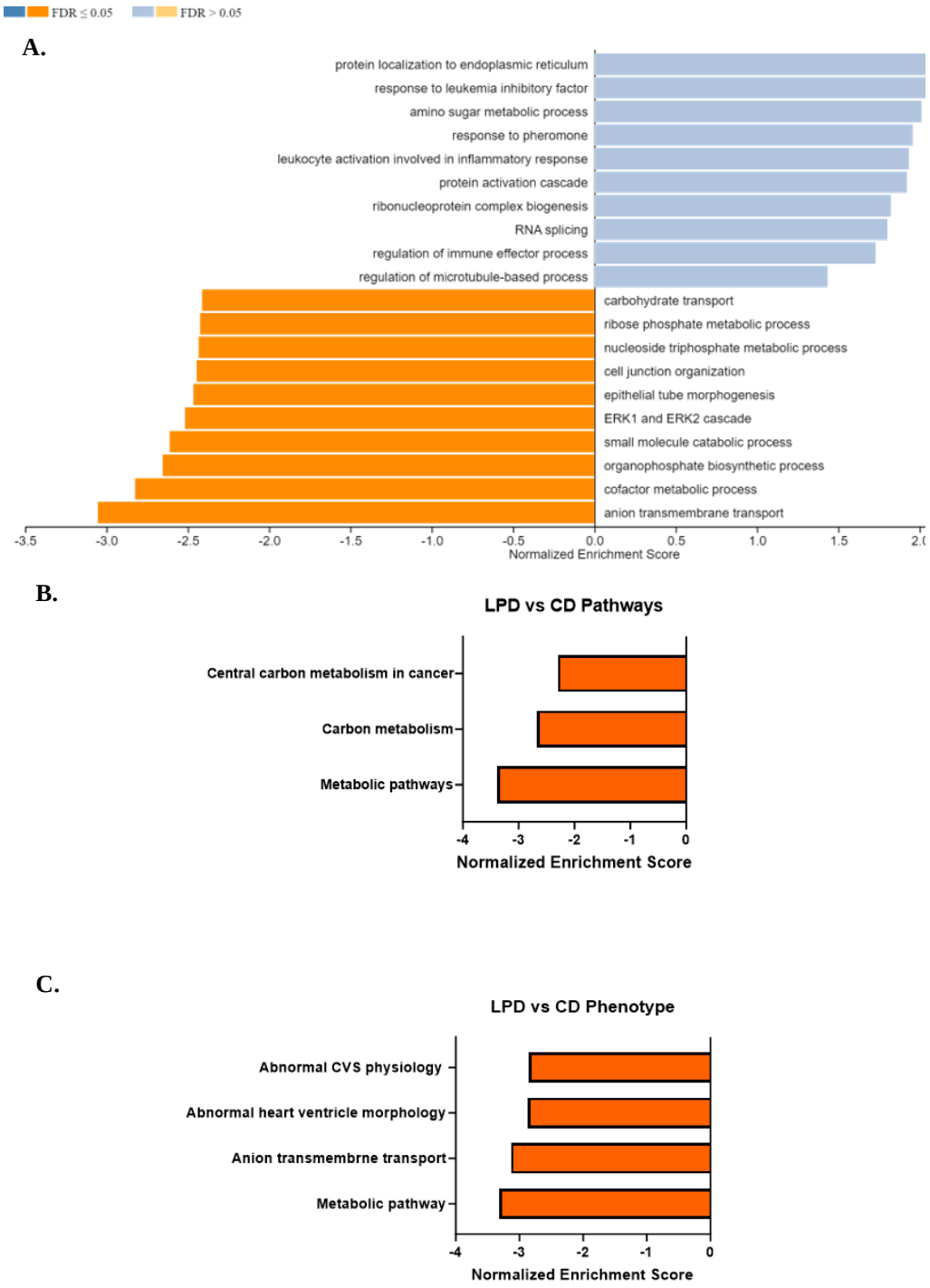


Figure 4-7 Displays Gene Ontology (GO) analysis of differentially expressed genes between CV and LPD fetal hearts (A), indicating the downregulation of genes involved in ERK1 and ERK2 cascades and epithelial tube morphogenesis. Preliminary Pathway analysis of differentially expressed genes (B) in LPD fetal heart demonstrates a downregulation of genes involved in pathways such as cofactor metabolic process, when compared to CD. Phenotype analysis of the downregulated genes in LPD fetal hearts (C).

In addition, pathways analysis of DEGs in LPD vs CD revealed a significant downregulation in a series of pathways. These include metabolism and metabolic pathways, including a total of 288 DEGs. Furthermore, carbon metabolism exhibited a total of 38 DEGs and biosynthesis of amino acids included a total of 24 DEGs. Notably, some of the central genes involved in metabolism were significantly expressed in all three analyzed pathways or they represented the highest fold change which was more than 1.4. Some the significant genes belong to each pathway have been added in Table 4-2.

Table 4-2 Displays the down-regulated genes based on multiple genes in a pathway being altered, however some of the genes associated with three key pathways in LPD vs CD, have been added to this table. Central genes crucial to metabolism pathways have emerged in all three of these pathways. Each of the listed genes plays a significant role in metabolism.

Metabolic pathway	Carbon metabolism	Biosynthesis of amino acids
Akt1	*Pfkl* 6-phosphofructokinase liver type	*Pfkl* 6-phosphofructokinase liver type
Insr	*Mthfr*	*Shmt1*serinhydroxymethyltransferase1
Nos3	*Hk1*	*Pkm* pyruvate kinase, muscle

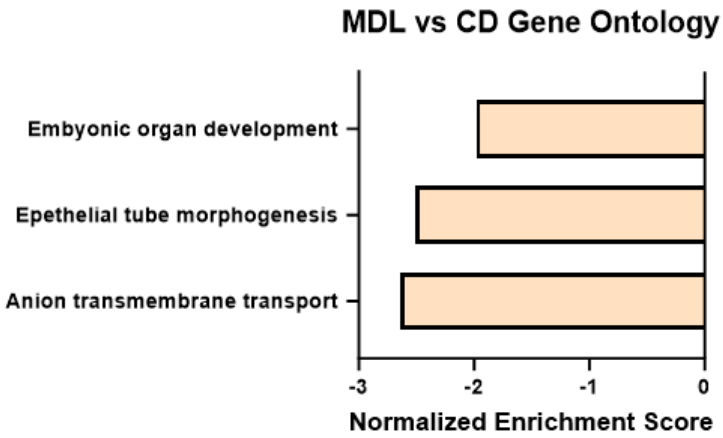
4.5.2.2.2 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDL vs CD

To examine the impact of the supplementation of methyl donor groups to the suboptimal diet (LPD), the comparative analyses were performed for LPD vs MDL. Next, the genes differentially expressed in MDL fetal hearts as compared to CD hearts were analyzed. Among significantly expressed genes, upregulation in various Gene Ontology (GO) pathways revealed a set of significantly expressed genes associated with pathways which downregulated non-significant

with (FDR> 0.05 and P <0.05 with regards to the pathway analysis as all the genes were differentially expressed using an FDR <0.05).

The down-regulated pathways were associated with anion transmembrane transport, epithelial tube morphogenesis and embryonic organ development (Figure 4-8 B) when compared to fetal heart samples derived from CD-fed fathers. In addition, preliminary pathways analysis of DEGs in MDL vs CD revealed, a non-significant downregulation in a range of genes involved in metabolism and metabolic pathways central genes such as *Mgll, Atp6ap1, Pfkl*, calcium signaling (central genes such as *Atp2a2, Mylk3, Cancna1c, Erbb2*), adrenergic signaling in cardiomyocytes (genes such as *Akt1, Atf6b, Atp1b3, Adrb1, and Mapk3*) and P53 signaling (genes such as *Bcl2l1, Cdk1, Chek1, Cdkn2a*) (Figure 4-8 B). As well as the phenotypical pathways that display the down-regulated pathways in MDL vs CD, including abnormal cardio-vascular system physiology, abnormal heart ventricle morphology, prenatal lethality, and embryonic lethality (Figure 4-8 C).

A.



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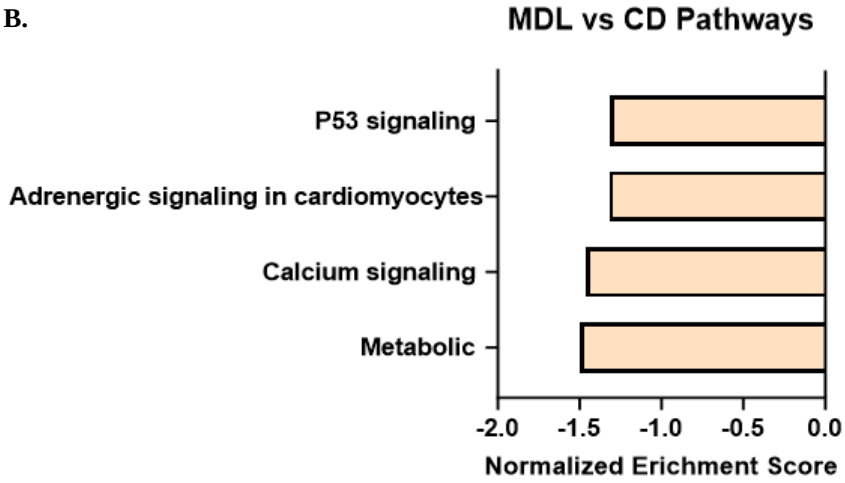

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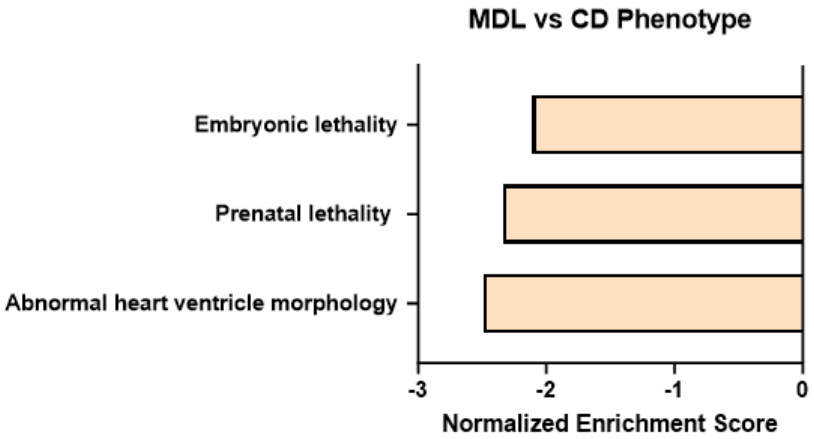
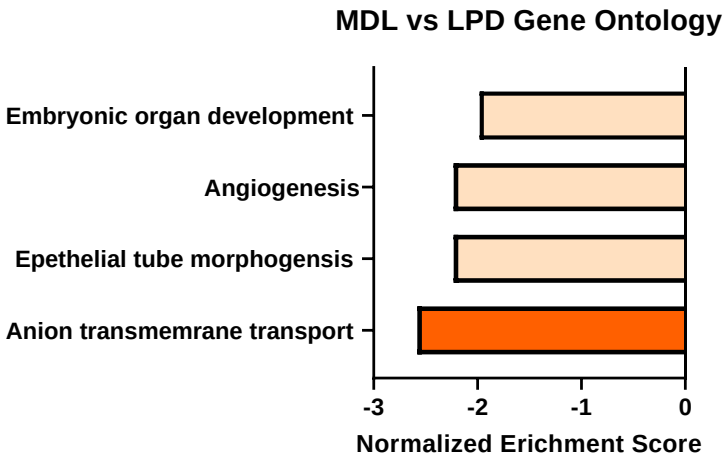


Figure 4-8 Displays, the downregulation of genes based on fold-change involved in different pathways of embryonic organ development, epithelial tube morphogenesis, anion transmembrane transport were expressed non-significantly in preliminary Gene ontology (A), P53 signaling, adrenergic signaling in cardiomyocytes, calcium signaling and metabolic pathways (B), abnormal heart ventricle morphology, prenatal and embryonic lethality (C) analysis in paternal MDL vs CD.

4.5.2.2.3 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDL vs LPD

Comparing the DEGs identified in the LPD hearts with those in the MDL hearts provides additional insight into the impact of the methyl donors on fetal cardiac gene expression. Here, the downregulation of some of the pathways that were identified in the LPD group was no longer significant in the MDL group. These included DEGs involved in angiogenesis (118 genes) including central regulatory angiogenesis genes such as *Ace*, *Vgefb*, *E2f8*, embryonic organ development (118 genes) including genes such as *Nkx2-5*, *Slc9a3r1*, *Gata2*, *Foxc2*, skeletal system development (116 genes), transmembrane receptor protein serine/threonine kinase signalling pathway (79 genes), glycolipid metabolic process (75 genes), and phospholipid metabolic process (89 genes) overall were observed in GO analysis of fetal heart from MDL-fed fathers when compared to CD (Figure 4-9 A). In addition, preliminary KEGG pathway analysis of 1456 DEGs in MDL vs LPD revealed, a non-significant downregulation in metabolic pathway and carbon metabolism (Figure 4-9 B). The preliminary assessment of the phenotype pathways showed a significant down regulation of genes involved in pathways such as CVDs, prenatal lethalities, and metabolism phenotypes (Figure 4-9 C) while pathways associated with mRNA processing (117 genes), RNA splicing (98 genes), DNA recombination change (34 genes) andncRNA metabolic process (108 genes), were downregulated significantly with normalized enrichment score (NES) more than -2, where a negative NES means that the gene sets are more present in the compared group (Figure 4-9). Here the graph (Figure 4-9 B) represents the pathway in MDL fetal hearts upregulated and down-regulated significantly, and interestingly two similar pathways with MDL were observed with the similar NES $\geq$ 3 for both angiogenesis and phospholipid metabolic process pathways. The other Gene Ontology analysis belonged to the LPD vs MDL group (Figure 4-9), the only significant common pathways between these two groups was anion transmembrane transport pathways which was downregulated by NES $\geq$ -2.5.

A.



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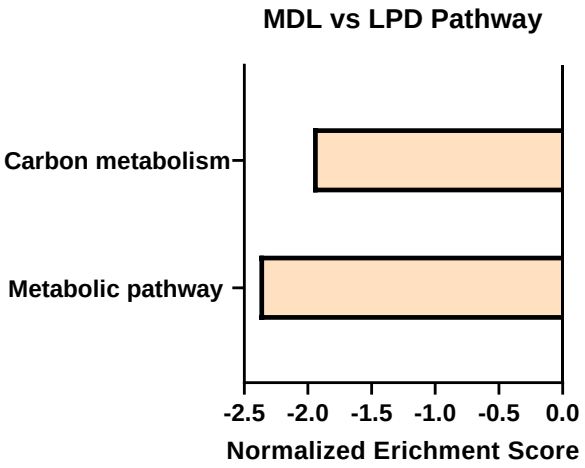


C.

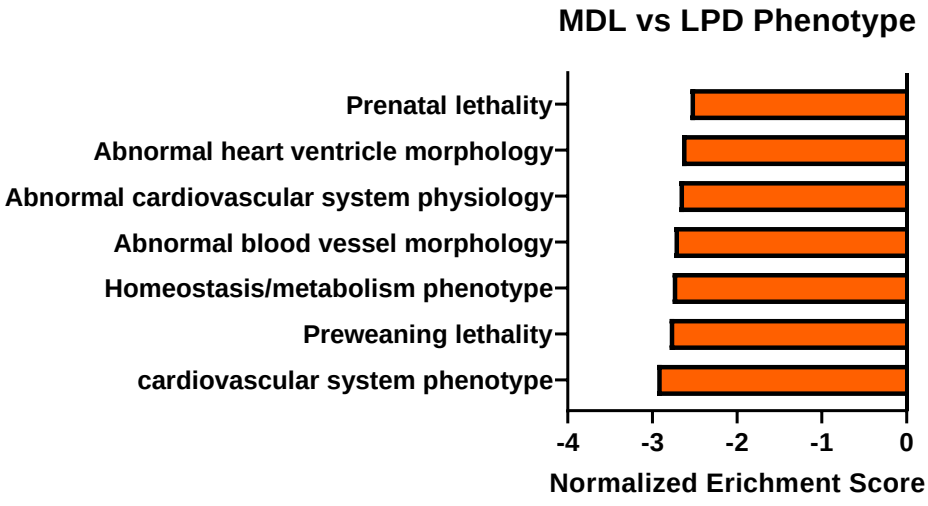


Figure 4-9 Represents downregulation of genes based on fold-change involved in different pathways embryonic organ development, epithelial tube morphogenesis, anion transmembrane transport was expressed non-significantly in preliminary Gene Ontology (A), metabolic pathways, and carbon metabolism pathways analysis (B), and significantly downregulated pathways involved in abnormal cardiovascular and embryonic lethality (C) analysis in paternal MDL vs LPD.

4.5.2.2.4 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal WD vs CD.

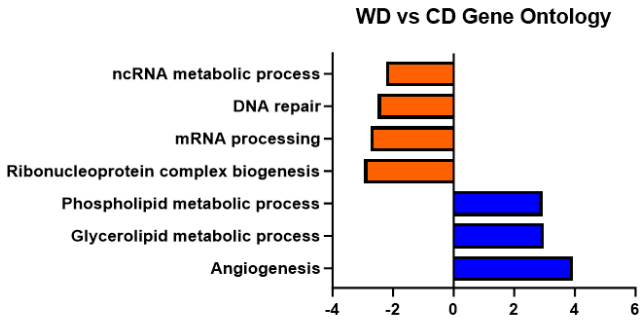
Among the significantly expressed genes, upregulation in the GO analysis pathways was associated with angiogenesis, embryonic organ development, skeletal system development, cell surface receptor signaling pathways involved in cell-cell signaling, epithelial tube morphogenesis and regulation of cellular response to growth factor stimulus were observed. The angiogenesis pathway including 79 genes such as *Ace, Foxc2, Vegfb,* and *Gata2* up-regulated by 3.8633 NES. This pathway revealed the expression of significant genes involved in angiogenesis. However, some of these genes such as *Adgrb2, Mmp2,* and *Notch1* (which is involved in angiogenesis and negatively regulates endothelial cell proliferation and migration and angiogenic sprouting), along with *Fn1*contribute to a network of molecular interaction involved in this biological pathway. In addition to the significant down regulation of pathways involved in transcriptomic regulations, such as ribonucleoprotein complex biogenesis with 88 genes such as *Cdkn2a, Drosha, Nol11, Gtpbp4,* as well as DNA repair pathways with 80 down regulated genes such as *Ube2w, Chek1, Cdkn2d* were shown (Figure 4-10 A). Analysis of WebGestalt pathways revealed a significant upregulation in cancer pathways with 57 genes such as *Bcl2l1, Vegfb, Col1a1,* Relaxin signaling pathway with 33 genes, such as *Vegfb, Col1a1, Col1a2, Map2k7* and pathways in metabolic pathways such as MAPK signaling with upregulated 48 genes *Akt1, Vegfb, Erbb2, Insr.* As well as phosphoinositide 3 kinase PI3K-Akt signaling with 57 genes such as *Bcl2l1, Vegfb, Col1a1.* (Figure 4-10 B).

Analysis of WebGestalt GO pathways also shows significant upregulation in angiogenesis, embryonic organ development, and glycerolipid metabolism. On the other hand, significant downregulation was observed in pathways such as mRNA processing including 84 genes such as *Adarb2, Snrnp35, Ccnb1, Tra2b, Zmat2.* DNA repair and non-coding RNA.

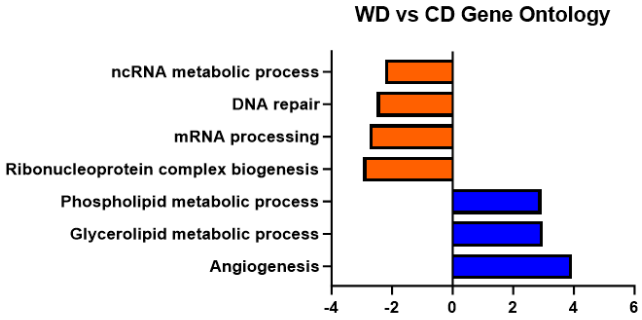
Preliminary analysis of pathways involved in phenotype using WebGestalt identified a significant upregulation in pathways involved in abnormal

cardiovascular system. Pathways, including abnormal heart morphology (242 genes) such as *Fbln5, Eln, Ace, Foxc2, Vegfb, Col1a1, Gata2*. Abnormal vascular development with 124 genes *Nkx2-5, Gata2, Eln, Foxc2.* Abnormal circulating cholesterol level (56 genes), with the upregulation of genes such as *Slc24a4, Ap2a2, Por, G6pc3*. As well as abnormal blood vessel physiology with upregulation of 55 genes such as, *Fbln5, Eln, Zfp36, Gata2, Fbn1, Col3a1* in fetal hearts of WD when compared to CD (Figure 4-10 C).

A.



B.



C.

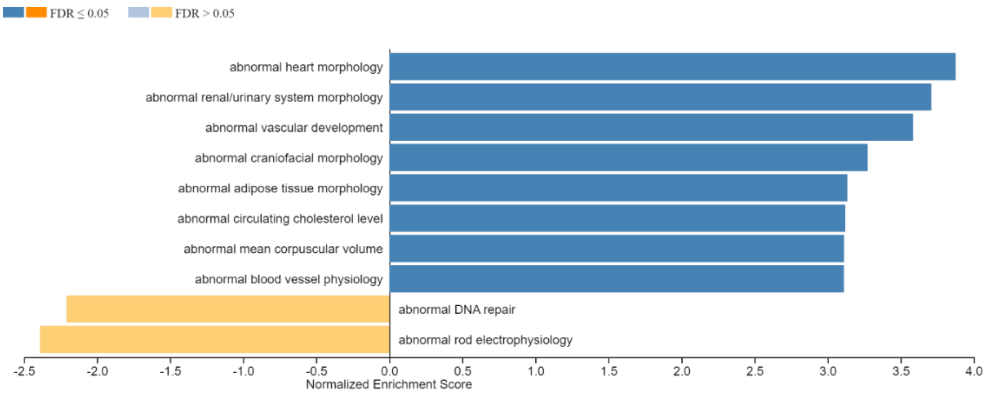


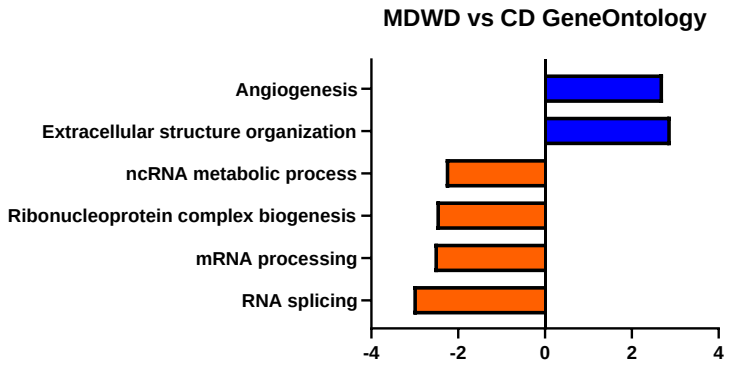
Figure 4-10 Displays downregulation of genes based on fold-change involved in different pathways embryonic organ development, epithelial tube morphogenesis, anion transmembrane transport was expressed non-significantly in preliminary Gene Ontology (A), metabolic pathways derived from GO analyzes, and carbon metabolism pathways analysis (B), and significantly downregulated pathways involved in abnormal cardiovascular and embryonic lethality (C) analysis in paternal WD vs CD.

4.5.2.2.5 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDWD vs CD.

More than 4000 genes (4094) were differentially expressed in MDWD fetal hearts when compared to CD. In the preliminary transcriptomic assessment of MDWD fetal hearts, a few pathways were significantly expressed. Upregulation in various pathways of GO is involved in angiogenesis and extracellular structure organization. Angiogenesis pathways with 91 genes such as Ace, Foxc2, Vegfb, and *Gata2* were up regulated by NES 2.7191. In addition of significant down regulation of central pathways in transcriptomic regulations, such as RNA splicing, with 70 genes were downregulated significantly, NES -3.0304, genes such as Aff2, Sfpq, Cdk13 and i. As well as mRNA processing with 100 down regulated genes, ribonucleoprotein complex biogenesis including 91 genes, and ncRNA metabolic process including 86 genes were down regulated significantly in MDWD fetal hearts when compared to CD hearts (Figure 4-11 A.). Analysis of WebGestalt pathways identified a significant upregulation in metabolic pathways such as MAPK signaling pathway including 56 genes, Cholesterol metabolism with 7 significantly upregulated genes, Metabolic pathway with 199 genes, and Glycosylphosphatidylinositol (GPI)-anchor biosynthesis with 9 genes, were significantly upregulated in MDWD fetal hearts compared to CD (Figure 4-11 B).

Preliminary analysis of pathways involved in phenotype using WebGestalt tool, identified a significant upregulation in pathways involved in abnormal cardiovascular system. Pathways, including abnormal blood vessel morphology with 199 up regulated genes such as *Foxc2, Foxc2, Vegfb, Slc2a10, Col1a1, Nkx2-5.* cardiovascular system phenotype with 302 upregulated genes *Foxc2, Nkx2-5, Vegfb, Tgfb1i1, Gata2, Eln, Nos3 Igfbp2*, abnormal cardiovascular development and 56 genes, with the upregulation of genes such as *Slc24a4, Ap2a2, Por, G6pc3.* As well as abnormal cardiovascular system morphology and abnormal artery morphology including 145, 298 and 76 genes respectively. Genes such as, *Fbln5, Eln, Zfp36, Gata2, Fbn1, Col3a1* were significantly upregulated in MDWD hearts when compared to CD hearts (Figure 4-11 C).

A.



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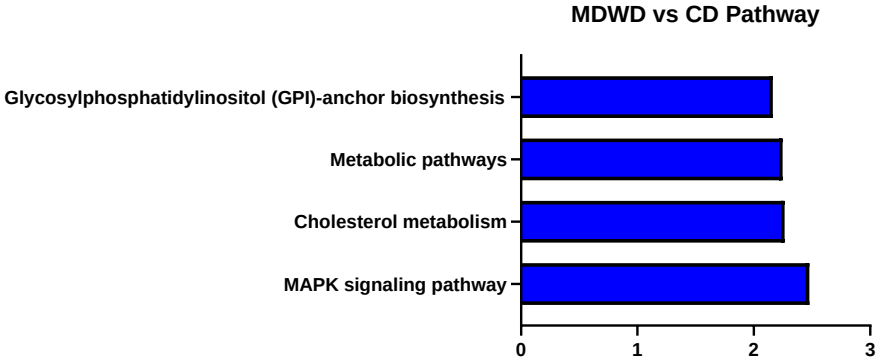


C.

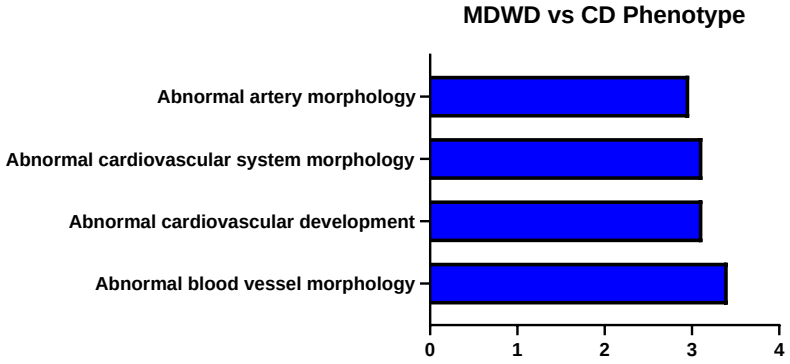


Figure 4-11 Displays downregulation of genes based on fold-change involved in different pathways angiogenesis and extracellular structure organization t, were Up-regulated and RNA splicing and ncRNA pathways were down-regulated significantly in preliminary Gene Ontology (A), metabolic pathways derived from GO analyzes, metabolic pathway and cholesterol metabolism pathways analysis which were Up-regulated significantly (B), and significantly up-regulated pathways involved in abnormal cardiovascular morphology and development (C) analysis in paternal MDWD vs CD.

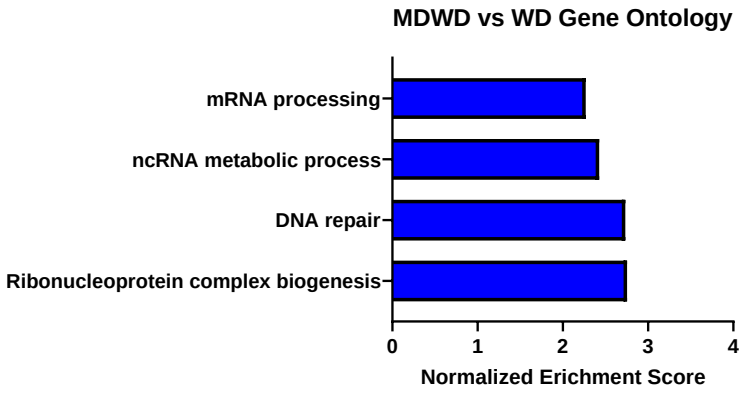
4.5.2.2.6 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDWD vs WD.

Preliminary analyses of MDWD vs WD revealed 1177 differentially expressed genes in MDWD vs WD fetal hearts. Among significantly expressed genes, upregulation in various pathways of GO including ribonucleoprotein complex biogenesis up regulated by 2.74 NES, with 25 genes such as *Dkc1, Gnl3l, Rpf2,* DNA repair with 37 genes such as *Chek1, Smarcad1, Cdkn2d, Ube2w, Fignl1*. In addition of, ncRNA metabolic process pathways including 21 genes such as *Dkc1, Rars2, Rpf2, Mettl3* were significantly differently upregulated. The other pathway was mRNA processing with 21 genes such as, *Ccnb1, Mettl3, Zfp326, Prpf3*. These pathways play a central role in preserving genomic integrity (Figure 4-12 A).

Preliminary analysis of the pathway in MDWD vs WD hearts revealed a significant upregulation of metabolic pathway. Within this pathway 51 genes such as *Atp6v0d2, Nos2, Cyp2c54, plcg2* were upregulated. Ubiquitin-mediated proteolysis pathway upregulated non-significantly, including 7 genes, such as the top three genes with the fold change over 1, *Ube2e2, Uba6, Siah1b*. The other pathways that came up were *PI3K-Akt* signaling with 18 genes and the cAMP signaling pathway with 7 genes, that down regulated non-significantly in MDWD fetal hearts when compared to WD fetal hearts (Figure 4-12 B).

The analysis of phenotype pathways in MDWD vs WD hearts, revealed pathways involved in embryonic lethality upregulated significantly in MDWD hearts when compared to WD. Three pathways including embryonic lethality with 77 genes such as Ccnb*1, Nepro, Chek1, Cdk1* were upregulated significantly. In addition, to embryonic lethality prior to organogenesis, 39 genes were significantly differentially expressed in this pathway such as *Bub1, Chek1, Ccnb1.* As well as prenatal lethality with 94 differentially expressed genes was significantly upregulated in MDWD hearts (Figure 4-12 C).

A.



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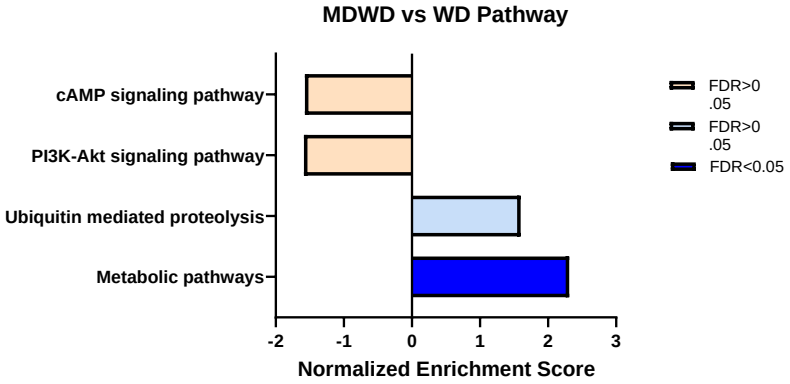


C.

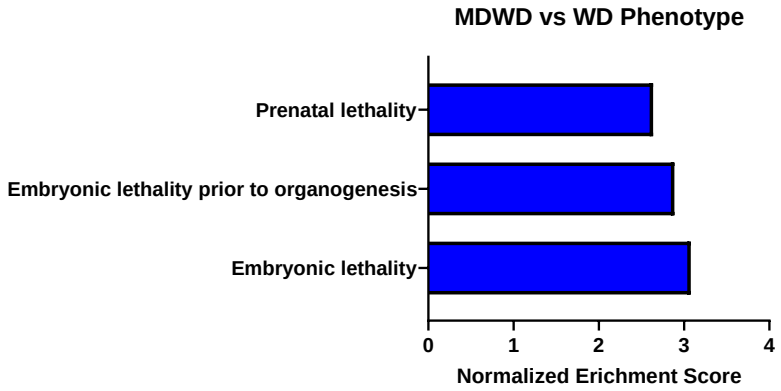


Figure 4-12 Displays, differentially expressed pathways were upregulated significantly in GO analysis related to the DNA structural mechanisms were expressed, analysis of this treatment group using Web Gestalt were revealed in GO (Figure. A), metabolic pathways derived from GO analyzes, metabolic pathway and ubiquitin mediated proteolysis pathways analysis which were Up-regulated significantly and non-significantly, respectively (B), and significantly up-regulated pathways involved in prenatal lethality, embryonic lethality prior organogenesis and embryonic lethality (C) analysis in paternal MDWD vs WD.

