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# IMPACT OF QUERCETIN-3-O-RUTINOSIDE ON BIOCHEMICAL AND REPRODUCTIVE PROFILE OF RATS PRENATALLY EXPOSED TO HIGH FAT DIET


BY

**TOLUWALOPE E. ADEYEMI**

**(217081454)**


*Submitted in fulfilment of the requirements for the degree of PhD Health Science (Human Physiology) in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal.*


**Supervisor: Dr. Anand Nadar**

**Co-supervisor: Professor M.L Channa**



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## DECLARATION

I, Toluwalope Esther Adeyemi (217081454), hereby declare that the work described in this thesis entitled:

"Impact of Quercetin-3-O-rutinoside on biochemical and reproductive profile of rats prenatally exposed to high fat diet"

is the result of my own investigations and has not been submitted in part or in full to any other university or tertiary institution for the purpose of obtaining a degree. Information taken from the work of others were duly acknowledged. This research project was carried out under the supervision of Dr A. Nadar and Professor M.L Channa.

[redacted] 18/11/2020

Toluwalope E. Adeyemi Date

[redacted] 18/11/2020

Dr. Anand Nadar Date

[redacted] 18/11/2020

Prof. ML. Channa Date

## DEDICATION

During my academic journey, I have lost loved ones, and I have also gained more. I dedicate this work to the Almighty God and my family.

*To my dad, late Dcn. I.A. Adeyemi. You told me never to limit God. You gave me the best gift a father can give- education. Your unquenchable thirst for excellence and godliness kept me going. You sacrificed everything to educate me. Thank you, daddy.*

*To my late mum Mrs. J.O. Adeyemi. My gold and pillar of strength. You mean so much to me. Thank you for all the sacrifices you made.*

*My dear brother, Late Lt. J.O Adeyemi. An embodiment of grace and excellence. Words cannot express how much I miss you. I love you, dearly. Thank you*

*Oluwatosin Adeyemi, you keep me going, and you are special to me. Thank you for your constant encouragement and prayers.*

***Especially to the almighty God, My all in all.***

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*"Being thankful not only shows good manners, but a simple expression of thankfulness can go a long way in relationships and communication with others. It not only enhances our own lives, but makes other people feel appreciated." – Daniella Whyte*

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*"Feeling gratitude and not expressing it is like wrapping a present and not giving it." – William Arthur Ward.*

Thank you to everyone who has contributed to this achievement.

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**LIST OF ABBREVIATIONS**

| | |
|---|---|
| AGA | Appropriate for gestational age |
| ANOVA | Analysis of Variance |
| ARC | Arcuate nucleus |
| AREC | Animal Research Ethic Committee |
| BMI | Body Mass Index |
| CAT | Catalase |
| CHS | College of Health Sciences |
| CMKLR1 | Chemokine-like receptor-1 |
| DETAPAC | Diethylenetriaminepentaacetic |
| DHEA | Dehydroepiandrosterone |
| DHT | 5α- dihydrotestosterone |
| DNA | Deoxyribonucleic acid |
| DOHaD | Development origin of Health and disease |
| DTNB | 5,5'-dithio-bis (2-nitrobenzoic acid) |
| ELISA | Enzyme linked immunosorbent assay |
| FPG | Fasting plasma glucose |
| FRAP | Ferric reducing antioxidant power |
| FSH | Follicle stimulating Hormone |
| GD19 | Gestation Day 19 |
| GLP-1 | Glucagon-like peptide-1 |
| GLUT4 | Glucose transporter 4 |
| GnRH | Gonadotropin Releasing Hormone |
| GSH | reduced Glutathione |

| | |
|---|---|
| HD | Hydroxydopamine |
| HDL | High density lipoprotein |
| HFD | High fat diet |
| HOMA-IR | Homeostatic Model Assessment of Insulin Resistance |
| HPG | Hypothalamic pituitary gonadal |
| HT | Hypothalamus |
| IL-1β | Interleukin-1 beta |
| LDL | low density lipoprotein |
| LH | Luteinising Hormone |
| MDA | Malondialdehyde |
| mRNA | messenger Ribonucleic acid |
| MS | Metabolic syndrome |
| ND | Normal diet |
| NFκB | Nuclear factor kappa B |
| NIH | National Institute of Health. |
| NO | Nitric Oxide |
| OGTT | Oral glucose tolerance test |
| PCOS | Polycystic ovarian syndrome |
| PND | Postnatal day |
| PP21 | Post-Partum day 21 |
| QR | Quercetin-3-O-rutinoside |
| RARRES2 | Retinoic acid receptor responder 2 |
| ROS | Reactive oxygen species |
| RPM | Revolutions per minute |

| | |
|---|---|
| SD | Sprague Dawley |
| SEM | Standard error of mean |
| SGA | Small for gestational age |
| SOD | superoxide dismutase |
| TAC | Total antioxidant activity |
| TBA | Thiobabituric acid |
| TBARS | Thiobabituric acid reactive substance |
| TC | Total cholesterol |
| TCA | Trichloroacetic acid |
| TNF-α | Tumour necrosis factor- Alpha |
| TPTZ | 2,4,6-tripyridyl-s-triazine |
| UKZN | University of KwaZulu-Natal |
| VLDL | Very low-density lipoprotein |

## THESIS OUTLINE

The principal findings of this PhD research study have been compiled into an article format and presented as a thesis by manuscript.

Chapter One Provides background information with a brief review of selected topics relevant to the study. Study aims and objectives, hypotheses and potential benefits of this research are also highlighted.

Chapter Two Forms part of the literature review and describes maternal nutritional manipulation and programming of reproductive functions. This manuscript is currently under review (REPBIO-S-20-00344) in Reproductive Biology.

Chapter Three Reports on diet induced oxidative stress in direct consumers and diet naïve offspring. This forms study 1 of the PhD experimental research and is currently under review by Heliyon (HELIYON-D-20-07205).

Chapter Four Forms part of study 2 to investigate the impact of maternal HFD on reproductive profile of diet naïve offspring. This manuscript is currently under review by Life Sciences (LFS-D-20-06711).

Chapter Five Titled “Primed sensitization to low-grade neuroinflammatory changes in rats prenatally exposed to high fat diet”.

Chapter Six This chapter forms the last chapter of the thesis and it provides the synthesis of all experimental findings, conclusion, limitations of study and recommendation for future research.

## THESIS ABSTRACT

The increasing prevalence of infertility and obesity over the last few decades have become a major public health challenge among individuals within the reproductive age. Consumption of a high-fat diet (HFD) is a harbinger for many metabolic alterations and diseases including infertility and subfertility. Studies have shown that the reproductive health of an individual can be programmed prior birth since exposure to certain environmental factors especially during intrauterine life play significant roles in transcriptional and epigenetic alterations in pivotal genes. However, understanding the molecular mechanisms linking oxidative stress caused by adverse environmental conditions to intrauterine alterations at critical periods of development might help in the clinical management of diet-induced infertility problems. This study therefore aimed at investigating the impact of maternal HFD consumption on sex-linked differences in the reproductive hormone profiles of diet unexposed offspring and examined the therapeutic potential of 150 mg/kg Quercetin-3-O-rutinoside (QR) against the HFD-induced biological changes. Adult female Sprague Dawley rats were randomly divided into two groups and fed either 45% HFD or normal diet (ND) for eight weeks before mating with male rats fed ND. Thereafter, the pregnant rats were divided into four dietary treatment groups: ND, HFD, ND+QR, and HFD+QR. At gestation day 19 (GD19), n=7 animals per group were sacrificed. Blood and tissue samples were collected and stored at -80$^0$C for biochemical and molecular analyses. The remaining dams were allowed to litter naturally and sacrificed. The pups were also sacrificed at postnatal day (PND) 21, 28 and 35. Blood and tissue samples were collected and stored for subsequent analyses. Using standard laboratory procedures, we measured oxidative changes in the liver, placenta and brain tissues by assessing levels of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase and nitric oxide (NO). Concentrations of hypothalamic gonadotropin releasing hormone (GnRH), serum luteinizing hormone (LH), testicular testosterone, and brain tumour necrosis factor (TNF-α) and glucagon-like peptide 1 (GLP-1) were assessed via enzyme linked immunosorbent assay (ELISA) technique. HFD-induced transcriptional changes in chemerin, chemokine-like receptor (CMKLR 1), TNF-α, GLP-1, interleukin-1 (IL-1β) and nuclear factor kappa B (NFκB) in the hypothalamic and testicular tissues were assessed by reverse transcriptase polymerase chain reaction (RT-PCR). After eight weeks of maternal HFD consumption, lipogram test indicated decreased plasma total cholesterol (TC) level, hypertriglyceridemia and increased low-density lipoprotein (LDL) levels. Our findings also

showed that offspring of HFD-fed dams had delayed fur appearance and lower body weight compared to those from the control (ND) dams. These morphological changes were accompanied by elevated MDA levels in placenta, liver and brain tissues of HFD-fed dams and their diet-naïve offspring. Furthermore, there was evidence of hepatic nitrosative stress, time-dependent and sex-linked differences in hepatic SOD and brain GSH levels in the offspring. Also, hypothalamic GnRH and serum LH levels were significantly reduced at PND 28 and 35 in the offspring. Moreover, testicular testosterone was decreased at PND 35 in offspring of HFD-fed dams. Upregulation of chemerin, TNF-α, IL-1β mRNA transcripts in the hypothalamic-gonadal axis of male offspring indicates possible HFD-induced tissue inflammation and consequences for dysregulated steroidogenic and/or reproductive functions. Elevated brain GLP-1 may be linked to activated bioenergetic and homeostatic responses to HFD-induced oxidative stress. Overall, maternal HFD exposure led to induced oxidative stress, low-grade tissue inflammation and decreased levels of gonadotropins and androgens in their diet naïve offspring, whereas QR has little or no significant effects on these parameters.

Keywords: Developmental programming, High-fat diet, Oxidative stress, infertility, male reproduction, quercetin-3-O-rutinoside, Chemerin, CMKLR1, TNF-α, GLP-

# CHAPTER ONE

## LITERATURE REVIEW

### 1.1 Background of the Study

Over the last four decades, the concept of developmental origins of health and disease (DOHaD) has received heightened attention in epidemiological and animal studies owing to the prevalent increase in onset of certain diseases and metabolic disorders at later childhood and adult life. Developmental origins of health and disease explains the link between maternal diseased state, nutritional imbalance, environmental changes, stress, lifestyle choices, (1, 2) and programming of metabolic disorders which leads to epigenomic modification and altered phenotype in the naïve offspring. Compounding this health challenge is the relationship between the foetus and intrauterine environment which further breeds phenotypic alterations that may consequently affect the health status of the offspring. Some of these changes may present immediately after birth. Maternal nutrition and lifestyle play crucial roles during the periconceptual period, which is a highly controlled and timed period for the offspring. Several epidemiological and animal studies have explained the scourge of maternal nutritional imbalance and adverse intrauterine environment during the periconceptual period. There is a constellation of evidence that maternal obesity and lifestyle changes engrain significant effects on several systems of the body which forms the nidus for metabolic disorders and diseases such as diabetes, obesity, neurodevelopment, cardiovascular, gastrointestinal and liver diseases in the offspring at critical developmental window periods (3).

During mitosis, through interaction with the environment the homogenous genes gives rise to stable structural and functional heterogenous heritable traits without alteration to the DNA sequence, during fetal development and even in matured adult under environmental influence, this process is referred to as epigenetics (4, 5). It is used to describe fixed changes or alterations in gene expression. Epigenetics which literally means ‘outside the normal genetics’ (5, 6) was coined by Conard H. Waddington a developmental biologist after his experiment using drosophila pupae. In his study, he exposed the pupae to heat for a while and noticed that there was an alteration in the wing vein pattern. These alterations did not only become permanent in the adult drosophila, it was also pass on to subsequent generations (5, 7-9), it is however not clear how long it takes for the heritable phenotype to emerge during critical windows of development. Histone modifications, DNA methylation and small-interfering RNAs are the three major mechanisms involved in epigenetic modifications which are also inheritable across

generations. Epigenetic modifications clearly reveal the link between gene expressions and disease vulnerability (10). Therefore, early disease diagnosis, management, and proper treatment will be easier with a broader understanding of the osmotic relationship between epigenetic processes and endogenous or exogenous stressors. Alterations in DNA methylation patterns has been linked to aging (11), maternal or environmental stressors (5, 10) provides a strong evidence to support for its association in the development of adult diseases.

Chemerin is a novel pleiotropic acting chemoattractant adipokine encoded by the retinoic acid receptor responder 2 (*RARRES2*) gene. It plays a significant varying degree of impact on the immune system (anti and pro-inflammatory properties), glucose metabolism and adipocyte differentiation (12-14). Chemerin is largely expressed in the liver, moderately expressed in the lungs with low expression in the heart, kidney and ovaries (15, 16). Interest in this novel adipokine was kindled by its multilevel effect and association with the metabolic syndrome, obesity and its comorbidities including; diabetes and cardiovascular diseases and regulation of reproductive functions (17) through a G protein- coupled receptor called chemokine-like receptor 1 (CMKLR1). It is therefore important to also study its novel role in obesity and regulation of the hypothalamic pituitary gonadal axis. DNA methylation controls the constitutive expression of chemerin. There was low methylation of Chemerin in unstimulated adipocyte and hepatocytes of murine in relation to IL-1β cytokines(12, 18). Its novel role was also observed in the decrease of pancreatogenic diabetes mellitus in human and mice with increased serum level of tumour necrosis factor (TNF-α) and IL-1β which was upregulated by treatment with chemerin-9 and CMKLR1 agonist. Chemerin plays an important role in the modulation of adipokine secretion from the adipose tissue following treatment with TNF-α, there was a significant increase in gene expression of chemerin with a consequent decrease in CMKLR1 levels in bovine adipocyte (13) and bovine intramuscular matured adipocyte(19) with increased cellular lipid droplets preadipocyte differentiation (16). With increased mRNA expression and decreased DNA methylation in neonate foreskin tissues, chemerin was also shown to be a possible link between later life obesity and maternal smoking during gestation (20) and as an implicit therapeutic target for obesity (21). A study conducted in children with obesity and metabolic syndrome shows elevated levels of chemerin in obese children compared to normal children irrespective of age and gender. In addition, decreased level of chemerin was associated with weight loss and significant improvement in the parameters of metabolic syndrome (22, 23). The expression of chemerin was assayed in the placenta of female Sprague

Dawley rats at gestation days 8, 12, 16,19 and 21 and found that its serum level was higher at the early stage of gestation and it decreased significantly as gestation advances from day 19 (24). This study is suggestive of the crucial metabolic role of chemerin in maintaining maternal-fetal energy homeostasis during gestation(17). It is however not clear whether the expression of chemerin is mediated by sex steroids or other endocrine chemicals in relation to gonadal functions. Although, in gonadectomised rats there was a divergent expression of the ligand and its receptor with an independent increase at post pubertal compared with pre pubertal rats (21).

## 1.2 Developmental Programming

Programming which refers to the process by which an external stimulus at critical periods of development gives rise to permanent changes with effects on the structure and function of the organism, was first introduced by Lucas Alan in 1991 (1, 25, 26) when he examined the long-term effect of early nutrition in man. In his study, Lucas stated that 'programming may occur as a normal part of biological development or in response to unphysiological events, it may also occur under the influence of genetically determined triggers or due to external stimuli at critical periods' (25). However, it is important to note that the concept of programming was first identified in a descriptive study carried out by a group of ethologists, psychologist and zoologist on the imprinting of bird's behaviour between 1873- 1953 where one of the scientist, Spalding D.A, examined why and how physical and behavioural (learned and instinctive) determinants are inherited (1, 27). The 'Developmental origin of health and disease' or 'fetal origin of adult disease' hypothesis proposed by Baker gained much impulse after the Dutch famine cohort epidemiological studies between October 1944 – April 1945 (28, 29). Their study showed a correlation between maternal starvation and increased risk of metabolic and cardiovascular diseases in offspring when maternal nutrient supply does not commensurate with nutrient demand. It was recorded that offspring with low birth weight developed coronary heart disease and other biological risk factors such as; stroke, hypertension and type II diabetes (non- insulin dependent diabetes mellitus) which was made possible via plasticity (30-32).

Subsequent research further unveiled the importance of maternal nutrition during fetal tissue and organ differentiation at critical periods of development. The alteration in a single genotype at early life gives rise to several inheritable phenotypic (physical and functional) and morphological changes in response to environment changes, referred to as developmental plasticity (1, 32). This scourge of scarce nutrient strips some organs of essential nutrients

necessary for development. The body therefore naturally responds in a prudent manner by diverting available nutrient to the development of some critical organs. This phenomenon is referred to as the 'thrifty' hypothesis. In a study using rodent models (33, 34), maternal undernutrition during gestation and overfeeding at postpartum led to altered glucose- insulin metabolism in offspring. Furthermore, to fully unmask the effect of perinatal life insult, another adverse condition/ insult is required at postnatal life for the development of adult diseased condition, this is referred to as the '2-hit' hypothesis. Genetic vulnerability together with perinatal life insult leads to alteration in organ system structuring which may not be enough to alter adult phenotype is referred to as the first hit. Insults/stressors at postnatal life may result in endocrine imbalance which then unveils underlying diseased condition is the second hit. The two hit hypothesis has been implicated in Alzheimer's disease, it was reported that either oxidative stress or mitotic disruption play a crucial role in the pathogenesis of Alzheimer's disease (35, 36). The pathogenesis of polycystic ovarian syndrome (PCOS) was explained using the two hit hypothesis, because there is a perinatal and postnatal event necessary for activation of PCOS (37). Furthermore, there appears to be a synergistic relationship between the '2-hit' hypothesis and the theory of mismatch. As the organism develops, it adopts a phenotype to match supposed demands at later life, such that at adult life, the individual remains healthy. There is however a mismatch theory where the adopted phenotype does not meet up with demands at later life due to stressors, environmental changes, lifestyle, and diet. With a mismatch, there is a very high chance of susceptibility to disease risk at adult life (5, 38).

### 1.3 Nutritional drift and Developmental Programming.

Over the last decades, rapid increase in maternal obesity and its associated non-communicable metabolic diseases have become a global public health challenge (39, 40) with increase in premature mortality and morbidity rate (41). Changes in lifestyle and diet has been primarily attributed to be the leading cause of obesity, diabetes and other related metabolic disorders (42). Over the years, incidence of obesity or overweight among the affluent people in urban cities in Africa has greatly increased. Studies have shown that in both African and white population, there is a link between obesity and prevalence of its associated metabolic disorders such as diabetes (43), cardiovascular diseases (44-46) and premature mortality (47). Interestingly, there has also been a surge of this public health challenge among South African women due to the rapid promotion and acceptance of unhealthy western diet and lifestyle. This

lifestyle is perceived to be a show of affluence among most South African while some ignorantly imbibe this unhealthy lifestyle. Unfortunately, the prevalence of obesity will continue to increase in South Africa (39, 48) until its citizens are properly enlightened and realise the imminent dangers it ingrains on transgenerational health status.