4.5.2.2.7 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal LPD vs WD.

A significant downregulation of genes involved in CVD in LPD hearts and upregulation of these genes in WD heart samples was observed. The overlap of genes between these groups was assessed, and some overlap in cardiovascular genes within LPD and WD were observed.

Overall, 1483 common genes were differentially expressed between LPD and WD hearts, these DEGs were involved in the cardiovascular system, such as *Nkx2-5, Nos3 Erbb2.* Additionally a similar number of DEGs (22) were observed for apoptosis such as *Bcl2, Mapk3, Foxo3, E2f1* and metabolism pathways such as *Akt1, Hk1, Slc25a10 and Glut4*. Significant and central regulatory genes in metabolism such as *Akt1, Insr, Srebf1, Hk1* which downregulated in LPD and upregulated in WD group. Also, central genes involved in apoptosis such as *Bcl2, Foxo3, Mapk3* came up in this pathway but interestingly a much larger number of regulatory underlying transcriptional genes such as *Tet3, Commd1, Notch1, Smad3, and Gata4* emerged, (Figure 4-13).

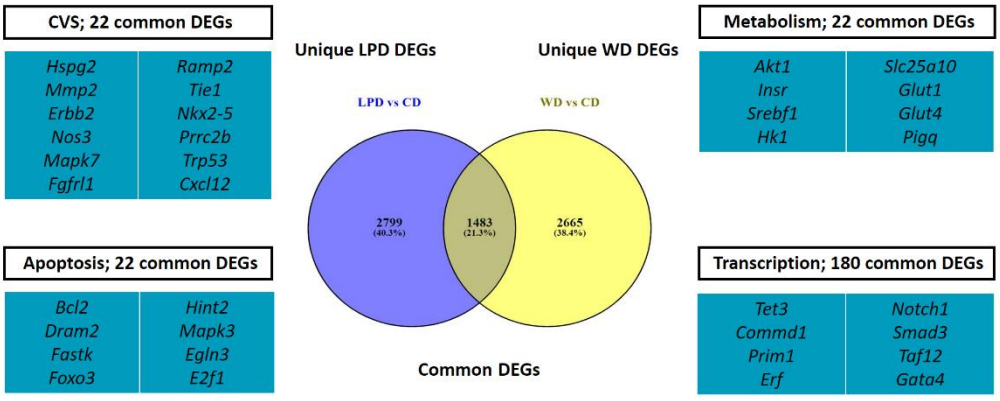
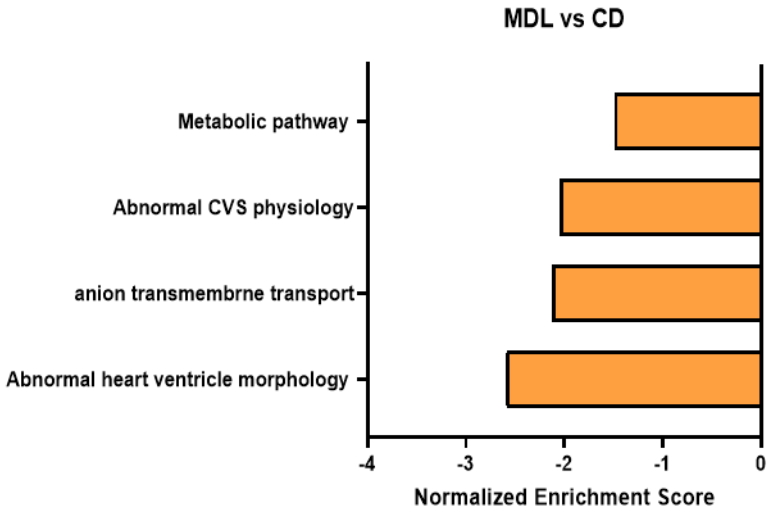


Figure 4-13 Revealed the overlap genes between LPD and WD differentially expressed. The common genes were analyzed using WebGestalt to find the pathways and the most significant genes in these pathways presented in the presented tables.

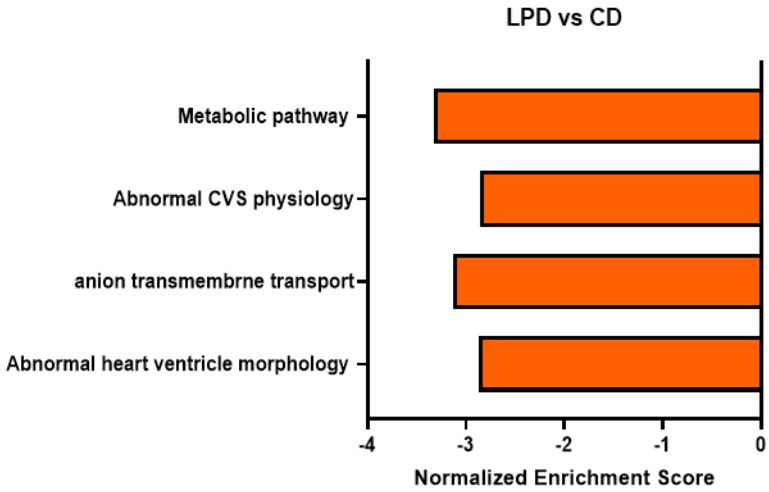
4.5.2.2.8 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal Methyl Donor Supplementation on sub-optimal diets.

The impact of methyl donor supplementation to the suboptimal LPD and WD diets was investigated. Particularly, the effects on pathways involved in cardiovascular diseases and metabolic pathways. Following the addition of methyl donors to both suboptimal diets, a decrease in the effect size of the CVD pathways were observed in MDL hearts (Figure4-13 A), meaning that the addition of methyl donors led to a reduction in the strength of the effects related to these pathways. Interestingly, there were no longer significant changes in terms of the FDR in the MDL group. However, the addition of methyl donor supplementation to WD did not mimic such effects in MDL and did not affect the effect size of these CVD pathways (Figure4-13 D). Therefore, in this specific example, methyl donors negate the negative effects of the LPD, but do not necessarily have the same effect on the WD.

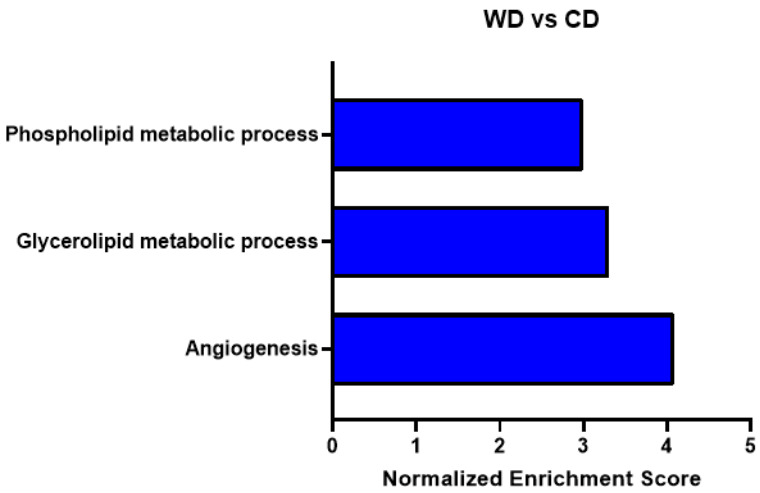
A. FDR>0.05



B. FDR<0.05



C. FDR<0.05



D. FDR<0.05

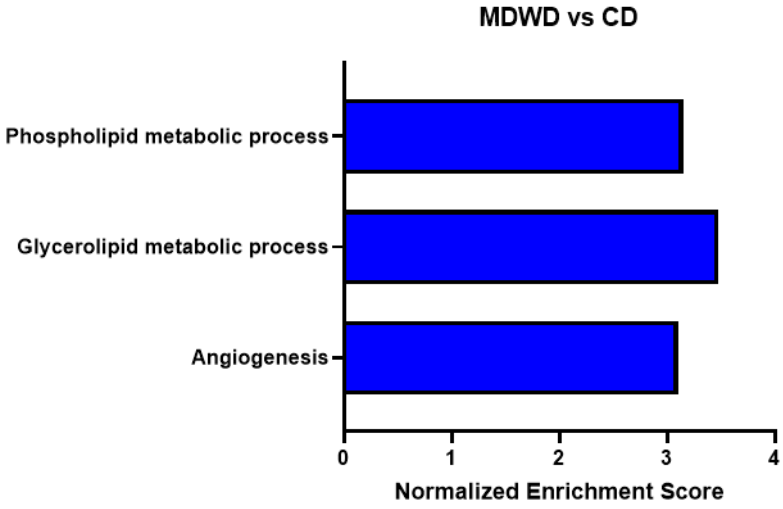


Figure 4-14 (A-D) Represents the analysis of up/down regulation of DEGs in LPD and WD diet compared to MDL and MDWD to investigate the impact of the methyl donor supplementation according to FDR value.

4.6 Analyses of the profile of fetal cardiac gene expression in response to paternal diet in a sex specific manner.

4.6.1 Analysis of the profile of cardiac gene expression in response to paternal diet was conducted, comparing males versus females.

4.6.1.1 Analysis of the profile of cardiac Gene Expression in Response to Paternal diet including comparison of CD male's vs CD females

To further understand the impact of paternal diet on the fetal cardiovascular system, the effect of fetal sex in conjunction with paternal diet was analysed. This involved comparing male and female responses to different paternal diets, as well as to a control diet (CD) group. Furthermore, potential differences in differentially expressed gene pathways within the same sexes in response to various paternal diets were explored. Preliminary analyses of CD male's vs CD females revealed 2583 DEGs in CD male's vs CD female fetal hearts, of which 1394 genes were upregulated, and 1189 genes were downregulated. The list of the up-regulated or down-regulated genes across different treatment groups when comparing male's vs females were listed in (Table 4.3). Among significantly expressed genes, upregulation in various pathways of GO including negative regulation of intracellular signal transduction 3.4218 NES, with 55 genes, process utilizing autophagic mechanism with 3.2487 NES, including 52 genes, positive regulation of catabolic process with 2.9773 NES, including 57 genes, negative regulation of transferase activity with 2.9326 NES, including 41 genes, mitochondrion organization with 2.9057 NES, including 60 genes, regulation of mitotic cell cycle with 2.8491 NES, including 70 genes, proteasomal protein catabolic process with 2.8121 NES, including 47 genes, regulation of protein stability with 2.7336 NES, including 39 genes, post transcriptional regulation of gene expression with 2.6767 NES, including 57 genes, were observed in these fetal hearts (Figure 4-15 A).

Preliminary analysis of the pathway in CD male's vs CD female's hearts revealed a significant up regulation in pathways associated with Cell cycle with 2.2985 NES, including 22 genes, Mitophagy with 2.2963 NES, including 15 genes, Ribosome with 2.2578 NES, including 27 genes, Non-alcoholic fatty

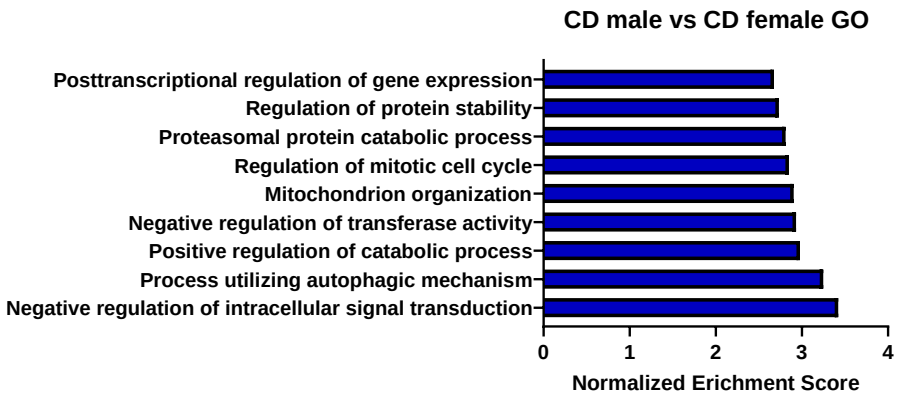
liver disease (NAFLD) with 2.2407 NES, including 27 genes, SNARE interactions in vesicular transport with 2.1899 NES, including 7 genes were observed (Figure 4-15 B).

The analysis of phenotype pathways in CD, LPD male's vs CD, and LPD female's hearts revealed significant upregulation of pathways, involved in prenatal lethality with 4.6118 NES, including 280 genes, embryonic lethality with 4.4176 NES, including 207 genes, abnormal survival with 4.2223 NES, including 543 genes, mortality/ageing with 4.2054 NES, including 589 genes, embryo phenotype with 3.5331 NES, including 222 genes, abnormal prenatal growth/weight/body size with 3.5226 NES, including 151 genes, abnormal embryo size with 3.3802 NES, including 94 genes, abnormal embryonic growth/weight/body size with 3.3708 NES, including 121 genes, abnormal embryo development with 3.2289 NES, including 117 genes, decreased embryo size with 3.0813 NES, including 73 genes, were observed (Figure 4-15 C).

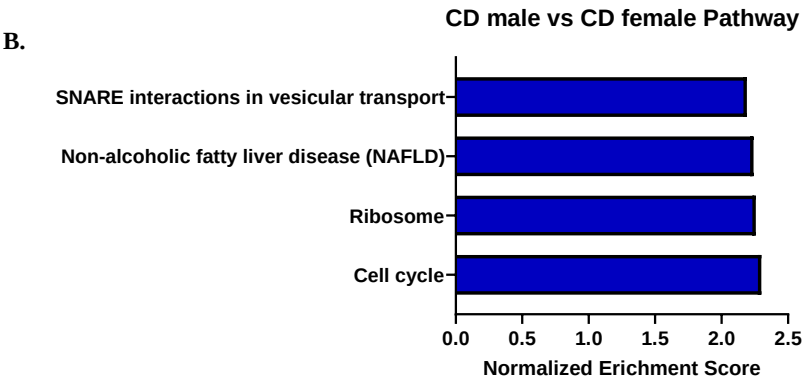
Table 4-3 Displays the number of fetal cardiac differentially expressed genes in each treatment group when compared to CD and each other in response to paternal diet.

Diets	Total Genes	Up-regulated Genes	Down-regulated Genes
CD male vs CD female	2583	1394	1189
LPD male vs LPD female	1,583	460	1,122
MDL male vs MDL female	1,007	592	415
WD male vs WD female	1,409	712	697
MDWD male vs MDWD female	793	359	434

A.



B.



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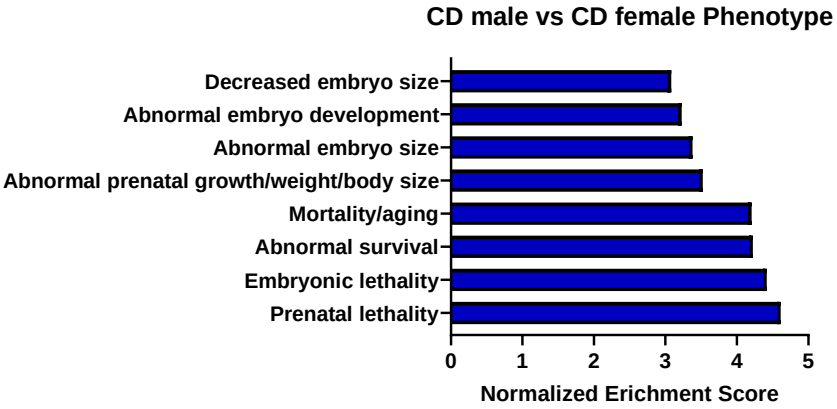


Figure 4-15 (A-C) Represents the analysis of up regulation of DEGs in CD males compared to CD female hearts to investigate the impact of the effect of fetal sex on the expression of sexually dimorphic genes, according to FDR value.

4.6.1.2 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal diet including comparison of LPD male's vs LPD females

Preliminary analyses of LPD male's vs LPD females revealed 1,583 DEGs in LPD male's vs LPD female fetal hearts, which 460 genes were upregulated, and 1122 genes were downregulated. Similar to the analysis comparing CD males to CD females, no significant pathways were observed in the preliminary assessments of the GO, when comparing LPD male to LPD female fetal hearts. However, when comparing LPD males to LPD females using the webGestalt pathways analysis, a significant downregulation of pathways involved in olfactory transduction, with -3.1434 NES, including 69 genes was observed. Interestingly, the pathways identified in the preliminary phenotype analysis represent a high similarity to those observed in CD males when compared to CD females. Upregulation of the following pathways including preweaning lethality with 3.1335 NES, including 285 genes, abnormal survival with 3.1196 NES, including 313 genes, prenatal lethality with 3.0389 NES, including 163 genes, embryonic lethality with 3.0218 NES, including 126 genes, mortality/aging with 2.6279 NES, including 336 genes, embryonic lethality prior to tooth bud stage with 2.4080 NES, including 54 genes,, abnormal extraembryonic tissue morphology with 2.3972 NES, including 54 genes, abnormal exocrine gland physiology with 2.3944 NES, including 13 genes, were observed (Figure 4-16).

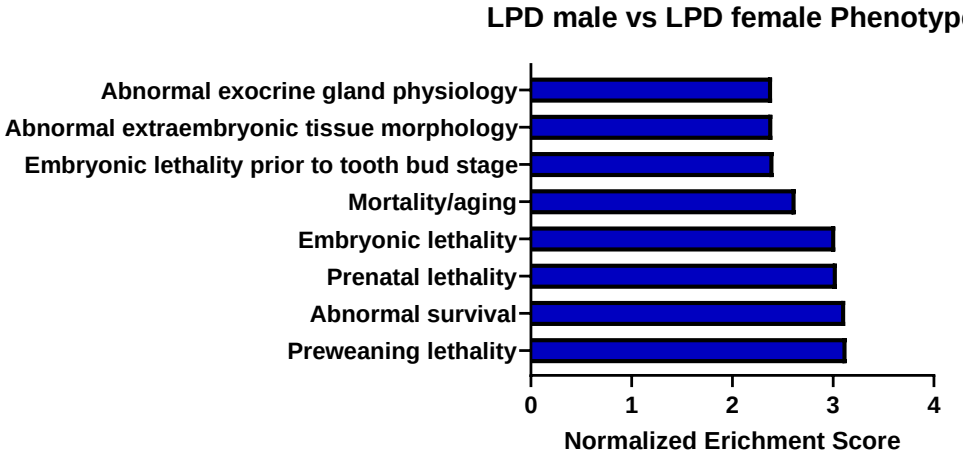


Figure 4-16, Represents the analysis of up regulation of DEGs in LPD males compared to LPD female hearts to investigate the impact of the effect of fetal sex on the expression of sexually dimorphic genes to FDR value.

4.6.1.3 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal Diet Including Comparison of MDL Male's vs MDL Females

Analyses of MDL male vs MDL female fetal heart gene expression using the WebGestalt tool, revealed 1007 differentially expressed genes in MDL male's vs MDL female fetal hearts. Among these number of DEGs, 592 genes were upregulated while 415 genes were downregulated. Among significantly expressed genes, upregulation in pathways of GO including humoral immune response with 2.6469 NES, including 20 genes were observed. Preliminary analyses of pathways represented the upregulation of olfactory transduction pathways, with 2.4699 NES, including 54 genes. Additionally, analysis of phenotype pathways in MDL male's vs MDL female's hearts revealed no significant pathways up/down regulated.

4.6.1.4 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal diet including comparison of WD male's vs WD females

Analyses of WD male's vs WD females revealed 1409 differentially expressed genes in WD male's vs WD female fetal hearts. Among these number of differentially expressed genes, 712 genes were upregulated while 697 gene were downregulated. Among significantly expressed genes, preliminary analysis of

the pathways in WD males versus WD females' hearts revealed a significant up regulation in pathways involved in GO, and phenotypes. GO analysis represented upregulation of pathways such as cilium organization with 2.8886 NES, including 16 genes, microtubule bundle formation with 2.6746 NES including 10 genes, microtubule-based movement with 2.6469 NES including 19 genes, morphogenesis of a branching structure with 2.5619 NES, including 12 genes, sperm motility with 2.4998 NES, including 9 genes, respiratory gaseous exchange with 2.4030 NES, including 6 genes and, respiratory system development with 2.4030 NES, including 13 genes (Figure 4-17 A). Preliminary analysis of the phenotype pathways assessment in WD male hearts compared to female WD hearts represented, the upregulation of numerus abnormal pathways associated to the pulmonary system morphology and function, including abnormal pulmonary alveolar system morphology with 2.5466 NES, including 10 genes, abnormal trachea morphology with 2.5418 NES, including 8 genes, abnormal pulmonary acinus morphology with 2.4241 NES, including 9 genes, abnormal pulmonary alveolus morphology with 2.2460 NES, including 6 genes, abnormal lung morphology with 2.3137 NES, including 14 genes, abnormal pulmonary alveolus epithelial cell morphology with 2.2460 NES including 6 genes, abnormal type II pneumocyte morphology with 2.2460 NES, including 6 genes, abnormal pulmonary alveolus epithelium morphology with 2.2460 NES, including 6 genes, abnormal respiratory conducting tube morphology with 2.2308 NES, including 6 genes, and atelectasis with 2.2308 NES, including 6 genes were observed (Figure 4-17 B).

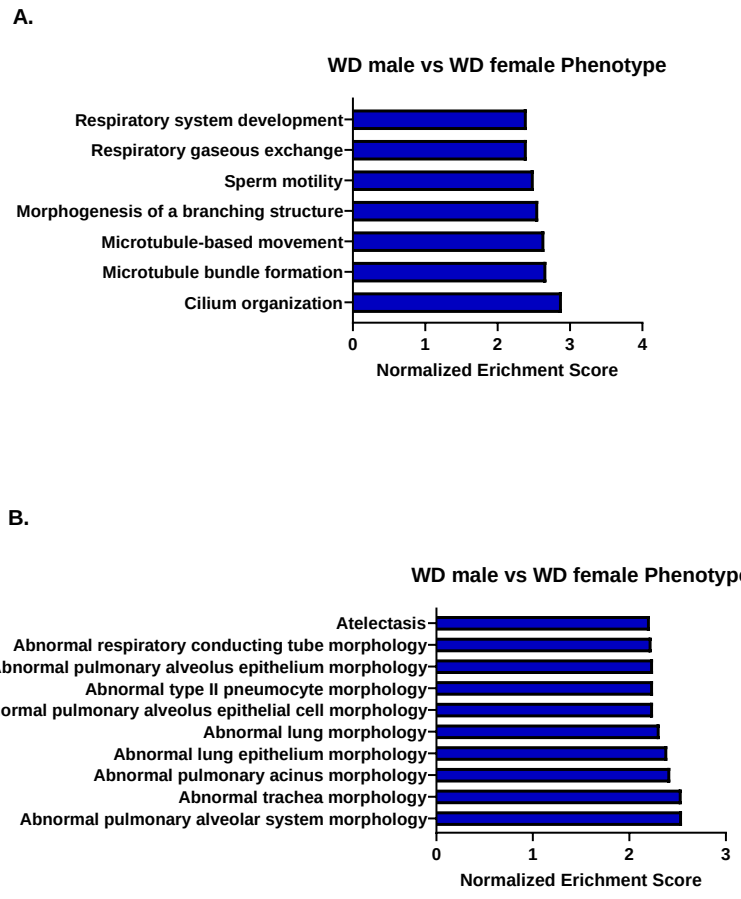


Figure 4-17 (A-B) Represent the analysis of up regulation of DEGs in WD males compared to WD female hearts to investigate the impact of the effect of fetal sex on the expression of sexually dimorphic genes to FDR value.

Additionally, preliminary analysis of the MDWD males compared to MDWD females represent no significant up/down regulated pathways.

4.6.2 Analysis of the profile of cardiac gene expression in response to paternal diet was conducted within the same sex (male).

4.6.2.1.1 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal CD vs LPD in males (same sex).

Preliminary analyses of CD vs LPD males revealed 2793 differentially expressed genes Among these number of differentially expressed genes, 1506 genes were upregulated while 1287 gene were downregulated. A comprehensive description of the total number of DEGs, along with the computation of upregulated and downregulated genes in male fetal heart sired from different paternal-fed diets, compared to CD, was provided in (Table 4-4). Preliminary

analysis using WebGestalt tool, represents upregulation in various pathways of GO including RNA splicing up regulated by 2.53 NES, with 64 genes, mRNA processing with 71 genes. Multiple pathways involved in the metabolic pathways and metabolism as well as angiogenesis were significantly downregulated in LPD male fetal hearts when compared to CD in the same sex. Among significantly expressed genes, down-regulation in various pathways of GO including pyruvate metabolic process with -2.6618 NES, including 25 genes, cofactor metabolic process with -2.5076 NES, with 62 genes, generation of precursor metabolites and energy with -2.4818 NES, including 47 genes, nucleoside triphosphate metabolic process with -2.3833 NES, including 39 genes were observed. As well as angiogenesis -2.3833 NES, with 39 genes, represented in (Figure 4-18 A).

Preliminary analysis of the pathway in LPD male's vs CD male hearts revealed a significant down-regulation of metabolic pathway, with -2.8720 NES. Within this pathway 126 genes were downregulated. Carbon metabolism downregulated significantly -2.6417 with NES, including 21 genes. The other pathways that significantly downregulated were Glycolysis / Gluconeogenesis with -2.4572 NES with 19 genes and Biosynthesis of amino acids pathway with -2.3445 NES with 17 genes, that downregulated in LPD male fetal hearts when compared to CD male's fetal hearts, represented in (Figure 4-18 B).

Table 4-4 Displays the number of differentially expressed fetal cardiac genes in each treatment group, both compared to CD and to each other, in response to paternal diet in male fetal hearts.

Diets	Total Genes	Up-regulated Genes	Down-regulated Genes
LPD male vs CD male	2,793	1,506	1,287
MDL male vs CD male	1,698	796	929
WD male vs CD male	1,560	1,014	546
MDWD male vs CD male	4,448	2,244	2,204

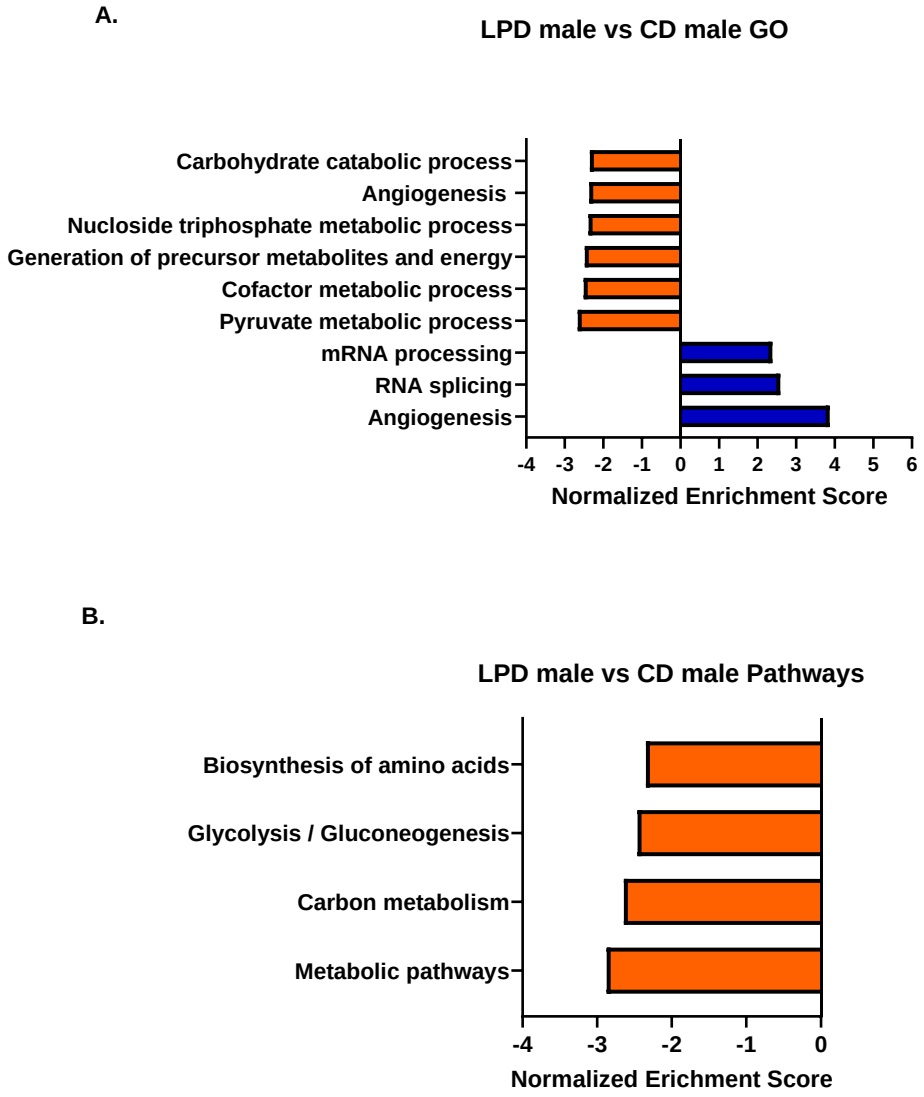


Figure 4-18 (A-B) Represents the analysis of up/down regulation of DEGs in LPD male when compared to CD males to investigate the impact of both sex and paternal diet according to FDR value.

4.6.2.1.2 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal CD vs MDL in males.

Preliminary analyses comparing CD and MDL males' fetal hearts identified 1698 DEGs. Among these, 769 genes exhibited upregulation, while 929 genes showed downregulation. However, initial analysis of GO, pathways, and phenotype using WebGestalt revealed no significant alterations in pathways in MDL male fetal hearts compared to CD male fetal hearts.

4.6.2.1.3 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal CD vs MDL in males

Preliminary analyses of CD vs MDL hearts in males revealed 1699 differentially expressed genes in CD vs MDL male fetal hearts. Among significantly expressed genes, upregulation in various pathways of GO including protein activation cascade by 3.7119 NES, with 60 genes, DNA repair by 3.5072, with 408 genes, regulation of DNA metabolic process by 3.0181 NES, with 391 genes, DNA recombination by 2.9760 NES, with 243 genes, cell cycle phase transition by 2.7545 NES, with 352 genes, coagulation by 2.6909 NES, with 154 genes, , protein maturation by 2.6454 NES, with 274 genes were up regulated by 2.6806 NES, with 33 genes, mRNA processing upregulated by 2.5325 NES with 37 genes very similar to the previous mentioned pathway (Figure 4-19).

Preliminary analysis of the pathway in MDL male's vs CD male hearts revealed a significant upregulation complement and coagulation cascades by 5.0628 NES, with 88 genes, cell cycle by 2.3380 NES, with 123 genes, and cholesterol metabolism by 2.2972 NES, with 48 genes, as well as a significant downregulation in Cytokine-cytokine receptor interaction by -2.7573 NES, with 284 genes.

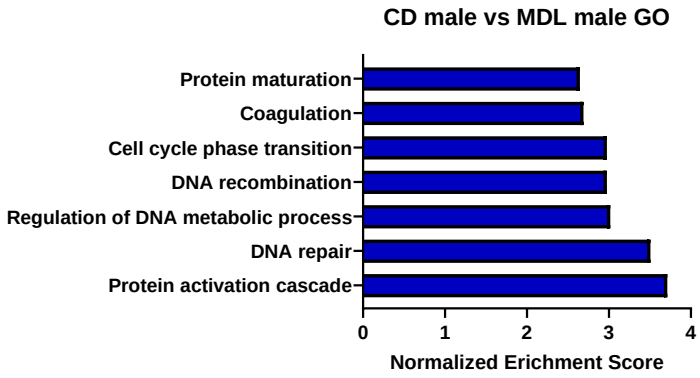


Figure 4-19 (A-B) Represents the analysis of up/down regulation of DEGs in MDL male when compared to CD males to investigate the impact of both sexually dimorphic genes and paternal diet, according to FDR value.

4.6.2.1.4 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal WD vs CD in males

Preliminary analyses of CD vs WD in males revealed 1560 differentially expressed genes in WD vs CD male fetal hearts, among these DEGs 1014 genes were upregulated while 546 genes were downregulated. Among DEGs, upregulation in various pathways of GO including negative regulation of intracellular signal transduction by 2.4264 NES, with 33 genes, negative regulation of establishment of protein localization by 2.3272 NES with 14 genes. The other upregulated pathways were including regulation of G protein-coupled receptor signaling pathway by 2.235 NES with 7 genes (Figure 4-20).

Preliminary analysis of the pathway in WD male's vs CD male hearts revealed a significant down-regulation of systemic lupus erythematosus with -3.2107 NES including 15 genes and, olfactory transduction pathway with -4.0548 NES, including 26 genes.

Preliminary analysis of the pathway in WD male's vs CD male fetal hearts revealed.