A wide range of epidemiological and animal studies have highlighted that physiological adaptations take place within the body of nutritionally manipulated naïve offspring to maintain homeostatic energy balance for survival. For instance, maternal undernutrition during early gestation to mid-gestation was shown to affect adipose tissue development in the fetus by increasing the number of adipocyte precursor cells (49). An increase in neurotensin, a localised enteroendocrine amino acid was correlated with risk of diabetes, cardiovascular diseases and mortality following a high fat diet (50, 51). Interestingly, in a study by Rincel et al., 2016 on impact of maternal high fat diet on programming of stress in offspring. They found out that maternal high fat diet alleviated stress in offspring separated from their mothers (52)

Liang et al. (2010) reported that consumption of high saturated fat diet prior to conception and throughout pregnancy may result in insulin resistance and placental vascular damage and that these abnormalities could result mainly from underlying oxidative stress (53). Chronic consumption of high fat diets has also been reported to cause significant decrease in uterine blood flow, placental inflammation and an increased fetal risk of developing non-alcoholic fatty liver disease, as evidenced by increased levels of liver triglycerides and increased hepatic oxidative stress (54, 55). Maternal obesity can also cause accelerated aging (56) and alterations in metabolic and endocrine system of naïve offspring in a sex dependent manner. In a study conducted by Rodriguez-Gonzalez G.L et al, 2019, programmed early and rapid aging in a gender specific manner was observed in the progenies of female obese wistar rat fed 20.5% high fat diet (57). Sixty percent (60%) high saturated fat diet administered throughout pregnancy and lactation was used to assess the risk of adult onset of diseases in C57BL/6 mice. They found out that offspring developed hypertension, hyperglycaemia, insulin resistance, and adult obesity at 6-12 months after birth (58, 59). Several studies have used percentages different of fat diet and duration to establish a model of maternal obesity and, it is therefore evident that energy content of maternal diet during the pre-and-periconception periods seems to play key roles in fetal development, cellular programming, and associated functions.

## 1.4 The Role of Oxidative Stress in Developmental Programming

Studies have shown that oxidative stress could be the possible link between developmental programming and increased risk of metabolic syndrome, obesity, and other disorder in offspring at early childhood or later life. Although the precise association between fetal energy imbalance and the resulting metabolic disorder has been established, the mechanism linking oxidative stress, adverse fetal growth and later risk of developing metabolic syndrome is yet to be fully understood. It is believed that oxidative stress can cause modulation of gene expression and or direct damage to cell membrane and other molecules during critical developmental period (60). Oxidative stress occurs as a result of an imbalance between oxidant and antioxidant defence system. It indicates an imbalance between the production of reactive oxygen species (ROS) and detoxification of reactive intermediates (61, 62). For instance, from a study on the antioxidant defence system in using umbilical cord blood taken during delivery, oxidative stress was shown to be increased in infants born small for gestational age (SGA) when compared with those born appropriate for gestational age (AGA). There was a significant decrease in the activities of superoxide dismutase and glutathione (intrinsic antioxidants) with an accompanying increase in the activity of malondialdehyde (MDA- marker of lipid peroxidation) (63, 64). Deficiency in nitric oxide (NO) system was also implicated in the programming of hypertension and kidney disease. Intervention to target the NO pathway could lead to reprogramming to alleviate programming of hypertension and kidney disease (65). Due to the low enzymatic antioxidant defence system of the pancreatic β cells (66), oxidative stress has been shown to alter insulin secretion which could eventually result in low-grade inflammation, metabolic syndrome (60, 67), cardiovascular diseases (68) and other related metabolic disorders. Although the precise association between fetal energy imbalance and the resulting metabolic disorder has been established, the mechanism linking oxidative stress, adverse fetal growth and later risk of developing metabolic and reproductive alterations is yet to be fully understood.

## 1.5 Programming of the Hypothalamic- Pituitary Gonadal (HPG) Axis

It was believed that humans are born as '*tabula rasa'* or '*clean slates'.* However, this is no longer the case as evidences from human and animal studies shows that experiences are gained during the periconceptual period (69). Studies have shown that nutritional challenge during intrauterine life will primarily affect placental and fetal hyperplasia and only impacts fetal weight at a later stage of gestation (70-72). Adverse fetal environment is the nidus for altered

reproductive functions in the offspring at later life. The HPG axis is one of the systems of the body that is adversely affected by altered growth rate. The hypothalamus, specifically the hypothalamus arcuate nucleus (ARC), plays a key role in the regulation of metabolic and reproductive functions which makes it a target organ of early life insult. It secrets gonadotropin releasing hormone (GnRH) which controls the release of luteinising hormone and follicle stimulating hormone from the anterior pituitary gland which are responsible for the synthesis of steroids in both sexes, folliculogenesis in female and spermatogenesis in male (69, 73, 74). In a particular study, dehydroepiandrosterone (DHEA) which was used to induce polycystic ovarian syndrome and HFDs caused metabolic alterations alongside reproductive alterations which includes, polycystic ovaries, irregular cycles, and hyperandrogenism in the 45% and 60% HFD rat models (75). Also, in a transgenerational study, 45% HFD fed to F0 mother had significant alterations in metabolic and reproductive changes in the intra testicular germ cell transcriptome although, it still remains unclear how long these effects could last (76).

Furthermore, it has been well documented in animal studies that fetal exposure to stressors early in life can lead to programming of not only the hypothalamic pituitary adrenal axis but also the hypothalamic pituitary gonadal axis. Studies have shown that kisspeptin together with other subset of neurons in the ARC referred to as KNDy neurons- kisspeptin (KP), neurokinin B (NKB), and dynorphin (DYN) act together to regulate reproductive functions (69, 74). To determine the effect of high fat diet on onset of puberty and regularity of oestrous cycle, 60% high fat diet was fed postnatally to three different groups of female rats. They observed 30% of the offspring exposed to HFD postnatally had irregular oestrous cycles also Kisspeptin (an upstream regulator of HPG axis) produced in the hypothalamus signals the release of luteinising hormone, follicle stimulating hormone and sex steroids was not affected by postnatal treatment of 60% HFD. Although, Rahim Ullah et al, 2017 observed that diet composition rather than the increased body weight increased expression of Kisspeptin which led to precocious puberty in pup fed 45% HFD postnatally (77-79). HFD fed offspring had increased oestradiol concentration and decreased luteinising hormone at 6 months. This study clearly indicates that maternal and post-parturition weaning on a high fat diet program affects the reproductive profile (HPG axis) in their female offspring (80). To have a better understanding of reproductive alterations and diseases, it is important to pay more attention to exposures and modulatory changes at their early life.

## 1.6 Animal Models of High Fat Diet

High dietary fat diet accounts for most of the reported cases of increased adiposity. Studies have shown that diet containing ≥ 30% of its energy from fat is capable of inducing obesity in humans and animal models (81). In view of this, we used 45% HFD in animal model for this study. Several animal models of high fat diet have been developed basically to mimic the same condition in humans. In animal studies it is easier to understand disease etiology, monitor disease progression and be able to pinpoint the various stages of programming at critical periods of development. This also allows for the use of interventions to improve the inherited pathophysiology. In the study of the DOHaD, rats (most common strain: Wistar rats or Sprague Dawley rats ) are preferred to mouse due to its several advantages which could probably be due to its ability to withstand insult, easier to handle and fetal size is bigger (82) hence, we decided to use rats for this study. Different models have been used to study the effect of programming on the reproductive axis. However, for our study the choice of our model was based on the average consumption of a western high fat diet by a woman at reproductive period. Sprague Dawley rats have shown to be effective models for high fat diet and DOHaD (83, 84)

## 1.7 Intervention

Several studies have focused on interventions targeted towards reprogramming of programmed metabolic disorders in mother and their naïve offspring. Dietary modification as an intervention has gained heightened attention. Dietary interventions including; diet modification, use of nutritional supplements (56), herbs and lifestyle changes. Studies using nutritional supplements which are rich in antioxidants has been used to accurately access beneficial long-term intake. Quercetin-3-O-rutinoside which is commonly called Rutin and also known as Rutoside or Sophrin is a bioflavonoid (85, 86) found in certain plants, vegetable and fruits. It is found in apples, citrus fruits, green tea, black tea and buckwheat. The dietary supplement database label database lists over 860 products containing Quercetin-3-O-rutinoside that are currently marketed in the United States (DSLD, 2016).

Quercetin-3-O-rutinoside is a very potent antioxidant (87-92) which also scavenges radiation induced free radicals (93, 94). Research has shown its multispectral pharmacological benefits for the treatment of various chronic diseases, such as cancer, diabetes, hypertension, and hypercholesterolemia (95, 96) Quercetin-3-O-rutinoside ameliorates high fat diet induced obesity (97, 98), prevents metabolic changes such as abdominal fat pads, glucose intolerance and reversed oxidative stress and inflammation (89) and diabetes (99, 100). Quercetin-3-O-

rutinoside exhibits significant antidiabetic activity, presumably by inhibiting inflammatory cytokines, and improved the antioxidant and plasma lipid profiles in high fat diet + streptozotocin-induced type 2 diabetic model (101, 102).

According to Jahan et al, 2016, Quercetin-3-O-rutinoside reduced hyperglycaemia and hyperlipidaemia. In their study, adult female Sprague Dawley rats were used to assess the ameliorative effect of Quercetin-3-O-rutinoside (100mg/kg and 150 mg/kg) against metabolic, biochemical and hormonal disturbances in polycystic ovary syndrome. They observed a dose dependent ameliorative response of Quercetin-3-O-rutinoside against clinical and biochemical features of polycystic ovarian syndrome (92). Recent studies have also shown the cytoprotective effect of rutin on Boaz sperm against oxidative attack (103), it alleviated testicular histopathological dysfunction and improved the testicular functions attenuation of inhibited testosterone and penile cGMP content in diabetic male rats (104). However, there appears to be a dearth of knowledge on the role of Quercetin-3-o-rutinoside on developmental programming associated with metabolic and reproductive alterations especially the sex steroids at critical developmental periods in high fat diet naïve rat offspring.

## 1.8 Rationale of Study

With the prevalent increase in the consumption of diets rich in high-fat and adoption of western lifestyle, descendants of high-fat consumers are more vulnerable to developing metabolic alterations leading to reproductive health issues and other diseases at a later stage in life. Of concern are the prevailing cases of male infertility which now presents a huge challenge in global health. Even though impact by constellation of factors including environmental and lifestyle changes have been theorized, but the underlined mechanisms linking male infertility to HFD-induced transcriptional and metabolic alterations, tissue oxidative stress and dysregulation in chemical signalling along the hypothalamic-gonadal axis remain poorly understood. Understanding the exact timing of alterations during developmental window periods would shed more light on the cryptic conditions associated with HFD-induced metabolic and reproductive pathologies. Also, Quercetin-3-0-rutinoside has been reported to have cytoprotective and antioxidant properties, but its therapeutic potential against HFD-induced trans-generational changes on reproductive profiles is yet to be validated.

## 1.9 Aim of the Study

The current study aimed to evaluate the effect of QR on the impact of maternal HFD consumption on sex-linked differences in the reproductive hormone profiles of diet unexposed offspring rats.

## 1.10 Objectives of the Study

We formulated six objectives for this study and are as follows:

1. To validate an existing rodent model of HFD-induced metabolic dysregulation using 45% high-fat and investigate whether maternal consumption of HFD throughout gestation could impact on the health of their progenies.
2. To examine changes in plasma lipid content (TC, HDL, LDL and TG), tissue oxidative and inflammatory changes and possible alterations in the expression of chemerin and CMKLR1 genes in the placenta, hypothalamus, and testes of diet-naïve offspring rats, influenced by maternal HFD consumption.
3. To investigate whether QR supplementation could possibly reverse the HFD-induced metabolic alterations and changes in reproductive hormone profile (GnRH, LH, testosterone) of offspring rats that were prenatally exposed to maternal HFD.

## 1.11 Brief overview of Methodology and Study Design

Sprague Dawley rats was the preferred choice for this project based on its suitability as reported in the literature (83, 84). Standard laboratory procedures and methods were strictly followed as illustrated in Chapters 3, 4 and 5. The animal experimental work was carried out at the Biomedical Resource Unit (BRU) as approved by the animal ethics committee (AREC) of University of KwaZulu-Natal (Approval no: AREC/005/018D) following guidelines of National Institutes of Health for the care and use of laboratory animals in South Africa. The overall study design is shown in the figure below:

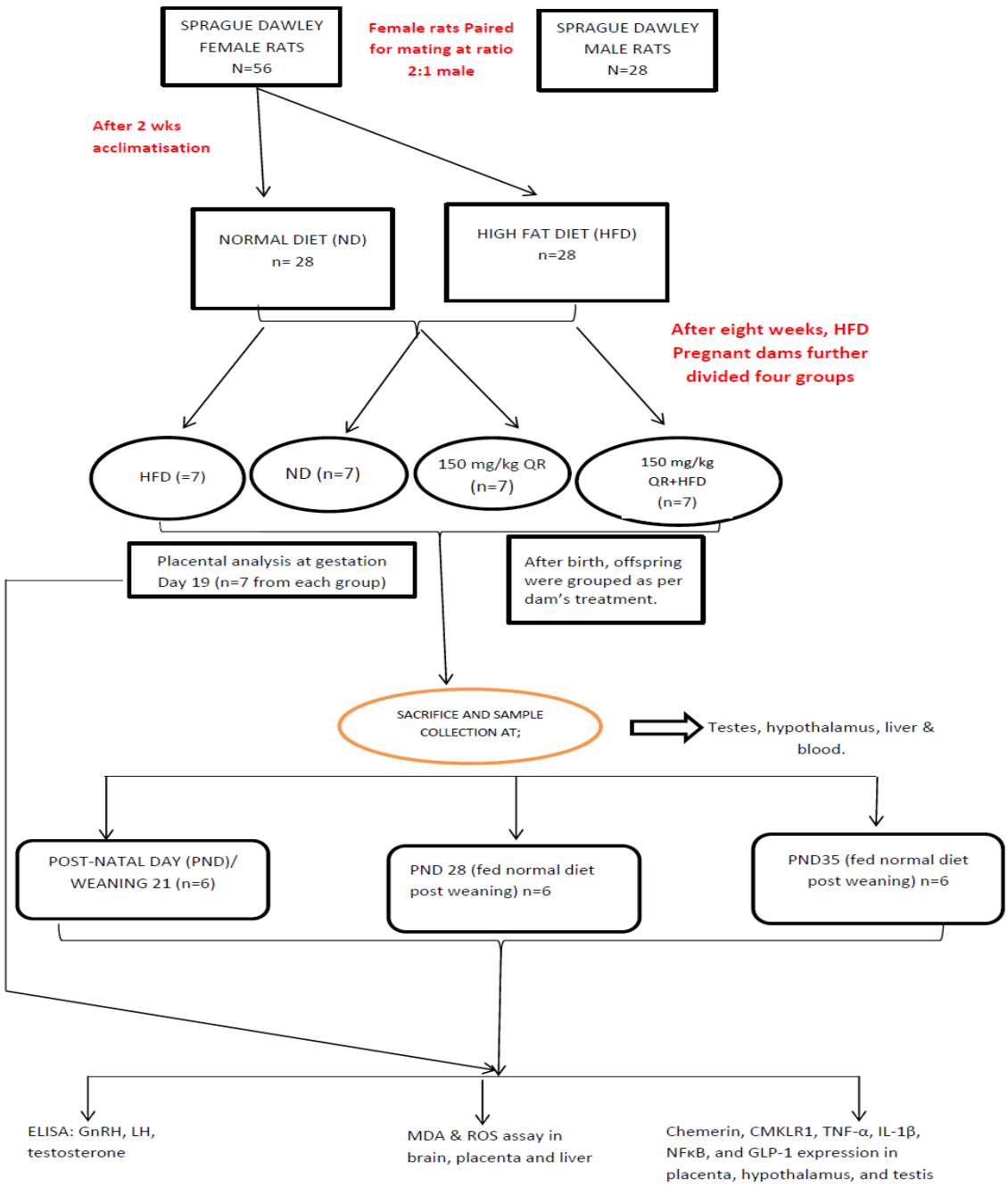


**Fig 1.1:** Showing the experimental design and workflow. As shown in the figure, two groups of Sprague Dawley female rats were fed either HFD or ND for eight weeks and paired with their male counterpart for mating (ratio 2:1). After conception, the female rats were further divided into four groups – two of which continued with their previous diets (ND and HFD) while the remaining two received diets supplemented with QR. At postnatal day (PND) 19, pregnant dams (n=7 per group) were sacrificed, while the remaining dams were allowed to litter naturally, and their pups were weaned and sacrificed (n=6) at different time points (PND

21, 28 and 35). In all killing time points, blood and tissue samples were collected and stored for subsequent biochemical analyses.

### 1.12 Potential Benefits of this research

The potential roles of QR on intrauterine development and postnatal health associated with HFD-induced alterations in metabolic programming is not clearly understood. It is therefore anticipated that the outcome of the present research study will provide beneficial information that may help improve progenitors' health and prevent lineal metabolic and biochemical changes in the offspring during active developmental window period.

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# CHAPTER TWO

This current chapter reviews the significance of maternal nutritional status and developmental programming of the hypothalamic-pituitary gonadal axis. It is presented in manuscript format titled: **"Nutritional Manipulation and Programming of Reproductive Functions"** which has been submitted for publication in Reproductive biology (Manuscript Number: REPBIO-D-20-00258) and is currently under review.


**Names of Authors**

Toluwalope E. Adeyemi*†, Mahendra L. Channa* and Anand Nadar*

**Affiliation of authors**

*Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.

**Corresponding Author:**

Toluwalope E. Adeyemi*†

*Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.