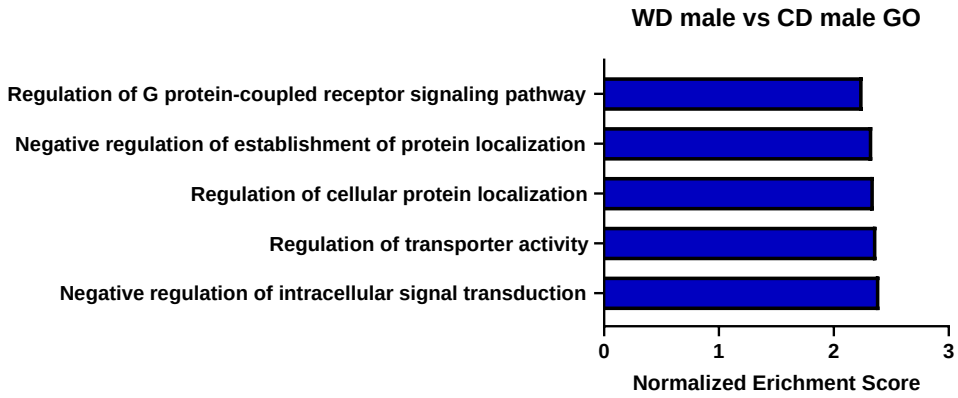


Figure 4-20, Represents the analysis of up regulation of DEGs in WD male when compared to CD males to investigate the impact of both sexually dimorphic genes and paternal diet according to FDR value.

4.6.2.1.5 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDWD vs CD in males

Preliminary analyses of CD vs MDWD in males fetal heart gene expression assessment revealed 4448 differentially expressed genes in MDWD vs CD male fetal hearts, among these DEGs 2244 genes were upregulated while 2204 genes were downregulated. Among significantly expressed genes, upregulation in various pathways of GO including cellular component disassembly with 1.2854 NES including 525 genes, small GTPase mediated signal transduction with 1.2652 NES including 552 genes, negative regulation of gene expression with 1.2228 NES including 1734 genes, negative regulation of cellular protein metabolic process with 1.1946 NES including 983 genes, negative regulation of protein metabolic process with 1.1898 NES including 1044 genes, vesicle-mediated transport with 1.1586 NES including 1942 genes, negative regulation of response to stimulus with 1.1568 NES including 1545 genes, positive regulation of catalytic activity with 1.1531 including 1380 genes, positive regulation of molecular function with 1.1508 NES including 1717 genes, regulation of intracellular signal transduction with 1.1463 including 1824 genes (Figure 4-21 A).

Multiple pathways involved in the metabolic pathways by 2.9990 with 191 genes, Ribosome with 2.8031 NES including 40 genes, pathways in cancer by 2.5812 NES with 101 genes, as well as AMPK signaling pathway with 2.5037 NES including 26 genes were significantly upregulated in MDWD male fetal hearts when compared to CD in the same sex (Figure 4-21 B).

The preliminary analysis of phenotype in MDWD male fetal hearts compared to CD male hearts revealed a significant upregulation of various pathways involved in abnormal vascular development with 4.0546 NES including 113 genes, abnormal heart morphology with 4.0333 NES including 304 genes, decreased cell proliferation with 3.4440 NES including 139 genes, abnormal angiogenesis with 3.4260 NES including 70 genes, abnormal cell proliferation with 3.2411 NES, including 231 genes, abnormal aorta morphology with 3.0224 NES including 59 genes, abnormal heart valve morphology with 2.9718 NES

including 41 genes, abnormal mean corpuscular volume with 2.9365 NES, including 53 genes, abnormal heart development with 2.9320 NES, including 84 genes (Figure 4-21 C).

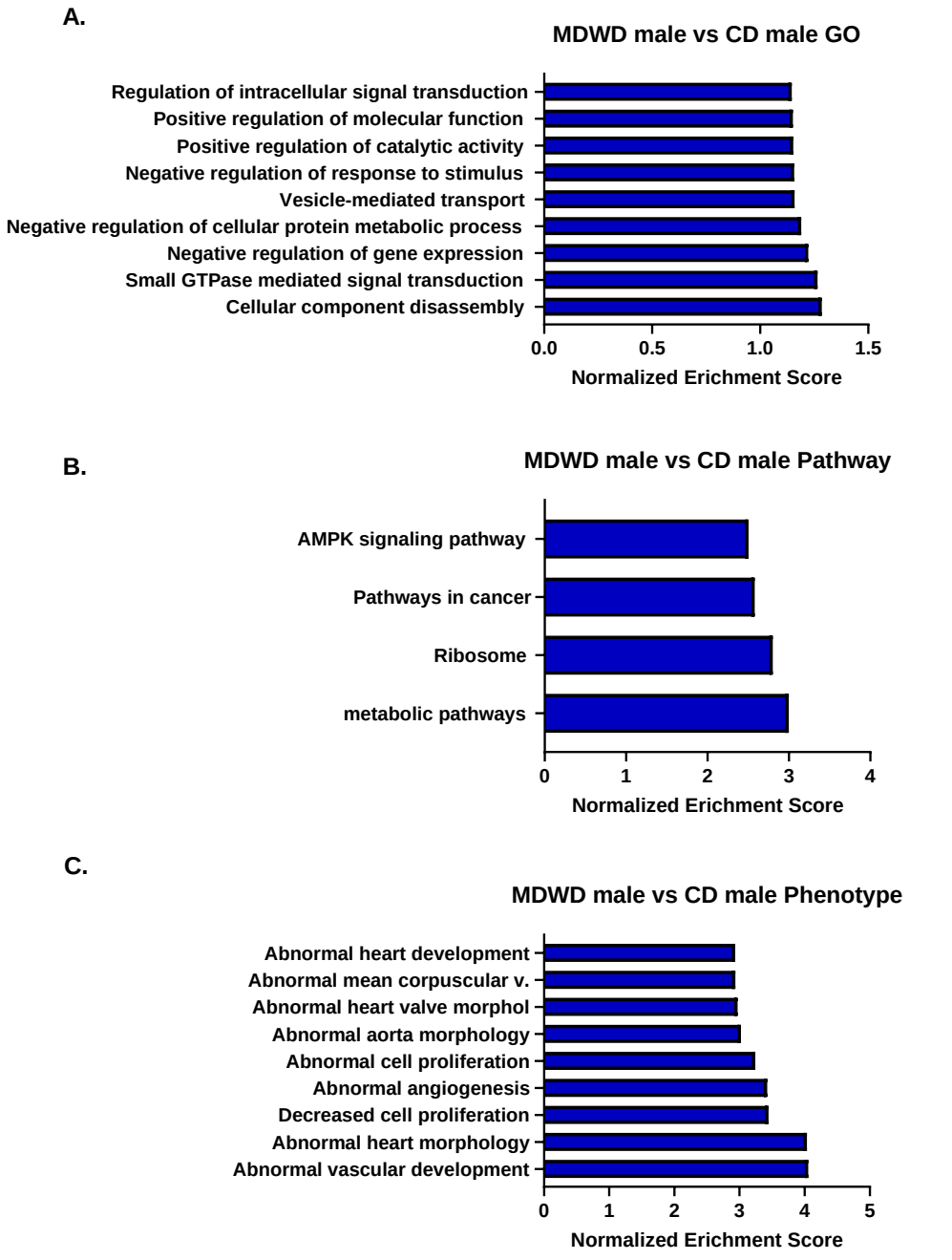


Figure 4-21 (A-C), Represents the analysis of up regulation of DEGs in MDWD male when compared to CD males to investigate the impact of both sexually dimorphic genes and paternal diet according to FDR value.

4.6.3 Analysis of the profile of cardiac gene expression in response to paternal diet was conducted within the females (same sex).

4.6.3.1.1 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal CD vs LPD in females.

Preliminary analyses of CD vs LPD in female hearts revealed 3784 differentially expressed genes in CD vs LPD female fetal hearts, among these DEGs 1180 genes were upregulated and 2603 genes were downregulated. (Figure 4-22 A). The list of the up-regulated or down-regulated genes across different treatment groups when comparing male's vs females were listed in (Table 4-5). Preliminary analysis of GO represents no significant either up or down regulated pathways in female fetal hearts derived from LPD fed fathers when compared to CD. One pathway involved in the metabolic pathways by -2.4497 NES with 268 genes was significantly downregulated in LPD female fetal hearts when compared to CD in the same sex.

Table 4-5 Represents the number of differentially expressed fetal cardiac genes in each treatment group, both compared to CD and to each other, in response to paternal diet in female fetal hearts.

Diets	Total Genes	Up-regulated Genes	Down-regulated Genes
LPD female vs CD female	3,783	1,180	2,603
MDL female vs CD female	2,515	1,075	1,440
WD female vs CD female	2,245	1,011	1,234
MDWD female vs CD female	1,487	652	835

4.6.3.1.2 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal CD vs MDL in females.

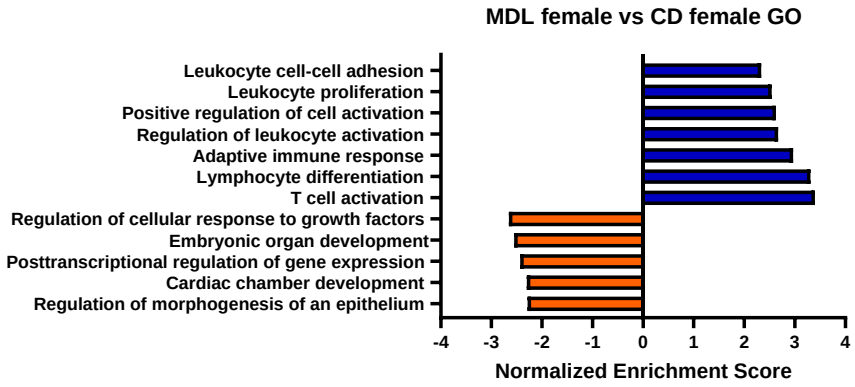
Preliminary analyses of CD vs MDL in females revealed 2516 differentially expressed genes in LPD vs MDL female fetal hearts. Among significantly expressed genes, upregulation in various pathways of GO including T cell activation with 3.3981 NES including 63 genes, lymphocyte differentiation with 3.3078 NES including 42 genes, adaptive immune response with 2.9662 NES including 34 genes, regulation of leukocyte activation with 2.6696 NES including 46 genes, positive regulation of cell activation with 2.6260 NES, including 35 genes, leukocyte proliferation with 2.5426 NES, including 30 genes, leukocyte cell-cell adhesion with 2.3349 NES, including 38 genes, neuropeptide signaling pathway with 2.2454 NES, including 10 genes, were observed. Additionally, downregulation of pathways of GO including, regulation of morphogenesis of an epithelium with -2.2868 NES, including 8 genes, cardiac chamber development with -2.3007 NES, including 28 genes, posttranscriptional regulation of gene expression with -2.4315 NES, including 67 genes, embryonic organ development with -2.5521 NES, including 68 genes, regulation of cellular response to growth factors with -2.6529 NES, including 34 genes, in female fetal hearts sired from MDL fed fathers compared to CD were observed (Figure 4-22 A).

The preliminary analysis of KEGG pathways revealed upregulation of multiple pathways related in primary immunodeficiency with 2.7533 NES including 7 genes, cell adhesion molecules (CAMs) with 2.6212 NES, including 27 genes, antigen processing and presentation with 2.6010 NES, including 15 genes, Th1 and Th2 cell differentiation with 2.5630 NES, including 16 genes, Th17 cell differentiation with 2.5361 NES, including 19 genes, T cell receptor signaling pathway with 2.4626 NES, including 25 genes, were observed in MDL female hearts when compared to CD female hearts (Figure 4-22 B).

The preliminary analysis of phenotype pathways revealed significant upregulation of multiple pathways including abnormal CD8-positive, alpha, beta T cell morphology, with 3.6914 NES including 56 genes, abnormal T cell

differentiation with 3.5918 NES, including 54 genes,, abnormal leukopoiesis with 3.4280 NES, including 85 genes, abnormal lymphopoiesis with 3.3648 NES including 73 genes, abnormal T cell sub population ratio with 3.3162 NES, including 9 genes, decreased T cell number with 3.3022 NES, including 88 genes, increased T cell number with 3.2673 NES, including 58 genes, abnormal T cell physiology with 3.1865 NES, including 78 genes, abnormal T cell proliferation with 3.1427 NES, including 45 genes, abnormal CD8-positive, alpha-beta T cell physiology with 3.1156 NES, including 18 genes, were observed when MDL female fetal hears compared to CD fetal heart in the same sex. In the same analysis, significant downregulation of pathways including embryo phenotype with -2.5764 NES, including 235 genes, abnormal blood vessel morphology with -2.5815 NES, including 152 genes, embryonic lethality during organogenesis, complete penetrance, with -2.6510 NES, including 107 genes, embryonic lethality during organogenesis with -2.7238 NES, including 126 genes, preweaning lethality, complete penetrance, with -2.8476 NES, including 122 genes, mortality/aging with -2.9947 NES, including 612 genes, embryonic lethality with -3.1121 NES, including 239 genes, prenatal lethality with -3.1363 NES, including 316 genes, abnormal survival with -3.2839 NES, including 582 genes, preweaning lethality with -3.3270 NES, including 512 genes, were observed (Figure 4-22 C).

A.



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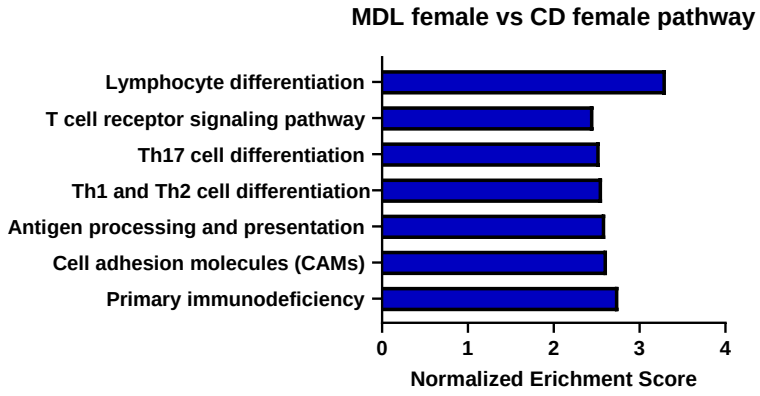

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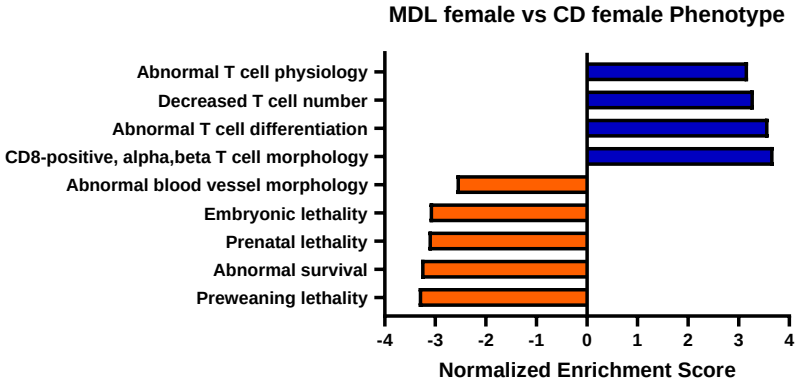


Figure 4-22 (A-C), Represents the analysis of up and down regulation of DEGs in GO, pathway and phenotype analysis in MDL female fetal hearts when compared to CD females to investigate the impact of paternal diet, according to FDR value.

4.6.3.1.3 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal WD vs CD in females.

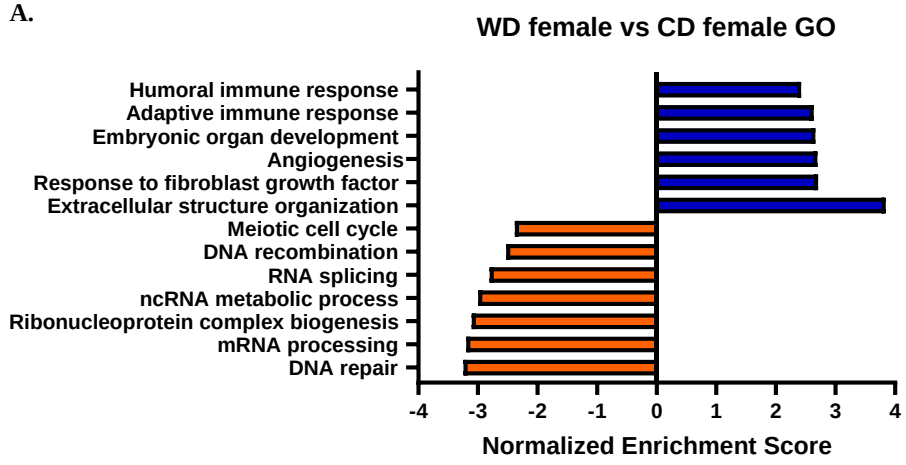
Preliminary analyses of WD vs CD in females revealed 2245 differentially expressed genes in WD vs CD female fetal hearts. Among significantly expressed genes, 1011 genes were upregulated, and 1234 genes were downregulated.

Preliminary analysis of pathways involved in GO using WebGestalt tool, identified a significant upregulation in various pathways of GO including extracellular structure organization with 3.8458 NES, including 45 genes, response to fibroblast growth factor with 2.7075 NES, including 16 genes, skeletal system development with 2.7000 NES, including 63 genes, angiogenesis with 2.6962 NES, including 62 genes, response to wounding with 2.6681 NES, including 70 genes, embryonic organ development with 2.6626 NES, including 54 genes, adaptive immune response with 2.6357 NES, including 32 genes, respiratory tube development with 2.5379 NES, including 27 genes, humoral immune response with 2.4232 NES, including 11 genes, were observed in female fetal hearts derived from WD fed when compared to CD in the same sex. Accordingly, significant downregulation in fundamental transcriptomic pathways including meiotic cell cycle with -2.3803 NES, including 27 genes, DNA-templated transcription with initiation -2.4167 NES, including 17 genes, covalent chromatin modification with -2.4260 NES, including 57 genes, female gamete generation with -2.5066 NES, including 13 genes, DNA recombination with -2.5290 NES, including 34 genes, RNA splicing with -2.8042 NES, including 48 genes, ncRNA metabolic process with -2.9976 NES, including 67 genes, ribonucleoprotein complex biogenesis with -3.1044 NES, including 66 genes, mRNA processing with -3.1966 NES, including 57 genes, DNA repair with -3.2425 NES, including 57 genes, were observed in female fetal hearts derived from WD fed fathers when compared to CD (Figure 4-23 A).

Preliminary analysis of pathways involved in KEGG pathway using WebGestalt tool, identified a significant upregulation in pathways involved in metabolism

of xenobiotics by cytochrome P450 with 2.5952 NES, including 11 genes, focal adhesion with 2.4431 NES, with 25 genes, protein digestion and absorption with 2.3498 NES, including 14 genes, complement and coagulation cascades with 2.2424 NES, including 7 genes, ECM-receptor interaction with 2.1202 NES, including 17 genes, arachidonic acid metabolism, antigen processing and presentation with 2.0940 NES, including 7 genes, were observed, in female fetal heart sired from WD fed fathers when compared to CD. Accordingly, to this assessment the other pathways were significantly downregulated in WD female fetal hearts when compared to CD in the same sex, were including the cell cycle with -2.2887 NES, including 19 genes and, spliceosome with -2.5497 NES, including 21 genes were identified (Figure 4-23 B).

A.



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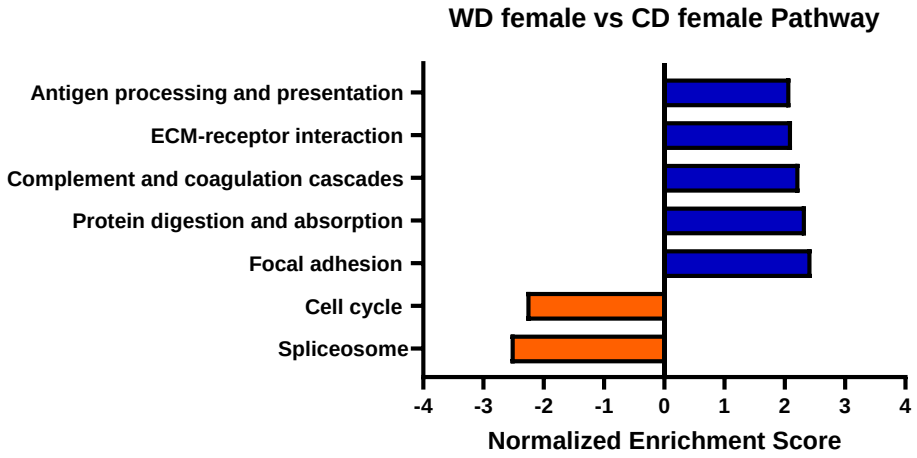


Figure 4-23 (A-B), Represents the analysis of up and down regulation of DEGs in GO and, pathway analysis in WD female fetal hearts when compared to CD females to investigate the impact of paternal diet, according to FDR value.

4.6.3.1.4 Analysis of the Profile of Cardiac Gene Expression in Response to Paternal MDWD vs CD in females.

Preliminary analyses of MDWD vs CD in female fetal hearts, revealed 1487 DEGs in MDWD vs CD female fetal hearts. Among significantly expressed genes, 652 genes were upregulated, and 835 genes were downregulated. Preliminary analysis of the GO, pathways and phenotype identified significant

upregulation of pathways including extracellular structure organization with 3.0184 NES, including 31 genes, muscle system process with 2.7440 NES, including 32 genes, regulation of ion transmembrane transport with 2.4907 NES, including 38 genes, protein autophosphorylation with 2.4746 NES, including 16 genes, multi-multicellular organism process with 2.4676 NES, including 17 genes, leukocyte mediated immunity with 2.4352 NES, including 18 genes, were observed in MDWD female fetal hearts when compared to CD fetal heart in the same sex (Figure 4-24).

Preliminary analysis of pathways involved in KEGG pathway using WebGestalt tool, revealed no significant pathways were up or down regulated in female fetal hearts derived from MDWD fed fathers when compared to CD, in the same sex.

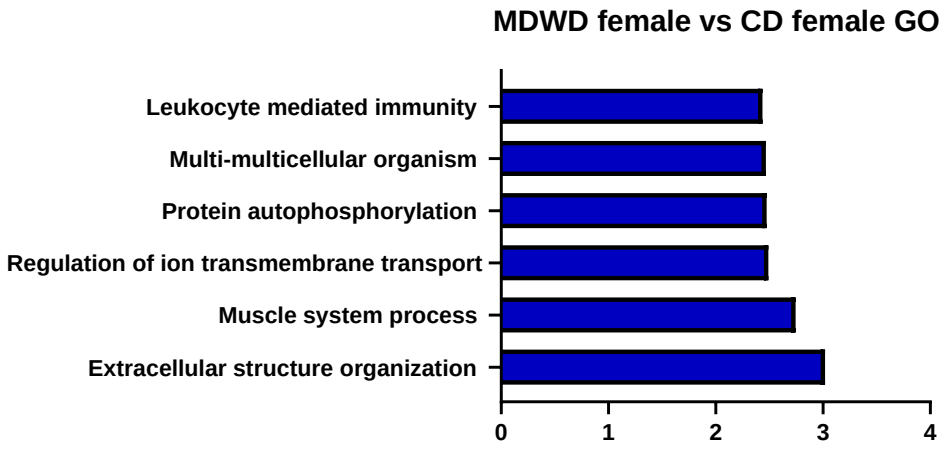


Figure 4-24, Represents the analysis of up regulation of DEGs in GO analysis in MDWD female fetal hearts when compared to CD females to investigate the impact of paternal diet, according to FDR value.

4.7 Placental gene expression and placental histomorphology

Placentas derived from different males on respective diets were collected at E17.5 to examine placental gene expression and morphological characteristics in response to paternal diet across different treatment groups.

4.7.1 Placental gene expression in response to paternal diet

Placental RNA was extracted from total placenta, to examine the relative expression of RAS components, apoptosis and 1-carbon metabolism pathways related genes in response to paternal diet. The expression of placental *Ace* was significantly reduced in WD (P=0.0157) and MDWD (P= 0.0468) compared to CD and in WD (P=0.0002) and MDWD (P=0.0006) versus LPD. No significant change in the expression of *Ace* was observed in LPD and MDL groups when compared with the CD group, or each other (Figure 4-25 A).

Placental *Ace2* expression was significantly decreased in the WD group when compared to CD (P=0.0363), LPD (P ==0.0033) and MDL groups (P=0.0445) (Figure 4-25 B). There was no difference in the expression of *Ace2* in the LPD and MDL groups when compared to CD group or each other.

Placental *Agtr1a* expression (Figure 4-25 C) was significantly reduced in the WD group when compared to LPD (P=0.0295) and MDL (P=0.0079) groups. Expression of *Agtr1a* was also reduced in MDWD versus CD (P=0.0275), LPD (P=0.0115) and MDL groups (P=0.0028).

Placental expression of *Agtr2* (Figure 4-25 D) was significantly increased in the MDWD group when compared to CD (P=0.0207) and MDL (P= 0.0107) groups.

The placental expression of *Atp6ap2* showed less variation according to diet, with reduced expression observed in the WD group compared to CD (Figure4-15 E).While placental *Ren1* expression showed no significant differences in response to paternal diet in any of the treatment groups (Figure 4-25 F).

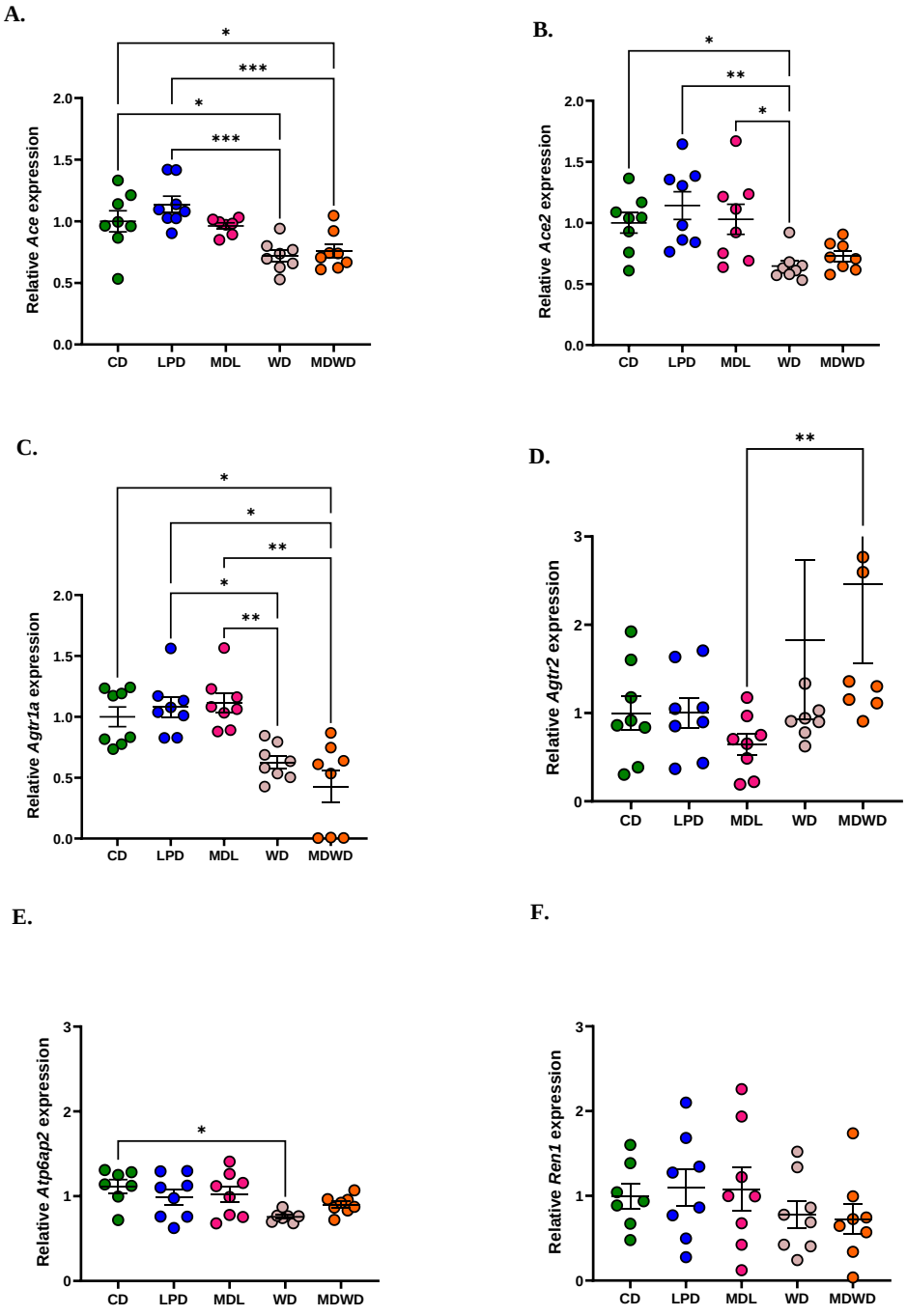


*Figure 4-25 Demonstrates the relative placental expression of genes involved in the components of RAS pathway in response to paternal diet, analyzed for Ace (A), Ace2 (B), Agtr1a (C), Agtr2 (D) Atp6ap2 (E), and Ren1 (F). The placental tissues were derived from females mated with stud males fed one of 5 different diets. (n=8 placentas per group, each from separate litters). The comparison of statistical significance for 5 different diet groups was determined using either one-way ANOVA with Tukey post-hoc test or Kruskall-Wallis with Dunns post-test. Data are mean ± SEM (n=8), *p<0.01, **<0.001, ***p<0.0001*

To determine the mechanisms underlying the potential influence of paternal diet in fetal programming as well as influencing maternal ill-health in late gestation the placental apoptosis pathways were examined. The results of placental apoptosis pathways gene expression have been represented in Figure 4-26 A-E.

Placental expression of *Bad* (Figure 4-26 A) was significantly decreased in the WD and MDWD groups compared to CD (WD vs CD, P=0.0018; MDWD vs CD, P=0.0355) and LPD (WD vs LPD, P=0.0005; MDWD vs LPD, P=0.0136). Expression of *Bad* was also significantly reduced in the WD group when compared to MDL (P=0.00078).

The relative expression of placental *Bax* (Figure 4-26 B) was significantly decreased in the WD group when compared to the CD (P=0.0003) and LPD (P=0.0016) groups.

A significant decrease in the relative expression of placental *Bcl2* (Figure 4-26 C) was observed in WD and MDWD when compared to CD (WD vs CD, P<0.0001; MDWD vs CD, P=0.0007), LPD (WD vs LPD, P<0.0001; MDWD vs LPD, P=0.0018).and MDL (WD vs MDL, P<0.0001; MDWD vs MDL, P=0.0001). A significant decrease in the relative expression of placental *Casp1* gene was observed in WD (P= 0.001) and MDWD (P=0.0009) groups when compared to MDL (Figure 4-26 D).

The relative placental expression of *Fas* was significantly decreased in WD (P=0.0018) and MDWD (P=0.0086) when compared to the LPD group (Figure 4-26 E).

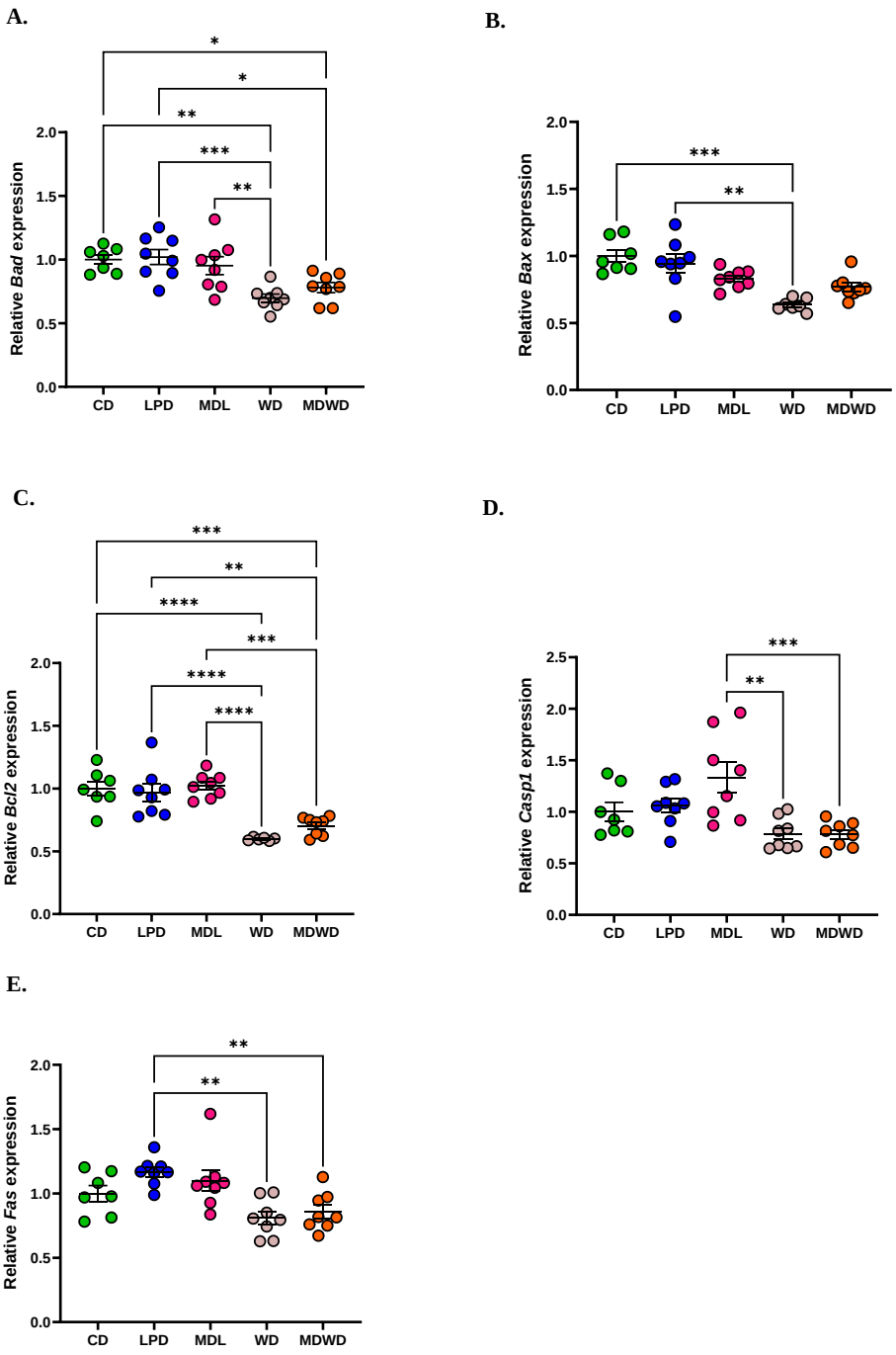


*Figure 4-26 Demonstrates the relative placental expression of genes involved in apoptosis pathway in response to paternal diet analyzed for Bad (A), Bax (B), Bcl2 (C), Casp1 (D) and Fas (E). The placental tissues derived from stud males were fed with 5 different diets. (n=8). The comparison of statistical significance for 5 different diet groups determined using either one-way ANOVA with Tukey post-hoc test or Kruskall-Wallis with Dunns post-test Data are the mean ± SEM (n=8), *p<0.05, **<0.01, ***p<0.001, ****p<0.0001.*

Analysis of placental 1-carbon metabolism pathway genes revealed significant alterations in expression in response to diet (Figure 4-27).

The expression of *Dhfr* (Figure 4-27 A) was significantly decreased in WD ($P<0.0001$) and MDWD ($P<0.0001$) groups, in comparison with CD, while *Dhfr* expression in LPD and MDL groups was not different from CD ($P > 0.05$). WD and MDWD expression was also decreased versus LPD (WD vs LPD, $P<0.0001$; MDWD vs LPD, $P<0.0001$) and MDL (WD vs MDL, $P = 0.0024$; MDWD vs MDL, $P=0.0023$), No significant difference in expression of *Dhfr* between WD and MDWD was observed.

The relative expression of *Mthfr* (Figure 4-27 B) was significantly decreased in WD ($P<0.0001$) and MDWD ($P=0.0014$) groups, compared to CD. In addition, the expression of *Mthfr* in WD was significantly decreased when compared to LPD ($P=0.0063$) and MDL ($P=0.0085$). Whereas such a pattern was missing in MDWD group.

The expression of gene *Mat2a* (Figure 4-27 C) was decreased significantly in WD when compared to CD ($P=0.0006$). No other groups were different to CD ($P>0.05$). The expression of *Mat2a* was significantly reduced in WD and MDWD compared to LPD (WD vs LPD, $P<0.0001$; MDWD vs LPD, $P=0.0232$) and MDL (WD vs MDL, $P<0.0001$; MDWD vs MDL, $P=0.0386$). No significant difference in expression of *Mat2a* was observed between WD and MDWD.

The expression of *Mat2b* (Figure 4-27 D) was significantly decreased in WD ($P=0.0138$) and MDWD ($P=0.0013$) groups, in comparison with CD, while *Mat2b* expression in LPD and MDL groups was not different from CD ($P> 0.05$). WD and MDWD expression was also decreased versus LPD (WD vs LPD, $P=0.0148$; MDWD vs LPD, $P=0.0012$). Additionally, expression in MDWD was decreased versus MDL ($P=0.0468$). No significant difference in expression of *Mat2b* was observed between WD and MDWD.

In a pattern similar to *Dhfr*, the expression of *Achy* (Figure 4-27 E) was significantly decreased in WD ($P<0.0001$) and MDWD ($P=0.0002$) groups, in comparison with CD, while *Achy* expression in LPD and MDL groups was not

different from CD (P>0.05). WD and MDWD expression was also decreased versus LPD (WD v LPD, P<0.0001; MDWD vs LPD, P=0.0002) and MDL (WD vs MDL, P<0.0001; MDWD v MDL, P<0.0001), No significant difference in expression of *Achy* between WD and MDWD was observed.

The last gene of interest from the 1-Carbon pathway was *Mtr* (Figure 4-27 F). All groups exhibited lower expression than CD (LPD, P=0.0326; MDL, P=0.0323; WD, P<0.0001; MDWD, P<0.0001). WD and MDWD expression was also decreased versus LPD (WD vs LPD, P<0.0001; MDWD vs LPD, P<0.0001) and MDL (WD vs MDL, P<0.0001; MDWD vs MDL, P<0.0001).

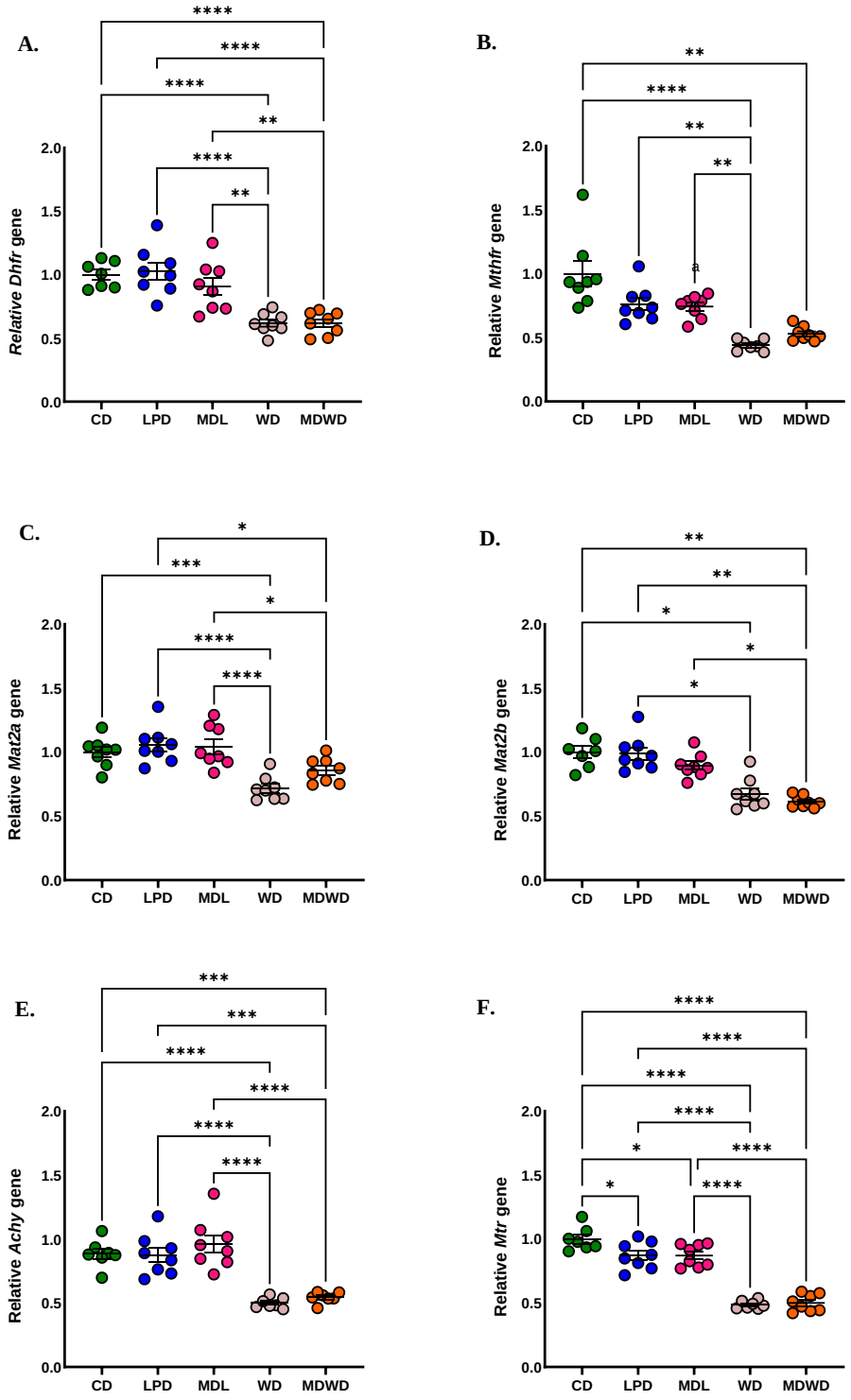


*Figure 4-27 Demonstrates the relative placental expression of genes involved in 1-C metabolism pathway in response to paternal diet analyzed for Dhfr (A), Mthfr (B), Mat2a(C), Mat2b (D), Achy (E) and Mtr (F). The placental tissues derived from stud males were fed with 5 different diets. (n=8). The comparison of statistical significance for 5 different diet groups determined using either one-way ANOVA with Tukey post-hoc test or Kruskall-Wallis with Dunns post-test Data are the mean ± SEM (n=8), *p<0.05, **<0.01, ***p<0.001).*

4.7.2 Commonly expressed genes between late gestation placental and fetal heart in response to paternal diet

As expected, a significant overlap exists in gene expression between the fetal heart and placenta. Therefore, the comparison below illustrates the shared expression genes between these two tissues to identify common alterations. Interestingly, most shared expressed genes between the placenta and fetal heart belong to the 1-C metabolism family. Notably, only one gene each from the RAS family (*Agtr1a*) and the apoptosis family (*Bcl2*) showed significant downregulation in both placenta and fetal heart.

Table 4-6 Displays the list of common expressed genes from RAS family, in both placenta and fetal heart, indicating their response to paternal diet.Genes exhibited significantly shared expression were highlighted in red.

Genes symbols	LPD vs CD		MDL vs CD		WD vs CD		MDWD vs CD	
(RAS)	P-value	Fold-change	P-value	Fold-change	P-value	Fold-change	P-value	Fold-change
Ace	-	-	-	-	-	-	-	-
Ace2	-	-	0.941	1.010	-	-	0.6	-1.0738
Agtr1a	-	-	0.006	-1.211	-	-	0.5	-1.028
Agtr1b	0.02	-1.16	0.751	1.112	0.02	-1.1	0.02	-1.16361
Agtr2	-	-	0.751	1.112	-	-	0.6	1.17085
Atp6ap2	-	-	0.955	1.002	-	-	0.1	1.06632
Ren1	-	-	0.54	-1.076	-	-	0.8	-1.0202

Table 4-7 Displays the list of commonly expressed genes from the apoptosis family, in both placenta and fetal heart, indicating their response to the paternal diet. Genes exhibited significantly shared expression were highlighted in red..

Genes symbols	LPD vs CD		MDL vs CD		WD vs CD		MDWD vs CD	
(Apoptosis)	*P-value*	*Fold-change*	*P- value*	*Fold-change*	*P - value*	*Fold-change*	*P value*	*Fold-change*
Bad	-	-	*0.83*	*-1.010*	-	-	*0.5*	*-1.03251*
Bax	-	-	*0.71*	*-1.017*	-	-	*0.9*	*-1.00443*
Bcl2	-	-	*0.008*	*-1.128*	-	-	-	-
Casp1	-	-	*0.082*	*1.197*	-	-	*0.1*	*-1.1405*
Fas	-	-	*0.22*	*1.090*	-	-	*0.3*	*-1.08258*

Table 4-8 Displays the list of commonly expressed genes from the 1-C metabolism family, in both placenta and fetal heart, indicating their response to the paternal diet. Genes that exhibited significantly shared expression were highlighted in red.

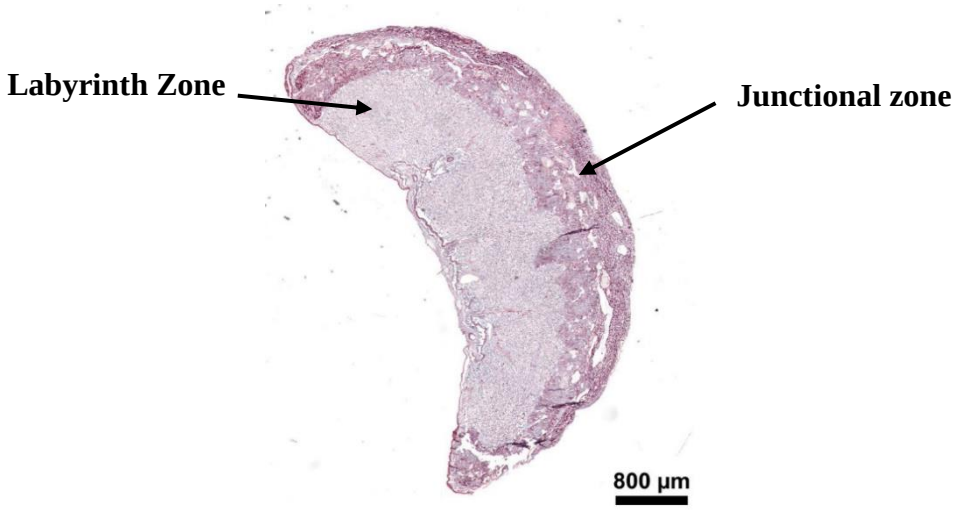
Genes symbols	LPD vs CD		MDL vs CD		WD vs CD		MDWD vs CD	
1-C Metabolism	*P-value*	*Fold-change*	*P-value*	*Fold-change*	*P - value*	*Fold-change*	*P value*	*Fold-change*
Dhfr	-	-	*0.70*	*-1.023*	-	-	*0.8*	*1.01*
Mthfr	*0.007*	*-1.15*	*0.07*	*1.079*	-	-	*0.2*	*-1.06*
Mat2a	-	-	*0.042*	*-1.05*	*0.0004*	*-1.1*	*0.3*	*-1.02*
Mat2b	-	-	*0.45*	*1.029*	-	-	*0.9*	*-1.00*
Achy	-	-	-	-	-	-	-	-
Mtr	*0.040*	*1.08*	*0.63*	*1.038*	-	-	*0.01*	*-1.10*

4.7.3 Placental histomorphology assessment

4.7.3.1 The impact of paternal diet on the morphological characteristics of the placenta

The mouse placenta is made up of three unique morphological layers, the decidua, the junctional (Jz) and the labyrinth zones (Lz). As the decidua originates from maternal uterine tissue, it was excluded from placental area measurement. The total area of the placenta is made up of the two layers of the Jz and the highly vascularized area of (Lz).

A.



B.

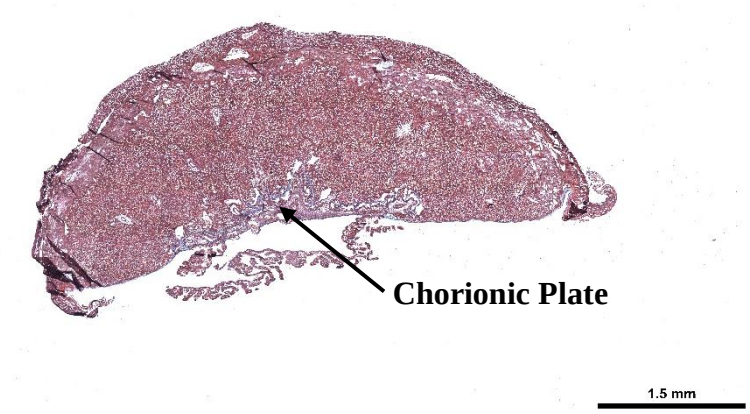


Figure 4-28 *Representative-stained placental sections. Highlighting the junctional, labyrinth and chorionic zones are shown as stained by periodic acid Schiff, from CD group at E17.5 gestation (A), and Masson's trichome, from CD group at E17.5 gestation (B).*

4.7.3.2 Late Gestation Placental Periodic Acid Schiff Staining Assessment in Response to Paternal Diet

The entire placental area was assessed without delineation of distinct zones. This presentation illustrates the proportion of staining relative to the total area examined. Overall, no significant changes in the whole placental cross-section area were observed in any treatment groups when compared to the control diet (CD) and each other (Figure 4-29 A). In contrast, Figure 4-29 4B presents the percentage of PAS-positive staining observed within various placental layers, accounting for the separation of placental layers, as well as whole placental areas, allowing for an analysis of the effects of paternal diets on placental composition and structure. No significant alterations in total, junctional, or labyrinth zone area were observed when compared to the CD, as well as the other groups (Figure 4-29 B). After further examination of the layer areas, no significant alterations in relative percentage of proportionally layers compared Jz to whole (Figure 4-29 C) Lz/ whole (Figure 4-29 D) and Jz/Lz were observed (Figure 4-29 E). However, LPD placentas demonstrate a reduction in % proportion of Jz/Lz ($P < 0.065$), (Figure 4-29 E).

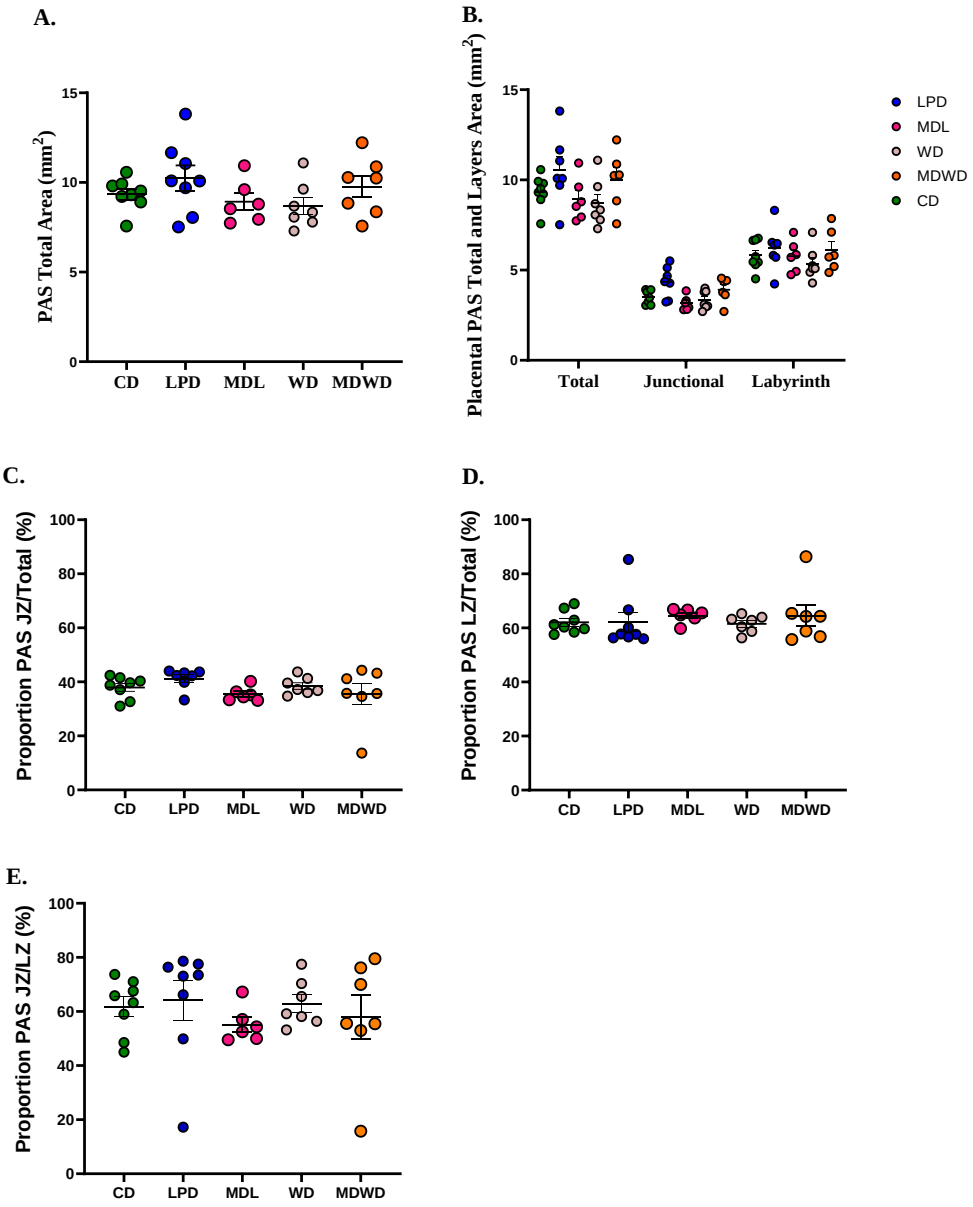


Figure 4-29 (A-E), presents data obtained from the analysis of PAS-stained placentas, specifically focusing on the junctional zone (JZ) and labyrinth zone (LZ) as depicted in (A). In (A), the entire placental area (total area) was measured. (B) displays the percentage of PAS-positive staining within different placental sections, presented as a proportion of the total area as well as different layers in response to paternal diets. (C) illustrates PAS-positive staining as a proportion of JZ/LZ, expressed as a percentage. Data were analyzed by D'Agostino & Pearson normality test was utelized followed by one-way ANOVA followed by Bonferroni posthoc test, or Kruskal–Wallis's test with Dunns multiple comparison tests where appropriate. Data are represented as mean ± SEM. Each data point represents an individual placenta (n=8).

4.7.3.3 Late Gestation Placental Masson's Trichrome staining assessment in response to paternal diet

Representative images of Masson's Trichrome (MT) stained placental sections, highlighting the junctional, labyrinth and chorionic zones are shown in (Figure 4-30 A). Overall, MDWD placentas had a significantly decreased chorionic placental cross-section area when compared to CD (P=0.0466), but no significant alterations were observed in LPD or MDL when compared to CD (Figure 4-30 A). WD and MDWD placentas did demonstrate a significant reduction in % of MT in the whole placental areas compared to CD respectively, (P = 0.0428), (P = 0.0420) (Figure 4-30 B). No differences in the % of MT staining in the junctional areas of placental sections were observed in any of the groups (Figure 4-30 C). MDWD placentas also demonstrated decreased areas in labyrinth zone compared to both CD (P = 0.0298) (Figure 4-30 D). The chorionic plate zone was not impacted by any of the paternal diets (Figure 4-30 E).

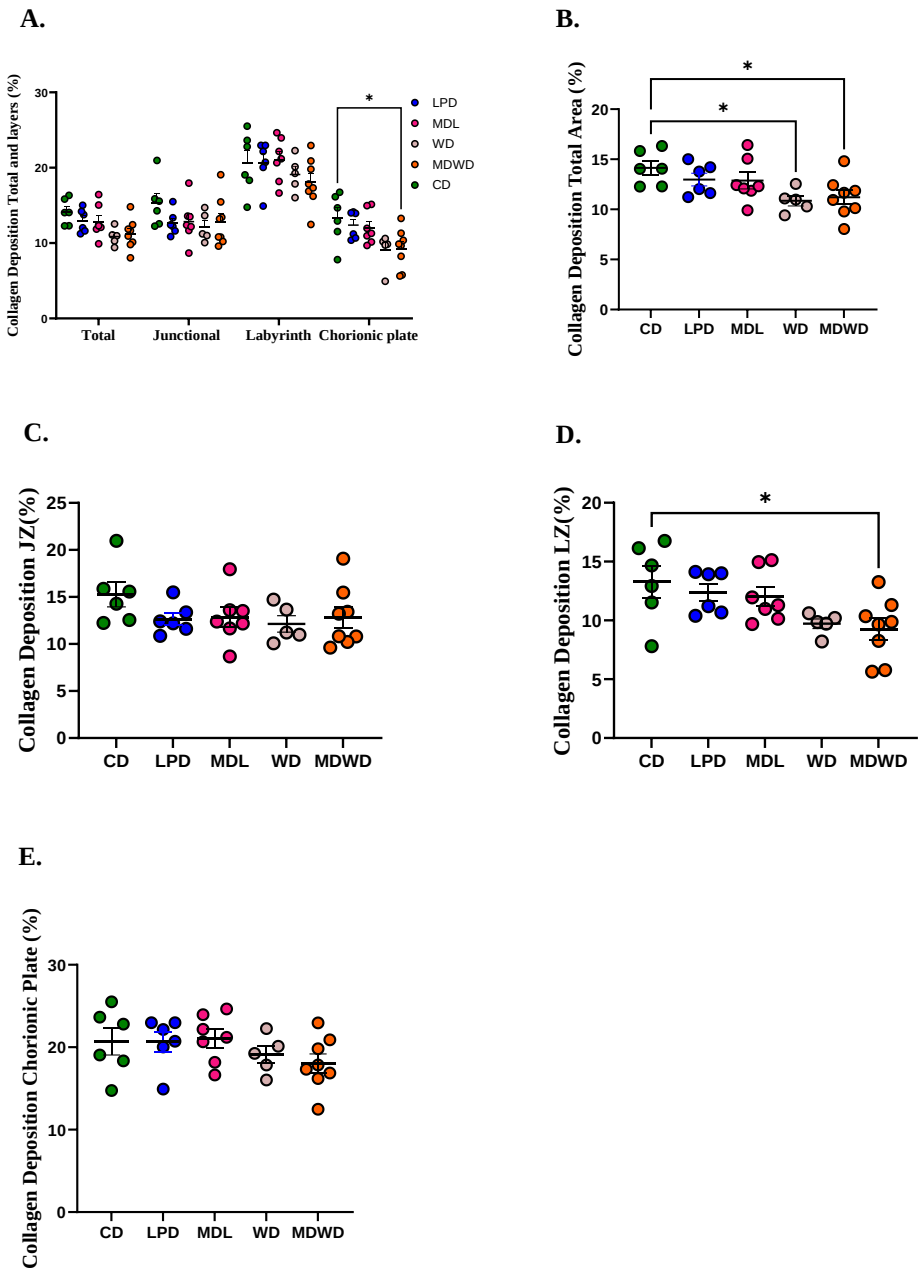


Figure 4-30 (A-E), the data obtained from analysis of the MT-stained placentas with junctional zone (Jz), labyrinth zone (Lz), and chorionic plate (CP), represented in Figure., (A-E). The whole placental area measured in pixels (A). The percentage of MT positive stating within placental different sections, displayed as a proportion of total area in response to paternal diets (B), and Figure C shows the positive PAS staining as proportion of Jz/Lz, expressed as percentage. Data graphed up using mean ± SEM. One-way ANOVA with Tukey post-hoc test, used for statistical analyses. Each data point shows a single placenta (n=8).

4.8 Discussion

The majority of existing studies that investigate the effect of diet intervention on offspring's cardiac developmental programming are primarily focused on maternal environmental manipulations. The well-established connection between disturbances in the maternal uterine environment and the subsequent development of cardiometabolic ill-health in her offspring has been documented (Batra et al., 2022). However, the implications of paternal diet on his offspring's cardiometabolic development and long-term health have remained largely neglected.

According to the Barker hypothesis proposed by Barker (1998), the increased risk of cardiometabolic illness later in life due to early life exposure to undernutrition has been well established. Fetal growth retardation, such as IUGR, in cases of maternal undernutrition, and fetal macrosomia in the case of maternal obesity or GDM, are both associated with CVD in the offspring. A 29% increased rate of early-onset CVD has been reported in babies (Ormazabal et al., 2022, Yu et al., 2019). Therefore, the adaptation mechanism is a logical mechanism that enhances fetal survival as it enables adaptation to adverse intrauterine conditions, thereby increasing the chances of survival despite suboptimal maternal nutrition.

This is because the developing fetus may undergo significant adaptations in response to the restricted nutrients, prioritizing nutrient availability for certain organs, such as neural system development, in sub-optimal intra-uterine conditions.

For example, in an unfavorable intrauterine environment, where brain development takes priority during fetal development, the effect of under nutrition has pronounced implications, which leaves substantial and conspicuous impacts on peripheral organ development such as the heart, liver and adipose tissue (Silva et al., 2019). As well as the cases of maternal obesity or diabetes such as predispose the fetus to macrosomia alongside short- and long-term consequences, which could be due to the brain sparing (Gu et al., 2012). However, animal models have revealed the detrimental implications of

perturbed paternal diet on fetal cardio-metabolic systems. For example, in a mouse model, a paternal low protein diet has been associated with hypotension as well as elevated heart rate in the offspring of LPD-fed males (Watkins and Sinclair, 2014a).

In the first part of these experiments, the gross morphological characteristics of the fetuses in response to paternal diet were studied. Examining any alterations in late gestational fetal growth, which mirrors birth weight, postnatal development, and the potential for adult disease risk, is crucial. Recent studies have shown the intriguing concept of intergenerational epigenetic effects caused by paternal nutrition (Li et al., 2022, Tiffon, 2018).

This study demonstrated that paternal diet had minimal impact on mean litter size or conceptus weight across different treatment groups when compared to CD. However, significant alterations in fetal, placenta, and yolk sac weight were observed across the different treatment groups, compared to CD and together, alongside alterations in their ratios with total fetal weight. A significant decrease in fetal weight in LPD and MDL was observed when compared to CD and WD. Therefore, it suggests that the supplementation of HFD diet does not necessarily reverse the negative effects of LPD and normalize the fetal weight in the MDL group.

No alteration in the size of litters derived from LPD-fed males was observed, which is in agreement with previous paternal LPD-fed studies in mice (Morgan et al., 2021, Watkins et al., 2018). Watkins et al. (2018), showed that the ratio of male to female offspring was decreased in favour of females, in response to paternal LPD. This could be a result of the alterations that LPD seminal plasma could exert on uterine environments by modulating the FRT cytokines, decreasing the uterine proinflammatory cytokines secretion. One potential reason to confirm the impact of diet on seminal plasma could be due to the alterations in motility and capacitation of sperm carrying X or Y chromosomes, or alternatively the LPD semen alters the uterine environment to be more favourable for implantation of the female embryos (Watkins and Sinclair, 2014a, Rosenfeld, 2011).

As well as LPD, the Western diet, often considered a poor-quality diet, did not result in any alterations in litter size when compared to CD. This indicates the WD did not influence the fertility of these stud males, as no changes in the number of offspring generated when compared to CD was observed. However, consumption of WD could still potentially impact the quality of sperm, potentially leading to alteration in post-ejaculation sperm maturation, such as capacitation and hyperactivation (Oliveira et al., 2017), as well as influencing the seminal plasma quality (Schjenken et al., 2021a). Larqué et al. (2023) showed that despite a significant reduction in sperm count and viability that HFD-fed males were capable of sireing offspring. No sperm count assessment has been conducted in our experiment to examine the effect of the WD on sperm count, however Larqué et al. (2023), has demonstrated perturbed reproductive parameters with no effect on fertility, in response to paternal HFD.

In contrast, there are studies reported the detrimental effects of paternal HFD on litter size and offspring numbers, which are not in accordance with our observations (Ghanayem et al., 2010, Bakos et al., 2011a). The fat percentage and the fat type consumed in our experimental model, in addition to the period of being on the experimental diets, differ from other studies that have reported the negative impact of the paternal HFD on litter size. There are reports which suggest keeping animals on HFD for a longer period (6 months) could drastically diminish their mating success and, consequently, litter size (Crean and Senior, 2019).

The central role of poor-quality sperm in the rate of implantation and embryo development has been documented. For instance, increased paternal BMI has been associated with decreases in the rate of blastocyst development, clinical pregnancy rates and live birth outcomes following IVF and infertility treatments (Bakos et al., 2011a). On the other hand, studies report no association between paternal BMI, sperm quality and pregnancy outcomes in humans (Jensen et al., 2004, Fejes et al., 2005). Therefore, more investigation is needed to define fully the impact of a paternal high fat diet on sperm quality and fertilization potential.

Conceptus weight is a combination of fetal, placenta and yolk sac weights (Noakes, 2009). No changes in conceptus weight were observed in response to paternal diet in this study, consistent with findings from other paternal studies. A paternal LPD reported no alterations in the conceptus weights derived from LPD compared to CD (Watkins et al., 2017).

The current study revealed a significant decrease in fetal weight derived from LPD-fed fathers compared to CD. Decreased fetal growth is a substantial risk factor for adult health, not only in the prenatal period but also, later in life (Ratnasiri et al., 2018). However, maternal studies have related this reduction in weight gain *in utero* due to imbalanced placental blood supply or inter-fetal blood transmission as a result of multiple births for an instance. Both of these factors result in inadequate oxygen and nutrient supply for the growing fetuses (Liu et al., 2019). The observed decrease in LPD fetal weight could be related to the decreased activity of placental 11 β-hydroxysteroid dehydrogenase (11 β-HSD), which serves to protect the fetus against being over-exposed to the maternal cortisol and increase the exposure of the growing fetuses to maternal cortisol. This reduction in 11 β-HSD may lead to increased fetal exposure to maternal cortisol, which has an adverse effects on fetal growth by inhibiting the fetal protein synthesis (Wu et al., 2012). Of note, no assessment regarding the cortisol assessment or any other pro-inflammatory factors was conducted in the present experiment. Additionally, the low protein fed diet in pregnancy in mice has been associated with decreases in the expression of central metabolic pathways gene expression such as *PI3K/PKB/mTOR/NO* signaling pathway which negatively impacts on blastocyst implantation and metabolism, could justify the reasons of the retarded fetal growth and development (Zeng et al., 2008). Interestingly, in mice paternal LPD resulted in the down regulation of mTOR and AMPK signaling in the blastocysts sired by LPD fathers. This reduction is due to decreases in the expression of genes such as adiponectin receptor (*Adipor1),* the facilitated glucose transporter 4 (*Slc2a4)* and the energy sensing non-catalytic subunit of *AMPK (Prkab1)* involved in the central cellular metabolic AMPK pathways in these blastocysts (Watkins et al., 2017). Additionally, paternal LPD has been related to sustaining mTOR pathway activity and consequently cell growth (da Cruz et al., 2018a).