E-mail: adeyemi.tolulope@yahoo.com

Alternative E-mail- 217081454@stu.ukzn.ac.za

**ABSTRACT**

The detrimental effects of energy imbalance due to maternal nutritional drift are not limited to the mothers but may also impact the vulnerability of their offspring to develop certain metabolic disorders. Fetal programming, early life exposure and environmental factors converge to influence gene expression patterns in the adult-onset of health and diseases. Although certain epidemiological and animal model studies have explained the link between fetal programming and early life exposure to maternal nutritional imbalances, while other studies have attempted to unravel the putative mechanism involved in the developmental origin of health and diseases (DOHaD), the exact and unifying mechanism of action of nutrition and metabolic disorders in different organs remains unclear. Using human and animal model data, in this review, we discuss the possible links between high-fat diet-induced obesity, subfertility, infertility, and other reproductive functions. Furthermore, we also reported possible mechanisms involved in the programming of metabolic disorders of the reproductive functions such as oxidative stress, insulin resistance, and its link to the hypothalamic pituitary-gonadal axis. We concluded that reproductive functions could be programmed through various prenatal life pathways, and maternal high fat diet forms the nidus of altered offspring reproductive functions.

Keywords: Nutritional manipulation, Hypothalamic-pituitary Gonadal axis, Developmental Programming, Metabolic disorder, High-fat diet.

## Background

The concept of Developmental Origin of Health and Diseases (DOHaD), as postulated by Baker and colleagues in the 1900s, established that there is a strong relationship between early life nutrition, infant mortality, and the development of cardiovascular diseases (Ischemic heart diseases) (1). They hypothesized that the intrauterine environment is essential in influencing the offspring's susceptibility to developing diseases at later childhood or adulthood, which implies a lifetime risk for obesity, diabetes, cardiovascular diseases, liver and kidney dysfunctions (1). Forsdahl in the 1970s used official statistical data on Norwegian counties and reported that poverty during adolescence, followed by prosperity, was positively correlated with the risk of death from coronary heart disease (2). Although Forsdahl did not observe any biological mechanism, he speculated that some form of permanent damage caused by the nutritional deficit might be involved. In 1986, Barker and colleagues began publishing reports on the association between an adverse intrauterine environment as determined primarily by low birth weight and an increased risk of coronary heart disease later in life (3). Dietary restriction during the periconceptional period has also been shown to shorten gestation, induce hypertension, and abnormal hypothalamic-pituitary-adrenal functions (4).

The underlying mechanism through which maternal nutrition and energy imbalance relates to developmental programming of reproductive functions is not fully understood. However, studies have shown that disruption of the endocrine milieu, hypothalamic neuronal circuits, and intrauterine epigenetic modifications at critical developmental periods translate to the programming of reproductive functions in naïve offspring (5). This review attempts to unravel current thoughts on the impact of maternal high fat diet-induced nutritional drifts on sex steroids, hypothalamic pituitary-gonadal axis at critical periods of development on reproductive functions in diet naïve offspring using data from epidemiological and animal model studies. It also further explains the underlying mechanisms involved in the offspring's developmental programming and reproductive health and diseases.

## Maternal nutrition and fetal development

The offspring's predisposition to metabolic and reproductive alterations results from maternal consumption of a high-fat diet before and throughout gestation and lactation(3, 5-7). Maternal high-fat diet feeding has been shown to increase the risk of obesity, metabolic syndrome, and impaired glucose tolerance in the offspring at later life (8-11). Hyperphagia was noticed in 10

weeks old offspring of rats fed junk food diet (rich in energy, fat, and sugar) during gestation and lactation (12-14). On the cardiovascular system, Taylor et al., 2005 also described an association between maternal high fat diet consumption and the cardiovascular system. There was reduced mitochondrial gene expression in the aorta of the six-month-old offspring of high fat-fed rat, which preceded a decline in whole-body insulin sensitivity (10). According to a cross-sectional study carried out in humans to ascertain the role of some markers of metabolic syndrome, hyperglycaemia, insulin resistance, and increased blood pressure on cognitive functions, the result showed that individuals with type 2 diabetes performed poorly in cognitive functions compared with those with prediabetes, which can be explained by hyperglycaemia and increased blood pressure. However, early glycaemic control can be a therapeutic way for the prevention of diabetes-related decrement in cognitive performance (15).

However, there is a growing body of evidence that reproductive functions are imparted during fetal development (5). The male and female reproductive functions are severely impacted by the environmental/intrauterine environment. Connor et al. (2012), in their study on the female offspring of rats prenatally exposed to the maternal high fat diet, had longer oestrus cycle (prolonged proestrus), and early maturation was reported (16). Less study has been conducted to investigate the impact of prenatal fat exposure on male offspring fertility, especially in animal models. Decreased sperm count and the decreased reproductive outcome was recently reported in male rat offspring prenatally exposed to a high-fat diet (17). Metabolic syndrome is strongly associated with the pathogenesis of infertility in males (18).

**Definitions and Prevalence of Metabolic syndrome.**

In the wake of global development and urbanization, high-fat diet-induced metabolic syndrome (MS) remains a major clinical and public health challenge. Metabolic syndrome is defined as a multiplex disease caused by several factors arising from insulin resistance, obesity, hyperglycemia, hypertension, and dyslipidemia. The International Diabetes Foundation defined metabolic syndrome as central obesity with ethnic variations plus any two of the following factors; raised triglycerides (> 150mg/dl), raised blood pressure (systolic BP > 130 or diastolic BP > 85 mmHg), reduced High-Density Lipoprotein (<40mg/dl in males and <50 mg/dl in females) and raised fasting plasma glucose (FPG > 100 mg/dl) (19). According to statistics, it is estimated that 22% of United States adults have metabolic syndrome (20). The

International Diabetes Federation also estimated that 25% of the world's adult population has metabolic syndrome.

It is reported that there is an increase in the prevalence of metabolic syndrome in young adults within the sixth and seventh grade of life in males and females (21) and a high prevalence among postmenopausal women between 32.6% to 41.5% (22). It is estimated that in the year 2030, up to 57.8% of the world's adult population (3.3 billion people) will be overweight or obese as a result of high-fat diet consumption (23).

Until recently, metabolic syndrome has been attributed to be a disease whose manifestation is seen in adulthood. (24). Although the estimation of all the symptoms of metabolic syndrome at childhood and early adolescence is difficult, the prevalence of obesity (which predisposes to metabolic syndrome) increases at adolescence (25). It is worthy of note that the symptoms and manifestations of this syndrome also present in childhood (26-28) (29). Metabolic syndrome started as a concept rather than a diagnosis, with over forty definitions reported in the literature (30). Kylin in the 1920s demonstrated the association between high blood pressure (hypertension), high blood glucose (hyperglycemia), and gout (31). Other scientists found out that upper body obesity (male type obesity) was associated with cardiovascular diseases, type 2 diabetes mellitus, and metabolic dysfunction (32, 33). In 1988, Gerald Reaven presented a lecture on the role of insulin resistance in human disease. He described the metabolic syndrome as a cluster of various conditions and named it "Syndrome X." with the emergence of different names for the metabolic syndrome, there have also been different definitions. Reaven described it in adult obese for the first time as a link between insulin resistance, hypertension, dyslipidemia, impaired glucose tolerance, and other metabolic abnormalities associated with the risk for atherosclerotic and cardiovascular diseases (34). It was renamed by another scientist as "Deadly Quartet" (35) and "Insulin Resistant Syndrome" (36). For a unifying definition of metabolic syndrome, in May 2004, the International Diabetes Federation held a workshop in the United kingdom sponsored by an educational grant from AstraZeneca Pharmaceuticals with participants from each of the five continents, World Health Organisation, National Cholesterol Education Program- Third Adult Treatment Panel (NCEP-ATP III) and experts from the field of diabetes, public health, epidemiology, lipidology, genetics, metabolism, nutrition, and cardiology (37). There was a consensus on a unifying definition from the constellation of different definitions by associations during the workshop. All groups agreed on the core components of metabolic syndrome as: obesity, insulin resistance, dyslipidaemia and

hypertension (37, 38) (Fig. 2.1). However, this definition might not be acceptable in all cases considering the current emergence of metabolic syndrome in children and adolescents (19, 39). According to Marie NG et al. (2014), about 2.1 billion adults are obese or overweight, and women of childbearing age occupy 38% of this population (40).

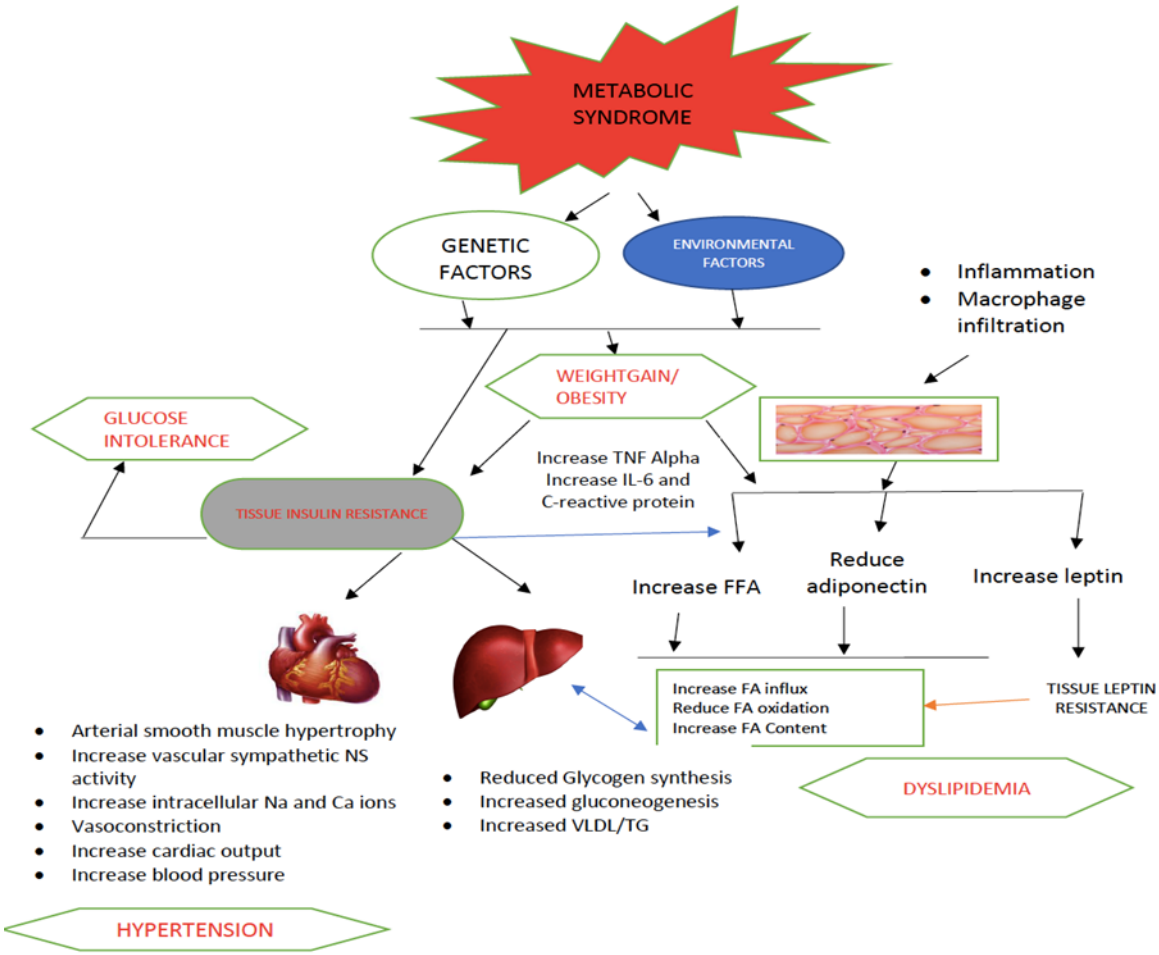


**Fig. 2.1** showing metabolic syndrome and associated disease pathways. Either through genetic or environmental factors, most of the symptoms of metabolic syndrome, which includes obesity/weight gain, increased tissue insulin resistance, and glucose intolerance, increased tissue leptin and free fatty acid (FFA). In the muscles and liver, causes dyslipidaemia, arterial smooth muscle hypertrophy, increased cardiac output, blood pressure in the heart causes hypertension. All these are expressed either in the tissue or specific organs.

**Nutritional manipulation phenotype induced by reprogramming of offspring machinery.**

Developmental changes arising before implantation are likely to affect many cell lineages. However, adaptations occur later during gestation, such as upregulation of placental nutrient and oxygen ($O_2$) transport to compensate for the early defects and normalize birth weight (41). Once placentation has begun, environmental signals' programming effects may be mediated via changes in placental development (42).

Programming of health and diseases is mostly due to placental insufficiency due to insufficient supply of nutrients, oxygen, and blood flow through and to the placenta (Fig. 2.2). Reduced oxygen delivery to maternal and fetal blood is accompanied by hypoxemia (43-45) and embolism of the umbilical artery (46-49), which is stress-induced due to maternal undernutrition or overnutrition. Pregnant rats that were exposed to a low protein diet had reduced pancreatic β cell mass at birth. They also had reduced insulin secretion in later life which may be due to dietary-induced reduction in proliferation rate and increased apoptosis of pancreatic β cells (50). Human and animal studies have shown that intrauterine life exposure to high sugar diets increases the risk of metabolic syndrome (51, 52). Diabetes during gestation places the offspring at risk of developing glucose intolerance and obesity in later life. In an epidemiological study of women born of diabetic mothers, 18-27 years old women had a risk of overweight, increased blood glucose, and high risk of metabolic syndrome compared with those born to non-diabetic mothers (53-55).

According to a study using rats, intrauterine life protein diet restriction may be the reason for later life preferences for fatty foods (56). Lipids and lipids disorders play a central role in developing metabolic syndrome and its associated diseases (57). Observational studies were performed in humans that consumed a high-fat diet during pregnancy and the effect on the health of the offspring. The risk of spontaneous abortion and miscarriages was associated with the consumption of high fats, butter, and oil where spontaneous abortion was inversely and significantly related to consumption of green vegetables, milk, cheese, eggs, fruit and fish while fat showed a direct association with risk of miscarriage(58). Oxidative stress is believed to link adverse fetal growth and later elevated risk of metabolic syndrome and other diseases. Oxidative stress results from an imbalance in the production and inactivation of reactive oxygen species. It can cause modulation of gene expression and direct damage to cell membranes and other molecules at critical developmental windows disorders (59). Multiple lines of inquiries indicate that oxidative stress is the link between adverse intrauterine

environment and phenotypic alterations. Liang et al. (2010) established that oxidative stress may link fetal exposure to metabolic syndrome. They developed a dietary-induced gestational diabetes mouse model, which demonstrated that consumption of a high saturated fat diet before conception and throughout pregnancy could result in insulin resistance and placental vascular damages, which could be a result of oxidative stress (60). The mechanism through which this occurs is not fully understood; however, obesity and inflammation have been implicated to be the signaling precursor for oxidative stress and its impact on the reproductive system (17, 61). An understanding of the mechanisms involved might provide a possible means for treating high-fat diet-induced fetal programming.

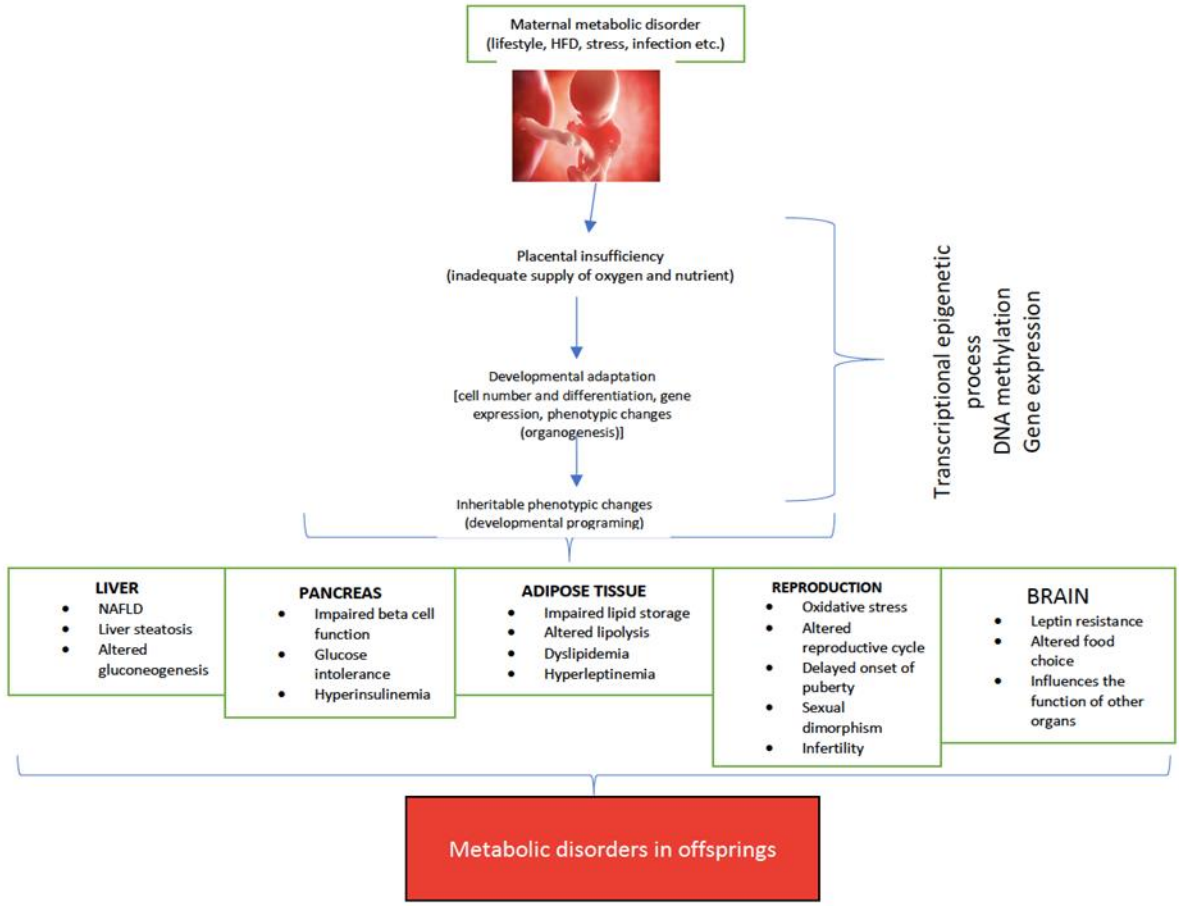


**Figure 2.2**: showing the impact of the maternal metabolic disordered environment on the health of the offspring. These impacts can be modified by the timing of exposure, diet, and lifestyle of the mother and offspring: HFD-high fat diet, NAFLD-non-alcoholic fatty liver disease.

**Oxidative stress and reproductive functions**

It has been proposed that oxidative stress and reactive oxygen species (ROS) account for one of the major idiopathic causes of infertility, miscarriage, pre-eclampsia, fetal growth restrictions, and pre-term labor (62, 63). Oxidative stress decreases fertility and facilitates poor embryonic development (64). According to a study by Desi et al. (2009), semen assessment of infertile men with high ROS levels had significantly lower sperm quality. High levels of ROS also altered the DNA sperm integrity because the spermatozoa have a limited defense against ROS induced DNA damage (65). It is worthy of note that the generation of reactive oxygen species at the normal level is beneficial to male fertility. It promotes capacitation, acrosome reaction, and sperm motility in mature sperm (66). A recent survey by Peters et al. (2020) on female fertility and oxidative stress found a strong link between autophagy, oxidative stress, and female fertility. They concluded that an understanding of the connection between autophagy and oxidative stress might provide a link to the pathology of oocyte aging (67). There is currently no unifying method for evaluating oxidative stress and the generation of metabolic disorders (62, 68).

**HFD-induced insulin resistance and associated reproductive alterations.**

Insulin is an anabolic hormone, that favors glycolysis and reduces gluconeogenesis and glycogenesis, secreted by the beta cells of the islet of Langerhans of the pancreas. It functions mainly in the mobilization of glucose from the circulation into the liver, muscle, and adipose tissue for conversion to fat and storage as glycogen through phosphorylation of glucose. Insulin is secreted mostly after a meal (mainly carbohydrate meal), and its action could either be within a few seconds, minutes, or hours after its secretion depending on the target. Insulin performs three (3) major functions in the cell; 1. It facilitates cell membrane permeability to glucose uptake into the cell by activating glucose transporter 4 (GLUT4) (mainly in skeletal muscle and adipose tissue), 2. Insulin alters cytosolic metabolic machinery, and 3. It also alters gene expression, leading to cell proliferation, growth, and differentiation through the activation of the mitogen-activated protein kinase pathway, which may link insulin resistance and programming of metabolic disorders in offspring (69, 70). In the adipose tissue, insulin increases the number of glucose transporters on the cell membrane, facilitating increased glucose uptake, fatty acid synthesis, glycerol phosphate synthesis, and potassium uptake (causing hypokalaemia) and activation of lipoprotein lipase (70). Insulin increases the glucose's entry into the liver (fig 2) by increasing the phosphorylation of glucoseglucokinasea activanase

(69). The liver cell membrane is permeable to glucose, which is made possible because of glucose transporter 2 (GLUT 2).

Studies have shown a sexually dimorphic effect of insulin resistance and reproductive functions' programming in the offspring. The male offspring of undernourished ewes increased the propensity to reduce insulin levels (71, 72). The male brain is more sensitive to insulin resistance and nutritional alterations compared to the female offspring. These alterations were linked to decreased expression of proopiomelanocortin in the hypothalamus. In females, insulin is regulated by estrogen, which elicits a compensatory to circumvent the effect of nutritional alterations (73, 74). In a recent study epidemiological study conducted by Ahmed et al. (2020), using 50 infertile male (idiopathic) patients aged 25-50 years and 50 healthy males as control, they observed a significant link between insulin resistance (using HOMA-IR), obesity, and idiopathic male infertility (75). Insulin resistance has been the underlying cause of obesity. Hyperinsulinemia and hyperandrogenism are associated with the manifestation of the polycystic ovarian syndrome (PCOS) by acting via insulin-like growth factor-I (IGF-I) secreted by the ovarian tissues with its receptors located on the ovaries (76). Another mechanism through which hyperinsulinemia affects steroidogenesis is exerting a stimulatory effect of the stromal cells over the synthesis of androgen- estradiol and lipid metabolism (77) through its receptors on the theca cells to produce androgens (76, 78).

**Obesity and Programming of hypothalamic pituitary gonadal Axis.**

Maternal nutritional status plays a significant role in the programming of health and diseases in their diet, naïve offspring. According to Black et al., 2008, maternal and child undernutrition is the cause of 3.5 million deaths globally (46). Data from epidemiological studies and animal models show that later life development of metabolic disorders is majorly caused by early-life exposure to maternal undernutrition or overnutrition (79-81). Increased nutritional availability to the offspring at postnatal like can also lead to the development of metabolic syndrome (82). Two-third of the women in the United States are overweight or obese at the time of conception (41, 83-85). Obesity, which results in an imbalance between food intake and energy expenditure, is considered a significant metabolic syndrome marker, and it is gradually becoming a worldwide pandemic. Central obesity (mainly abdominal obesity) is now a significant outbreak among children and adults due to a sedentary lifestyle, diet, and low energy output (86).

Nutritional insults from the mother to the offspring has been linked to perturbations in the appetite regulatory systems in the hypothalamus; hence, the development of obesity in the offspring is programmed by early stimulation of key neuronal circuits located in the arcuate nucleus of the hypothalamus responsible for appetite control (87, 88). The mechanism underlying this process was suggested to be the neurotransmitter signaling pathway such as the serotonergic (89) and dopaminergic routes (90) through the influence of circulating inflammatory cytokines, hormone (leptin and insulin), and key macronutrients (fatty acids, glucose, and triglycerides).

There appears to be an osmotic relationship between the early trigger of the neuronal circuits and obesity in naïve offspring. Obesity forms nidus for several metabolic disorders. It is generally associated with increased risk of depression, anxiety (91, 92), type 2 diabetes, cardiovascular diseases (19), and reproductive dysfunction (5, 93). Proopiomelanocortin (*Pomc*) enhancer region in the arcuate nucleus of the hypothalamus has been implicated in the programming of obesity and metabolic disorders in offspring exposed to maternal malnutrition early life (94). In a study using Sprague Dawley rats, female Sprague Dawley rats' progeny fed a high-fat diet throughout gestation. Lactation had increased body weight, increased adiposity, and hyperleptinemia. Offspring on high fat diet till weanling showed hypermethylation of the ARC *Pomc* gene (nPE1 and nPE2) enhancer region and not the promoter region which mediates the effect of leptin (94). Studies have shown that sex steroids regulate leptin and lipoprotein lipase through genomic or nongenomic mechanisms (93).

A study carried out in rodents showed that increased prevalence of metabolic disorders at menopause in correlation with the reduced incidence of metabolic diseases in women at reproductive age (less than 50 years) suggests a strong influence of steroid hormones. Ovariectomized female rats showed an increase in adipose tissue and increased lipoprotein lipase activity, which was decreased by estrogen replacement (95-100). Progesterone and androgen were also shown to increase the activity of adipose lipoprotein lipase (93). Leptin plays an important role in regulating food intake, body weight, sex steroid distribution within the adipose tissue, and energy expenditure. However, it is not clear if sex steroids regulate these effects. Leptin receptors have been reported in gonadal tissues and affect fertility and reproductive functions (101, 102). A high concentration of leptin has been reported in infertile males (103), and suppression of the arcuate nucleus by leptin inhibits the secretion of gonadotropin (103, 104). There appears to be a sexually dimorphic effect of leptin, as observed

in humans, which coincides with puberty. A cross-sectional study in humans showed that leptin is positively correlated with an increase in estrogen level and negatively correlated with testosterone levels (96, 105). Another study showed that sex steroids regulate the distribution of adipose tissue through the sex steroid receptors (genomic effect) and second messenger (nongenomic effect) that are present in the adipocytes which exists in most tissues (106, 107). The transcriptional effect of the sex steroid in the adipose tissue could either be up regulated or down regulated depending on the gene and the protein activated.

**Effects of placental insufficiency on intrauterine growth**

During intrauterine development, the placenta plays a vital role in exchanging substances between the mother and the fetus. It serves as the channel of communication between the mother and the developing fetus. The primary substance required for the fetus's development, among others, includes; oxygen, fatty acid, amino acids, etc. (108). The transport of these substances depends on the placenta's morphology, size, blood flow and vascularity, transporters' availability, apoptosis (physiologic cell death), autophagy, and insulin-like growth factors. Changes in the morphology and function of the placental results in a consequent decrease in fetal growth and intrauterine growth restriction (IUGR) (108). Failure of the placenta to deliver adequate nutrients to the developing fetus is called placental insufficiency, which results in intrauterine growth restriction affecting up to 5%-10% of pregnancies in developed countries (109, 110).

In placental insufficiency and intrauterine growth restriction, placental morphology, epithelial sites, oxygen supply, blood flow, and vascularity seem underdeveloped, leading to a reduction in oxygen and nutrient supply to the fetus (111, 112). There is also increased expression of insulin-like growth factors enhanced apoptotic factors (BAX, p53, and decreased expression of anti-apoptotic B- cell lymphoma 2) (113), increased expression of transporters like; FABPs (114), and reduced expression of some amino acid transporters (115) in placenta insufficiency. However, there are differences associated with the pattern of placental changes, as observed in human and animal models concerning placental insufficiency and IUGR (108). Developmental changes are altered at different critical periods of the developmental window, depending on the species. Various species have different responses to an external stimulus, duration of pregnancy, maturation of offspring, and birth. Humans are monotocous and altricial species. They give birth to one fetus that is matured at birth. Humans and nonhuman primates are also

referred to as precocial species. Rodents are altricial species, i.e., giving birth to many offspring following a short gestational period (116-119).

**Dietary restriction and Developmental Programming of Reproductive functions**

The reproductive system is the system in the body that is involved with producing offspring. Experimental research carried out in the early 1970s on nutrition and reproductive programming described the maternal and intrauterine environment's effect on offspring reproductive functions (5, 120-122). Prenatal exposure to stress has a programming effect on the offspring's reproductive health. In a study where rats were treated with glucocorticoids, the disappearance of sexual dimorphism of aromatase activity was observed in the offspring's hypothalamic preoptic area in early postnatal life. The critical period for sexual differentiation of the rodent's brain extends from late fetal life (gestational day 14-21) through the first two weeks of postnatal life. There was also a surge in testosterone's testicular secretion, which is essential for the masculinization of the brain on the $18^{th}$ and $19^{th}$ fetal days (123). Insulin resistance is associated with impaired female reproductive functions, which is involved in the pathogenesis of polycystic ovarian syndrome (124). Menstrual disorders and infertility are a result of insulin resistance and obesity. Insulin-resistant knockout female mice in gonadotropin- secreting pituitary cells showed increased infertility (125), and reduced serum level of luteinizing hormone was seen in mice that lacked the IRS-2 (126). There is evidence that shows that insulin in the brain has a direct impact on reproductive functions according to the study of Burks et al. (2000) using the NIRKO mice; they displayed hypothalamic hypogonadism and decreased fertility due to hypothalamic dysregulation of luteinizing hormone (124).

Human epidemiological and animal experimental studies have shown that overexposure to maternal stressors at an early stage of development negatively impacts male and female sexual development and reproductive functions (5). Maternal nutritional alterations can lead to the generation of oxidative stress, a proinflammatory state, and hence programming of metabolic syndrome and naïve offspring reproductive capacity alteration (127-135). However, it is now clear that offspring phenotypic changes in offspring include impaired reproductive functions (fig. 2.2). In animal models, hypocaloric maternal undernutrition produces DNA oxidative damage in fetal ovine oocytes (136) and delayed puberty in rat pups (137) and lowers fertility

in ewe lamb offspring (122, 138) as well as follicular and neuroendocrine alterations (137, 139, 140) in female pups. Maternal protein restriction during lactation reduces testicular weight when compared with testicular/body weight ratio in weanling rat offspring (141) and an ovarian development (135).

It is also important to note that the time and duration of exposure of stressors during and post intrauterine life influence the impact on the reproductive functions. Maternal nutritional drifts negatively impact the reproductive system through different mechanisms and time of exposure. The vulnerability of the reproductive system to intrauterine stress due to maternal dietary drift is mostly dependent on the insult's time and duration, which is species-specific. However, it is important to note that irrespective of the species type- either altricial species (rodent) or precocial species (human) are exposed and vulnerable to change in the internal milieu at different timing (135, 142). Early sexual maturation and longer proestrus phase were observed in female offspring of rats fed a high-fat diet throughout gestation and lactation. Studies on maternal nutritional stress and reproductive functions in humans are scarce; however, nutritional stress has been linked to cancer development, polycystic ovary, and ovarian cancer (5). In a rodent study, there was a delay in the testes' descent, reduced relative expression of P450 side-chain cleavage on postnatal day 21, and relative reduction in luteinizing hormone, sperm count, and fertility in adulthood (5, 135). Maternal obesity during pregnancy and lactation decreased sperm mobility, viability, and concentration in adult male offspring, increasing malondialdehyde levels, and reduced levels of superoxide dismutase and glutathione peroxidase production during intrauterine life (143).

Therefore, we conclude that maternal nutritional status before and during gestation and lactation forms the nidus for programming the reproductive status of their diet naïve offspring. However, male fertility requires more study/attention. There also appears to be a sexual dimorphic programming effect on the diet naïve offspring, which time dependent.

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# CHAPTER THREE

The study in this chapter has been accepted for publication in Heliyon (manuscript no: HELIYON-D-20-07205). Manuscript title: **"Maternal high-fat diet consumption induced persistent oxidative stress and developmental changes in the offspring"**. The current chapter examines high fat diet induced oxidative stress and biochemical alterations in direct consumers and diet naïve offspring.

**Name of authors:**

Toluwalope E. Adeyemi[1*], Duyilemi C. Ajonijebu [2], Mahendra L. Channa[1], Anand Nadar[1]

**Author Affiliation:**

[1]Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa. [2]Department of Physiology, School of Biomolecular and Chemical Sciences, Faculty of Science, Nelson Mandela University, Port Elizabeth, South Africa.

***Corresponding Author:** T.E. Adeyemi

[1]Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.

**E-mail:** 217081454@stu.ukzn.ac.za or adeyemi.tolulope@yahoo.com

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## ABSTRACT

Oxidative stress is usually associated with prolonged intake of high-fat diet (HFD). However, the impact of maternal HFD on endogenous modulation of antioxidant-defence-enzyme-network, its link to adverse fetal growth, and overall effects of Quercetin-3-o-rutinoside (QR) supplementation requires further investigation. Sprague-Dawley rats were initially assigned to ND or HFD for 8 weeks then mated. Post-conception, rats were further divided into four groups, of which two groups had diets supplemented with QR while others continued with their respective diets until delivery. Measurements include food and water consumption, physical parameters (body weight, BMI and fur appearance), oral glucose tolerance, lipid profiles, and placental/ liver oxidative changes. We observed that water consumption was significantly increased in dams fed HFD ($p<0.05$) without marked differences in food intake, body weight, BMI and glucose tolerance ($P>0.05$). Surprisingly, offspring of HFD-fed dams had reduced body weight marked by delayed fur appearance compared to the ND offspring. In dams, there were alterations in lipid profile. Lipid peroxidation was increased ($p<0.05$) in the placenta and liver of GD19 HFD-fed dams and their PND21 male offspring. There was evidence of HFD-induced nitrosative stress in dams and PND28 female offspring. Adaptive defence indicate decreased placenta and liver superoxide dismutase levels as well as differential changes in total antioxidant capacity and catalase activity in HFD treated dams and their progenies. Overall, the results indicate that intrauterine metabolic alterations associated with maternal high-fat consumption may induce persistent oxidative challenge in the offspring accompanied by mild developmental consequences, while QR supplementation has little or no beneficial effects.

**Keywords:** High fat diet, oxidative stress, metabolic changes, intrauterine, developmental alterations, quercetin-3-O-rutinoside.

## 1.0 Introduction

Studies have established the link between certain phenotypic alterations that result from early adverse life exposures and intergenerational susceptibility to poor health outcomes [1-3]. Increasing evidence clearly indicate that modulation of phenotypic traits is also of direct relevance to maternal nutritional imbalance with profound implications involving transmissible imprints and early life programming that could provoke metabolic dysfunctional states in the progenies [4-7]. Indeed, extensive experimental data suggests that maternal experience, such as chronic exposure to high-fat diet (HFD), may not only influence programmed effects in the fetus but also accompanied by dysregulated placenta development and renal functions [6], cardiovascular disorder [8] and other related metabolic dysfunctions including type II diabetes mellitus [9] and fetal obesity [10, 11].

Prolonged intake of HFD is commonly associated with cellular oxidative stress. Recent study by Yu et al (2018) showed that HFD-induced oxidative stress impaired lipid homeostasis in mice by blocking the activity of hepatic nuclear factor 4α, downregulated apolipoprotein B and reduced very-low density lipoprotein (VLDL) secretion [12]. These changes are linked to development of non-alcoholic fatty liver disease. On the other hand, rats fed on HFD and/or high-fructose diet can develop cluster of physiological abnormalities which include hyperglycaemia, hyperinsulinemia, glucose intolerance, hypercholesterolemia, hypertriglyceridemia and hypertension [13-15]. Other studies have shown that intrinsic antioxidant defence enzyme system, proteins and other essential cellular components are also compromised by poor and/or imbalanced maternal nutrition during gestation which leads to significant alterations in the balance between oxidative and antioxidant factors, coupled with excessive production of free radicals, leading to compromised fetal development [16, 17]. Although the precise association between fetal energy imbalance and the resulting metabolic disorder has been established, the mechanism linking oxidative stress, adverse fetal growth and later risk of developing metabolic syndrome is yet to be fully understood. An understanding of HFD-induced placental and/or *in-utero* oxidative and metabolic changes may hence help unravel the mechanisms involving maternal-foetal transmission.

Quercetin-3-O-rutinoside (QR) is a flavonoid glycoside popularly known for its antioxidant potential and has been used as nutritional supplements in the treatment of variety of diseases. Using voltammetric and flow cytometric methods, Zhang et al (2011) showed that quercetin increased the production of total antioxidant capacity (TAC) and decreased reactive oxygen

species (ROS) and nitric oxide (NO) production in lipopolysaccharide-stimulated human THP-1 acute monocytic leukemia cells [18]. Another study demonstrated that daily consumption of monoglucosyl-rutin inhibited HFD-induced visceral fat accumulation and prevented excessive weight gain by suppressing gastric inhibitory polypeptide secretion in mice [19]. It is worth noting that QR data from animal studies at times contradicts reports from human studies [20], hence further investigation is merited.

The current study aimed at investigating the impact of HFD-induced oxidative changes in the placenta and liver of Sprague Dawley rats [21] and addressed how *in utero* exposure to HFD influences offspring glucose homeostasis and lipid profiles, antioxidant enzyme network and fetal development. Overall effects of QR supplementation on HFD-induced metabolic and developmental alterations were also examined.

## 1.2 Materials and Methods

This study was carried out in accordance with the approved protocol (AREC/005/018D) by Animal Research Ethics Committee of the University of KwaZulu-Natal (UKZN), South Africa.