The decreased in fetal weight sired by LPD fathers in this thesis, is aligned with a study in male mice fed undernutrition with a global reduction in food intake, which led to a decrease in fetal weight due to lower consumption of protein driven by an alteration in fat accumulation and dyslipidemia. As well as an alteration in pancreas gene expression in their offspring. However, increases in weight gain in these offspring were reported later in their lives (McPherson et al., 2016). Indeed, in a mouse model, paternal LPD has been related to differential expression of specific miRNAs (miR-200c, miR-92a, and miR-451a), in the female offspring. These miRNAs modulate the expression of AMPK pathways known as energy-sensing pathways, in the female offspring of these fathers with mammary gland tumors, due to amino acid metabolism, leading to an increase in the level of glutamate amino acid, which suggest that tumor cells results from low protein diet demonstrates a higher level of consumption glutamine (da Cruz et al., 2018b). These findings are in contrast with the obtained results from paternal LPD-fed male mice, showing an increased in the offspring weigh, and interestingly they have reported a slower pattern of growth with less weight gain compared to the offspring sired from CD-fed fathers later in life (Watkins and Sinclair, 2014a, Watkins et al., 2017). In addition, the results of our study noted a significant increase in the weight of fetuses derived from WD males compared to LPD and MDL, with no observed significant changes when compared to CD. Of note, no significant alteration in fetal weight derived from MDWD-fed males was observed which could suggest methyl donor supplementation can normalize fetal growth. The reported observation also indicated a significant increase in fetal weights, by modulating the glucose metabolite in the uterus of mice mated with LPD fed males (Watkins et al., 2017). However, obtained results from this study regarding WD group is consistent with the decreased fetal weights in paternal high fat diet fed males (Claycombe-Larson et al., 2020).

In contrast, other experiments investigating the role of paternal diet have reported a significant decrease in LPD and MDL placentas when compared to CD placentas (Morgan et al., 2021). Therefore, it could be suggested that supplementation of the suboptimal diets with methyl donors has not been effective in reversing the adverse effects of the under/overnutrition diets on their

fetal growth characteristics. The lack of changes in placental weight in the WD group compared to the CD and other groups aligns with a study conducted on a paternal HFD in a mouse model. In this study, an increase in the surface area of the placenta in the HFD group was proposed as a reason for observing no changes in placental weight compared to the CD group, as placental weight does not describe the thickness or surface area (Barker et al., 2010). This increase in surface area was suggested to act as a protective mechanism against the negative effects of placental hypoxia. As blood vessel immaturity and hypoxia have been reported in placentas derived from paternal HFD (Pépin et al., 2022) and maternal HFD (Gohir et al., 2019). WD can induce the production of ROS in sperm due to sperm mitochondrial dysfunctions (Pereira et al., 2020). In this context, the paternal diet has minimal impact on male fertility.

One of the notable observations in this analysis was the contrasting patterns observed between fetal and placental weight in the LPD group, considering the significant decrease in fetal weight compared to CD, however, no significant changes in LPD placental weight were observed. Therefore, these conflicting results between the fetal and placental weight could be attributed to the fact that changes in fetal weight may not be immediately reflected in placental weight measurements at the time of assessment. Furthermore, it is essential to consider that the fetus and placenta exhibit independent growth processes (Roland et al., 2014).

The placenta, with its independent vascular network, can adapt and contribute to overall growth even if there has been a temporary halt in fetal growth (Malhotra et al., 2019). Alternatively, decreased in fetal weight sired from LPD-fed fathers could be a consequence the dam's uterine environmental response to the LPD-fed studs semen cytokines or perturbed blastocyst metabolic signaling, in addition on increased in the expression of mRNA of genes involved in the placental nutrient transportation including glucose (*Slc2a1*, *Slc2a4*), neutral amino acids (*Slc38a2*) and calcium (*Atp2b1*). Increased in the expression of these genes has been reported in perturbed pregnancies leading to fetal macrosomia, such as cases in women with type-1 diabetes (Watkins et al., 2017). No significant differences between the LPD vs MDL and WD vs MDWD

placental weight were observed, suggesting the supplementation of the suboptimal groups did not influence the placental weight.

The fetal weight/placental weight ratio was examined as an indication of placental efficiency. The feto-placental examining revealed a significant reduction in LPD when compared to WD, with decreased in fetal weight and no changes in placental weight. Feto-placental measurement of WD represented a significant increase compared to all the group. WD represented a significant increase in fetal weight when compared to LPD and MDL, and no changes compared to CD, as well as no changes in WD placental weight were observed when compared to CD and other groups. No changes in placental weight are considered as a proxy for placental efficiency. Maternal mice study with the same strain of mice demonstrated the fetoplacental weight is lower in HFD (Lager et al., 2014), which is in accordance with data derived from overweight or obese pregnant women (Wallace et al., 2012). However, the opposite pattern has been observed in our experiment. The obtained results are not in line with other paternal suboptimal diet studies which investigated the impact of paternal LPD on placental weight and feto-placental ratios in mice (Morgan et al., 2021). It could suggest, the period of male mice exposure to the suboptimal diet might have resulted in these differences.

Studies on parental HFD showed decreases in placental weight and fetal weight consequently in response to paternal HFD. An animal study conducted by Claycombe-Larson et al. (2020), showed, the reduction in placental expression of the *Slc38a2* amino acid transporter gene, known as *SNAT2*, expression of this gene in the placenta has been associated with paternal HFD and its influences placental metabolism. The same study also showed a potential reason for that could be related to the sperm miRNA and more specifically increase in the expression of sperm miRNA204 in response to environmental stress. Meanwhile, paternal HFD induced a decrease in sperm H3K9 protein expression, H3K9 is associated with adiponectin promoter region in adipose tissues, which could potentially impacts on offspring metabolic health (Claycombe-Larson et al., 2020). H3k9me3 and H3K27me3 have been associated with chromatin compaction and repression of transcriptional

modulators, which leads to dynamic and incorrect alterations in the structure of chromatin and its function, resulting in aberrant gene expression (Chung et al., 2015, Zhang et al., 2017a). Therefore, it means H3K4me3 in sperm plays a role in the transgenerational transfer of non-genetic characteristics. As sperm H3K9/H3K27 me3 dependent reprogramming of the metabolic genes is essential in the zygotic period (Öst et al., 2014).

It is plausible that paternal HFD increases the induction of the expression of placental IL-1β and TNF-α, which could potentially negatively impact nutrient transporter protein expression and activity (Jazwiec et al., 2022). No alteration in the weight of the placenta from WD in our study was observed, this may be the result of the fat percentages employed in the WD experiment model, and the short duration of WD exposure.

A significant increase in MDWD placental weight when compared to MDL was observed in response to the paternal diet. A study on male mice showed in a paternal obesity mouse model that a paternal high-fat diet had no impact on fetal or placental weights (Mitchell et al., 2011). In contrast, Binder et al, 2015, reported paternal diet-induced obesity led to a reduction in fetal and placental weight mediated through alterations in metabolic and cell signaling stress pathways (Binder et al., 2015b, Fullston et al., 2015). Spermatogenesis involves a remarkable shift to a different form of chromatin. This is necessary for adequate sperm motility and involves the replacement of nucleosomal histones with basic protamine's, resulting in a 10-fold greater compaction of DNA. It is believed that some histones (between 1-10%) are still retained close to the DNA and could be transferred to the next generation of his offspring (Carone et al., 2014, Erkek et al., 2013). Gametogenesis and embryogenesis cause substantial reprogramming of the genome, but certain areas referred to as imprinted domains must be actively protected by an epigenetic status. These areas are very vulnerable to epigenetic changes and disruptions Denomme et al. (2020), demonstrated paternal diet-induced obesity resulted in a significant decreased in placental weight, and placental total length. Sperm epigenetic intimately associated with the expression of the imprinted genes such as *Igf2, Igf2r, Peg3, Cdkn1c, H19* that are well known in placental development

(Jazwiec et al., 2022, Thomas et al., 2021). A paternal low protein diet-fed model in mouse, displays abnormal expression of various imprinted genes such as *Cdkn1c*, *Grb10*, *H19*, *Mest*, and *Snrpn* in placentas derived from LPD fetuses (Watkins et al., 2017, Morgan et al., 2020). However, no assessment of the imprinted gene expression was conducted on the placental samples.

The yolk sac, which evolved in fetuses to collect nutrients from their lipid-rich eggs, is phylogenetically the earliest of the extraembryonic membranes (Cindrova-Davies et al., 2017, Elmore et al., 2022). It originates from the inner cell mass of the blastocyst and appears to play a crucial role in modifying the primary pathways of embryonic development and fetal nutrition until it ceases to function. This transition occurs by the time the placenta takes over the major role, typically by the end of the first trimester in women, and with a fully functioning placenta observed at E12.5 with the maximum weight in mice, known as the definitive placenta (Elmore et al., 2022).

Nutrients are digested in a specific area of the yolk sac called the visceral yolk sac, where the function of micro and macronutrient transportation through active pinocytosis occurs. Consequently, the digested and degraded products, such as maternal sources of amino acids, are delivered to the embryo (Ornoy and Miller, 2023). Intriguingly, a significant portion of amino acid transportation to the growing fetus occurs even in late gestation through the yolk sac. (Beckman et al., 1998, Watkins et al., 2008). Furthermore, a larger yolk sac size has been associated with fetal loss, serving as a strong indicator of miscarriage in women(Detti et al., 2020, Boldeanu et al., 2019, Marin et al., 2021).

The analysis of fetal heart physiological alterations in response to paternal diet revealed no significant alteration in the mean weight of fetal hearts and fetal/heart ratio across the different treatment groups when compared to CD and each other. The obtained data are aligned with different animal model experiments findings (Watkins et al., 2017, Morgan et al., 2020b, Morgan et al., 2021, Furse et al., 2023). Maternal studies have reported a significant decrease in fetal heart weight from mothers fed high fat diets comprising more than 30% fat and could be the result of compromised cardiomyocyte development in a sex

specific manner (Wang et al., 2022). However, analysis of fetal weight revealed a significant reduction in the weight of fetuses sired by MDL and WD fed males when compared to CD. Studies have shown that both paternal under- (LPD) and over- (WD) nutrition have significant influences on adult offspring cardiovascular metabolic health (Watkins and Sinclair, 2014a). Furthermore, studies have shown that addition of methyl donors, either alone or to these diets, affects aspects of sperm epigenetic status and adult offspring development (Tiffon, 2018). While the long-term consequences of these diets on cardio-metabolic health have been defined, the time in development at which these changes are initiated remains unknown.

The key findings of the second part of this set of experiments investigated a set of fetal cardiac physiological and metabolic pathways whose expression was altered in response to paternal diet, in late gestation. Several studies in human (Hoy et al., 2006), and experiments in animal models (Langley-Evans et al., 1999, Woods et al., 2005) have reported an association between maternal undernutrition diets and cardiovascular and renal deficits in the offspring, later in life (Wood-Bradley et al., 2015, Lee et al., 2018).

These results revealed paternal diet has a wide range of effects on the expression of genes involved in the metabolism and morphological characteristics of cardiomyocytes in fetal hearts, sired from males fed different diets. Affecting the expression of multiple genes involved in metabolic/metabolism pathways, CVD, angiogenesis, cardiovascular morphology and relevant signaling cascades such as ERK1/2, epithelial tube morphogenesis, and cofactor metabolic process. In addition, LPD and WD had differential effects on the direction and magnitude of these gene expression changes, especially with respect to CVD pathways. Finally, while the LPD methyl donors' supplementations diminished the size effect of the LPD diet on differential fetal cardiac gene expression, no such effect was observed in the MDWD hearts when compared to WD hearts.

Previous studies have shown that the effect of paternal low protein diet on the offspring hearts are associated with disrupted testes 1-carbon metabolism pathways and sperm DNA hypomethylation in males (Watkins et al., 2018).

Consequently, the addition of methyl donor supplements was intended to determine whether this addition could counteract the negative alterations in the sperm epigenetic status and negate the negative impacts of the under/over nutrition diets. The analysis of DEGs revealed the largest difference occurred between LPD and CD hearts (4282 genes), while the smallest difference was between MDL and CD (2318 genes). Interestingly, CD vs WD and CD vs MDWD hearts displayed a similar number of differentially expressed genes to the CD vs LPD group.

Paternal LPD, reduced fetal heart expression of genes involved in central metabolic pathways such as carbon metabolism and biosynthesis of amino acids. Furthermore, analysis of the GO output revealed a significant downregulation of genes involved in central pathways responsible for the proliferation, differentiation and survival of cardiomyocytes such as *Notch, Erb, Akt1, Nkx2.5, Foxo1, and C-myc*. In addition to the significant reduction in the pathways associated with metabolism, there was also a notable decrease in the expression of genes involved in cell cycle regulation or pro-mitotic regulation such as *Cdk1* and *Cyclin A*, in fetal hearts derived form LPD-fed fathers. This observation was also seen in the fetal hearts derived from MDL-fed fathers. Indeed, *Cdks* are the key regulators genes for the growth of cardiomyocytes (Wagner et al., 2008). Blockage of the Notch-1 in a new born mice model has been followed by promoting the dilated cardiomyopathy coupled by high mortality rates (Urbanek et al., 2010, Watanabe et al., 2006). Interestingly, knocking out of the Notch-1 gene in fetal mice has been associated with repressing the mesodermal markers encompasses *GATA4, Nkx-2.5,* or α- and *β-MHC* (Nemir et al., 2006).

A significant down regulation of ERK1/2 pathways was observed in LPD hearts. The ERK1/2 pathway is an essential subfamily of mitogen-activated protein kinases (MAPK), playing a central role in cardiomyocyte physiology and function, cell survival, differentiation and apoptosis (Rose et al., 2010). In addition, dysregulation in the balance of ERK1/2 signaling has been shown in Atrial Septal Defect (ASD), which is a type of congenial heart disease (CHD) (Yeh et al., 2017).

On the other hand, down-regulation of the ERK1/2 pathways, and extra cellular signal-regulated kinase is a central mediator of size and shape in cardiomyocytes could results in a reduction in cardiomyocyte cells size which could possibly impact on cardiac contractility which would ultimately compromise the systolic phases of the cardiac cycle (Alharbi et al., 2022). Such changes could potentially result in diminishing cardiac parasympathetic stimulation, resulting in reduction in baroreflex constraint (Lohmeier and Iliescu, 2011). The underlying mechanisms resulting in alteration in the expression of ERK1/2 pathways have been related to the imbalance in activities of PKC- and PKA-mediated pathways, including ERK1/2, resulting in cardiomyocyte hypertrophy and electric cardiac remodelling, leads in changes in sympathetic tone and ultimately, resulting in hypertension in adulthood (Silva et al., 2014). The observed changes in ERK1/2 pathways suggest these alterations potentially could result in hypotension in these animals. This finding is inconsistent with results obtained from paternal LPD studies, which report hypotension in these fathers offspring later in life (Watkins and Sinclair, 2014a). The expected hypertension resultants of alterations in ERK1/2 pathways is attributed to the impairment of parasympathetic stimulation of cardiac tissues and the diminishing of baroreflex constrain (Bugenhagen et al., 2010).

Down-regulation of ERK1/2 has been associated with losing the structural support of the ECM, leading to abnormal tissue architecture and eventually altered cardiac remodeling (Liu et al., 2016). Downregulation of this pathway disturbs the balance between extracellular matrix synthesis and degradation, in addition to increased apoptosis in fetal cardiac tissue, resulting in abnormal remodeling of cardiac tissue and increased fibrosis (Sugiura et al., 2021). Moreover, involvement of the ERK1/2 pathway is essential for normal cardiac tissue angiogenesis , and so disruption of this pathway could result in cardiac ischemia (Rakhshan et al., 2022). Additionally, over expression of the ERK1/2 in cardiomyocytes results in cardiac muscle hypertrophy, thickening and stiffness and cardiac arrhythmia (Lian et al., 2015). Furthermore, over expression of this pathways in cardiac fibroblasts, results in fibrosis, highlighting that the outcomes of differential pathway expression can vary based on the specific cell type analyzed (Alharbi et al., 2022).

Paternal LPD also caused a significant down-regulation of genes associated with glucose transport (*Slca2)* and insulin signaling (*Insr)*, in line with significant down-regulation in genes involved in amino acid metabolism such as *Shmt1, Got2, Pfkm in* addition to 23 other genes in this pathway. Therefore, it could suggest that parental diets program the cardiomyocytes transcriptomic profile of their progeny.

Notably, in LPD hearts alterations in the expression of several central regulatory metabolic genes such as *Akt1, Hk1* and *Insr*, were identified. Down regulation of these genes, which are involved in key cell metabolism pathways, could have detrimental effects on these fetal hearts. Specially, the reduced expression of these central genes, such as *Insr* may lead to diminished glucose uptake by these hearts through the deactivation of PI3K enzyme (Bertrand et al., 2008), via GLUT4 translocations , resulting in impaired energy supply to cardiomyocytes (Mora et al., 2004) could indicate a deficiency in the growth of cardiac muscles and ultimately metabolic dysregulations in these hearts (Guo and Guo, 2017). Moreover, alterations in cardiomyocyte characteristics have been reported in response to insulin resistance in knockout insulin receptor mouse models. These changes include reductions in cardiomyocyte size, shifts towards increased glycolysis, decreased fatty acid oxidation, and the persistent expression of fetal myosin heavy chain isoforms, all of which collectively define characteristics of an immature heart (Belke et al., 2002). However, no morphological assessment in fetal hearts derived from LPD-fed fathers have been conducted to follow up the potential morphological alterations in response to paternal diet. Such metabolic dysregulation could potentially predict a plausible risk of permanent cardiovascular dysfunctions and disease later in life for these offspring. As well as the potential occurrence of the cardiac insulin resistance, a critical risk factor for cardiac failure (Tan et al., 2011).

Interestingly numerous differentially expressed genes associated with phenotypic pathways were related to a wide range of cardiovascular abnormalities. In LPD hearts downregulation of the *Insr, Akt, FoxO* and glucose metabolism pathways. Downregulation of *FoxO* gene expression in cardiomyocytes has been associated with cardiomyocyte differentiation and

function, which aligns with the down regulation of PI3K/AKT as its central regulator (Ronnebaum and Patterson, 2010). Down regulation of genes involved in *FoxO* could result in dysregulation of the cardiac contractility, increase the oxidative stress and damage to the cardiomyocytes and overall affecting cardiac development and growth considering the down regulation of *Akt* and its crucial role in cell proliferation and growth (Liu et al., 2007). Down-regulation of *FOXO1* gene was reported in cardiomyocytes of patients with type 2 diabetes, which could suggest that downregulation of these genes associated with metabolic stress in cardiomyocyte which leads in activation of FoxO1. The activation of FoxO1 has a central role in aberrant modulation of the metabolic enzymes and energy dependent of cardiac myocytes leading to diabetic cardiomyopathy (DCM) (Kandula et al., 2016).

Preliminary WebGestalt analysis demonstrated a significant downregulation of genes involved widely in metabolism such as metabolic pathways, carbon metabolism and biosynthesis of amino acids. Moreover, genes associated with the structural and organizational aspects of heart development, which influence the functioning and metabolism of cardiomyocytes in fetal hearts from LPD-fed males, were also found to be downregulated. These findings potentially suggest that both metabolic and functional deficits might occur in the hearts of LPD-fed fathers' offspring and may predispose the offspring to chronic cardiovascular disease and dysfunctions later in life (Muroya et al., 2021, Sun et al., 2016).

One of the significantly down-regulated genes in fetal hearts derived form LPD-fed fathers is *Nos.* Disruption in the function of this gene is associated with impairment of endothelium-dependent relaxation, which is further linked to hypertension and hypercholesterolemia (Abe and Berk, 2014). The down regulation of this gene in these fetal hearts may have the potential to predispose individuals to atherosclerosis later in life, and perhaps hypotension. This is because *NO* is associated with numerous intracellular. Therefore the down regulation in the expression of *No* results in vasorelaxation and endothelial regeneration (Napoli et al., 2006).

A significant downregulation in phenotype pathways including genes involved in cardiovascular system phenotype, *RAS* family pathway and insulin receptors were observed. The downregulation of these genes has been attributed to the modulation of genes involved in vascular smooth muscle contractions (VSMC) through the activation of the *NF-κB* (Kranzhöfer et al., 1999). This suggests that hypotension may have a potential impact on the health of these offspring later in life. Further monitoring of the cardiovascular status of these fetuses will be essential to a better understanding of the implications.

Additionally, as significant downregulation of the anion transmembrane transport pathway in LPD hearts was observed, this pathway included several genes involved in solute carrier families (*SLCs)*, which play a central role both in cardiomyocytes in terms of maturation as it could imply a role in transduction of fatty acids metabolism and vascular smooth muscle cells contraction. Down regulation of *SLC* family genes has been reported in cardiac dysfunctions through aberrant alterations cardiomyocyte transcriptional profile, morphology, Ca2+ handling activity, and contractility (Hewton et al., 2021). In the smooth muscle cells of vessels, transporter genes belonging to the SLC family are associated with the modulation of vascular smooth muscle cells (VSMCs). Downregulation of the SLC1A5 gene has been linked to significant attenuation of the TEA domain (TEAD), a transcription factor, mediated by the activation of mTORC1 signaling (Osman et al., 2019). This downregulation of *Slc* genes was observed not only in hearts affected by a low-protein diet (LPD) but also in hearts affected by a moderate-fat diet (MDL).

The precise timing of alteration in cardiomyocytes is various but it is clear that the fetal heart is one of the first organs to develop (Tan and Lewandowski, 2020). Therefore, the development of this organ could be influenced by alterations in the intrauterine environment in response to paternal seminal fluid. It has been reported that seminal plasma from LPD-fed fathers manipulate the intrauterine environment through blunting uterine cytokines in line with reductions in the level of cytokines as such, TNF, IL-1β, CSF3, MIP-1α, and IFN-γ which could potentially impact on the uterine spiral arteries remodeling, secretion of embryotropic modulators and finally the uterine immune system responses

(Watkins et al., 2018). The impact of paternal LPD disturbed blastocyst implantation through a reduction in intercellular levels of ATP as well as reduction in AMPK pathway gene expression, as well as adversely effect on the modulation of the *Adipor1* which plays a central role in glucose transportation and insulin receptors expression (Watkins et al., 2017). The role of paternal LPD in fetal programming and development has also been documented. Paternal LPD resulted in increased fetal and placental weight during late gestation, along with alterations in the relative layers of the placenta(Morgan et al., 2021). In a mouse model, paternal LPD was shown to influence fetal heart cardiovascular function, leading to relative hypotension and increased heart rate in offspring (Watkins and Sinclair, 2014a). The results of fetal cardiac gene expression in response to paternal diet strongly suggest the role of paternal LPD in offspring heart function. These molecular findings are consistent with observed alterations in cardiac morphology and indications of impaired cardiomyocyte metabolism. However, a notable distinction is that these pathways did not exhibit significant expression levels in the MDL hearts. This suggests that the addition of methyl donor groups to the paternal undernutrition diet (LPD) may have mitigated the negative effects of paternal LPD in these hearts. The mechanism underlying these differences remains currently unknown and requires further investigation.

Interestingly, the addition of the methyl donor group to the suboptimal low-protein diet revealed a decrease in the effect size of the cardiovascular disease's pathways in the fetal hearts derived from the MDL-fed fathers, as the changes in MDL hearts are no longer significant in terms of the FDR. These results are in line with another under nutrition study in a male mouse model fed 70% caloric restriction, designed to resemble the nutritional environments in developing countries. Supplementation of this undernutrition diet with vitamins and antioxidants in stud males negated the negative effects of reduced offspring weight, dyslipidemia adipose accumulation and altered pancreas gene expression, which could be due to the normalization of sperm epigenetic status in these males (McPherson et al., 2016).

The results from fetal hearts sired by LPD-fed males revealed a significant downregulation of pathways involved in carbon metabolism, which plays a

crucial role in fetal cardiovascular development during early stages (Korsmo and Jiang, 2021). It is well-documented that the addition or deficiency of methyl donor supplements can permanently alter the epigenetic status of genes involved in non-communicable diseases (Oikawa et al., 2020). The central, epigenetic mechanism involved in the programming of the phenotypic plasticity are microRNA (noncoding RNA), covalent histone modification in addition of DNA methylation (Randunu and Bertolo, 2020). Thus, the addition of the methyl donors to this sub-optimal low protein diet might have compensated for the deficits caused by the low protein diet in the epigenome of the sperms from LPD-fed fathers. The addition of methyl donor groups to the male mice diet, modulates the DNA, RNA methylation and histone modification as well as the sncRNA (Dimofski et al., 2021, Chleilat et al., 2021b). Since methylation processes around histones and gene promotor regions might influence transcription regulation, blocking transcription factors from reaching the promotor regions would have an impact on the transcription of genes. For example, a paternal LPD diet in mice led to alterations in the spermatogonia gene expression in testes as LPD may affect the sperm's maturational tRNA fragments composition, which controls the gene expression process (Yoshida et al., 2020, Sharma et al., 2016b). Furthermore, there is evidence that paternal-rich methyl donor diets can negatively impact offspring metabolism as well as the neurocognitive status (Ryan et al., 2018). So, any imbalance in the level of methyl donor groups is accompanied by adverse effects on offspring.

Low paternal dietary folate has been linked to sperm epigenetic transmissions, including histone H3 methylation and DNA methylation (Lambrot et al., 2013b). A low protein diet induces sperm DNA hypomethylation and downregulates 1-carbon metabolism pathways in the testes of males fed with a LPD (Watkins et al., 2018). Results from studying the expression of 1-carbon metabolism genes and enzymes in the testes of male mice fed a low protein diet revealed the disrupted expression of genes involved in carbon metabolism pathways such as *Dhfr*, *Mthfr*, and *Mtr* as well as DNA methyltransferases *Dnmt1* and *Dnmt3L* (Watkins et al., 2018). Therefore, the finding regarding the down regulation of the genes involves in the subgroup pathway 1-carbon metabolism in fetal hearts from LPD-fed diet fathers are in line with the previous studies on testis gene

expression data derived from LPD-fed male mice, as well placental 1-Carbon metabolism genes. The transgenerational effects of paternal diet on sperm small RNAs such as sperm small-tRNAs and manifestation of altered phenotype in their offsprings perhaps be able to justify this effect (Sharma et al., 2016b, Huypens et al., 2016, Grandjean et al., 2015).

There are several studies assessing the influence of a maternal high fat diet on fetal cardiovascular development, including studies in different mammalian models. For instance, the non-human primate model such as the baboon (Maloyan et al., 2013). These studies have extensively examined the association between maternal overnutrition and fetal heart disruptions, discussing the dysregulation of mRNA and miRNA expression involved in cardiomyocytes and vascular endothelium development. This could potentially suggest paternal programming operates through distinct mechanisms that are separate from those induced by maternal HFD. The results obtained from analyses of fetal hearts sired from WD-fed males revealed, dysfunctional transcriptomic regulation and down regulation of genes in central transcriptomic regulation pathways. In WD fetal hearts, all genes related to CV structure and function are upregulated, whereas we have seen that it is the opposite in LPD hearts,

On the other hand, in WD hearts a significant downregulation in the pathways such as mRNA processing, DNA repair and non-coding RNA metabolic process were observed, and we know that sperm ncRNA genes significantly influence post fertilization embryo development (Le Blévec et al., 2020). Moreover, in the WD heart, the downregulation of genes involved in the DNA repair pathway goes hand in hand with the upregulation of genes for angiogenesis and cancer. A study on maternal HFD in rats, showed that maternal HFD could modulate cardiac transcriptome remodeling as well as the cardiac metabolism (Preston et al., 2020). Interestingly, a significant down-regulation in PI3K/AKT pathways in HFD-fed mother's newborn hearts has been reported (Preston et al., 2020), which is in contrast with obtained observations from my research in WD hearts, as a significant upregulation in MAPK signaling, and PI3K/AKT pathways were observed. This suggests both maternal and paternal diet could imply changes on the offspring's cardiac gene expression however in an opposite direction.

Overexpression of the PI3K/AKT pathway has a central role in the modulation of pathogenesis, such as heart disease, by induction of regulating roles in survival and the size of cardiomyocytes, angiogenesis and inflammatory responses (Ghafouri-Fard et al., 2022). Therefore, over expression of this pathway in WD and MDWD hearts, could lead to cardiovascular disorders such as hypertrophy in cardiomyocytes and excessive angiogenesis which potentially results in abnormal vessel growth and remodeling (Oka et al., 2014, Shiojima et al., 2005). As well as, over expression in the pathway of PI3K/AKT has been attributed to CVD, metabolic dysfunctions such as insulin resistance and diabetes by interfering with insulin signaling and glucose metabolism, which could be seen in the cases of diabetic cardiomyopathy, by alteration in cardiac energy utilization and contractile functions (Fortunato, 2012).

Another significant finding from WD hears in comparison with CD samples revealed significant upregulation of genes associated with angiogenesis, however, some of these genes such as *Adgrb2, Mmp2, and Notch1* (Involved in angiogenesis; negatively regulate endothelial cell proliferation and migration of angiogenic sprouting), such as *Fn1* (Swaminathan et al., 2022). It is known that the *Fn1* gene governs the production of two types of the fibronectin-1 protein: soluble plasma fibronectin-1 and insoluble cellular fibronectin-1 and it has a significant role in blood clotting and wound healing, forkhead box family such as *Foxc1, Foxc2, Foxj2, Foxo4,* were found to be involved in angiogenesis inhibition (To and Midwood, 2011). In addition, changes in glycerolipid metabolism and pathways observed in the WD group also go hand in hand with cardiovascular changes.

Indeed, the underlying mechanisms of these alterations in cardiac gene expression have not been fully defined. However, alterations in metabolic environment of fetuses might direct significant alterations in cardiac gene expression in these offspring. For example, enhanced levels of central growth modulators such as increased levels of fetal insulin, growth factors metabolites, might lead in an increase in the expression of central PI3K/AKT pathway genes (Díaz del Moral et al., 2021). Which potentially suggests that the metabolic status of these fetuses could be altered in response to paternal WD. However, to

define and confirm the hypothesized potential cardiovascular deficit in these fetal heart further assessments are required.

Observations in examining, these offspring's blood pressure, electrocardiogram (ECG) assessment to test any aberrant changes in the cardiac cycles, as well as some metabolic profile assessments, including checking blood glucose level, glucose tolerance, insulin resistance as well as checking the liver enzymes, and lipid metabolism profile, could be very insightful to better understand the underlying mechanism in these hearts.

Studying, the addition of methyl donor groups to the WD does not change the effect size of negative impacts of the WD on these fetal hearts. However, when the methyl donors were added to the LPD, many of the pathways which had been altered by the LPD were no longer significant in extent of their enrichment.

The other repeating and common downregulated pathway was anion transmembrane transport, which was observed to be significantly downregulated in LPD vs CD, MDL vs LPD, and non-significant in MDL vs CD. Some of the very important genes in this pathway such as families of *Slca, Slcb* and *Clcn* were expressed. Genes involved in this pathway have crucial roles in the modulation of electrical activity, pH, cell volume and the transfer of osmolyte and metabolite levels. As well as the regulation of immune responses, cell migration, cell proliferation, and cell differentiation are all activities that anion transport proteins perform in mammalian cells (Hume et al., 2000). Any deficit of these genes has been related to heart failure (DUAN et al., 2005).

The precise mechanism of how paternal under- (LPD) and over- (WD) nutrition induces such significant changes in the transcriptomic profiles of their offspring's cardiomyocytes remains relatively ill-defined. Moreover, these alterations in fetal hearts do not appear to be a response to disturb maternal metabolic conditions. In the previous chapter of this thesis, observations revealed no notable changes in maternal glucose, insulin or lipid profiles. Nevertheless, it is evident that these epigenetic modifications in the offspring stem from their fathers at the time of conception, likely exerted through

alteration in sperm epigenetic status. This stands out as the most plausible scenario, considering that the father's influence on maternal metabolic changes during pregnancy is limited, as evidenced by maternal cardiometabolic changes that respond more noticeably to maternal diet than paternal. Thus, the epigenetic changes occurring in the sperm preconceptionally and at the time of conception exert the shifts observed in the transcriptomic profiles of these offspring cardiomyocytes.

In addition, a recert pioneering study discovered a protein called ATF7 in mouse model, which is a transcription factor which affects by modulating the H3K9me2 and small RNA contents in spermatogonia germ cells in testis. Under/over nutrition conditions in fathers, such as paternal LPD, lead to reactive oxygen species (ROS) production in the testis. This results in the phosphorylation of ATF7, which is associated with a decrease in H3K9me2/3 levels. These epigenetic changes play a crucial role in affecting gene expression in the offspring of these fathers (Yoshida et al., 2020).