### 1.2.1 Animals and diets

Male and female Sprague-Dawley rats (180-200g) used in this study were obtained from the Biomedical Resource Unit (UKZN). They were bred and housed under standard laboratory conditions (50-60% humidity, 23±2˚C room temperature, and 12h light/dark cycle with lights on at 06h00). Regular rat chow (ND) and modified diet containing energy from dietary lard-based fat were used in this study. The normal rat chow was composed of grain and grain by-products, forage products, plant protein products, animal protein products, oils and fats, minerals, vitamins and registered stock remedies, with approximately 18% protein, 2.5% fat, 6.0% fibre, 1.8% calcium, 0.7% phosphorus and 12% moisture (EPOL, South Africa). The pelleted lard-based diet which is hereafter referred to as HFD was composed of normal food and sugar containing approximately 45% fat, 20% protein and 35% carbohydrate (Tshwane University of Technology, South Africa) [22, 23]. Rats had free access to their diets and water, except where mentioned otherwise. Food and water intake were recorded during the first eight weeks of dietary treatments.

Table 3.1: Composition of Lard fat

| **Lard** | **Nutritional Value per 100g** |
|---|---|
| Saturated | 39.2g |
| Monounsaturated | 45.1g |
| Polyunsaturated | 11.2g |
| Energy | 4730 (kcal/kg) |
| Sugar (including NLEA) | 0 |
| Fat | 45% |
| Protein | 20% |
| Carbohydrate | 35% |

*NLEA: The Nutritional Labeling and Educational Act, g: Gram, kcal: kilocalorie, kg: Kilogram

### 1.2.2 Experimental design

After two weeks acclimatization, the female rats were randomized to two treatment groups (*n*=28 per group) that either received ND or HFD for eight weeks and body weight changes were monitored. At estrus, the nulliparous female rats were paired with their male counterparts (previously fed ND) at a ratio of 2:1 (two females to one male per cage) for mating. Pregnancy was confirmed by the presence of spermatozoa in the vaginal smear when viewed under light microscope. Thereafter, the male rats were removed, and the dams were housed individually. After fertilization, some of the pregnant rats continued with their respective diets (ND or HFD; *n*=7 per group), while the remaining group of rats received QR (150 mg/kg) orally as co-treatment (ND+QR and HFD+QR; *n*=7 per group), until delivery (Fig. 1). Half of the pregnant rats (*n*=7 per group) were sacrificed on gestation day 19 (GD19) and tissues were collected. The remaining female rats gave birth at approximately 21 days post-conception, littering was carefully monitored to ensure age matching while QR treatment was discontinued and the rats were maintained on ND. Post weaning, the pups were fed normal diet, and housed by gender. After weaning, post-partum (PP) day 21 dams were killed and their offspring were sacrificed at postnatal day (PND) 21, 28 and 35.

### 1.3 Tissue collection

Rats were killed by inhalation of anaesthesia (Isofor) in an airtight chamber, and both placentas (GD19 dams) and livers (GD19 dams, PP21 dams and all offspring) were collected, weighed and snap-frozen in liquid nitrogen for subsequent protein and biochemical analyses.

### 1.4 Oral glucose tolerance test (OGTT)

OGTT was performed before and after 8-week HFD treatment. After an overnight fast female rats (*n=10* per group) randomly selected from ND and HFD groups were mildly bled by puncturing the tail vein and blood glucose was determined using Accu-chek active glucometer (USA) at baseline, 15, 30, 60 and 120 mins. Between the basal and the 15 min time interval, the rats were given oral glucose solution (2g/kg bw) to induce spiking effect.

### 1.5 Quantification of plasma lipid content

Blood was also collected into lithium heparinized or plain tubes by milking the punctured tails [24] of ND and HFD female rats. Blood samples were used for total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low-density lipoprotein (LDL), performed by Global Clinical and Viral Laboratories, Durban.

### 1.6 Measurement of lipid peroxidation in liver and placenta

Lipid peroxidation in the rats was determined using a standard laboratory procedure to assay for the concentration of malondialdehyde (MDA). MDA levels was assayed using thiobabituric acid reactive substance (TBARS) colorimetric method [25, 26]. Tissues were homogenised with 0.2% phosphoric acid and centrifuged for 10 mins at 10000rpm. Phosphoric acid (2% and 7%), butylated hydroxyl toluene and thiobabituric acid (TBA) solution were added while the resultant solution was transferred to a water bath and heated at 100℃ for 15 mins. Thereafter, butanol was added, and the top phase was transferred to 96 well plate in triplicate. Absorbance was measured at 532nm and 600nm. Final concentration was calculated using the formula below:

$$\text{Concentration} = \frac{A_{532}-A_{600}}{1.56}$$

Where A = Absorbance.

## 1.7 Quantification of antioxidant defence system in liver and placenta

### 1.7.1 Superoxide Dismutase

The tissue was homogenised and centrifuged in a cold centrifuge at 10000 rpm for 10 mins to obtain the supernatant. Determination of superoxide dismutase activity was based on the premise that hydrogen peroxide produced from the dismutation of superoxide ion by SOD oxidized 6-hydroxydopamine (6-HD) to produce a coloured product and 0.1mM diethylenetriaminepentaacetic acid (DETAPAC) was used to inhibit aerobic autoxidation of 6-HD. 1.6mM 6-HD was prepared using Miliq water and hydrochloric acid which was sonicated to remove air bubbles by negative pressure. The resulting 1.6mM 6-HD was wrapped in aluminium foil and stored on ice for immediate use. SOD assay buffer was used for blank. Absorbance at 490nm was recorded for 5 minutes in 1 minute interval using at spectrophotometer 96 well plate reader [27, 28]. The activity of SOD was calculated using the following formula:

Activity = 1000 x {(A1-Ab) / ε490} x 0.5 nmol/min/µg protein.
ε490=Molar absorptivity at 490nm= 1.742/m/M/cm, A1 and Ab= reaction rate for sample and blank respectively.

### 1.7.2 Catalase

Assessment of catalase activity was based on the principle that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide, and perchromic acid (unstable) is formed as an intermediate compound. The catalase preparation could split hydrogen peroxide from different time. Chromate/acetic acid mixture was added to stop the reaction while the remaining hydrogen peroxide mixture was determined by measuring chromic acetate colorimetric after heating the reaction mixture for 10 mins in boiling water. Standard concentrations at 10, 20, 30, 40, 60, 80 and 100 µmoles hydrogen peroxide was used. Absorbance was read at 570nm. A standard curve was plotted and catalase activity was extrapolated from the standard curve [27, 29].

### 1.7.3 Reduced glutathione concentration

Reduced glutathione (GSH) assay was based on a previously modified method by Oyebode et al. 2018 [27]. After precipitating with 10% trichloroacetic acid (TCA), supernatants were transferred to 96-well plate. 0.5mM DTNB (5,5'-dithio-bis (2-nitrobenzoic acid)) and 0.2M

sodium phosphate buffer (pH 7.8) were added to supernatant or standard and incubated for 15 mins. Absorbance was read at 415nm. The concentration of GSH was extrapolated from the standard graph.

### 1.7.4 Determination of nitric oxide

An indirect diazotization technique was used to assay for NO concentration in placenta and liver tissues. Briefly, the concentration of nitrites in the tissue homogenates were measured based on Griess reaction method, as previously described [30].

## 1.8 Total antioxidant capacity

The concentration of total antioxidant in placenta and liver tissues were determined using commercially obtained enzyme linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, USA. Catalog No: E-BC-K225). The kit uses the FRAP (ferric reducing antioxidant power) method for colorimetric quantification of antioxidant levels, such that Fe-TPTZ (2,4,6-tripyridyl-s-triazine) are reduced by antioxidants under acid conditions. Total antioxidant capacity (TAC) in the samples were detected at 593nm wavelength.

## 1.9 Statistical analysis

Results are presented as mean ± SEM and were statistically analysed using Student's t-test (water intake and lipogram), one-way analysis of variance (ANOVA) (MDA, NO, TAC, SOD, GSH, and catalase in placenta), two-way ANOVA (food intake, body weight, BMI, OGTT), and two-way repeated measures ANOVA (MDA, NO, TAC, SOD, GSH and catalase at PND 21, 28 and 35), followed by Bonferroni post-hoc analysis, where appropriate. Shapiro-Wilk normality test was used to test if the data were normally distributed. Values were considered statistically significant when $p<0.05$. Statistical procedures were performed using GraphPad Prism software (version 5.0, USA).

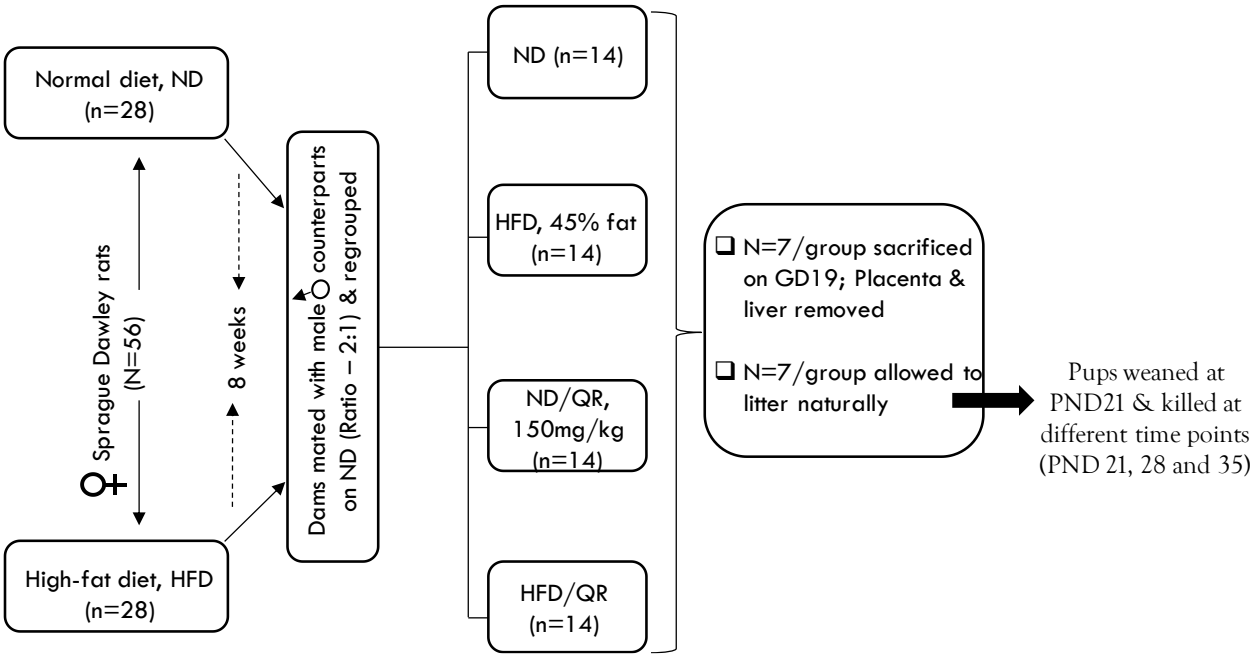


**Fig 3.1:** Schematic showing experimental design and timeline of the study. Female Sprague Dawley rats were randomly assigned to dietary treatment groups, ND-fed or HFD-fed groups (n=28/group) for approximately 8 weeks. Thereafter, these groups of rats were mated with their male counterparts who received normal rat chow and regrouped after conception, based on the dietary treatments supplemented with or without QR. It is worth noting that dietary treatments continued throughout gestation. Half set of the pregnant rats (n=7/group) were killed at gestation day 19 (GD19), their placenta and liver were harvested. The remaining pregnant rats were allowed to litter naturally and hereafter referred to as PP21 dams. Both PP21 dams and one-third of their progenies were sacrificed at postnatal day 21 after weaning. The remaining two-third of the offspring were sacrificed at PND 28 and 35 (n=6 per group). At all the killing time-points, liver was removed and preserved for subsequent biochemical analyses.

## 1.10 RESULTS

### 1.10.1 HFD-induced changes in food and water consumption, body weight composition and fur appearance.

To confirm the hypothesis that QR treatment could protect against transgenerational homeostatic imbalance owing to maternal nutritional drift through exclusive high-fat intake, we initially exposed adult female Sprague-Dawley rats to ND or HFD for 8 weeks, mated and subsequently fed with QR supplemented diets. Fig. 3.2 a indicates no significant difference in the amount of food consumed by both ND (control) and HFD-treated rats during the first 8 weeks of dietary exposure ($p>0.05$). Surprisingly, water consumption during this period was greatly increased in dams fed with HFD compared to the control dams ($p=0.0006$; Fig. 3.2 b).

Moreover, we observed time-related progressive increases in body weight of both HFD fed dams and the controls ($F_{(8, 96)} = 376.1$, $p=0.001$; Fig. 3.2d) without marked differences in their weight gain and body mass index ($p>0.05$; Fig. 3.2d, 3.2c). Strikingly, two-way ANOVA detected significant interaction ($F_{(6, 40)} = 2.885$, $p=0.0197$; Fig. 3.2e) between effects of HFD and time on body weight gain in male offspring rats (HFD treatment, $F_{(3, 40)} = 15.07$, $p<0.0001$; time, $F_{(2, 40)} = 607.2$, $p<0.0001$; Fig. 3.2e), but not in the females ($F_{(6, 40)} = 2.166$, $p=0.0667$; Fig. 3.2f). However, body weights of all male and female offspring of HFD-treated dams were significantly lower than offspring of control dams (ND and ND+QR) when assessed post-weaning to early adulthood ($p<0.05$; Fig. 3.2e, 3.2f). No difference in body weight composition was observed between the offspring of HFD and HFD+QR-treated dams ($p>0.05$; Fig. 3.2e, 3.2f), suggesting that maternal 150 mg/kg QR treatment has no impact on HFD-induced weight changes in the progenies.

Differences in diet composition of the offspring rats also coincide with changes in their fur appearance. After birth, almost all offspring of HFD or HFD+QR dams had delayed fur appearance that persisted till PND 28 (Fig. 3.2gii), as opposed to normal fur appearance in offspring belonging to ND or ND/QR groups (Fig. 3.2gi).

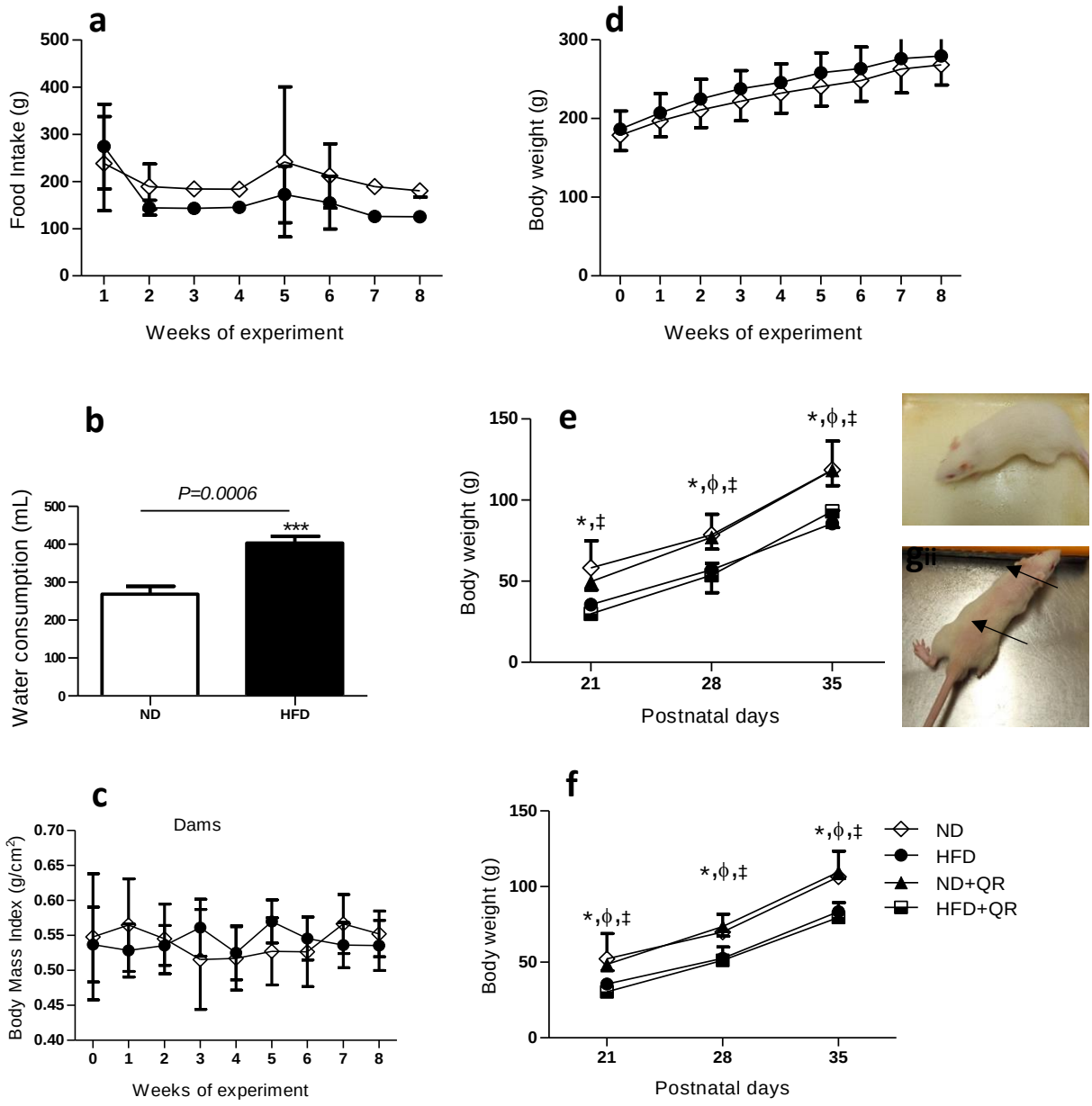


**Fig. 2.2:** Indicate food intake (a) and total water consumed (b) by dams during 8 weeks of HFD exposure. Also, body weight (d) and body mass index (c) of dams, and differences in body weight of male (e) and female (f) offspring rats were measured at PND 21, 28 and 35. Differences in fur appearance of offspring of control dams (ND or ND+QR; gi and HFD-fed dams (HFD or HFD+QR; gii) observed at PND 28. Data shown represents mean ± SEM; dams, n= 6 or 7 per group; offspring, n=6 per group. **P<0.05*, ND vs. HFD, HFD+QR; $^{\phi}$*P<0.05*, HFD vs. ND/QR; $^{\ddagger}$*P<0.05*, ND+QR vs. HFD/QR; Student's t-test; Two-way ANOVA,

followed by Bonferroni *post hoc* comparison test. Arrows indicate regions of late fur appearance.