The other aspect of the fetal heart experimental analyses, which has a potential role in fetal cardiac development, is the assessment of the effect of fetal sex (Ramamoorthi Elangovan et al., 2023, Muralimanoharan et al., 2017). The effect of fetal sex was analysed both between and within diets in this experiment. Fetal cardiac development may demonstrate sex-specific patterns and trajectories, even under the same dietary interventions.

Differences in healthy male and female hearts in mice, humans and other species have been associated with characteristics including alterations in lipid metabolism, rhythmicity, fibrosis, and regenerative capacity, even the size and weight of the heart have been affected in response to the offspring sex (Ventura-Clapier et al., 2017, Norheim et al., 2019, Karp et al., 2017).

Traditionally, the concept of sexual dimorphism has been connected to the effects of sex chromosomes and sex hormones (Dean and Mank, 2014). However, epigenetics has a potential role in directing the tissue function differences between the two sexes. Particularly, genes such as *CTAG2, RPS4X*

which escape the process of X chromosome inactivation are involved in the development of cardiac pathologies, which could be established in early embryogenic stages (Deegan et al., 2019). The distribution of sexually dimorphic genes across the genome is not random; rather, these genes tend to cluster within specific genomic regions, indicating coordinated regulation by epigenetic mechanisms (Oliva et al., 2020).

The influence of paternal diet on offspring cardiovascular health through sperm epigenetic modifications and seminal plasma factors has been reported (Bodden et al., 2020, Watkins et al., 2018, Morgan et al., 2020b). Given the significant transcriptomic changes during embryo genome activation and zygote genome coordination (Chen et al., 2017, Schulz and Harrison, 2019), sperm small non-coding RNAs (sncRNAs) are proposed as potential mediators transferring epigenetic traits from sperm to the embryo. This transfer may lead to metabolic alterations in offspring later in life (Zhu et al., 2021). It is suggested that sperm sncRNAs, functioning as mobile agents of intercellular communication, could transmit paternal information to the egg before zygote formation (Ayaz et al., 2021).

Interestingly, sncRNAs, including miRNAs and lncRNAs, play pivotal roles in normal heart development and serve as regulatory factors in stress responses during adulthood. These sncRNAs are crucial for early cardiac development in embryogenesis. For instance, the lnRNA Braveheart (Bvt) activates gene regulatory networks determining cardiovascular cell fate. Bvt operates upstream of mesoderm posterior (MesP1), a critical gene governing cell commitment to the cardiovascular lineage (Philippen et al., 2015).

While most sperm sncRNAs act as transient regulators, their potential impact on cardiac gene expression may be heightened during fetal heart development, which initiates early embryogenesis. For example, a transcript derived from human testes, DKK2, suppresses the expression of WNT, implicated in developmental pathways and cardiac axis formations (Ostermeier et al., 2005). Therefore, since spermatozoa contain testes transcripts (Ostermeier et al., 2002),

they may serve as vehicles for transferring sperm sncRNAs into zygotes and developing embryos.

The initial comparison of differential DEGs between CD males and CD females group revealed a significant up-regulation in GO pathways related to regulation and negative regulation of the cellular and intracellular mechanism and process. Analyses of the phenotype pathways represent a significant upregulation of various pathways involved in abnormal embryonic development mortality/ageing in CD males when compared to CD female hearts. The overexpression of certain genes, such as *Tead1*, in CD male fetal hearts has been linked to the development of age-dependent cardiac dysfunction such as septal wall thickening, and fibrosis (Tsika et al., 2010). This condition predisposes to a decrease in left ventricular power output, which could be correlated to an increase in βMyHC protein levels in these male hearts later in life (Tsika et al., 2010).

Overexpression of genes such as *Smad7* in the male fetal heart plays an essential role in the alteration of multiple signaling pathways such as TGF-β involved in cardiomyocytes cell cycle modulation and proliferation alongside cardiac development and function. Smad 7 is a negative modulator of the Tgf-β superfamily as well as the BMP superfamily. The Tgf-β superfamily signaling is involved in the most substantial transcriptional changes in the transition phase of proliferative to non-proliferative stage of mammalian cardiac development (Mohamed et al., 2018, Sorensen and van Berlo, 2020). The over-expression of this gene in the heart has been related to misregulation of the migration of neural crest cells which are essential for heart morphogenesis. Overexpression4 of *Smad7* by downregulation of the *Tgf-b and Bmp* through restriction of *Wnt1-Cre; Smad4*$^{loxp/loxp}$ has been reported in the congenital pathological cases such as craniofacial, pharyngeal, cardiac defects such as ventricular septum defect and in utero lethality (Tang et al., 2010, Büchmann-Møller et al., 2009, Nie et al., 2008). Therefore, the occurrence of the dysregulation of fundamental cellular pathways in male cardiomyocytes which could potentially enhance the occurrence of pathological cardiovascular lesions later in life is higher than in female heart in the CD group. These changes could be related to the sex-specific

alteration in metabolic pathways, as well as the effect of the hormonal factors and molecular mechanisms involved in genetic and epigenetics, which eventually lead to the different programming the myocardial hypertrophy and fibrosis (Villar et al., 2009), and consequently contribute in the biomechanical sex differences. However, the molecular mechanism associated with cardiac ageing remains not fully defined yet, concerning biological sex. These mechanisms are likely more intricate than solely estrogen-related, as indicated by observations that sex differences persist beyond menopause in women and or in mice that are incapable of reproduction (Iorga et al., 2017).

Significant downregulation of sex-linked genes such as *Eif2s3y, Uty,* and *Ddx3y* in LPD female hearts was observed when compared to LPD male hearts. These genes encode for male-specific minor histocompatibility antigens that contribute to inflammatory disease (Ocañas et al., 2022), as were observed in the LPD male hearts. Indeed, proper cilium organization is crucial for cellular communication mainly in calcium signaling which plays a crucial role in the contractility of the heart, mechanosensation, and response to extracellular cues during cardiac development (Pala et al., 2017).

Furthermore, the overexpression of genes in the *Foxo* family was noted in LPD male fetal hearts, potentially associated with thin myocardium, reduced cardiomyocyte proliferation, and early fetal lethality, when compared with LPD female hearts (Payan et al., 2020). Additionally, instances of upregulation were observed in genes within the solute carrier family (*Slc*), such as *Slc25a40*, which have been implicated in fetal heart dysfunction and mitochondrial impairment. (Ogunbona, 2018). Enhanced transportation of dicarboxylates into mitochondria may disrupt mitochondrial function, affecting metabolite flux through the citric acid cycle and oxidative phosphorylation (Frezza, 2016). The consequences of mitochondrial dysregulation may disrupt cellular energy metabolism and ATP production within mitochondria, thereby adversely affecting fetal cardiac proliferation (Ramaccini et al., 2021). This dysregulation could potentially increase the predisposition of individuals to heart failure, arrhythmias, and other cardiovascular disorders in adulthood such as dilated cardiomyopathy (Lewis et al., 2005, Gutiérrez-Aguilar and Baines, 2013). In humans overexpression of

SLC family genes coincides with increased ROS in mitochondria, leading to hypoxia and cell apoptosis (Shi et al., 2023). The overexpression of *Slc1a5* mimics the function TEAD1 in promoting mTORC1 activation of VSMC proliferation (Osman et al., 2019). Overexpression of VSMC is associated with increased vascular pathologies and remodeling (Pollman et al., 1998, Wang et al., 2004), which could potentially prone these fetuses to vascular and cardiac remodeling, later in life.

Similar to the significantly overexpressed pathways identified in the phenotype assessment of CD males compared to CD females, analogous pathways were also significantly upregulated in the phenotypic assessment of LPD males versus females' fetal hearts. These findings potentially suggest that the upregulated pathways in both CD and LPD males, when compared to females, may be driven by sexually dimorphic genes. This implies that the upregulation of these pathways could occur irrespective of the effect of the paternal diet.

No significant either up or downregulated pathways were observed within the analyses of MDL males vs MDL females. The observation indicates that male fetal hearts sired from LPD-fed fathers revealed a downregulation of genes involved in pathways associated with CVD as well as pathways related to metabolism, such as carbon metabolism and amino acid biogenesis. However, this downregulation was not observed in female fetal hearts sired from LPD-fed fathers. This suggests that the impact of paternal diet may be more pronounced and evident in male fetal hearts compared to female fetal hearts. Interestingly, among the pathways that were downregulated in LPD males, only metabolic pathways showed downregulation in female fetal hearts from LPD-fed fathers.

When comparing WD males to WD females, significant upregulations were observed in pathways associated with cilium microtubule bundles and those related to abnormal respiratory morphogenesis and function. These heightened pathways could potentially be associated with dysregulated gene superfamilies governing cellular differentiation, such as the homeobox family (e.g., *Hoxa5, Nkx2-1*), zinc finger family (e.g., *Zmynd10*), and Fox family (e.g., *Foxf2, Foxk2, Foxn1*). These gene families, particularly the Fox family, play crucial roles in

cellular developmental processes such as cell cycle progression, proliferation, migration, apoptosis, and DNA damage response.(Carlsson and Mahlapuu, 2002). Additionally, they are involved in cell differentiation, tissue organization, and structural patterning during embryonic development. (Holland, 2013).

Genes belonging to the forkhead (Fox) family act as transcriptional modulators, functioning both as activators and inhibitors of gene expression. They interact with various transcriptional regulators and epigenetic effectors(Lam et al., 2013). Overexpression of homeobox family genes such as *Nkx2-1*, an early marker of cardiac lineage, has been implicated in congestive heart failure.(Azakie et al., 2006). This suggests that paternal WD may contribute to the overexpression of genes involved in morphological and structural dysfunction in both the heart and respiratory system. Over expression of the abnormal respiratory structure and morphology is indicative of structural abnormalities within the respiratory system. While they may not directly cause cardiovascular disease, they can contribute to respiratory conditions that subsequently impact cardiovascular health. Heightened levels of connective tissue within the alveolar-capillary units, alongside an elevated count of type II alveolar cells have been reported in patients diagnosed with chronic heart failure, pathology investigations (Lee, 1979, Nugent et al., 2019). These alterations signify an adaptive reaction within these individuals. Additionally, increased levels of the surfactant type A and B *(Stfpa1, Stfpb)* which overexpressed in WD male hearts have been reported in patients with acute cardiogenic pulmonary edema and its correlated with enhanced clinical severity of the heart failure (De Pasquale et al., 2003, De Pasquale et al., 2004). Therefore, overexpression of the genes involved in the abnormal type II pneumocyte morphology, and abnormal pulmonary alveolar system morphology could potentially mimic a similar condition in the male offspring derived from WD fed diet when compared to the WD females.

No other significant up or downregulated pathways were observed when comparing male fetal hearts from MDWD groups to female fetal hearts based on DEGs.

The difference within the same sex in response to paternal diet is another aspect of the fetal heart transcriptomic assessment that was considered, in this analysis. Interestingly, when male fetal hearts sired from LPD-fed fathers compared to CD males, a significant downregulation in the pathways involved in metabolic pathways such as carbon metabolism, glycolysis, and metabolic pathways was observed. This observation was accompanied by significant upregulation in pathways involved in fundamental transcription such as mRNA and RNA splicing. Upregulation of fundamental transcriptomic pathways in the fetal heart has been associated with fetal cardiac hypertrophy, as alteration in splicing has been associated with upregulation and development in the tropomyosin 3 gene *Tpm3* (Ames et al., 2013).

The concept of fetal cardiac hypertrophy appears paradoxical when considering the concurrent downregulation of metabolic pathways in cardiomyocytes, as cardiomyocyte hypertrophy could be considered as a compensating response to pathological stressors (Van Berlo et al., 2013). For example, left ventricular hypertrophy represents a potent and autonomous risk factor for cardiovascular morbidity and mortality during adulthood. An increase in left ventricular mass persists from fetal development through childhood into adulthood (Geelhoed et al., 2009). However, it could potentially serve as a compensatory mechanism in response to the downregulation of key metabolic pathways, particularly those involved in carbohydrate metabolism and amino acid biosynthesis. These downregulated pathways may lead to reduced cardiomyocyte growth and proliferation. In male fetal hearts sired from fathers fed an LPD when compared to CD males, a notable downregulation in the expression of genes associated with metabolic pathways and metabolism such as pyruvate metabolic process, carbohydrate catabolic pathway including genes such as *Pkm, Insr, Prkag3, Gck* process, and nucleotide phosphorylation pathway was observed. Nucleotides are in high demand during rapid cell division (Perez-Ramirez et al., 2024). Therefore, downregulation of the nucleotide phosphorylation pathway could be associated with disruption in normal cardiomyocyte proliferation This downregulation was not evident in female fetal hearts.

Overexpression of genes involved in apoptosis such as *Casp3,4,8,12* and *Bclaf1* which codes *Bcl2* protein were observed in male LPD fetal hearts when compared to CD males. Overexpression of these genes is associated with an increased level of apoptosis, especially in response to oxidative stress (Yu et al., 2022). Increased levels of apoptosis could activate the expression of the fibroblasts involved in the stimulation of fibrosis. In addition, overexpression of genes such as collagen families particularly, collagen type 1 and III has been reported in cardiac pathologies such as hypertensive as well as aortic stenosis pathologies (López et al., 2014, Echegaray et al., 2017). Elevated blood pressure initiates a cascade of events wherein ECM deposition is induced, ultimately disrupting the mechanical milieu within the heart over time, consequently could potentially result in diastolic dysfunction (Di et al., 2023). Extracellular matrix deposition in perivascular and interstitial areas could potentially impair cardiac function and could be a potential risk factor for heart failure (López et al., 2021).

Nonetheless, this assumption requires further investigation to provide a deeper understanding of the underlying mechanisms, which may be influenced by fetal sex and paternal diet., coincidentally. A significant downregulation of pathways involved in metabolism was comparing male fetal hearts sired from the LPD-fed fathers' group with those males from the CD group, the previously downregulated pathways observed in LPD hearts were no longer significantly downregulated in male MDL hearts, when compared to CD males. This suggests that the addition of methyl donors to a paternal poor diet may have mitigated the negative effects of paternal LPD in male MDL fetal hearts.

The comparison of male WD vs male CD, the gene ontology assessment represent a significant upregulations in signal transduction and transportation pathways such as positive regulation of catabolic process, regulation of transporter activity, negative regulation of transport, negative regulation of intracellular signal transduction signal transduction in absence of ligand, negative regulation of establishment of protein localization, Overexpression these pathways with expression of genes such as *Mapk3, Akt2, Insr, Mapk9,*

Ubqln2, Ppargc1b suggests the paternal WD in male fetal hearts could suggest by dysregulation of nutrient uptake could potentially put these offspring in cardio pathologic risks later in life. For example, myocardial lipid accretion due to increased fatty acid uptake would result in myocyte apoptosis and cardiomyopathy, a lipotoxic effect which potentially could mimic the diabetic cardiomyopathy. However, in female WD hears totally different pathways were upregulated pathways such as extracellular structure organization skeletal system development response to wounding response to fibroblast growth factor angiogenesis. Overexpression of genes such as *Eln, col1a1, Col11a2, Egfl6, Lox3* and *Fmod* were observed. These genes actively involved in the process of collagen synthesis which overexpression of these gene have been reported in the arterial stiffness and cardiac fibrosis due to collagen deposition, and cardiac matrix remodeling (Li et al., 2016b, Li et al., 2018). Conversely, a significant downregulation of pathways involved in the fundamental transcription process were observed in female WD hearts when compared to CD hearts.

Pathways such as DNA-templated transcription, initiation meiotic DNA recombination RNA splicing cRNA metabolic process, biogenesis mRNA processing DNA repair were significantly downregulated. Expression of genes such as *Hnrnpf* has been associated with postnatal normal heart development and function (Ye et al., 2015). Overexpression of genes such as Gpm6a which is involved in the activation of MAPK and *Src* signaling pathway, has been associated with the pathology of cardiac failure (Mathiyalagan et al., 2019, Gao and Wang, 2020). However, none of the mentioned pathways were up or downregulated in the male WD hearts.

Analysis of DEGs in phenotype pathways assessment in WD male hearts when compared to WD female hearts represent, the upregulation of numerus abnormal pathways associated to the pulmonary system morphology and function. Downregulation of fundamental genes in the development of respiratory system such as *Spry2, Nkx, Mapk3, 4, 9, 14, Nkx2-1, Hoxa5.*Downregulation of these transcriptomic pathways drastically effect on the expression of functioning proteins in female fetal hearts derived from WD fed fathers.

These observations suggest no matter of the fetal sexual dimorphism the fetal hearts both in male and female would be prone to potentially develop the cardio pathological lesions even though through the expression of different pathways. In addition, sexual dimorphism directs the effect of paternal diet through the expression of different gene expression pathways in these fetal hearts.

Comparing female LPD fetal hearts with female CD fetal hearts, no significant up- or downregulated pathways in response to paternal diet were observed. This finding could suggest the presence of protective effects conferred by the sex chromosome in females, which may support female fetal hearts against the adverse effects of paternal LPD, as a significant downregulation of cytokine-cytokine receptor interaction was observed in LPD male fetal hearts when compared to CD male fetal hearts. This analysis displays that male and female hearts differed significantly in the transcription of genes involved in the regulation of the immune system. In a normal female heart expression of the pathways involved in the immune system both innate and adaptive has been associated with the development and progression of cardiovascular disease (Hansson, 2005, Fernández-Ruiz, 2016).

In LPD female hearts no pathways related to the immune system were expressed. The absence of the expression of these pathways related to the protective effects of the immune system could potentially prone these females to cardiovascular disease later in life. For instance, the role of inflammation in the occurrence of atrial fibrillation and myocarditis resultant of viral infection has been reported due to dysfunction of the immune system (Hu et al., 2015). Therefore elimination of these pathways involved in the suppression of inflammation such as expression of regulatory T cells including Treg cells and modulation of the anti-inflammatory macrophages (Meng et al., 2016) could be potential protective factors against particular atherosclerosis and abdominal aortic aneurysm (Meng et al., 2016) the suppression of these pathways in LPD female fetal heart when compared with females CD hearts, was observed.

In addition, by ageing the protective effect of estrogen on endothelium and against CVD initiates fading (Clarkson, 2018, Somani et al., 2019) and

potentially these offspring may represent a similar incidence of CVD later in their lives,

The role of the expression of genes involved in immune system pathways in the heart has been associated with various biological mechanisms including inflammation regulation, tissue remodeling, cell communications as well as modulation of signaling pathways in the heart (Lafuse et al., 2020). The overexpression of genes involved in the immune pathway could be discussed as a potential response to the existing inflammation due to the cardiac tissue damage caused by systemic inflammation, arrhythmia and conduction defects (Hu et al., 2015). Due to the consumption of the paternal LPD and MDL in female fetal hearts. Even though no such responses in the male fetal heart were observed it could be potentially a sign of the occurrence of cardiac deficit later in life in the male offsprings of LPD and MDL-fed fathers.

Interestingly, in the female MDL heart, significant upregulation of the abnormal pathways involved in the immune system was observed when compared to CD female hearts, coincidentally with the downregulation of pathways involved in embryonic and preweaning mortality and abnormal blood vessel morphology. The other significant genes involved in inflammatory cytokines Cx3cl1 and Vcam1 were observed in MDL females when compared to CD females (Chang and Chen, 2016, Apostolakis and Spandidos, 2013).

The significance of chemokines like Ccl4 lies in their pivotal role in modulating the migration and adhesion of monocytes and lymphocytes, which in turn greatly influences left ventricular remodeling in response to injury (Nemska et al., 2016). Overexpression of *Il-7* has been associated with enhanced recruitment of macrophages and monocytes through activation of the PI3K/AKT dependent signaling by stimulating the expression of mRNAs for MCP-1 and CAMs into the endothelium which the accumulation of these leukocytes potentially leads in inflammation of the endothelium contributing in the atherosclerosis, which plays an active role in the atherogenic leading to the cardiac dysfunctions (Li et al., 2012).

The mentioned cytokines were expressed in female MDL hearts when compared to female CD hearts, and none of them were expressed neither in female LPD fetal hearts or male MDL hearts. This observation could shed light on fascinating findings that paternal LPD governs the expression of genes involved in immune system pathways.

This observation potentially suggests that paternal LPD could govern the expression of the normal sex-specific pathways in this female fetal heart. Additionally, it suggests fortification of the paternal LPD diet could manage to ameliorate the negative effects of the poor paternal LPD resulting from autosomal genes in MDL female fetal hearts, but not the negative effect of paternal LPD in these female fetal hearts regarding the sex-linked genes.

However, we should consider we might not see significant effects of cardiovascular malfunctions in these offspring. However, follow-up experiments such as assessment of the lipedema and cholesterolemia which represent the lipid metabolic status of these offspring later in life could be insightful.

Totally different pathways were upregulated in WD male hearts when compared to CD, including upregulation pf pathways involved in cellular mechanism, however female WD heart when compared to CD female hearts displayed a significant upregulation in pathways involved in immune system and conversely a significant down regulation of pathways involved in metabolic process and metabolism in response to paternal WD diet. It could suggest both male and female sex could be affected by suboptimal paternal diet however in the different pathways through expression of different genes and pathways under the control of sexually dimorphic genes and other factors such as influence of sperm epigenetics in modulation of the transcriptional initiation and post-transcriptional gene expression regulation, by impacting the metabolic process and pathways. It could suggest a significant finding regarding the governing the expression of sexually dimorphic genes in different sex in response to paternal diet.

Overall, it could be concluded that the observed effects in fetal cardiac sired from LPD and WD fed fathers could be associated to the effect of diet rather than the effect of sex, however, the presence of sexually dimorphic genes in males amplifies the negative effects of the paternal diet.

The final concluding section of this series of experimental assessment relates to the histomorphology examining of the placenta in response to paternal diet. The obtained results show paternal diet has minimal impact on placental morphology in late gestation.

Paternal diet significantly decreased the expression of genes involved in RAS, apoptosis and 1-carbon metabolism in the placentas derived from WD and MDWD-fed fathers. The uteroplacental *RAS* components pathways play a central role in placental development alongside the glomerular filtration during pregnancy (Yart et al., 2021). It is essential for the proper modulation of uteroplacental blood flow, prostaglandin synthesis, and estradiol secretion, which are all essential for a successful pregnancy (Nitert et al., 2013, Lin et al., 2019). Furthermore, impairment of uteroplacental *RAS* function has been associated with pregnancy complications such as IUGR and preeclampsia (Mistry et al., 2013). However, in this study, neither the WD nor the MDWD group exhibited fetal implications in response to the significant decrease in the expression of RAS component genes, such as decreased fetal or placental weight, when compared to the control diet. This outcome does not follow the anticipated trend seen in pregnancy complication associated with RAS impairment. As such, could be suggested that this diminished level of RAS components genes expressions in response to paternal WD and MDWD, did not lead to placental insufficiency. Collectively, redundancy in the systemic RAS could potentially compensate for the reduction that observed in the placenta. Indeed, no change in maternal serum ACE activity was observed. Therefore, this may counteract any placental changes.

Placental *RAS* component genes are highly expressed in early and mid-pregnancy (Wang et al., 2018, Arthurs et al., 2019, Gheorghe et al., 2006). Conversely, a study on rats demonstrated that *Ace* and *Ace2* expression

increased dramatically at late gestation (gestational day 17.25), indicating that the pathway is highly active at this stage of gestation (Vaswani et al., 2015). *ACE2* has a substantial role within the central nervous system in modulating blood pressure (Mendoza and Lazartigues, 2015). However, in contrast, existing literature indicates a decrease in the level of serum *ACE* as the only RAS component in normotensive pregnancies in women (Irani and Xia, 2011). This indicates the controversial documents regarding the expression of placental *ACE* at late gestation. Thus, the decrease in the relative expression of *Ace* that was observed in the WD and MDWD groups could not be necessarily related to the pregnancy complications in this study, as no significant decrease in fetal and placental weight derived from WD and MDWD fathers was observed. However, the analysis of fetal cardiac gene expression revealed valuable results in which paternal diet significantly and on a large scale directs the fetal cardiac gene expression, given that the obtained data indicate a potential risk of long-term CVD in the offspring sired from suboptimal diets-fed males. As mentioned, placental and fetal weights have not been affected. Therefore, these data indicate that using traditional proxy measures of impaired in-utero development may not be applicable when it comes to defining the impacts, or evidence, of paternal programming.

This observation is not consistent with the maternal HFD studies. Maternal HFD studies reported a significant increase in the expression of mRNA of *Ren*, *Atp6ap2*, *Ace,* and *Agtr1a*, which results in the alteration of placental RAS function (Lin et al., 2019). Additionally, an increase in the level of RAS activity has been associated with the pregnancy complications, such as fetal growth retardations, IUGR and preeclampsia. In contrast, a significant decrease in the expression of RAS components within the placentas derived from WD and MDWD fed fathers was observed. This suggests that the impact of paternal WD on placental gene expression does not necessarily mirror the maternal HFD.

The present results derived from placental gene expression have demonstrated a significant down-regulation of genes responsible for apoptosis regulation in placentas derived from WD and MDWD-fed fathers.

In a study aimed at assessing apoptotic markers in placental samples from women diagnosed with pregnancy complications such as PE and GDM, it becomes evident that *Bcl-2*, an anti-apoptotic gene, and *Bax*, a pro-apoptotic gene, are among the most significant genes involved in the regulation of placental apoptosis. For example, down regulation of *Bcl-2*, and up-regulation of *Bax*, have been reported in pregnancy complications such as GDM and preeclampsia. Therefore, it could suggest that placental *Bax* protein level could be a significant marker in defining apoptotic alterations in pregnancy complications such as GDM and PE (Gokalp-Ozkorkmaz et al., 2018).

In a normal pregnancy, trophoblast apoptosis increases with placental growth which depends on influential invasion of placental trophoblast into the maternal decidua, and advancing gestation to ensure providing the developing baby with a sufficient amount of nutrients (Sharp et al., 2010). In human, syncytiotrophoblast layer of placenta and in the rodent labyrinth zone undergo apoptosis and invades to the uterine spiral arteries (Silva and Serakides, 2016). A normal placental development in humans is dependent on extravillous trophoblast invasion of the maternal decidua and subsequent remodeling of the maternal spiral arteries to provide stability to the placenta and effective utero-placental blood flow (Sharp et al., 2010). The key point in placental development is a balance between the pro and anti-apoptotic factors, to prevent any gestational difficulties. Oxidative stress, defined as an imbalance between free radicals and antioxidant protection (Silvestris et al., 2016), is associated with abnormal revascularization in the placental spiral arteries, resulting in impaired angiogenesis. One mechanism that explains this detrimental effect is the reduction in VEGF, which plays a role in antiapoptotic activities by influencing the expression of antiapoptotic proteins, as reported in the study by (Kasture et al., 2018). Interestingly, in a mice study investigated the impact of paternal HFD-fed on placental function in response to this perturbed diet. They showed, paternal HFD-fed has been associated with placental hypoxia and placental vascular impairment as well as VEGF and VEGFR-2 at E14.5, but intriguingly, not at E18.5 (Jazwiec et al., 2022). Therefore, perhaps impairment manifestations in placenta have been resolved by late gestation. Thus, there is a

possibility that we may observe more significant evidence of placental function in response to paternal diet in mid-gestation.

In a normal pregnancy increased expression of *BCL-2* in the third trimester and at term is expected to be increased as an anti-apoptotic factor, so that decreased in the expression of this gene results in enhanced level of apoptosis in placenta, and ultimately may lead in placental insufficiency, and gestational conditions related to this such as IUGR and Preeclampsia. However, no signs of placental insufficiency were observed in fetuses derived from these placentas.

However, in a normal pregnancy diminishing the expression of *Bcl2* should be expected. Decrease in the expression of *Bcl2*, has been associated with gestational complications such as premature rupture of the membrane (Tanir et al., 2005) and preterm birth (Daher et al., 2008). The decreased level of RAS activity, has been attributed to the elevated placental apoptosis demonstrated in pregnancy complications such as IUGR and preeclampsia, which is manifested with a decreased level of *Bcl-2* (Cali et al., 2013, Sharp et al., 2010). It is further possible that maternal food restriction-induced placental apoptosis in both zones may result in a defective placental barrier enhancing exchange across the maternal/fetal Interface perhaps exposing the fetus to excess maternal glucocorticoids which impact fetal growth and development (Belkacemi et al., 2009).

The major placental gene pathways are up- or down- regulated by maternal protein deprivation (Gheorghe et al., 2009). Maternal malnutrition in mice, such as maternal LPD, resulted in changes in placental gene expression within apoptosis, growth inhibition and epigenetic modification pathways (Watkins et al., 2015). Maternal protein restriction showed up-regulation of the genes responsible for the negative regulation of cell growth and metabolism in the placenta (Gheorghe et al., 2009). IUGR is relevant with restricted fetal growth, reduced size at birth and adiposity in later life. More studies are needed to investigate the potential association of paternal diet on any impairment in placental apoptosis genes and eventually consequent implications in placental

structures and functions, subsequently their impacts on fetal development as well as implications in both short- and long-term life.

The expression of 1-carbon metabolism pathway genes in placentas derived from males fed different diets was not different in LPD and MDL groups compared to CD groups, while expression of these genes in WD and MDWD was decreased significantly.

Throughout pregnancy in women, there is a correlation between the reduced level of folate and hyperhomocysteinemia with highlights risk of pre-term delivery, low birth weight, preeclampsia, restricted fetal growth, as well as placental abruption or infarction (Thakur and Bhalerao, 2023).

Folic acid and vitamins B2, B6 and B12 are essential one-carbon metabolites involved in DNA, histones and RNAs methylation and act as methyl donor groups eventually, they affect the programming of the offspring's (Vanhees et al., 2014). Suboptimal diets such as over/under nutrition leave substantial negative impacts on pregnancy outcomes and fetal growth. A mouse study suggested dams fed with HFD, that also received 1% betaine which acts as a methyl derivative of glycine, before and during the gestation, demonstrated no difference in fetal and placental weight when compared to the control group (Joselit et al., 2018). This shows addition of betaine has negated the negative impacts of HFD on placental development such as placental dysfunction that could be seen in pregnancy complications, including IUGR and GDM. The decreased level of MTHFR expression of this gene in humans is associated with fetal development and neural tube dysfunction, considering maternal *MTHFR*, plays a central role as a determinant of folate status and 1-C metabolism (van Mil et al., 2014). Our data revealed a significant reduction in the relative expression of this gene in WD and MDWD, in which paternal HFD mirrors the maternal HFD relevant to the expression of the *Mthfr* gene in the placenta. As well as no amelioration of methyl donor group in MDWD was observed.

MTHFR A1298C polymorphism is a significant risk factor for vascular related pregnancy complications. Women with *MTHFR A1298C* polymorphism, or

elevated homocysteine levels, have an increased risk of placental vasculopathies by damaging the vascular endothelium through endothelial dysfunction (Klai et al., 2011) which subsequently may lead to IUGR, which may lead to a significantly increased risk for type 2 diabetes mellitus, obesity, hypertension, dyslipidemia, and insulin resistance (Longo et al., 2013). The one-carbon cycle and homocysteine recycling play a significant role during the preimplantation stage. Such supplementation is highly recommended as it can significantly reduce the risk of placental dysfunction resulting from deficiencies in the 1-carbon metabolic pathways. Additionally, it can help mitigate the risk of metabolic syndromes like obesity and diabetes. For instance, a decreased level of folate in the mother is associated with adiposity and insulin resistance in the offspring, which means that insulin resistance and adiposity in offspring are intimately associated with alteration in maternal 1C metabolism (Solé-Navais et al., 2016). As studies have reported micronutrient deficiency in rats, there is a close association between maternal serum and placental micronutrient deficiencies such as folic acid and vitB12, This deficiency leads to diminishment in the expression of *MTHFR* and *MTR* mRNA levels, resulting in alterations in the re-methylation of homocysteine in the placenta (Khot et al., 2014, McCaddon and Hudson, 2007). This is aligned with the obtained results regarding the decreased expression of the *Mthfr, Mtr, Dhfr, Achy, and Mat2b* from the placentas derived from WD and MDWD-fed fathers. Therefore, it could suggest that deficiency of these core elements in methyl donor groups in mother and father are influencing the placental gene expression in the same way. In addition, supplementation of the methyl donor groups in MDWD did not compensate for the decrease in the expression of these pathways in MDWD placentas. Micronutrient deficiency in the dams affected by the expression of placental phosphatidylethanolamine methyl transferase (PEMT) which is essential for the transportation of docosahexaenoic acid (DHA), which is essential for the baby's neurodevelopment to the developing fetus (Khot et al., 2014).