### 1.10.2 Effects of HFD on fasting blood glucose levels and plasma lipid profiles of dams

We also examined the impact of HFD consumption on glucose homeostasis and plasma lipid profiles. After 8 weeks of dietary treatments, fasted blood glucose levels in HFD-treated rats were similar to those of ND-fed rats, indicating that glucose tolerance was unaffected by 45% lard-based high-fat during the test period ($p>0.05$, Fig. 3.3a). Lipid analyses indicated that HFD-fed dams exhibited hypertriglyceridemia accompanied by high plasma level of low-density lipoprotein (LDL), when compared to control dams ($p<0.05$; Fig. 3.3c, 3.3e). Whereas, plasma total cholesterol and high-density lipoprotein (HDL) levels are significantly lower in HFD-fed dams compared to ND-fed dams ($p<0.05$; Fig. 3.3 b, 3.3.d).

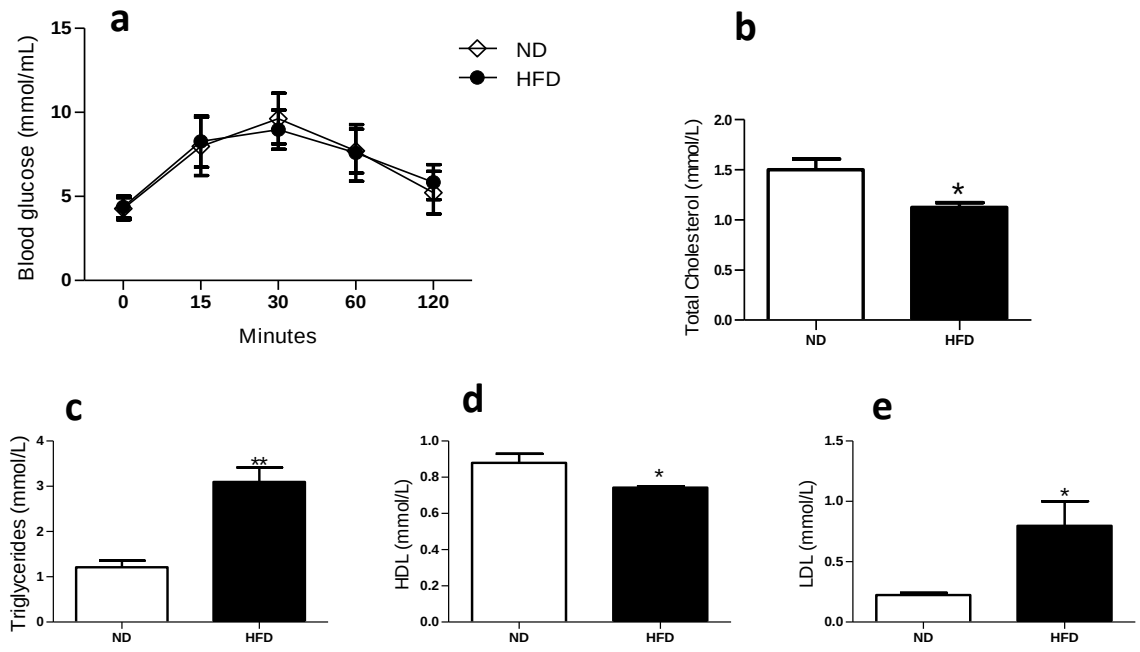


**Fig. 3.3:** OGTT and lipogram results obtained from dams after 8 weeks of HFD consumption. Fasting blood glucose (a), total cholesterol (b), triglycerides (c), high-density lipoprotein (HDL) (d) and low-density lipoprotein (LDL) (e) levels in dam's blood post high fat meal. Data shown represents mean ± SEM; OGTT, n=10 per group; Lipids, n=4 per group. **P<0.05*, HFD versus ND; Student's t-test; Two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

### 1.10.3 MDA levels in placenta and liver

MDA concentration was measured in GD19 dams' placenta and liver as well in the liver of PP21 dams and their offspring. Our findings show that MDA concentration was significantly increased by HFD in placenta and liver of GD19 dams compared to ND-treated group ($p$<0.05; Fig. 3.4 a, 3.4 b). Also, liver and placenta MDA levels were significantly reduced in groups fed with QR supplemented diets compared to HFD-fed rats ($p$<0.05; Fig. 3.4 a, 3.4 b), whereas QR treatments did not reverse HFD-induced changes in these groups of rats ($p$>0.05; Fig. 3.4 a, 3.4 b). Moreover, both HFD and QR supplement had no effect on MDA concentration in the liver of PP21 dams ($p$>0.05; Fig. 3.4 b). Strikingly, only PND 21 male offspring of HFD-fed dams exhibited significantly elevated hepatic MDA levels which was depressed in offspring of all QR-treated dams ($p$<0.0001; Fig. 3.4 c). This variable was not altered in the female offspring ($p$>0.05; Fig. 3.4 d).

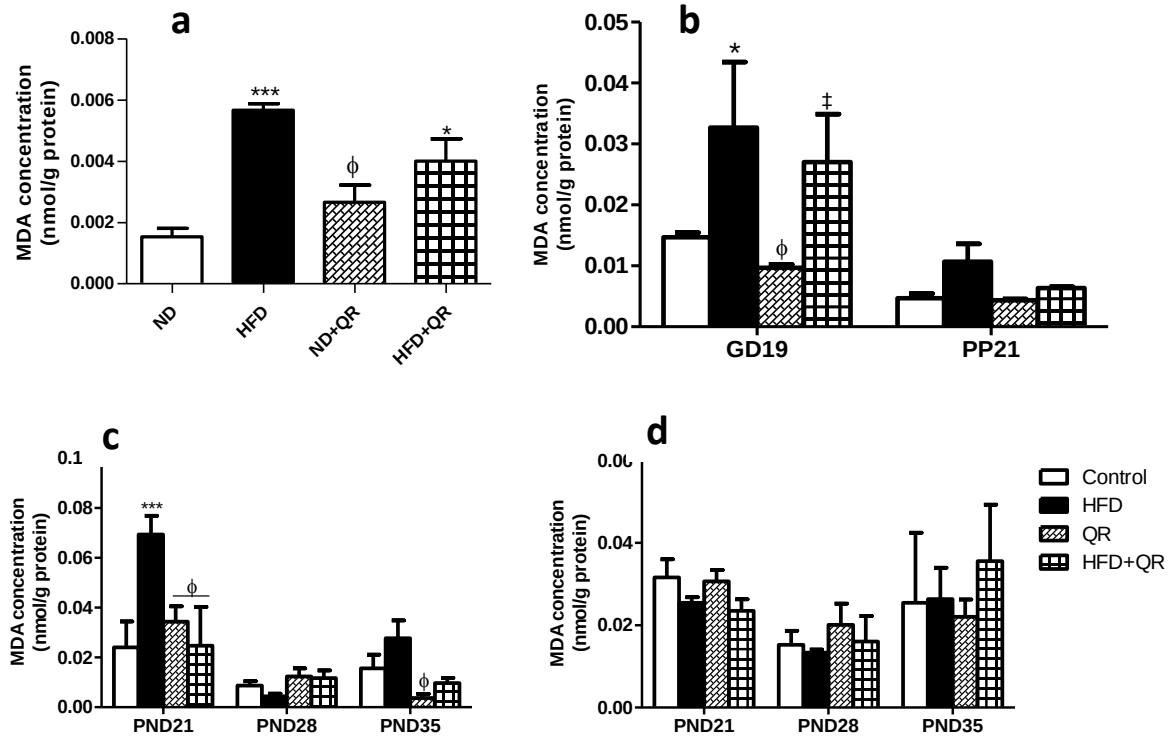


**Fig. 3.4:** Lipid peroxidation profile indicating MDA concentration in (a) placenta, (b) liver tissues of GD19 and PP21 dams, (c) liver of male and (d) female offspring rats at PND 21, 28 and 35. Data shown represents mean ± SEM; n=6 per group. *$P<0.05$, ***$P<0.0001$ compared to ND; $^{\phi}P<0.05$ compared to HFD; $^{\ddagger}P<0.05$, ND+QR vs. HFD+QR; One-Way or Two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

### 1.10.4 Placenta and liver NO concentration in dams and offspring

Our data showed significant main effect of treatments on NO concentration in GD19 placenta ($F = 8.051$, $p<0.001$; Fig. 3.5a), while comparison test indicated only significant decrease in placenta NO of HFD/QR ($p<0.05$; Fig. 3.5a) and not HFD group compared to ND-fed rats ($p>0.05$; Fig. 3.5a). This suggest that placenta NO was unaffected by maternal HFD consumption. However, this was not the case in GD19 and PP21 dams' liver as HFD consumption provoked NO production which appears prevented by QR treatment ($p<0.05$; Fig. 3.5b). Overall, NO concentration was greater in GD19 liver compared to PP21 (Fig. 3.5b). Similar to dams, hepatic NO was also significantly increased only in PND 28 female offspring of HFD-dams and decreased in offspring of QR-treated dams compared to control, ND ($p<0.05$; Fig. 3.5d). There was no change in liver NO of all groups of male offspring ($p>0.05$; Fig. 3.5c).

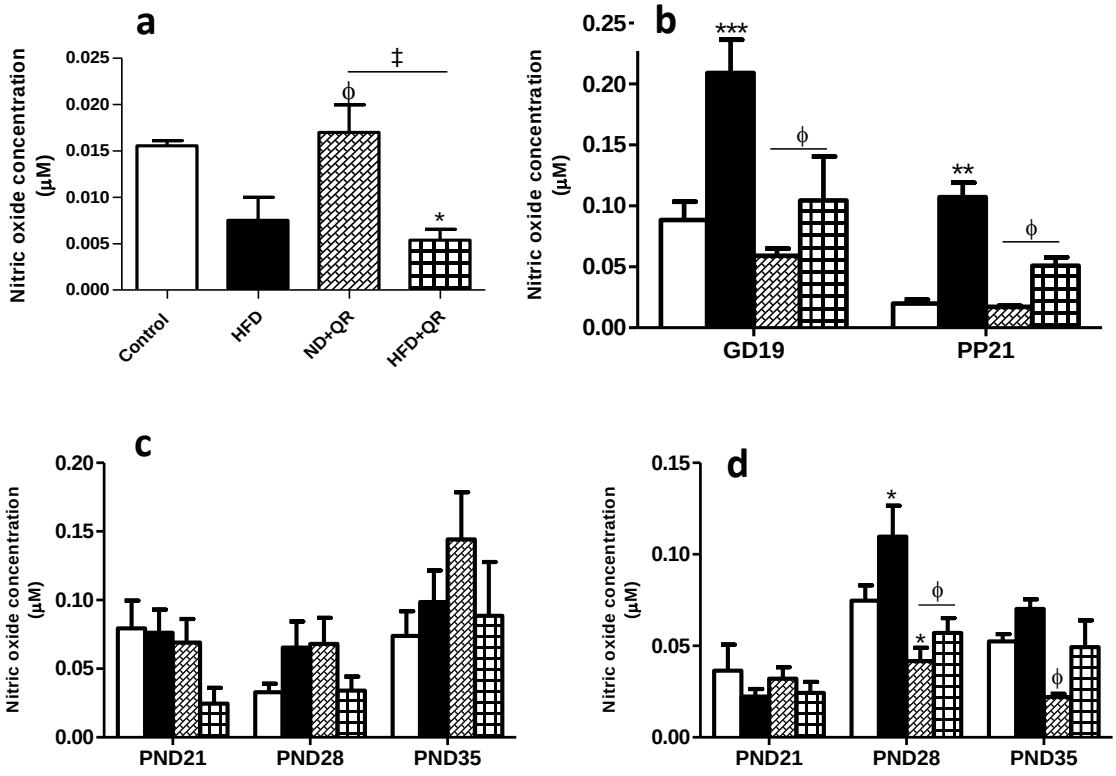


**Fig. 3.5:** Concentration of nitric oxide in (a) placenta, (b) liver tissues of GD21 and PP21 dams, (c) liver of male and (d) female offspring rats at PND 21, 28 and 35. Data shown represents mean ± SEM; n=6 per group. *$P<0.05$, ***$P<0.001$ compared to ND; $^{\phi}P<0.05$ compared to HFD; One-Way or Two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

### 1.10.5 Antioxidant status of dams and offspring

As shown in table II, we observed significant decrease in placenta SOD concentration of all HFD and QR-treated GD19 dams compared to ND ($p<0.05$), without any change in placenta CAT, GSH and TAC ($p>0.05$). Also, GD19 liver SOD concentration was similar to the placenta profile, indicating decreased liver SOD in HFD and QR treated dams ($p<0.05$, Table III). In contrast, liver SOD was significantly increased in PP21 dams fed either HFD or diets supplemented with QR ($p<0.05$, Table III). In the offspring, liver SOD was significantly increased in PND 35 HFD females only compared to ND group ($p<0.05$, Table IV), without significant change in other groups ($p>0.05$, Table IV). We also observed that co-exposure to HFD and QR significantly increased liver CAT and TAC in GD19 and PP21 dams, respectively, compared to ND-fed dams ($p<0.05$, Table III). Male offspring of HFD-fed dams had increased liver concentration of CAT and TAC at PND 21 and 28, respectively ($p<0.05$, Table IV), while liver GSH was significantly increased in offspring of all HFD-fed dams only at PND 28 and 35 ($p<0.05$, Table IV). Moreover, there was also a significant increase in liver GSH of PND 21 and 35 male offspring of dams that received normal diets supplemented with QR (ND/QR group) compared to ND group ($p<0.05$, Table IV). In the female offspring, there was no significant alterations in liver CAT, GSH and TAC levels at PND 21, 28 and 35, except an observed significant increase in liver TAC concentration of PND 35 female offspring of HFD-fed dams ($p<0.001$, Table IV).

**Table 3.2:** Antioxidant profile in Placenta of GD19 dams

| Parameters | ND | HFD | ND+QR | HFD+QR |
|---|---|---|---|---|
| CAT ($H_2O_2$/ mg protein) | 53.44±1.1540 | 52.57±2.3540 | 55.47±0.4080 | 56.24±1.1250 |
| SOD (nmol/min/µg protein) | 81.74±9.6030 | 41.26±3.573* | 43.41±12.42* | 16.50±1.456** |
| GSH (mM) | 0.0255±0.0003 | 0.0256±0.0002 | 0.0252±0.0000 | 0.0254±0.0002 |
| TAC (µM) | 0.5693±0.0185 | 0.8257±0.1325 | 0.6887±0.0837 | 0.6267±0.0800 |

Values are presented as mean±SEM (n=6 per group). *p<0.05, **p<0.01, ***p<0.001 (vs. ND).

**Table 3.3:** Liver antioxidant profile of GD19 and PP21 dams

| Parameters | GD19 | | | | PP21 | | | |
|---|---|---|---|---|---|---|---|---|
| | ND | HFD | ND+QR | HFD+QR | ND | HFD | ND+QR | HFD+QR |
| CAT ($H_2O_2$/ mg protein) | 37.07±2.5230 | 40.77±0.9503 | 33.97±1.5980 | 73.57±7.4850*‡ϕ | 26.31±1.665 | 41.32±2.792* | 34.08±5.018 | 35.04±3.210 |
| SOD (nmol/min/µg protein) | 763.8±56.47 | 583.4±25.49* | 498.3±36.02** | 520.9±25.08** | 159.3±32.01 | 449.1±23.90* | 562.3±32.96** | 245.2±77.22‡ |
| GSH (mM) | 0.0283±0.0009 | 0.0287±0.0003 | 0.0277±0.0010 | 0.0306±0.0019 | 0.0309±0.0012 | 0.0331±0.0020 | 0.0324±0.0000 | 0.0276±0.0006‡ |
| TAC (µM) | 1.367±0.0511 | 1.689±0.0365 | 1.211±0.1338 | 1.626±0.0726 | 0.8533±0.0353 | 1.003±0.0599 | 0.6240±0.1461 | 1.977±0.1753*** |

Values are presented as mean±SEM (n=6 per group). *p<0.05, **p<0.01, ***p<0.001 (vs. ND); ϕ*P<0.05* (vs. HFD); ‡*P<0.05* (ND+QR vs. HFD+QR).

**Table 3.4:** Liver antioxidant profile of male and female offspring rats.

| | Parameters | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ND | HFD | ND+QR | HFD+QR | ND | HFD | ND+QR | HFD+QR |
| PND 21 | CAT ($H_2O_2$/ mg protein) | 31.61±1.109 | 38.77±4.638* | 34.77±2.232 | 31.11±5.696 | 15.64±3.922 | 24.31±3.949 | 9.707±1.291 | 19.91±5.370 |
| | SOD (nmol/min/µg protein) | 104.8±17.02 | 101.9±6.999 | 128.1±15.26 | 126.8±32.27 | 30.92±5.644 | 31.86±7.014 | 37.68±1.760 | 24.23±3.181 |
| | GSH (mM) | 0.0246±0.0004 | 0.0252±0.0003 | 0.0349±0.0027*** | 0.0250±0.0006 | 0.0209±0.0004 | 0.0218±0.0055 | 0.0223±0.0014 | 0.0217±0.0003 |
| | TAC (µM) | 0.7047±0.0243 | 0.7254±0.0712 | 1.093±0.1018 | 0.8877±0.0296 | 0.1333±0.0456 | 0.0747±0.0362 | 0.4510±0.0769 | 0.1763±0.0484 |
| PND 28 | CAT ($H_2O_2$/ mg protein) | 30.77±1.896 | 30.04±3.725 | 38.31±2.751 | 27.54±1.552 | 10.64±0.1934 | 9.573±0.8044 | 9.173±0.2951 | 8.173±0.0422 |
| | SOD (nmol/min/µg protein) | 348.9±87.28 | 449.9±89.99 | 359.7±34.62 | 322.1±94.99 | 59.05±10.48 | 65.50±19.97 | 47.36±8.428 | 58.86±5.927 |
| | GSH (mM) | 0.0304±0.0010 | 0.0251±0.0003** | 0.0291±0.0013 | 0.0256±0.0004** | 0.0222±0.0007 | 0.0231±0.0007 | 0.0212±0.0002 | 0.0232±0.0000 |
| | TAC (µM) | 1.077±0.0404 | 1.162±0.1513 | 1.382±0.0207 | 1.108±0.0736 | 0.4133±0.0509 | 0.5530±0.0563 | 0.4030±0.0147 | 0.4917±0.0165 |

| PND 35 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CAT ($H_2O_2$/ mg protein) | 54.61±2.106 | 62.37±10.46 | 48.11±3.115 | 34.74±1.818 | 9.040±0.1265 | 11.84±0.8764 | 10.24±1.409 | 10.37±1.106 |
| SOD (nmol/min/µg protein) | 718.9±32.55 | 829.2±18.57 | 629.8±146.2 | 529.6±83.65 | 45.87±2.368 | 97.82±9.809*** | 24.59±1.087 | 33.33±6.699 |
| GSH (mM) | 0.0272±0.0006 | 0.0311±0.0009* | 0.0313±0.0008* | 0.0310±0.0010* | 0.0222±0.0005 | 0.0219±0.0002 | 0.0221±0.0004 | 0.0229±0.0003 |
| TAC (µM) | 1.655±0.2479 | 1.601±0.0776 | 1.178±0.1080 | 1.288±0.0440 | 0.5007±0.0320 | 0.9393±0.0592** | 0.4917±0.0345 | 0.4920±0.1085 |

Values are presented as mean±SEM (n=6 per group). *p<0.05, **p<0.01, ***p<0.001 (vs. ND).