Besides the crucial impact of maternal folate status, it appears that paternal folate status plays a key role in fetal programming and long-term life consequences through sperm epigenetic modification (Lambrot et al., 2013b). Additionally, in

our study, diminished expression in WD and MDWD placentas was observed. As the same changes were seen in both the WD and the MDWD, such, the decreased expression suggests there is a fundamental consequence of the WD and not the paternal 1-C status. In the present experiment, no significant changes in the expression of folate cycle genes were observed in the LPD group while, Watkins et al, 2018 demonstrated that paternal LPD perturbed testicular expression of the folate-cycle enzymes *Dhfr, Mthfr,* and *Mtr* and of the DNA methyltransferases *Dnmt1*, *Dnmt3L,* which means LPD diet may lead to disruption of the testicular folate cycle, sperm DNA hypo-methylation. However, it is important to note that this experiment cannot provide conclusive findings, as no further analyses, such as Western blot analysis for further assessments have been conducted. Paternal folate deficiency exerts it's impacts on placental developmental impairment and abnormalities through epigenetic information for genomic imprinting from the father (Lambrot et al., 2013b). However, no significant alterations between the WD and MDWD compared to CD were observed. Suggesting supplementation of the WD group did not negate the downregulations of the 1-carbon metabolism in MDWD placentas. In the MDWD the adequate amount of folate so the effects are not due to a lack of folate but rather the fundamental effects of the WD, which has not been mitigated by supplementation of the methyl donors.

The impact of parental folate status in rat placental as well as analyzing the expression of folate transporter-α (FRα) in placenta reported a significant decrease in the placental content of folate by 35% and a significant upregulation of the FRα receptor by 130% in the placentas derive from folate-supplemented when compared to the folate-deficient placentas. Suggesting a positive correlation between paternal folate content and placental folate content. Indeed, the level of folate throughout the preconception period can have an impact on the lasting epigenetic programming of genes, including imprinted genes (Tserga et al., 2017). This influence has the potential for adverse effects on both embryonic and placental development after conception. A father's folate-deficient diet can lead to changes in the sperm epigenome, including histone modifications such as histone H3K4/H3K9 me2/3. These modifications have been demonstrated to differentially mark genes in human and mouse

sperm(Delaval et al., 2007, Lambrot et al., 2013b). Ultimately, these epigenomic alterations affect the expression of genes like *Cav1*, a cell regulator, and *Txndc16,* which is expected to be highly expressed in the placenta and plays a central role in cellular homeostasis. These changes have implications for development and the risk of chronic diseases (Lambrot et al., 2013b, Ly et al., 2017)These modifications have been demonstrated to differentially mark genes in human and mouse sperm(Delaval et al., 2007, Lambrot et al., 2013b). Ultimately, these epigenomic alterations affect the expression of genes like *Cav1*, a cell regulator, and *Txndc16,* which is expected to be highly expressed in the placenta and plays a central role in cellular homeostasis. These changes have implications for development and the risk of chronic diseases (Lambrot et al., 2013b, Ly et al., 2017). Possible paternal programming pathways encompass including changes in testicular and sperm epigenetics, such as (DNA methylation, histone modification miRNA expression), seminal plasma composition, as well as FRT responses, including immunological responses and uterine vascular remodeling, which regulates early embryo development and have long-term implications for offspring health. Indeed, further assessments, such as examining the level of protein synthesis derived from central genes in fetal hearts through western blotting could be insightful.

Initially, placental morphology was assessed using Periodic acid-Schiff (PAS) staining to define the relative placental layer cross-section areas and highlight cells positive for glycogen.

No significant changes were observed in placental PAS staining. These results are not consistent with existing literature that focuses on the impact of paternal diet on placental morphology. The findings from the PAS staining experiment contradict a study conducted by Morgan et al. (2021), which demonstrated significant alterations in placentas derived from LPD and MDL males. This study reported reduced areas of Jz layers in LPD and MDL placentas (Morgan et al., 2021). Further assessments, such as examining the expression levels of key genes involved in glycogen storage, including glycogen synthase, glycogen synthase kinase 3 (GSK3), and, most importantly, AKT signaling as a pivotal regulator of glycogen storage in the placenta, should be conducted (Tunster et

al., 2020). Ultimately, it would not be surprising to observe a decrease in PAS staining in the Jz of LPD placentas, given the decrease in fetal weight in the LPD group. This is because placental glycogen content and fetal weight at late gestation are interconnected (Roberts et al., 2008).

Several factors could contribute to and cause an increase in the placental surface with remaining the placental weight with no changes. These factors include the increase in villous branching, and finger-like projections which increase the surface area, the plausible reason could result from the thinning of the placental villi, which has been reported in the cases of GDM and type 1 maternal diabetes, with a significant impact on increasing maternal nutrition transportation to the fetus (Jirkovská et al., 2012, Castillo-Castrejon and Powell, 2019). Moreover, a significant level of oxidative stress in the placentas derived from pre-existing diabetic mothers or GDM mothers has been reported which potentially modulates the placental structure and could result in increased fetal growth or macrosomia (Phipps et al., 2016, Wu et al., 2016).

However, a significant decrease in fetal growth in fetuses sired from LPD-fed fathers was observed. The final factor that could be listed as a potential candidate factor for this disproportion between the placental weight and surface area could be related to angiogenesis, which could increase the placental surface area, which suggests they might still maintain the same weight but with enlarged surface areas. Nevertheless, it cannot be argued confidently, that further assessment such as examining the expression of angiogenic factors including vascular growth factors, inflammatory cytokines, adhesion molecules, and nitric oxide could demonstrate a deeper understanding regarding these placentas. Indeed, it is clear that disturbance in the balance of the angiogenetic factors in the placenta is associated with maternal pathologies such as IUGR and preeclampsia (Cerdeira and Karumanchi, 2012). The obtained results are consistent with the placental weight data, as no significant alteration across different groups except the MDWD group was observed which was significantly heavier than the MDL group. However, an assessment of the glycogen content could have been conducted earlier in gestation as, according to the literature, the

glycogen content drops by E18.5 by 50%, due to a significant decrease in the population of glycogen cells (Aykroyd et al., 2020).

The additional staining method employed to evaluate placental morphological changes in response to the paternal diet was Masson's Trichrome staining. This technique was utilized to assess alterations in connective tissues and collagen deposition within these placental samples. As, the content of connective tissues, particularly collagen, is essential for the integrity and proper functioning of the placenta.

Notably, a significant reduction was observed in the overall intensity of Masson trichrome-positive staining, indicative of a decrease in collagen content deposition, in the placentas originating from WD and MDWD-fed fathers. Decreased placental collagen content has been reported in the cases of placental insufficiency in pregnancy complication cases such as IUGR, preeclampsia and fetal growth restriction (Warrander and Heazell, 2011). Evaluation of the placental connective tissues, specifically collagen, can be insightful as the decrease in the placental surface has been reported in pregnancy complications as well as other maternal insufficiencies such as the number of villi and capillaries in the placentas derived from IUGR and preeclampsia (Zhang et al., 2016b).

Alongside decreased in the total surface areas of WD and MDWD placenta, no significant changes in trends of fetal weight derived from these placentae were observed, suggesting no placental insufficiency could have occurred in these fetuses.

As placental connective tissue disturbance could potentially, influence the placental structure integrity, maintaining the integrity and function of placental vessels, malfunction of the placental barrier role, and ultimately, influencing the fetal development and growth. As well as increasing the expression of the proinflammatory cytokines in the placenta with the connective tissue's dysfunctions (Claycombe-Larson et al., 2020).

Also, the footprint of paternal diet has been reported in pregnancy complications such as preeclampsia, upregulation of expression of LncRNA alongside an enhanced level of H3K4me3 has been reported in preeclampsia (Sun et al., 2020). The significance of alterations induced by paternal HFD in sperm H3K4me3 is linked to the established connection between H3K4me3 in the placenta and embryonic tissues, along with its role in gene regulation within these tissues (Pepin et al., 2022).

Paternal obesity in mice models was associated with changes in placental DNA methylation, impairment cell lineage allocation to trophectoderm (TE), hypoxia, dysfunctional placental vasculogenesis, increased expression of inflammatory cytokines, and reduced food transporters (Pépin et al., 2022). These studies are in line with maternal HFD impacts on placental development (Gohir et al., 2019, Wallace et al., 2019).

Studies in maternal HFD have made a positive correlation between HFD and placental inflammatory cytokines. As well as, increased placental hypoxia and following enhanced angiogenesis, such as enhanced vessel density and decreased vessel functionality and maturity, which potentially influence the nutrient transport to the growing fetus (Wallace et al., 2019). A significant decrease in the labyrinth zone of the MDWD placenta was observed. As it is clear the role of LZ is very essential for successful nutrient transportation to the developing fetus. Interestingly, the obtained result is in line with the other people's observation regarding the decreased placental labyrinth zone in response to paternal HFD when compared to CD placenta, which could be related to the decreased cell division in this layer (Deshpande et al., 2023). The other potential factor could be related to the increased apoptosis in this specific layer in the placental gene expression experiment, a significant reduction in the expression of the *bcl-2* gene was observed, as *bcl-2* as a strong anti-apoptotic protein could have a substantial role in enhancing the apoptosis in the zone of the placenta.

The MDWD placentas mirrored the existence pattern in WD placentas, which could suggest that the addition of the methyl donor supplements to ameliorate

the detrimental impacts of paternal HFD plays a minimal role. This agrees with obtained results from cardiac gene expression in WD and MDWD groups, by showing similar patterns. The minimal effect of methyl donor groups on WD, consistent with the paternal LPD on mice which they showed a minimal impact of MDL on negating the negative impact of paternal LPD on placenta areal size and weight when compared to CD (Morgan et al., 2021).

4.9 Conclusion

The findings of these series of experiments confirm that paternal under (LPD) and over (WD) nutrition had no impact on male fertility as examined based on litter size. Paternal under-nutrition (LPD) and over-nutrition (WD) affected fetal cardiac gene expression profiles drastically in a diet-specific manner. Paternal LPD regulates the expression of genes involved in cardiovascular morphology, central metabolic processes, and ageing. Paternal WD regulates the expression of genes involved in cardiovascular morphology, lipid metabolism and embryonic organ development. Both paternal under/overnutrition can stimulate perturbations in the expression of transcriptional factors involved in the control of amino acid, carbohydrate, and lipid metabolism, in line with significant alterations in cardiovascular transcriptomic genes. Methyl donor supplementations ameliorated the impact of LPD on fetal CVD and metabolism gene expression, while they had minimal impact on WD hearts. It suggests that the negative impacts of paternal WD on fetal growth, placental and cardiac gene expression as well and placental morphology cannot be mitigated just simply by addition of the methyl donor supplements. However, the stage of fetal heart development is important as not all the genes become expressed in the heart at any gestational time (Li-Villarreal et al., 2023). Therefore, current analysis has not covered all the different parameters that could impact on fetal cardiac transcriptomic profile.

Studies are ongoing to explore further sex-specific influences of paternal LPD, WD and methyl donor supplementation on fetal heart gene expression. Paternal undernutrition with and without methyl donor supplementations impacts placental gene expression however paternal under/overnutrition with and

without supplementation has minimal impacts on placental histomorphology at late gestation.

Further monitoring of cardiovascular changes in these offspring, their growth trajectory, and the metabolic parameters of the progenies could prove immensely valuable in enhancing the current findings of the impacts of the paternal diet impacts on these fetuses' later lives. Therefore, it is essential to monitor postnatal factors including birth weight, growth rate, blood pressure, heart rate and metabolic profiles in these offspring. As well as assessment of the expression of microRNA miR-1 of sperm from these males could be substantially insightful for better understanding of these sperm's molecular status. Also examining the level of sperm H3K4me3 which serves as a metabolic sensor could bring a link between the paternal diet and the offspring's phenotype through the placenta, which could bring a high level of certainty regarding the influence of the paternal diet and his offspring's cardiometabolic ill-health.

These assessments will help to determine the impact of a suboptimal paternal diet, both with and without methyl donor supplementation intervention, on later life outcomes.

5 Chapter 5 General Discussion

5.1 The impact of paternal diet on maternal cardiometabolic health in the late gestation mouse

The present thesis focuses on the impact of paternal diet on maternal cardio-metabolic health and fetal development in late gestation (E17.5). This research is considered a novel study in the field of reproductive health, developmental biology and nutrition.

Pregnancy is a transformative period for a female's body, significantly affecting her cardio-metabolic and immune systems. Aberrant maternal adaptation during pregnancy, by diminishing the uterine environment milieu due to inappropriate level of maternal provision, may have a substantial implication on her offspring's cardiometabolic ill-health, both in the short- and long-term. Maladaptation in maternal physiology during pregnancy results in aberrant alterations in uteroplacental and circulatory function, negatively impacting placental development and fetal growth. Among such pregnancy complications are conditions like gestational diabetes (GDM) and preeclampsia.

Abnormal fetal growth has been associated with a wide range of cardiometabolic diseases later in the offspring's life (Barker, 1997a). The placenta plays a central role in this process. It is worth noting that the placenta is a shared organ, with genetic contributions from both the mother and the father. While most of the research has focused on the mother's influence, there is a growing body of evidence suggesting that the father's lifestyle and health can also impact the development and growth of his offspring by influencing the uterine environment. Therefore, the role of the father in maternal health has not been looked at extensively. The role of poor paternal health in pregnancy complications such as preeclampsia and GDM has not been studied extensively, and indeed, these complications have been associated with long-term health consequences for the mother and her offspring.

The results from this set of experiments in this thesis are novel and could create a strong base for future research on the field of male fertility and reproductive

fitness, as well as the impacts of paternal reproductive ill-health on their partners and future offspring. This research could be insightful for translational medicine in the field of reproductive and developmental biology.

The main findings include:

1. Paternal diet had minimal impact on stud male growth. However, the different diets resulted in a significant alteration in stud male organ weights, such as liver, kidneys and seminal vesicle weight, alongside organ/ body weight ratios.
2. Paternal diet had a subtle impact on maternal weight gain and organ size in late gestation.
3. Paternal diet had a significant impact on increasing the number of SGA fetuses both in MDL and WD groups when compared to CD.
4. There was little impact of paternal diet on maternal metabolic profiles, except in liver triglycerides/ serum insulin at late gestation. Additionally, minimal changes in maternal hepatic gene expression for central metabolic pathways were observed.
5. Poor paternal diet significantly reduced placental gene expression in RAS, apoptosis, and 1-carbon metabolism pathways in WD and MDWD groups.
6. Suboptimal paternal diet led to a significant reduction in the total area of staining for Masson's Trichrome (MT) to investigate any alterations in the connective tissues content as well as a significant reduction in the amount of MT staining within the labyrinth zone of WD and MDWD placentas.
7. The influence of paternal diet on offspring outcomes remains unaffected by maternal physiological changes.
8. There was a significant alteration in fetal cardiac transcriptomic profile in LPD and WD, as well as the methyl donor-supplemented groups (MDL and MDWD) in response to the paternal diet.
9. There were significant differences in the pathways expressed in response to paternal diet between male and female fetal hearts. However, the data indicated that the impact of the expression of sexually dimorphic

genes alongside of paternal diet might be amplified in male WD fetal hearts, potentially contributing to suggest they could have compromised cardiovascular health in adolescence. The addition of the methyl donor to the suboptimal diet did not mitigate the adverse effects of the suboptimal diets in either sex, which is noteworthy.

10. A novel finding was that the addition of methyl donor supplementation of the LPD (MDL) ameliorated the detrimental impacts of the LPD on the fetal cardiac transcriptomic profile. However, methyl-donor supplementation had less of an impact on fetal heart gene expression when added to the WD.

11. A paternal suboptimal diet had an adverse effect on the offspring, regardless of a father's weight status, resulting from either overnutrition (WD) or undernutrition (LPD). This impact is observed through alterations in the gene expression of the fetal heart.

This thesis aimed to investigate the role of paternal under/overnutrition, with and without supplemented methyl donors, on maternal cardiometabolic ill-health in late gestation. No significant alteration in the stud male growth trajectory was observed in this thesis. However significant alterations in paternal organ weight in suboptimal diet-fed groups were observed which were in line with the existing literature (Kerley-Hamilton et al., 2012, Bodden et al., 2021, Fan et al., 2015a).

No significant differences in fetal weight were observed between the maternal MDL and WD groups when compared to the CD. However, a notable increase in the number of SGA fetuses was observed in both the MDL and WD groups. Numerous rodent studies reported the relevance of a paternal high fat diet and decreased fetal weight (Capobianco and Pirrone, 2023, Binder et al., 2012, Claycombe-Larson et al., 2020). This finding is intriguing, particularly because WD fathers were not obese; yet the highest number of SGA fetuses was observed in the offspring of WD-fed fathers. Previous rodent studies have highlighted the relevance of a paternal high-fat diet in reducing fetal weight, underscoring the significance of these findings in clinical nutrition. SGA, defined as birth weight

below the 10th percentile, has been linked to an increased predisposition to chronic non-communicable cardiometabolic diseases such as type 2 diabetes and cardiovascular disease due to heightened metabolic risks in these offspring.

An elevated expression of placental FATP4 and SNAT2 proteins in response to a maternal high-fat diet in rodents, suggesting a compensatory mechanism in these fetuses' (Song et al., 2018). Additionally, an interesting cohort clinical trial analysed data and reported an increased incidence of SGA in the offspring of obese fathers compared with those with a normal BMI (Lin et al., 2022). The occurrence of SGA not only negatively impacts the long-term prognosis of these infants but also has maternal-neonatal consequences such as an increased rate of cesarean section and psychological distress in mothers of SGA neonates. It's worth noting that this effect is exacerbated when both parents are obese, suggesting that optimizing paternal BMI could significantly improve the health of future generations (Lin et al., 2022, Bellido-González et al., 2019, Guo et al., 2023) Furthermore, the occurrence of SGA delivery in underweight fathers, as observed in the MDL group of this experiment, suggests that the addition of methyl donors may not mitigate the negative effects of a low-protein diet. Although LPD fathers were not underweight in this study, fetuses derived from LPD-fed fathers were significantly lighter than those in the CD group. One potential mechanism could be related to the effect of paternal seminal fluid derived from LPD fathers, which may dysregulate uterine environments and modulate immunological, cell signaling, and vascular remodeling processes.(Watkins et al., 2018).

The significant influence of paternal diet on fetal cardiac gene expression has been discussed in the previous chapter. However, paternal diet had minimal impacts on maternal cardio-metabolic ill-health in late gestation. The fetal cardiac transcriptomic results are a strong confirmation for the hypothesis of this thesis as none of the stud males in these experiments showed any weight changes, however, still the negative implication of this over/undernutrition diet affected the gene expression in their fetal hearts. Therefore, no manipulation of the adverse effects of obesity metabolites has been involved in the transcriptomic profiles in the WD hearts. These findings from

under/overnutrition diets could be very insightful for the men who are seeking to become a father and have healthy babies. It could be suggested suboptimal diets have adverse effects on male reproductive fitness even before any gross physiological changes become manifest.

How can the paternal diet direct his offspring's phenotype? The answer to this question remains elusive. However, we could hypothesise that poor paternal diet modulates the sperm epigenome, seminal plasma microbiota as well as its interaction with the female reproductive tract (FRT), or a mother's assessment of the quality of a potential partner, such as the phenotypic traits (Daxinger and Whitelaw, 2012, Rando, 2012) which could link poor paternal diet with his offspring's development and metabolism.

Sperm DNA methylation and histone modifications, such as methylation or acetylation of histones on H3K9me2/3 and sperm and seminal plasma RNA modifications are potential routes that a father's lifestyle could impact on the ill/health of his offspring both short and long-term (Miller and Grant, 2012). A study on male mice conducted by Watkins and colleagues examined the effects of a suboptimal diet on sperm and seminal plasma concurrently, comparing it with seminal plasma from fathers on a normal diet using artificial insemination (Watkins et al., 2018). The observations demonstrated that a paternal low-protein diet significantly increased the expression of RNA methylation modulators in F1 adult offspring testes, including *Fto*, *Mettl3,* and *Mettl14.* Furthermore, it was found to impact factors related to histone modification, such as *Hdac1, Hdac2,* and *Kdm3a,* along with factors involved in DNA methylation like Dnmt1 and Dnmt3b (Morgan et al., 2020b). Therefore, it could be suggested that flexible epigenetic processes may potentially have a greater impact on the phenotype of offspring, than fixed and inheritable alteration to DNA sequences.

5.1.1 Paternal high-fat diet

Sperm from overweight male mice exhibit an increased methylation of CpG sites within the *Peg9* promoter (Mitchell et al., 2017). This heightened methylation subsequently results in reduced *Peg9* expression. This reduction in *Peg9*

expression plays a role in oocyte fertilization challenges and perturbs proper zygotic reprogramming, ultimately causing issues in placental tissue development (Bakos et al., 2011a). Additionally, when fathers consume a high-fat diet before mating, it alters the process of spermatogenesis by modifying the epigenetic profile of sperm through sncRNA-mediated mechanisms. These changes make the offspring more susceptible to metabolic disturbances (Klastrup et al., 2019). Additionally, studies involving men have explored the association between WD and male fertility. For example, a cross-sectional study conducted on Iranian men undergoing infertility treatment reported an association between the higher consumption of the Western diet among infertile men (Haeri et al., 2021).

Apart from the more established sperm epigenomic mechanisms (DNA methylation and histone modifications) which are influenced by diet, different types of sperm miRNAs could be potential agents for the paternal programming of offspring. In addition to playing a significant role in offspring programming, sperm miRNAs can also affect placental gene expression by crossing the placental barrier (Kasimanickam et al., 2022). Therefore, sperm miRNAs can play a central role in regulating maternal-placental-fetal gene expression—differential sperm miRNAs which have a role in the occurrence of idiopathic gestational difficulties such as preeclampsia. Indeed, differential expression of miRNAs, such as miRNA (miR-210 and miR-182), has been reported in the placenta of patients with preeclampsia and SGA (Pineles et al., 2007). Therefore, in the subsequent studies of the placental experiments in this thesis, tracing the presence of any sperm miRNA would be highly insightful.

It can be suggested that making a switch from a suboptimal diet to a balanced and healthy diet might help reduce the negative effects of the suboptimal diet on sperm and its potential impact on future generations. Transitioning from a high-fat diet (HFD) consumed during childhood, puberty, and early adulthood (crucial periods for male reproductive development) to a normal diet might mitigate the adverse effects of the HFD on the health of the next generation (Qi et al., 2022). This transition could potentially prevent the development of metabolic diseases in offspring.

However, it is important to note that this dietary change may not fully reverse the damage already inflicted on sperm, and HFD could lead to irreversible effects on spermatogonia cells. This, in turn, may have detrimental consequences on various sperm quality parameters due to the prior consumption of the HFD. The damage to sperm quality parameters might persist because of factors like oxidative stress and increased levels of substances such as glutathione and acetate produced during lipolysis (Crisóstomo et al., 2019). A systematic review and meta-analysis investigated the effects of bariatric surgery on male reproductive function, including sex hormones, sperm quality parameters like sperm volume, concentration, motility, and morphology, as well as sexual function (Lee et al., 2019). The results following bariatric surgery showed a significant improvement in erectile function due to enhancements in sex hormone levels, favouring male hormones over female hormones. However, weight loss after the surgery did not seem to impact sperm quality parameters or the levels of dehydroepiandrosterone (DHEA), androstenedione, and inhibin B. A recent study reported a quick response of human sperm by changing a healthy diet to one, rich in sugar, as detected by changes in both sperm motility and sperm tRNA-derived small RNA (tsRNA) content (Nätt et al., 2019). This study suggests that there is a significant and rapid response of human sperm to dietary interventions, which could potentially suggest an association between the sperm sncRNA code and sperm motility. Through the function of sperm sncRNA, it could be interpreted that parental dietary intervention strongly influences intergenerational metabolic response. There is evidence sperm sncRNA could act as a mobile source of cell-to-cell interaction, which means it has the potential to conduct sperm information to the egg prior to the formation of the zygote (Ayaz et al., 2021).

The intergenerational effects of paternal HFD in occurrence of the breast cancer in female offspring of male mice fed HFD (Fontelles et al., 2016) could draw attention toward human studies. However, no studies in humans have investigated the association between paternal diet and breast cancer in their daughters. Therefore, it could press the point that the clinical research on breast cancer needs to pay more attention to the field of POHaD. Perhaps, monitoring the daughters of obese fathers and investigating any initial signs of breast cancer

such as the mammary gland density in puberty, as well as monitoring the epigenetic changes in their fathers' sperm could be insightful.

5.2 The impact of methyl donor supplementation and male fertility

Elevated levels of reactive oxygen species (ROS), often triggered by chronic diseases, obesity, genetic variations, medication use, ageing, and unhealthy dietary and lifestyle choices, can result in oxidative stress (Sharifi-Rad et al., 2020). Oxidative stress is one major reason for sperm DNA damage. A crucial role of the 1-C cycle is to counteract the effects of these ROS through the production of the antioxidant glutathione. Glutathione is synthesized from folate in conjunction with homocysteine. Importantly, an inadequate dietary intake of folate can lead to insufficient glutathione production. It is worth noting that glutathione is formed only when there are adequate concentrations of methionine and, particularly, folate (Bailey et al., 2015).

Sulfur-containing amino acids play a significant role in preserving cellular systems by affecting cellular redox balance and the ability to detoxify harmful compounds, free radicals, and reactive oxygen species, thereby maintaining cellular integrity. Methionine and cysteine are the two primary sulfur-containing amino acids in mammals (Townsend et al., 2004). Folate, through its role as a methyl group donor, plays a central role in S-adenosylmethionine synthesis and cellular metabolism Methionine and cysteine are the two primary sulfur-containing amino acids in mammals. A novel study on paternal diet proposed that the occurrence of intestinal, skeletal, and muscular abnormalities such as craniofacial deficits, when paternal folate intake is deficient. This deficiency is primarily mediated through perturbed levels of DNA methylation and one-carbon metabolism, potentially leading to these developmental issues (Lambrot et al., 2013a, Padmanabhan et al., 2013). On the other hand, excessive intake of paternal methyl donors has been associated with its adverse effects. Such as, in a mouse model, when paternal consumption of dietary methyl donors was increased before mating and then discontinued, it resulted in offspring which experienced deficiencies in their ability to learn and remember tasks dependent

on normal hippocampal function. Additionally, these mice displayed impaired synaptic plasticity in the hippocampus and reduced theta oscillations in this brain region. Examining gene expression, researchers observed altered activity in the methionine adenosyl transferase *Mat2a* and the BK channel subunit Kcnmb2. (Ryan et al., 2018). These changes were linked to modifications in the Kcnmb2 promoter's methylation status in MD F1 mice MeDIP-chip analyses of sperm of MD fathers in this study showed a significant increase in processes associated with the sense of smell within the group of genes linked to the regions with increased methylation in both MD sperm and the hippocampal tissue of MD-enriched offspring (Ryan et al., 2018). Therefore, the role of paternal dietary elements could be related to the programming of their offspring's mental ill-health in their future generations. In a study involving humans, Pauwels and colleagues employed comprehensive food diaries to compute the consumption of methyl donors in men. They then investigated the links between the intake of methyl donors such as methionine, choline, and betaine, and birth weight (Pauwels et al., 2017). Their findings indicated that methyl donors might be linked to variations in birth weight. Specifically, betaine and methionine showed a negative connection, while choline exhibited a positive association (Morgan et al., 2021, Pauwels et al., 2017). For example, similar patterns in fetal weight among offspring sired from paternal LPD in my experiments were observed.

The significant animal and human findings regarding the influence of paternal dietary methyl donor deficiency or supplementation during preconception and their effects on the fetal/placental development, and indeed the ill-health of the offspring, could be extended to the field of fertility treatment. A recent study of couples who were referred to a fertility clinic with a male infertility primary factor (Martín-Calvo et al., 2019), explored the influence of parental supplementation with folate (folic acid) in both men (989 μg/day) and women (1797 μg/day). The results showed paternal folate intake during the preconception period was associated with a slight an elongation in the duration of pregnancy (2.6 days), in babies conceived through ART. The level of paternal red blood cell folate content has been related to the embryonic growth trajectories (Hoek et al., 2019). Therefore, it may suggest a shift from maternal

preconceptionally folate fortification to both maternal and parental-based approaches would be more beneficial.

On the other hand, another study focusing on human sperm enhancement found no changes in the sperm epigenome including DNA methylation, when men had a low dose of folic acid intake, specifically 1000 μg/day. This lower dose showed no harmful effects on sperm epigenome. However, it is worth noting that a higher dosage of folic acid fortification, at 5 mg/day, was reported to have an impact on sperm epigenome methylation in cases involving male infertility (Chan et al., 2017). Consequently, these findings highlight the importance of considering both the safe levels of methyl donor supplementation and the underlying fertility issues at the same time, for men. For women who are considering becoming pregnant, the taking of specific supplements, such as folic acid, has been recommended by health practitioners for several decades due to its role in preventing congenital abnormalities such as neural tube defects.

The understanding of how paternal environmental factors influence the offspring's health is expanding. This includes examining the combined effects of maternal and paternal physical activity that contribute to enhanced glucose tolerance in their ageing offspring, leading to alterations in the endocrine function of the offspring's pancreas. These findings hold significant implications for the prevention and management of type 2 diabetes in future generations (Zheng et al., 2020). Therefore, such findings would suggest a synergistic relationship between parental lifestyle factors, such as exercise, which can imply additive effects and maximize their effects on their progenies. Furthermore, it is possible that if both parents maintain either a balanced or sub-optimal diet, it may intensify their effects on their offspring. Consequently, monitoring and enhancing parental lifestyle can play a substantial role in preventing the occurrence of intergenerational metabolic diseases.

A study in breeding roosters has shown, that positive associations with growth and organ development of their offspring have been associated with dietary folate intake which folate impacts on sperm piRNAs profile. The beneficial effects of paternal folate fortification occur through targeting and changing the

expression of sperm piRNAs profile (Wu et al., 2019). piRNAs have a substantial role in maintaining the silencing of transposons in the genome of the germlines as well as in the post-transcriptional gene silencing pathways in spermatogenesis and male reproduction (Carmell et al., 2007, Senti et al., 2015, Gebert et al., 2015). Additionally, sperm piRNAs are types of RNAs that are involved in a phenomenon called paramutation (an epigenetic interaction between two alleles of a locus) in non-vertebrate animals. They have been recognized in a specific RNA cluster that influences epigenetic inheritance (Ronsseray, 2015).

Providing men of reproductive age diagnosed with idiopathic infertility, with a high dose of folic acid 5mg/day for 6 months showed, alteration in sperm DNA methylation through modulating the MTHFR enzyme, however, the study revealed that semen parameters such as semen volume, sperm motility and sperm chromatin integrity remained unaltered in response to high dose folic acid supplementation, however, a significant global loss of methylation in sperm epigenome methylation was observed with increased effects in patients with homozygous for the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism (Aarabi et al., 2015). Semen quality, characterized by parameters such as volume, convention, total sperm count, vitality and WHO-defined normal morphology can provide valuable insights into male fecundity (Louis et al., 2014). In contrast, another study has shown that folic acid supplementation in a short period is ineffective on human sperm methylome (Chan et al., 2017). Therefore, it could suggest that high-dose folic acid supplementation should be accompanied by caution considering factors such as the ethnicity and genetic background of the recipient.

5.3 The impact of paternal low protein diet

A pioneering study reported that progeny derived from LPD-fed fathers, displayed a perturbed expression of genes involved in lipid and cholesterol metabolism in neonatal livers, relative to offspring of control diet-fed fathers (Carone et al., 2010). Also, increased glucose intolerance, cardiovascular dysfunction and enhanced levels of tumour necrosis factor-α have been

identified in the offspring derived from paternal LPD (Watkins and Sinclair, 2014a). One potential hypothesis for the transmission of paternally mediated pathologies in LPD could be related to the increased level of ROS in the testicular germ cells, following changes in the modifications in transcription factor binding capabilities and the related histone marks, which are preserved in sperm cells (Oikawa et al., 2020). Additionally, accompanied alterations in the biogenesis of sperm small non-coding RNAs, might collectively convey intergenerational impacts (Yoshida et al., 2020). Additionally, a huge alteration in the subgroups sncRNA subtypes such as miRNAs, piRNAs and tRFs tRF-Gly-CCC, -TCC, -GCC tRF-Lys-CTT tRF-His-GTG Down miRNAs, piRNAs and tRFs miRNA-let7 family has been reported in response to paternal LPD (Sharma et al., 2016b).

Indeed, the upregulation of sperm tsRNA in a paternal low protein diet has been reported. Enhanced levels of 5′-tsRNA might occur as a natural reaction to decrease protein production in response to a shortage of amino acids (Nätt et al., 2019). The variability in the content of small non-coding RNAs (sncRNA) among different sperm cells from the same individual could potentially explain some of the variations in inherited epigenetic traits observed among offspring.

Among the subclasses of sncRNAs, piRNA, tsncRNA, and miRNA are the ones most associated with the inheritance of metabolic diseases. A relative increase and decrease, respectively, in these different subtypes of ncRNAs in paternal WD and LPD has been reported (Fullston and Ohlsson-Teague, 2016, Sharma et al., 2016b, Grandjean et al., 2015). While the fine-tuning of gene regulation through miRNA and piRNA is relatively well understood as a mechanism for epigenetic inheritance, the mechanisms underlying tsncRNA-mediated epigenetic inheritance are still in the early stages of investigation.

Studies conducted in mice have demonstrated that 5′-GlyGCC, a molecule found in high quantities in sperm and influenced by a low-protein diet, inhibits the activity of genes essential for the growth of murine endogenous retroviruses in both embryos and embryonic stem cells (Sharma et al., 2016a). A pioneering study on human sperm in response to diet has demonstrated that human sperm

exhibits the ability to adapt and modify its small sncRNA code in reaction to quick environmental shifts, a mechanism observed in other species as a result of transmitting information to the succeeding generation. Of particular significance is the correlation between changes in sperm motility and alterations in the sncRNA code, suggesting a potential underlying cause linking male fertility and intergenerational metabolic reactions (Nätt et al., 2019).