## 1.11 DISCUSSION

The current study comparatively examined HFD-induced oxidative changes in the placenta and liver of obesity-resistant rats and addressed how *in utero* exposure to HFD influences offspring glucose homeostasis, lipid profiles, fetal growth and antioxidant enzymes. Potential alleviation of HFD-induced metabolic and developmental alterations by QR was also investigated.

As documented in the literature, body weight gain and abdominal obesity are prominent features of metabolic syndrome (MS) generally associated with excess consumption of fat [31]. Here, we show that food consumption by Sprague Dawley (SD) rats that had exclusive access to 45% HFD for 8 weeks matched those of the control rats fed normal chow. These observations partly contrast with previous findings that SD rats fed lard-based HFD or diets rich in high-fat and high-sugar for either 4 or 8 weeks had lower food intake but consumed more calories, accumulated fat mass and exhibited increased body weight gain than rats fed with control diet [31]. Even though the 45% fat content used in this study falls within the range of 30-60% energy from saturated fat recommended for use in animal models [32, 33], it appears still that the fat content is not extreme enough to provoke severe metabolic disorder as many other studies that reported significant metabolic and weight changes actually used higher energy content and allowed prolonged consumption. There is also accumulating evidence that SD rats adapt to long-term feeding of high fat or high fructose diets without significant weight changes or prominent features of metabolic disorder [34], thus suggesting another possible reason for lack of morphometric changes in the current study. To further support this claim, several animal studies have indicated that rat strains vary widely in their propensity for diet-induced weight gain [14, 35], most especially outbred SD rats known to exhibit bimodal weight gain pattern [35]. Despite no significant differences in the physical parameters of dams, we observed that HFD offspring had delayed fur appearance and reduced weight gain which may suggest transgenerational impact of maternal fat consumption on neurobiological development of the pups. In agreement with our findings, Santillan and colleagues (2010) have previously observed that female mice fed soy oil-enriched diet (SOD) or sunflower oil-enriched diet (SFOD) throughout gestation and lactation were not different in food consumption and body weight compared to mice fed commercial diet, however both SOD and SFOD offspring had

shorter length and exhibited early simple reflexes while pubertal onset was delayed only in SFOD offspring [36].

Most earlier studies have reported the adverse effect of consumption of high-fat and/or high sugar diets on glucose metabolism and lipid profiles. For example, Huang et al (2004) fed SD rats with 20% lard-based high-fat or 60% high-fructose diets for 8 weeks and observed increased plasma glucose concentrations in both groups of mice from the seventh week, while high-fat diet increased plasma cholesterol level and amylase activity group fed high-fructose diet showed higher fasting insulin and triglyceride concentrations [15]. Another study reported that C57BL/6 mice fed 58% HFD for 20 weeks had increased fasting blood glucose, pronounced glucose intolerance, and were more insulin resistant than mice fed 11% low fat-diet [37]. Unexpectedly, our OGTT result showed that rats fed HFD for 8 weeks did not develop glucose intolerance but exhibited decreased plasma cholesterol and HDL accompanied by hypertriglyceridemia and increased LDL. Undisturbed glucose homeostasis in this study may suggest intact functioning of the pancreatic β-cells and non-resistance of the liver and other peripheral tissues to insulin signal following chronic high-fat consumption. Our data also suggest that critical threshold concentration of fat needed to induce adiposity in peripheral tissues may not have been reached by consumption of 45% high-fat for 8 weeks. HFD-induced changes in blood triglyceride, HDL and LDL are clear indications of underlying mild pathological and/or metabolic alterations.

Long-lasting metabolic perturbations induced by high-fat feeding also implicate cellular dynamics involving changes in tissue oxidative state and mitochondria bioenergetics [38, 39]. Indeed, high levels of oxidative damage markers, most especially lipid peroxidation, and decreased antioxidant defences are often associated with oxidative imbalance in animal models of HFD. Milagro et al (2012) previously demonstrated that liver MDA concentrations were increased in male Wistar rats fed HFD for 56 days (8 weeks), and that the MDA levels correlated positively with body weight gain, serum leptin and homeostasis model assessment [40]. Similarly, we show that consumption of HFD for more than 8 weeks induced lipid peroxidation (increased MDA levels) in the placenta and liver of GD19 dams but without any correlation with body weight. Insignificant changes in PP21 liver MDA levels further support

previous observation that SD rats adapt to prolonged high fat feeding [34]. However, increased liver MDA concentration in HFD offspring clearly suggest persistent *in-utero* HFD-induced oxidative changes. This further indicates that lipid deposition in the placenta and hepatic fatty infiltration may contribute to harmful prenatal/perinatal changes, as previously posited [41]. HFD-induced increases in liver NO concentration of GD19 and PP21 dams agree with a previous study which supports that increased plasma and hepatic NO levels contribute to nitrosative stress in animal models of HFD [42]. While NO flux at PND 28 in female HFD offspring may be linked to developmental and/or pubertal changes, future studies should address sexual dimorphic effects of nitric oxide synthase activity in HFD models of metabolic syndrome. Consistent with previous studies [43], the observed increase in oxidative stress parameters (MDA and NO) might be associated with decreased placenta and liver SOD and increased TAC/CAT activity in HFD treated dams and their progenies. Differentially increased SOD activity in the PP21 dams may also indicate normalisation of hepatic response to the high-fat challenge subject to adaptive changes. Like SOD, GSH is another crucial antioxidant produced naturally to reduce oxidative stress. We thought that GSH activity would be decreased by chronic consumption of HFD, as previously reported, and reversed by QR treatment. Contrary to our expectations, the current data show that GSH activity was not altered by HFD in direct consumers, however, unexplained are observed increases in liver GSH activities in the offspring of dams fed HFD and those supplemented with QR. The stability of GSH has been correlated to these changes partly provide additional support for possible adaptive responses to prolonged high-fat consumption.

Several studies have reported the beneficial effects of quercetin in animal models of HFD, these include modulation of gut microbiota to prevent development of non-alcoholic fatty liver disease and obesity [44, 45], amelioration of steatohepatitis [46], reduction of HFD-induced fat accumulation, inflammation and oxidative stress [47-49]. Despite this, the current study indicates that QR has little or no effect on HFD-induced changes in physical parameters and tissue homeostasis. These observations agree with the previous report of McAnulty et al (2008) that chronic quercetin ingestion does not protect trained athletes from exercise-induced oxidative stress and inflammation [20]. Although, strain and dose differences can be attributed

to response changes in our study compared to human and other *in vivo* experiments. It is also possible that lack of impact by test compound QR may result from unaltered baseline metabolic parameters particularly in the HFD+QR groups.

In conclusion, this investigation provides evidence that chronic consumption of HFD may not induce prominent obesity and/or MS-related features in SD rats, however maternal HFD exposure engages metabolic and pathological alterations *in-utero* leading to persistent oxidative changes accompanied by mild developmental consequences. Whereas, 150 mg/kg dose of QR tested in this study has very little or no beneficial effects on HFD-induced metabolic and/or oxidative changes.

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**Declaration of interests.**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Authors contribution:**

**Toluwalope E. Adeyemi:** Conceptualization, methodology, funding acquisition, data curation, statistical analysis, manuscript drafting/writing.

**Duyilemi C. Ajonijebu:** Statistical analysis, data curation, manuscript proof-reading and editing.

**Mahendra L. Channa:** Manuscript review.

**Anand Nadar:** Manuscript proof-reading and editing.

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## Compliance with ethical standards

All animal procedures were approved by the University of KwaZulu-Natal's Animal ethics committee (AREC/005/018D) in accordance with National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

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# CHAPTER FOUR

This current chapter examined the effect of HFD-induced inflammatory and hormonal changes linked to alterations in hypothalamic-pituitary-gonadal functions in male rats while seeking to understand the therapeutic roles of QR in this phenomenon. This current chapter has been presented in an article format titled: "**Evaluation of maternal high-fat diet and Quercetin-3-O-rutinoside treatment on the reproductive profile of diet naïve male offspring".** This article has been published in Life Sciences.

**Authors name:** Toluwalope E. Adeyemi[1*], Mahendra L. Channa[1], Anand Nadar[1]

**Authors Affiliation:**

[1]Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa.

***Corresponding Author:** T.E. Adeyemi

**E-mail:** 217081454@stu.ukzn.ac.za or adeyemi.tolulope@yahoo.com

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**ABSTARCT**

Male infertility and reproductive dysfunction have become a major global health. Several factors are involved in male infertility or subfertility although, the developmental environment of the offspring together with alterations in chemical transmitters seems to play a significant role. In this present study, we sought to investigate the putative role of Quercetin-3-O-rutinoside (QR) and relationship between sex steroid hormones, adipokines, and pro-inflammatory cytokines on developmental male infertility. Fifty-six pregnant rats (previously fed normal diet (ND) or 45% high-fat diet (HFD) were maintained on supplemented chow (plus 150mg/kg QR administered orally) – ND/QR, HFD/QR throughout gestation. Subsequently, dams (n=7) and offspring (n=6) were sacrificed at post-natal day (PND) 21,28 and 35 respectively, and the blood, placenta, hypothalamus (HT), testis samples were processed for Gonadotropin-releasing hormone (GnRH), Luteinizing hormone (LH), testosterone biochemical analysis and expression of chemerin, CMKLR1, TNF-α, IL-1β and NF-κB. We observed a significant decrease in GnRH level in the HFD group at PND21 and PND28 in male offspring and PND21 in female offspring and treatment with QR significantly reduced GnRH. There was a significant reduction in LH levels in the HFD group at PND21 in the male offspring accompanied by a significant decrease in testosterone level ($p<0.05$) at PND 28 and PND35 which appears to be age dependent. In the HT, chemerin and CMKLR1 was significantly ($p<0.05$) upregulated in the HFD group at PND 21 and PND 35 respectively while CMKLR1 was significantly ($p<0.05$) downregulated in the HFD group of the placenta and testis at PND 21. TNF-α, IL-1β and NF-κB were also expressed in the placenta, HT and testis at PND 21. In conclusion, male fertility is affected by maternal HFD and treatment with QR had little or no ameliorative effect.

Keywords: Male fertility, Developmental Programming, High-fat diet, Sex hormones, pro-inflammatory cytokines, Adipokines.

## Introduction

Multiple lines of inquiry indicate that obesity can adversely impact reproductive functions associated with cases of subfertility and infertility (1-3). Obesity impacts fertility through various pathways, some of which include adipokines (4), sex steroids (5), leptin and localized or systemic oxidative stress (2). Since the mid-1900s, there has been a progressive inverse relationship between sperm count and a global increase in obesity. A clinical study conducted by Lui Y. and Ding Z. (2017), they showed that obese or overweight men had reduced sperm quality, including sperm concentration, sperm motility, decreased acrosome reaction, increased DNA damage, and lower embryo implantation rates compared to men with normal BMI (2). It is worthy of note that male infertility accounts for 70% of infertility cases as sperm count has decreased by over 50% over the last 40 years in western countries (6, 7). Although the primary reason for this decline in male fertility has not been fully established but has been however attributed to the effect of perinatal life environmental modifications which could be direct or through epigenetic modifications (8). According to an extensive epidemiological study conducted by Skakkebaek N.E et al, (2015), they observed that issues related to male fertility are relatively consistent with changes including environmental and lifestyle factors rather than a build up of inherited genetic alterations (8-10).

Epigenetically, exposure to high dietary fat could possibly affect components of sperm and endocrine contents of the seminal fluid which consequently impacts early life development (11). Connor et al. (2012) had previously investigated the relationship between 45% high-fat diet (HFD) consumption throughout gestation and lactation on maternal care and reproductive functions in dams and their offspring. The authors observed that female offspring of HFD fed dams had more fat mass accompanied by altered and prolonged estrous cycle associated with ovarian aging and infertility. Their study supports the hypothesis that the nutritional environment plays a significant role in offspring development with probable impact on reproductive functions (12, 13). Another related study revealed that HFD induced maternal obesity during gestation and lactation caused testicular and sperm oxidative stress in the male progenies, marked by significant alterations in the sex steroid hormones (14). Aromatisation and reduction of testosterone to 5α- dihydrotestosterone (DHT) is an important step in male

sexual functions (15). However, a decline in testosterone occurs via the negative feedback loop when the excessive production of oestrogen inhibits the secretion of pituitary gonadotropins, mainly follicle stimulating hormone (FSH) and luteinizing hormone (LH), and hypothalamic gonadotropin- releasing hormone (GnRH) due to hyperactivity of aromatase- cytochrome P450 (16).

Also, it has been reported that adipokines play significant roles in the regulation of fertility and reproductive disorders (17). Recent studies have shown that the novel adipokine-chemerin and its receptors are associated with obesity and reproductive functions through endocrine or paracrine routes (18-20). The mechanism involved in male subfertility, infertility, and increase adiposity remains poorly understood. Chemerin's expression and its receptor in human and animal hypothalamic-pituitary-gonadal axis (HPG axis) have been shown to regulate the secretion of GnRH and other sex steroid hormones (4, 17). Long-term treatment of human Sertoli cells with chemerin, irisin, nicotinamide phosphoribosyltransferase (Nampt), resistin and progranulin in high concentration similar to what is observed *in vitro* in obese male significantly downregulated the expression of FSH and upregulated cytochrome P450 CYP26A1 which induced the characteristic phenotype of pre-puberty (18). These cytokines negatively impacted Sertoli cells' maturation and the manifestation of testicular dysfunction associated with obesity (18). Chemerin and its receptor chemokine-like receptor 1 (CMKLR1) also plays a vital role in the regulation of male steroidogenesis. Li et al. (2014) reported that chemerin and its receptors are developmentally regulated and highly expressed in the Leydig cells of rat's testis while treatment with human chorionic gonadotropin (HCG) induced testosterone production from the Leydig cells (19).

In addition, pro-inflammation cytokines have been linked to some of the several factors involved in male subfertility or infertility (21, 22). Pro-inflammatory cytokines such as tumour necrosis factor- alpha (TNF-α) and interleukin-1 have physiological roles in male reproduction (23), when secreted at normal levels. Extreme production of these cytokines could provoke inflammatory changes that may affect spermatogenesis (24) which could be as a result of impairment of accessory gland function, obstruction to sperm transport and dysregulation of sperm formation. TNF-α and IL-1 are produced in the Leydig and Sertoli cells of the testis.

Inflammation in the reproductive tract is also associated with increased oxidative stress. Increased level of IL-1 was associated with decreased sperm quality accompanied by increase in oxidative stress and lipid peroxidation (25).

Quercetin-3-O-rutinoside (QR), also known as rutin (26), is a flavonoid with potent antioxidant properties (27). Only few studies have highlighted the effects of QR on male reproductive functions. Jahan et al (2016) studied the ameliorative effect of QR (100mg/kg and 150mg/kg) on metabolic and hormonal imbalances in polycystic ovarian syndrome (PCOS) in adult female Sprague dawley rats (28). They observed a dose dependent ameliorative response in the animals on the clinical features of PCOS. Inhibition of testosterone and penile cGMP content by QR was observed in diabetic male rats (29) and the cytoprotective role of QR was observed in Boaz sperm against oxidative damage (30). A limited study has been carried out to investigate the role of chemerin and its receptor-CMKLR1 and androgens on developmental programming of male reproduction (4). This study was therefore undertaken to investigate the developmental expression of chemerin, CMKLR1 receptor and pro-inflammatory cytokines on HFD induced maternal reproductive alterations in diet naïve male offspring rats. We further investigated the cytoprotective role of QR on HFD induced reproductive alterations.

## Materials and Methods

### Ethics and Animal Treatment

All animal procedures were ethically approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal (approval no: AREC/005/018D). Fifty-six adult female Sprague Dawley rats weighing between 180g-200g obtained from the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, were used for this study. All animals were housed in plastic cages within the test room and allowed to acclimatize for two weeks prior to the start of the experiment. Standard laboratory conditions of 23±2 ˚C room temperature, 50-60% humidity and 12h light/dark cycle with lights on at 06h00 were maintained throughout the study period. The animals had unrestricted access to food and water.

**Drug and Dietary Treatment**

The animals were acclimatized for two weeks. Thereafter they were divided into two groups. The first group were fed the standard rat chow (ND) while the other group was fed 45% high fat diet for eight weeks. They were mated with ND fed male and diet supplemented with 150mg/kg QR which was administered to the animals orally throughout the period of gestation.

**Experimental Design**

Fifty-six female Sprague Dawley rats (180-200g) were divided into 2 groups (*n*=28/group) and they were fed either normal diet (ND) or 45% high fat diet HFD for eight (8) weeks (fig 1). The oestrous cycle of the animals was monitored. After the eighth week, the animals were mated on a ratio 2:1 (two females to one male) with male Sprague Dawley rats who were fed the normal diet (ND). Day one pregnancy was taken on the day of appearance of spermatozoa in the vaginal smear as seen under a microscope and the pregnant dams were housed individually. Post conception, rats were then further divided into four (4) groups each, of which two (2) groups continued with ND or HFD while the remaining two (2) groups received either ND + 150mg/kg of Quercetin-3-O-Rutinoside (QR) or HFD+QR. The dams were sacrificed with anaesthesia in batches with the first set (n=28) on gestation day (GD) 19, this group constituted the Placenta group. The animals were euthanised with Isofor and we harvested the placenta from the dams. The second batch which constitute the dams that were allowed to litter naturally and sacrificed on post-partum day 21, their pups (n=6/gender) were fasted for 12 hours and sacrificed at postnatal day (PND) 21, 28 and 35 respectively. The testis and hypothalamus were harvested and immediately snap frozen in liquid nitrogen and later transferred to -80$^{o}$c bio-freezer for biochemical analysis.