A diet restricted by reducing the diet access gradually in three weeks, and marinating on 70% with no restriction for the rest of the study for an additional 14 weeks, in male mice, has reported no alteration in fertility parameters such as sperm, count, motility and morphology, which are the most applicable male fertility assessment in fertility clinics (McPherson et al., 2016). Nonetheless, diet restriction led to a reduction in the quantity of sperm capable of attaching to an MII oocyte. This suggests a potential decline in the later stages of sperm development after ejaculation, including capacitation and hyperactivation, which are essential for sperm binding (Suarez, 2008). Therefore, paternal undernutrition could be one of the potential risk factors for a successful fertility treatment.

Yoshida and his team have documented that a paternal LPD leads to an increase in reactive oxygen species (ROS) within the germ cells of mouse testicles. This, in turn, results in changes to the activity of transcription factors and the associated histone modifications, which are then preserved in sperm. Additionally, this diet alters the process of small non-coding RNA (sncRNA) production in sperm (Yoshida et al., 2020). Collectively, these alterations could potentially carry over as intergenerational effects. The spermatozoa plasma membrane contains a considerable amount of polyunsaturated fats, making it susceptible to damage from ROS, in addition to losing a significant amount of the scavenging enzymes during spermiogenesis (McPherson et al., 2016).

The consumption of plant-based proteins has been shown to improve ovulatory infertility in up to 50% of women (Chavarro et al., 2008). While there are some suggestions that a wide range of proteins, iron, vitamin D, calcium, iodine, omega 3, and vitamin B12 deficiencies can arise from the consumption of strict

vegan and vegetarian diets. It has been reported that well-planned whole-food regimes considering the plant-based proteins, could be beneficial in terms of preventing the inadequate supply of essential nutrients (Sebastiani et al., 2019).

The type of protein has a substantial impact on sperm and male fertility, as plant-based proteins have a lower level of sulfur-based amino acids such as methionine cysteine, in addition to phenylalanine in comparison with animal proteins. The aforementioned amino acids may potentially impact sperm motility (Ferramosca and Zara, 2022). One of the key amino acids with positive effects on sperm motility and trophic effects on Sertoli cells is leucine. Lucien can be found abundantly in casein. Leucine by improving the efficacy of the PI3/AKT signalling pathways in the testis and suppressing the autophagy process by improving the expression of P62 and LC3-II has been demonstrated in leucine therapy in zebrafish sperm (Zhang et al., 2017b). Indeed, the activation of the PI3/AKT signalling pathways has been reported to play a role in the activation and motility of human sperm, as described (Sagare-Patil et al., 2013).

However, it should be noted that the synthesis of glutathione depends on the availability of cysteine (Sadeghi et al., 2023). A sufficient level of glutathione is essential for sperm viability, as glutathione is an endogenous antioxidant that plays a central role in maintaining the redox balance in sperm, as sperm are particularly vulnerable to ROS (Zhu et al., 2022). Therefore, a balanced level of the amino acids involved in oxidative and reductive stress is essential for sperm chromatin integrity.

The source of protein used in the current experiment is casein. Casein is a β-Casein A2-5P, which is a reference protein for the β-casein family, and it is a dairy-based source of protein derived from bovine milk, which provides a range of essential amino acids (Huppertz et al., 2018). Casein is considered a high-quality protein as it contains essential amino acids such as arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine. The availability of leucine has an essential role in spermatogenesis.

5.4 The potential impact of other diets on male fertility parameters

So far, the implications of suboptimal diets such as under/overnutrition with and without methyl donors have been discussed extensively in this thesis. However, there are significant changes in the type of food and drinks that people consume, particularly among younger generations. Excessive consumption of sugar-sweetened beverages (SSBs) is widely recognized as a major factor in the obesity epidemic. However, its impact on behavioural changes in offspring remains an area that requires further investigation. The assessment of sperm and seminal plasma parameters in young men who consumed the most SSBs revealed elevated levels of oestradiol (E2) in their serum (Chiu et al., 2014). No significant deteriorated parameters in semen quality were reported. Interestingly, men in the highest quartile of intake had a higher percentage of sperm with normal morphology (37.4%) compared to men who consumed the least SSBs (Kiwitt Cárdeas et al., 2020). Another study on 2,935 young men and SSB intake, who were unaware of their reproductive hormone levels and semen quality, reported an inverse link between semen quality such as lower sperm concentration, lower total sperm count, sperm motility and semen volume as well as a lower ratio of inhibin/FSH in their serum samples, about SSB intake (Nassan et al., 2021). A decreased inhibin/FSH ratio suggests an adverse impact on spermatogenesis as the ratio of inhibin B/FSH is a strong marker of Sertoli cell function and could indicate a primary reduction in spermatogenesis.

Studies in male mice-fed fructose/glucose solution, mimicking exposure to SSBs, observed a negative impact on fertility with an observed decrease in average litter size by 25% from control males (Ruff et al., 2013). Indeed, more research related to the impact of SSB is required, as the long-term effect of these types of drinks on male fertility is unclear.

No alterations in male fertility have been reported after the consumption of a high-fructose diet (HFru; 45% energy) in a parental mouse model after being fed for 8 weeks. Despite the absence of any observable changes in body mass, significant alterations in the hormonal profile were noted, including increased

leptin and decreased adiponectin levels in the stud males. Additionally, there was an increase in genital fat pad mass and blood pressure among their offspring, in a sex-specific manner such that males were affected to a greater extent than females (Ornellas et al., 2020). This raises the possibility of the predisposition of these offspring to CVD later in life, by deteriorating the metabolism and blood pressure of these offspring.

The abundance of glucose and fructose in spermatogenic cells as well as a high level of the polyunsaturated fatty acids in sperm, make sperm susceptible to glycation reaction and Advanced glycation end-products (AGEs) formation. AGEs are other metabolites of unhealthy and imbalanced diets. The formation of this metabolite is the result of attaching sugar to proteins. AGEs are generated via a non-enzymatic process called glycation/Maillard, where reducing sugars (e.g., glucose and fructose) react with proteins, lipids, or nucleic acids. Availability of the high level of reactive oxygen species or oxidative stress in the body as well as a high level of inflammation underlies the production of these factors. A study conducted on high-fat 60% AGEs fed non-obese and non-diabetic C57BL/6 male mice, reported a significant increase in sperm protamine deficiency as well as membrane and cytoplasmic peroxidation in these animals (Akbarian et al., 2021). Therefore, it suggests combination of high sugar with a high-fat diet could worsen the production of ROS.

Mediterranean diet so-called MedDiet is characterized as the consumption of low levels of dairy products, red meat, processed meats, and sweets; a high intake of olive oil, fruit, nuts, legumes, vegetables, and whole grains; and a moderate amount of fish, poultry, and wine. effectively has been associated with improving semen quality and sperm parameters including, higher sperm concentration, total sperm count and sperm motility, due to the higher levels of antioxidants such as Vit E, C and beta-carotene, which have the potential to improve sperm motility in young healthy men (Zareba et al., 2013, Salas-Huetos et al., 2019, Jurewicz et al., 2018, Mínguez-Alarcón et al., 2012). Additionally, antioxidants have a protective role against the elevated level of ROS in sperm and improve sperm fertilization capacity in humans (Ko et al., 2014). However, the impact of sperm ROS levels on fertilization rate is controversial, as ROS is

an essential factor for sperm development and capacitation (Dutta et al., 2020). Therefore, elevated levels of ROS could be detrimental to male reproductive fitness. Indeed, a high level of anti-inflammatory agents and low pro-inflammatory nutrients in the Mediterranean diet MedDiet could play a protective role against inflammation which has the potential to impact the process of reproduction by either changing the structure or function of the male accessory glands (La Vignera et al., 2013).

5.5 Male diet supplementation and Assisted Reproductive Technology

Perturbed sperm epigenetic status has been related to male infertility (Stuppia et al., 2015). Current estimates indicate that 15% of couples are struggling with infertility, of which almost 25% are due to decreased quality of sperm and semen (Salas-Huetos et al., 2017). Sub-fertility requires more attention to be considered as it is a complex case combination of different factors including genetic, environmental and lifestyle parameters. Additionally, aberrant sperm epigenetic functions in spermatozoa have been related to long-term congenital diseases in babies who were born through ART (Marzouni et al., 2022).

Further understanding regarding the sperm epigenome status, such as abnormal sperm methylation at imprinted loci with poor seminal plasma quality (Tang et al., 2018) derived from the men with sub-fertility compared to the men with normozoospermic males has been reported (Boissonnas et al., 2010, Marques et al., 2004, Poplinski et al., 2010). Particularly men with idiopathic infertility and the cases such as oligozoospermia are more likely the cases who suffer from sperm epigenetic alterations. Conventional semen analysis relies on measurements and parameters that were defined decades ago, before our understanding of epigenetics evolved. This raises the question of whether our assessment of sperm quality is outdated, considering that sperm from fertile males can still have post-fertilization consequences (Watkins et al., 2020). Recent studies have reported aberrant methylation within control regions of imprinted genes, such as H19, GNAS, and DIRAS3 (Tang et al., 2018),

suggesting the need for a more comprehensive approach to evaluating sperm quality.

Obesity is increasingly recognized as a fundamental contributor to various non-communicable diseases. Consequently, proposing strategies to optimize dietary patterns could significantly enhance the reproductive health and fitness of individuals aspiring to parenthood. Several studies have investigated the impact of obesity and elevated BMI on male fertility outcomes following fertility treatments (Thomsen et al., 2014, Bakos et al., 2011b). In addition, it is essential to define BMI categories in men. BMI is typically divided into three categories: normal BMI (18.5–24.9 or 20–24.9 kg m^{-2}), overweight BMI (25–29.9 kg m^{-2}) and obese BMI (≥30 kg m^{-2}). A meta-analysis study found that paternal obesity had minimal implications on pregnancy rate and live birth rate in couples undergoing infertility treatments such as in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) (Le et al., 2016). In addition, studies in human and mouse models have reported a positive association between the paternal BMI and reduced sperm motility, as well as an increase in sperm DNA fragmentation, due to enhanced levels of oxidative stress in spermatozoa (Peel et al., 2023). Altogether, it could suggest a decrease in pregnancy outcomes could occur (Salas‐Huetos et al., 2021). However, some studies provided strong support for the hypothesis of negative implications of male-raised BMI in terms of the success rate of clinical pregnancy via IVF, though not ICSI. However, they reported no detrimental implications for embryo quality (Merhi et al., 2013). In addition, a fascinating study, known as the Longitudinal Investigation of Fertility and the Environment (LIFE), is the first prospective cohort study with the assessment of anthropometric characteristics and semen quality (Louis et al., 2014).

This study is the first of its kind to relate body size, BMI and waist circumference (WC) with assessments of semen samples in men without any signs of infertility. Therefore, it might suggest, that BMI alone may not be a direct reason for infertility in men within this study. Instead, the distribution of body fat appears to be a critical factor in semen quality.

In this thesis, abnormal fat distribution was observed in stud males fed WD. Therefore, this observation suggests men who are of normal BMI and a healthy weight and are considered healthy and fertile, maybe physically unfit due to consumption of suboptimal diets and a sedentary lifestyle. While their sperm may be capable of oocyte fertilization, these individuals could still exhibit an altered sperm epigenetic composition.

Therefore, in the cases of male infertility treatments sperm selection could be a bypass for natural sperm selection in FRT substantial step for better and maybe promising outcomes from ART cases, due to treatment of cases associated with male infertility. In conventional sperm selection methods, the sperm are selected using practices such as swim-up (for choosing the most motile sperm), or density gradient centrifugation (DGC). DGC has the potential to increase sperm ROS (Organization, 2010), which suggests it is not a perfect way to select the most motile and fertile sperm. Furthermore, morphology does not guarantee sperm viability and DNA status which plays a central role in sperm fertility (Samuel et al., 2016). Therefore, new non-invasive methods in sperm selections such as microfluidics, nano purification, Raman spectroscopy, and transcriptomic profiling at the single- or few-cell level, or a combination of these methods could offer very promising methods to select the most viable sperm prior to ART (Štiavnická et al., 2017).

Utilizing the analysis of sperm epigenetic status, while an invasive procedure, offers a novel approach to enhancing the quality of sperm utilised in ART procedures. Importantly, unlike for women, collecting sperm samples is non-invasive for males, and it is both feasible and cost-effective to obtain multiple samples for analysis before subsequent interventions or ART selection. The majority of ART clinics prefer ICSI over IVF, due to its higher fertilisation rate, particularly in cases involving both maternal and paternal infertility, with an emphasis on parental factors (Merhi et al., 2013). Avoiding the selection of sperm with disrupted epigenetic status could prove immensely beneficial (Präg and Mills, 2017), but also significantly enhances the likelihood of a successful pregnancy. Moreover, it holds the potential to prevent the development of non-communicable diseases in their offspring later in life.

In the field of ART treatments, the majority of research has traditionally centred around improving oocyte quality (Gürtin and Tiemann, 2021). Consequently, directing attention towards enhancing sperm quality offers valuable insights regarding the sub-fertility treatments. Given that, a full cycle of spermatogenesis and spermiogenesis in men spans approximately three months (Picut et al., 2018), there exists the potential to mitigate the adverse effects of poor diet and certain environmental exposures within a relatively short timeframe. Furthermore, men can produce a substantial number of sperm samples; a fertile normospermic man can generate approximately one thousand sperm per heartbeat (O'Donnell et al., 2015). Therefore, considering the limited quantity of high-quality oocytes in women, assessing sperm in both fertile and infertile men represents a significant opportunity that merits further research.

5.6 Sperm and seminal plasma exosomes

In addition to sperm DNA integrity and epigenetic status, there are other pathways through which a father could impact his offspring's development, and consequently, their risk of metabolic health issues later in life. Seminal plasma proteomic analysis derived from obese men has shown significant enhancement in the level of factors such as proinflammatory cytokines, and components of the apoptotic pathways, which results in oxidative stress (Wang et al., 2020). This corresponds with alteration in sperm quality parameters such as increased non-progressive motility, morphology, acrosome integrity, mitochondrial activity, and increased sperm DNA fragmentation. Additionally, seminal plasma contributes to sperm function and affects the intrauterine milieu by influencing endometrial cytokines and embryotropic agent secretion through both its exosomes and microbiota (Javurek et al., 2016).

The epididymosomes and prostasomes exosomes which are extracellular vesicles containing agents such as protein and RNAs, have been shown to contribute to sperm function, interaction with the FRT and finally fertilization by binding to the sperm surface (Samanta et al., 2018). Interestingly, exosomes can deliver to the target cells. This genetic material may contain protein-encoding messenger RNAs (mRNAs) and small noncoding RNAs, which play

substantial roles in the regulation of gene expression (Yáñez-Mó et al., 2015). Dicer1 knock-out mice were generated using ICSI (for injecting sncRNA-deficient sperm and sperm RNA supplementation) to study the role of these sperm-derived RNAs in terms of fertilization success and preimplantation development. It has been reported that this deletion of Dicer 1 generation altered sperm morphology and motility, mimicking the human oligo-astheno-teratozoospermia (OAT) syndrome (Yuan et al., 2016). Interestingly, these exosomes have the potential to bond with sperm tsRNAs therefore, the link between the somatic and germline epigenetic transmission can be justified (Bodden et al., 2020). However, the precise mechanism of transferring the different types of RNA through these exosomes into the mature sperm has not been fully understood yet. Of note, the paternal diet's role in modulating the alteration in different types of RNAs has been discussed. Therefore, paternal diet through modulation of the RNAs influences embryo development and fetal programming (Grandjean et al., 2015, Bodden et al., 2020, Claycombe-Larson et al., 2020). In addition, the role of seminal plasma exosome in interaction with FRT and promoting the modulation of the proinflammatory cytokines and embryo trophic agents can programme embryo implantations and development (Wang et al., 2021, Gholipour et al., 2022).

Currently, semen sample assessment primarily focuses on the methylation pattern within the entire semen sample. Notably, sperm DNA methylation assessment is not a routine part of standard semen analysis in in fertility clinics, however, it may be considered for more comprehensive sperm evaluations. Therefore, there is some level of uncertainty about whether environmental factors affect sperm epigenetic patterns consistently or if certain spermatogonia are particularly sensitive to these influences. Indeed, findings in ART have shown that ART possibly can lead to premature vascular ageing, even in seemingly healthy young adults who do not exhibit traditional cardiovascular risk factors (Meister et al., 2018). This ageing process can advance the development of arterial hypertension. There is a growing body of evidence suggesting that epigenetic mechanisms, similar to those observed in experimental animals, play a role in the alteration of the offspring's cardio-metabolic and neurological characteristics induced by ART (La Rovere et al.,

2019, Norrman et al., 2021). Applying the insights gained from these mechanisms in mice to humans may open up possibilities for preventing ART-induced changes in cardiovascular characteristics and reducing cardiovascular risks in the countless future babies who will be borne by ART (Meister et al., 2018, Scherrer et al., 2015).

Therefore, finding this knowledge could potentially lead the field to develop new diagnostic tools which could differentiate sperm based on their epigenetic profiles. Such techniques would represent an innovative way to select the sperm with the highest fertility potential and could be one way to ensure the well-being of future generations. In addition, such approaches would be of significance for the field of farming and husbandry of large farm animals as well as horses.

This thesis has raised several interesting questions. It would be of interest to conduct further experimentation such as cardiac RNA sequencing, RT-qPCR analysis and validation on fetal heart tissues, and immunostaining of different proteins expressed in the heart would be insightful. know the physiological characteristics of the cardiac gene expression in these fetuses. As well as analysing the effect of sex in the manifestation of cardiac transcriptomic in male and female offspring. And indeed, further investigation of the F2 generation would be substantially valuable.

5.7 Conclusions and strength of this thesis

The present set of experiments demonstrates that suboptimal paternal diet, either under or over-nutrition, directs fetal and organ development and growth, as well as placental gene expression for RAS, 1-carbon metabolism, and apoptosis pathways. In addition, poor paternal diet modulated fetal cardiac gene expression pathways in late gestation. However, paternal diet had minimal effects on maternal cardiometabolic health in late gestation.

This thesis has not aimed to directly determine whether alterations in sperm epigenetic status or seminal plasma composition occurred in response to paternal diet. However, the majority of findings from these experiments are in line with

other studies demonstrating that offspring growth and metabolic health could be directed via alterations in sperm epigenetics.

Indeed, findings in this thesis could be extremely insightful as a strong scientific backup and related to human studies to shed light on the reproductive health of men who are seeking to become biological fathers, as well as their female partners. While advice on taking methyl donor supplements, it's important to consider that they may not reverse all the sperm epigenetics damages caused by taking the HFD for a long time. Consumption of HFD has adverse effects on sperm epigenome through multifactorial mechanisms, including, enhanced levels of oxidative stress leading to alterations in sperm DNA methylation patterns, as well as inflammation. Proinflammatory cytokines can actively target sperm DNA methyltransferase enzymes.

Addressing the underlying inflammatory conditions, as well as antioxidant supplementations alongside the fortification may offer more benefits for men who have been consuming WD for an extended period. Fortification alone may not be sufficient, to fully reverse all the adverse effects of the WD on sperm epigenome. Of note, considering the individual genetic and epigenetic variability potential impacts on the results from fortifications and antioxidant therapy should not be neglected.

Findings from this thesis could be particularly valuable in the field of obstetrics helping to find effective ways to improve the conditions of pregnancy complications like gestational diabetes (GDM) and preeclampsia, and incidence of increased risk of SGA in babies from fathers with WD and overweight. It is important to note that preeclampsia is still often considered an unexplained maternal and fetal condition. Knowing the other environmentally based sensitive epigenetic mechanisms, such as non-coding RNA exosomal loading and expression in seminal plasma and epididymosomes will move this field forward.

Recognizing the significance of enhancing fertility and achieving positive pregnancy results is crucial not only for supporting paternal preconception well-being but also for laying the foundation for the long-term health of his offspring.

We are aware of that paternal factor, including lifestyle, nutrition, and overall health, play a significant role in reproductive success and can impact the health outcomes of offspring later in life. Therefore, prioritizing paternal preconception health can lead to improved pregnancy outcomes and contribute to the long-term health and well-being of future generations.

5.8 Limitations

These findings are limited to their focus on the influence of paternal diet on fetal heart transcriptomic profiles and do not consider the phenotypical alterations that may manifest in the adulthood cardiometabolic characteristics of these offspring, in a sex-specific manner. Studies have reported a higher incidence of vascular dysfunction as well as hypertension in a sex-specific manner in favour of female offspring from mouse dams that were fed LPD in the pre-implantation period (Watkins et al., 2011, Watkins et al., 2008). These would require additional experiments to investigate comprehensively.

Another notable limitation in this thesis is the absence of an analysis of fetal sex-specific effects on fetal/placental assessment. This could provide important insights into potential variations between male and female offspring. Significant differences in gene expression between males and females have been observed in both humans and animals, even within identical organs. The emphasis on these sex-based differences in gene expression is highlighted by (Deegan et al., 2019). Genes encoding regulatory factors associated with the X and Y chromosomes are actively expressed in cardiac progenitor cells during early embryogenesis, and this expression continues throughout different stages of development (Burgoyne and Arnold, 2016, Wijchers et al., 2010). For example, the occurrence of congenital cardiac defects and their differential impacts in terms of the morbidity and mortality rate among the different sexes could justify the sexual dimorphism of cardiac disease (Deegan et al., 2021). Additionally, knowing the fetal sex would provide valuable insights into justifying the differences in fetal weight observed in fetuses derived from LPD compared to other studies, which have reported increased fetal weight from LPD-fed diets (Watkins and Sinclair, 2014a, Morgan et al., 2021). As male fetuses are more

prone to be born as SGA (Monier et al., 2022), and it could potentially skew the data. Therefore, considering the sex of the fetus would enhance the understanding of the observed weight differences and their implications in LPD-fed offspring.

Furthermore, there was no assessment of changes in sperm methylation patterns and the expression of paternally imprinted genes, which play a significant role in embryonic development, placental function, fetal growth, and post-natal metabolism. Paternally imprinted genes are highly expressed in the placenta and are essential for the normal growth of offspring. While this thesis did not detect any changes in the growth trajectory of the stud male, investigating the response of the sperm methylome to various diets could yield valuable insights into the underlying mechanisms.

Moreover, valuable data could potentially be generated by investigating the impact of paternal diet in early gestation which could yield findings regarding seminal plasma agents and maternal responses, providing insights into the early stages of pregnancy's response to the impact of paternal diet.

In summary, while this thesis provides valuable insights into the transcriptomic profiles of fetal hearts influenced by paternal diet, there are several important limitations to consider for a more comprehensive understanding of the broader implications and mechanisms involved.

It is worth noting that the original plan for this project was to assess the influence of paternal diet on maternal cardiometabolic health during three different stages of pregnancy: late (E 17.5), mid (E 9.5), and early (E 3.5) gestation. Additionally, we aimed to study the impact of various diet groups on the seminal fluid proteome using mass spectrometry. This research aimed to investigate how seminal fluid affects preimplantation and early embryo development, uterine environment, vascularization, inflammatory cytokines, and eventual uterine adaptation. As well as following up the maternal cardio-metabolic alterations in response to paternal diet in mid and late gestation. Unfortunately, as a result of the recent COVID-19 pandemic, I was unable to carry out the planned research,

losing a significant amount of time. I was not authorized to work in the lab and generate data for a total of 11 months.

I particularly believe in exploring the effects of paternal diet during early gestation. This interest arises from the fact that, in mice, most maternal metabolic adaptations have typically stabilized by the mid-gestation stage. Therefore, studying the impact of paternal diet on the period of early gestation could potentially yield more meaningful and accurate metabolic assessments. As well as assessment of the endocrine profile of dams and the pro-inflammatory cytokines secreted in response to any potential alterations caused by the influence of paternal diet. Addressing this limitation could significantly enhance the comprehensiveness of my research.

Appendices

Appendix Table 1, components of male mice diets

Dietary supplement	CD (g)	LPD(g)	MDL(g)	WD (g)	MDWD (g)
Casein	18.0	9.0	9.0	19.5	19.5
Corn Oil	10.0	10.0	10.0	10.0	10.0
Starch Maise	42.5	48.5	48.5	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Milk Fat Anhydrous	-	-	-	20.0	20.0
Maltodextrin	-	-	-	10.0	10.0
Sucrose	21.3	24.3	24.3	33.9	33.9
Vitamins (AIN76)	0.5	0.5	0.5	1.0	1.0
Minerals (AIN76)	2.0	2.0	2.0	3.5	3.5
Choline Chloride 50%	0.2	0.2	0.5	-	0.5

D, L-Methionine	0.5	0.5	1.25	-	1.25
Cholesterol	-	-	-	1.5	1.5
Folic Acid	-	-	0.0015	-	0.0015
Vitamin B12	-	-	0.00015	-	0.00015
Betaine	-	-	15	-	15
Dietary supplement	CD (g)	LPD(g)	MDL(g)	WD (g)	MDWD (g)
Casein	18.0	9.0	9.0	19.5	19.5
Corn Oil	10.0	10.0	10.0	10.0	10.0
Starch Maise	42.5	48.5	48.5	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Milk Fat Anhydrous	-	-	-	20.0	20.0
Maltodextrin	-	-	-	10.0	10.0
Sucrose	21.3	24.3	24.3	33.9	33.9
Vitamins (AIN76)	0.5	0.5	0.5	1.0	1.0

Minerals (AIN76)	2.0	2.0	2.0	3.5	3.5
Choline Chloride 50%	0.2	0.2	0.5	-	0.5
D, L-Methionine	0.5	0.5	1.25	-	1.25
Cholesterol	-	-	-	1.5	1.5
Folic Acid	-	-	0.0015	-	0.0015
Vitamin B12	-	-	0.00015	-	0.00015
Betaine	-	-	15	-	15

Appendix, Table 2: List of primers used for quantitative RT-qPCR studies, including apoptosis, RAS pathway, and one-carbon metabolism primers.

Gene symbol	Gene name	Accession Number	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon length
Bad	BCL2 Associated Agonist Of Cell Death	NM_007522.3	gccctaggcttgaggaagtc	catactctgggctgctggtc	90
Bax	BCL2 Associated X, Apoptosis Regulator	NM_007527.3	agtgtctccggcgaattg	ccacgtcagcaatcatcct	69
Bcl2	B-cell lymphoma 2	NM_009741.5	gtacctgaaccggcatctg	gctgagcagggtcttcagag	130
Casp1	Caspase 1	NM_009807.2	caagttgacctcagagaaatgaag	ggcagcaaattctttcacct	114
Fas	Fas Cell Surface Death Receptor	NM_007987.2	caagtgcaagtgcaaaccag	gggttccatgttcacacga	86

Ace	angiotensin-conv+C164:C188erting enzyme	NM_207624.	tctgcttccccaacaagact	aggatgttggtgagctctgg	61
Ace2	Angiotensin I Converting Enzyme 2	NM_001130513.1	tgtagaacgtaccttcgcagag	gggctgatgtaggaagggta	99
Agtr1a	angiotensin II receptor, type 1a	NM_177322.3	cgccagcagcactgtaga	ctcagacactgttcaaaatgcac	62
Agtr1b	angiotensin II receptor, type 2	NM_175086.3	caattgcagatgccttcaac	gggggtgaattcaaaatg	93
Agtr2	angiotensin II receptor, type 1b	NM_007429.4	ggagctcggaactgaaagc	ctgcagcaactccaaattctt	131
ATP6AP2	ATPase H+ Transporting Accessory Protein 2	NM_027439.4	gggtggataaactggcacttc	tggaatttgcaacgctgtc	93
Ren1	Renin 1	NM_031192.3	cccgacatttcctttgacc	cccgacatttcctttgacc	96

Mthfr	methylenetetrahydrofolate reductase	NM_001271353.1	gaagaacataatggcgctgag	ggcatagctgaagcctcctt	76
Mtr	methionine synthase	NM_001081128.3	caagtgctctgcagatgtgg	ccacaaacctcttgactcctg	74
Mat2a	Methionine Adenosyltransferase 2A	NM_145569.4	ccgagtctgtaggggaaggt	gggtcttgttgaaggtgtgc	85
Mat2b	Methionine Adenosyltransferase 2B	NM_134017.2	caattgcagatgccttcaac	tcgagctgagcatttttgg	96
Achy	Adenosylhomocysteinase	NM_016661.3	taccctgttggggttcactt	cagcttcacattcagcttgcc	84
Dhfr	Dihydrofolate reductase	NM_010049.3	cacgtttttcccagaaattga	ttcctcctggacctcagaga	82

Appendix, Table 3: List of primers used for maternal liver quantitative RT-qPCR studies.

Gene symbol	Gene name	Accession Number	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon length
Cyp27A1	Cytochrome P450 Family 27 Subfamily A Member 1	NM_024264.5	tcccggatcatagaaaagga	atagtggcagaacacaaactgg	78
Akt2	AKT Serine/Threonine Kinase 2	NM_001110208.1	gaataccaggcacccttc	cacaaagcataggcggtca	70
Adipor1	Adiponectin receptor - insulin sensitivity	NM_028320.3	ggtgctgacgcttcctct	aacgtccctcccagacct	80
G6pc	Glucose-6-Phosphatase, Catalytic	NM_008061.3	tctgtcccggatctaccttg	gaaagtttcagccacagcaa	85

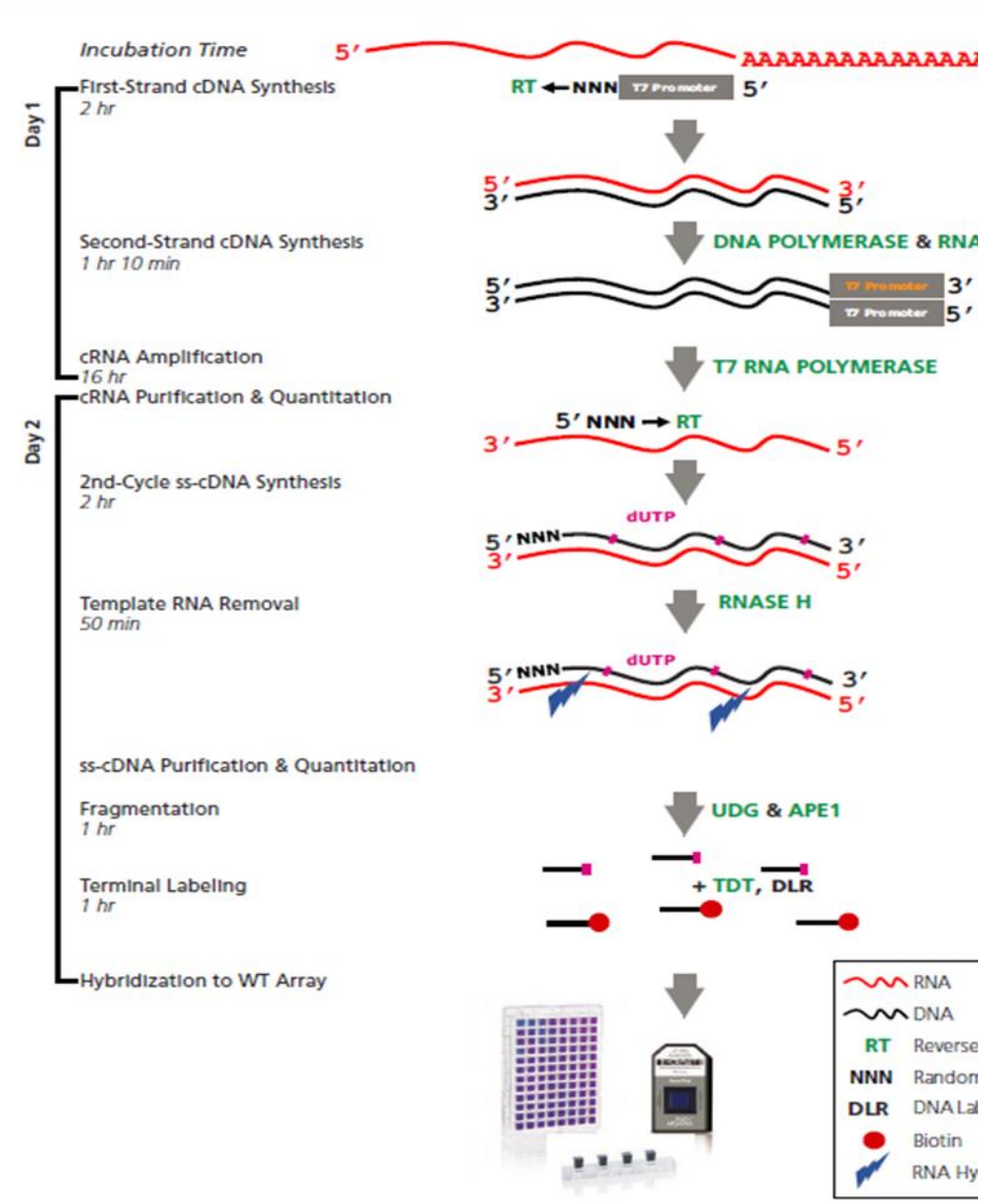


Figure 0-1 WT PLUS Amplification and Labelling Process (ThermoFisher Scientific).

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