**Determination of hormonal profile using Enzyme-Linked Immunosorbent Assay (ELISA) Technique**.

Gonadotropin releasing hormone (GnRH), Luteinising hormone (LH) and Testosterone in tissue supernatants were determined using sandwich ELISA kits (Elabscience Biotechnology, USA. Catalog No: E-EL-R0450, E-EL-R0026, and E-EL-0072 respectively). Tissue samples

were homogenised and prepared according to the assay procedures provided. Optical density (OD) value was determined using a microplate reader set to 450 nm.

**Analysis of mRNA expression**

Total mRNA was extracted from the placenta, HT and testis using the modified Trizol reagent (Zymo Research Corp.) protocol (31, 32). Tri reagent was used to isolate total RNA which effectively dissolves DNA, RNA and protein when homogenised. 1ml of trizol per 50-100 mg of tissue was used to homogenise thereafter, chloroform was added and centrifuge at 13500rpm for 5 minutes at $4^0$C. The mixture separates into 3 phases and RNA is contained in the clear aqueous upper phase which was transferred to a clean test tubes for extraction and washing of the RNA using isopropanol and 75% ethanol respectively. RNA purity and concentration were determined using the thermoscientific nanodrop 1000 spectrophotometer. Total RNA of 1µg was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratory (Pty) Ltd. USA) using the manufacturer's protocol.

Real time polymerase chain reaction (RT-PCR) was done using the lightcycler 96® (Roche Diagnostic Germany). For the RT-PCR the mixture was subjected to $95^o$c denaturation/preincubation cycle for 10 minutes, three- steps amplification (45 cycles at $95^0$c for 15s, $60^o$c for 20s and $72^o$c for 20s) with a single fluorescent measurement. SYBR Green (Luna universal qPCR master mix, New England BioLabs Inc.) used as fluorescent dye and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house keeping gene. The Cq values were chosen within linear range while differences in expression between samples were determined using comparative Cq method as previously described (33).

Table 4.1: Oligonucleotide primer sequence.

| Genes | Forward Primer | Reverse Primer |
|---|---|---|
| **TNF-α** | 5'- CAGCCGATTTGCCATTTCA -3' | 5'- AGGGCTCTTGATGGCAGAGA -3' |
| **IL-1β** | 5'- TGACAGACCCCAAAAGATTAAGG -3' | 5'- CTCATCTGGACAGCCCAAGTC-3' |
| **Chemerin** | 5'- TGTGCAGTGGGCCTTCCA-3' | 5'- CAAAGGTGCCAGCTGAGAAGA-3' |
| **CMKLR1** | 5'- CAAGCAAACAGCCACTACCA-3' | 5'- CAAGCAAACAGCCACTACCA-3' |
| **NF-κB** | 5'-GCGGCCAAGCTTAAGATCTGCCGCCGAG TAAAC-3' | 5'-CGCTGCTCTAGAGAACACAATGGCCACTTG-3' |
| **GAPDH** | 5'-TGACAACTCCCTCAAGATTGTCA-3' | 5'-GGCATGGACTGTGGTCATGA-3' |

**STATISTICAL ANALYSIS.**

Data was analysed using GraphPad Prism statistical software version 5.0. All values are presented as means ±SEM and data were analysed using analysis of variance (ANOVA) followed by post-hoc tests (either Bonferroni or Dunnett's tests as appropriate) for multiple comparison, Shapiro-Wilk normality test was used to test if the data were normally distributed, and the level of significance was set at $p<0.05$

## RESULTS

### Expression of TNF-α, IL-1β, and NFκB.

We investigated the effect of maternal HFD treatment on TNF-α, IL-1, and NFκB expression in the placenta, HT and testis. In the placenta, we observed a significant increase ($F_{(3,20)}$=32.32, p<0.0001) in the expression of TNF-α in the HFD group when compared to the other groups (Fig.4.1a) which was accompanied by a non-significant decrease of TNF-α in the HFD+QR group compared to control. At PND 21 TNF-α expression was significantly increased ($F_{(3,20)}$=6.756, p<0.0001) in the HT when compared with other groups (Fig.4.1b), while at PND 35 QR treatment alone caused a significant increase ($F_{(3,20)}$=6.578, p<0.0001) in TNF-α expression with no significant changes in TNF-α expression in the HFD group when compared with the other groups (Fig.4.1c). In the testis, TNF-α was significantly expressed ($F_{(3,18)}$=12.30, p< 0.001) in the HFD group at PND 21 (Fig.4.1d), which was accompanied by a non-significant increase in TNF-α expression in HFD group at PND 35 (Fig.4.1e). Furthermore, we observed a significant increase ($F_{(3,20)}$=33.93, p<0.0001) in the expression of IL-1β in the HFD group in the placenta (Fig.4.1f). There was a non-significant increase in the expression of hypothalamic IL-1β in the HFD group at PND 21 (Fig.4.1g) accompanied by a non-significant decrease in IL-1β expression in the HFD group compared to the control group at PND 35 (Fig.4.1h). In the testis, we observed a non-significant increase in the expression of IL-1β in the HFD group when compared to the control at PND 21 (Fig.4.1i) and PND 35 (Fig.4.1j). In addition, NFκB was significantly expressed ($F_{(3,20)}$=33.09, p< 0.0001) in the HFD+QR group of the placenta when compared with other groups (Fig.4.1k). In the HT, NFκB expression was significantly decreased ($F_{(3,20)}$=34.78, p<0.0001) in the HFD group at PND 21 (Fig.4.1L), while at PND 35 there was no significant change in NFκB expression in the groups (Fig.4.1m). We also observed a non-significant increase in the expression of NFκB in the testis at PND 21 (fig.4.1n) and PND 35 (fig.4.1o).

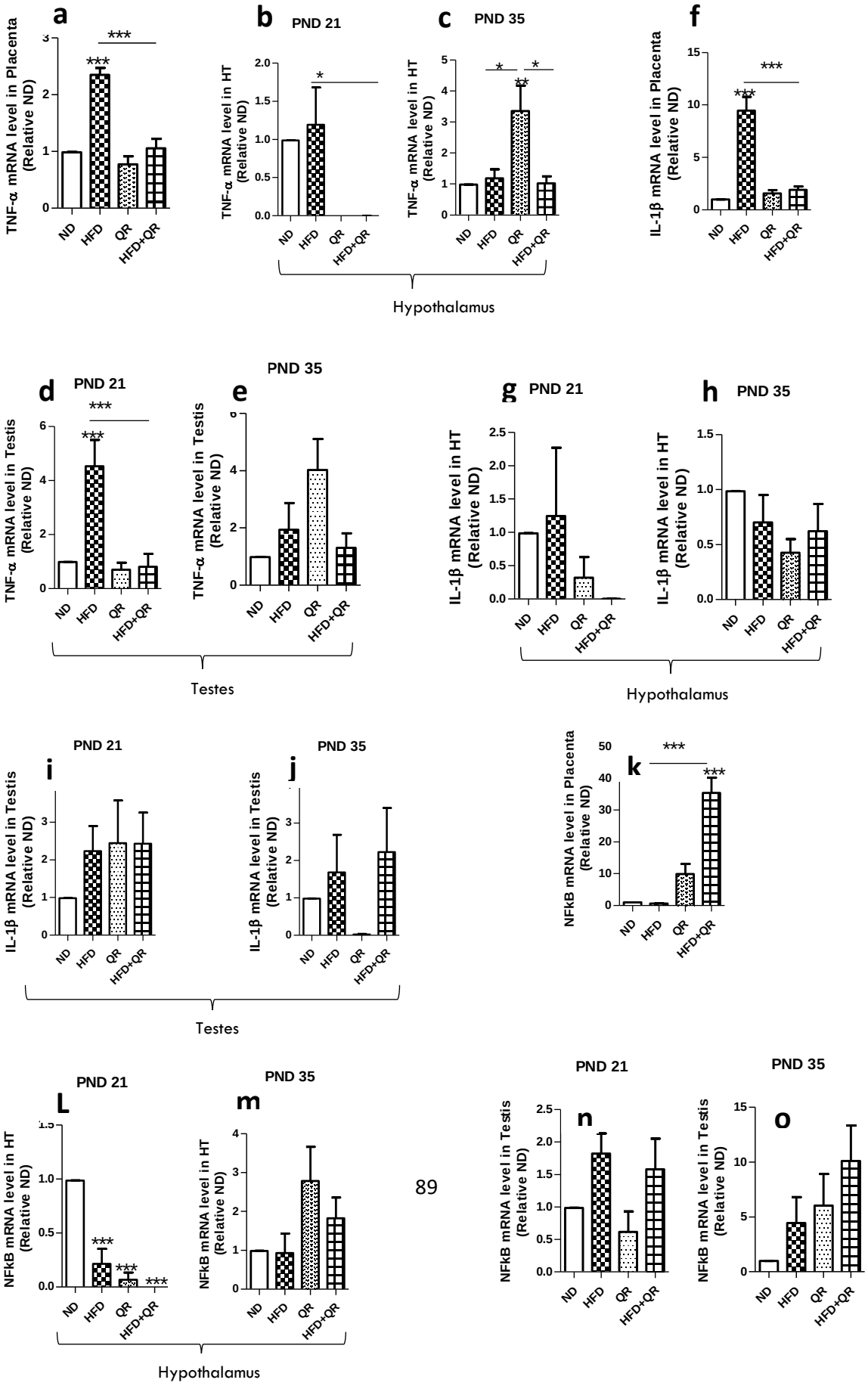
a
b
PND 21
c
PND 35
f
TNF-α mRNA level in Placenta (Relative ND)
TNF-α mRNA level in HT (Relative ND)
IL-1β mRNA level in Placenta (Relative ND)
ND
HFD
QR
HFD+QR
Hypothalamus
d
PND 21
e
PND 35
g
PND 21
h
PND 35
TNF-α mRNA level in Testis (Relative ND)
IL-1β mRNA level in HT (Relative ND)
Testes
Hypothalamus
i
PND 21
j
PND 35
k
IL-1β mRNA level in Testis (Relative ND)
NFkB mRNA level in Placenta (Relative ND)
L
PND 21
m
PND 35
n
PND 21
o
PND 35
NFkB mRNA level in HT (Relative ND)
NFkB mRNA level in Testis (Relative ND)
Hypothalamus

**Fig. 4.1:** Showing mRNA expression of inflammatory markers in the placenta of HFD fed dams, hypothalamus (HT) and testis of HFD naïve male offspring. Gene expression changes in placental TNF-α (Fig. 4.1a), IL-1β (Fig.4. 1f) and NFκB (Fig4. 1k) transcripts are indicated above. Also, transcriptional changes in hippocampal TNF-α, IL-1β and NFκB in the male offspring at PND 21 (b, g and l) and 35 (c, h and m), respectively. Whereas, sımılar changes in testicular TNF-α, IL-1β and NFκB in PND 21 (d, i and n) and 35 (e, j and o) were also examined. n=6 per group; *$p$<0.05, **$p$<0.01, ***$p$<0.001.

Testes

**Effects of maternal HFD and QR treatments on GnRH, LH and testosterone levels in the male offspring rats**

To investigate the effects of maternal HFD consumption and impact of QR supplementation on the reproductive hormonal profile of their offspring, we measured tissue levels of GnRH, LH and testosterone in the male offspring rats. There appears to be a significant interaction ($F_{(6,24)}$ = 123.4, p<0.0001) between time ($F_{(2,24)}$ =94.11, p<0.0001) and treatment ($F_{(3,24)}$ =20.34, p<0.0001) using two-way ANOVA. As shown in Fig.4.2b, we observed that maternal HFD significantly reduced the level of GnRH in the offspring rats at early childhood (PND 21) and adolescent stage (PND 28), compared to control. Surprisingly, QR supplementation further decreased the GnRH levels at PND 28 (p<0.0001) and 35 (p<0.05) when compared to the HFD group (Fig. 4.2a). Assessment of tissue LH concentration in the naïve offspring (Fig. 4.2c) indicates significant interaction ($F_{(6,24)}$ =3.994, p=0.0065) between treatment ($F_{(3,24)}$ =9.934, p=0.0014) and time ($F_{(2,24)}$ =1202, p<0.0001). There was a significant decrease in LH at PND 21 across all treatment groups compared to control (p<0.01, Fig. 4.2c). Whereas at PND 28 and 35 there was no significant change observed in the HFD groups compared to control except that HFD+QR group displayed a higher LH levels compared to HFD and QR groups at PND 28 (Fig. 4.2c). Having established that the impact of maternal HFD consumption on the levels of GnRH and LH in the naïve male offspring, we decided to investigate further the effects of the treatments (maternal HFD consumption and QR supplementation) on the offspring testosterone levels. We observed a significant interaction ($F_{(6,24)}$ =7.831, p<0.0001) with

treatment effects ($F_{(3,24)}$ =12.78, p=0.0005, Fig. 4.2d). Our data indicated an increase in testosterone levels in offspring of HFD/QR fed dams (p<0.001) and a significant reduction in testosterone level in QR group (p<0.001) at PND 21, while offspring of dams fed only HFD displayed significant decrease in testosterone levels at PND 28 compared to control (p<0.001) and other treatment groups (p<0.01, Fig. 4.2d). No differences were observed in testosterone levels at PND 35 (p>0.05, Fig. 4.2d).

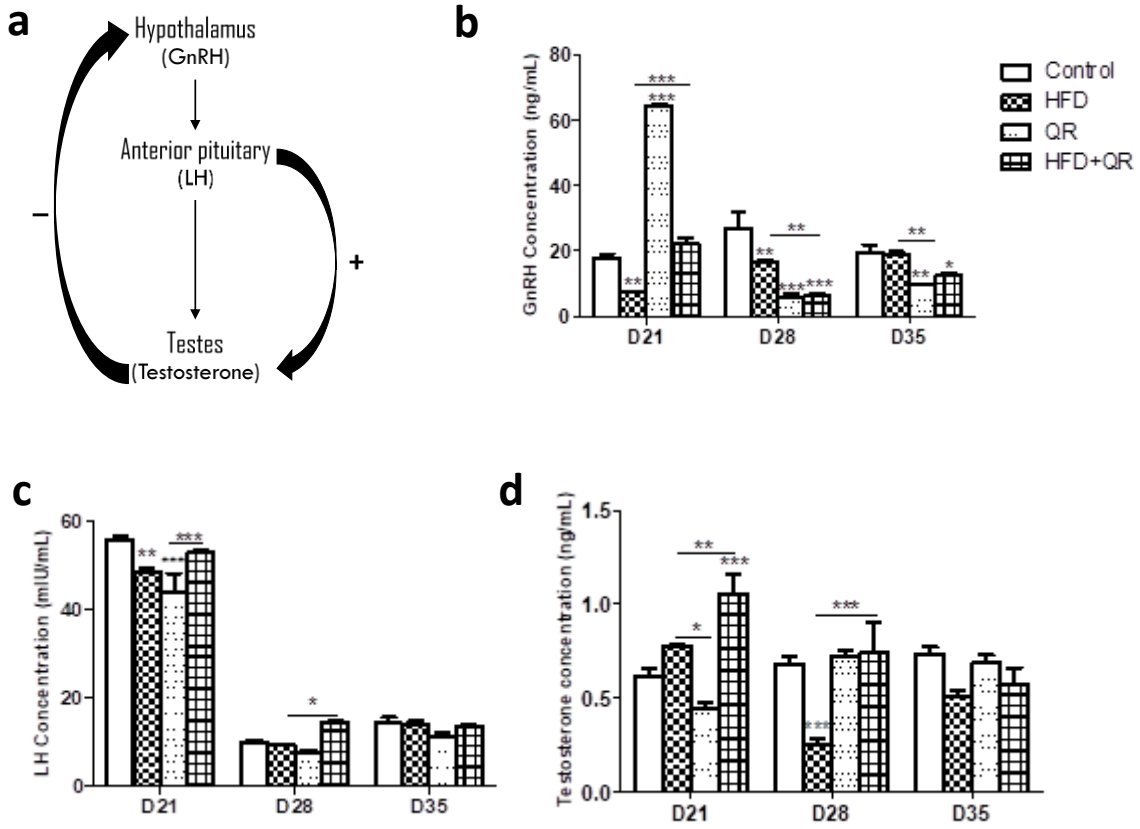


**Fig 4.2:** Shows the effect of maternal HFD consumption on GnRH, LH and testosterone in male offspring at PND 21, 28 and 35. The link between the hypothalamic-pituitary gonadal axis in the regulation of reproductive hormone through positive or negative feedback mechanism is shown in fig.4.2a above. Expression of GnRH (fig.4.2b), LH (fig.4.2c), and testosterone (fig.4.2d) at PND- D21, D28 and D35 in the male HT, plasma and testis of the male offspring are shown in the figure above respectively.

**HFD-induced changes in hypothalamic-gonadal chemerin/CMKLR1 expression**

There is substantial evidence that adipokines, especially chemerin, could affect reproductive functions by modulating GnRH-induced LH and FSH release from the anterior pituitary cells (34). However, the biological functions of chemerin/CMKLR1signaling associated with HFD-induced transgenerational changes in the reproductive profiles of F1 offspring remains unclear. To unravel the biological functions, we quantified mRNA transcripts of chemerin and CMKLR1 in the dams' placenta and offspring's hypothalamic and testicular tissues (Fig. 4.3a). As shown in Fig. 4.3b, there was no significant change in the expression of placental chemerin mRNA of HFD-fed dams when compared to ND group ($p>0.05$). Moreover, ANOVA indicates significant effects of HFD-treatment on hypothalamic expression of chemerin ($F_{(3,20)}=231.2$, $p<0.0001$) which was greatly increased in the HFD group only at PND 21 compared to ND and other treatment groups ($p<0.0001$, Fig.4.3d), without significant changes at PND 35 ($p>0.05$, Fig. 4.3f). Surprisingly, we observed downregulation of CMKLR1 expression in the placenta ($p<0.001$, Fig. 4.3c) and testicular tissues of all HFD and QR offspring at PND 21 ($p<0.01$, Fig. 4.3i) without any change in the HT of PND 21 offspring ($p>0.05$, Fig. 4.3e). On the contrary, offspring of HFD dams displayed significant increase in hypothalamic CMKLR1 mRNA expression compared to other groups ($p<0.01$, Fig. 4.3g). At PND 35, QR group exhibited increased CMKLR1 expression ($p<0.05$) while maternal HFD exposure had no significant impact on the testicular CMKLR1 changes at this time ($p>0.05$, Fig. 4.3k).

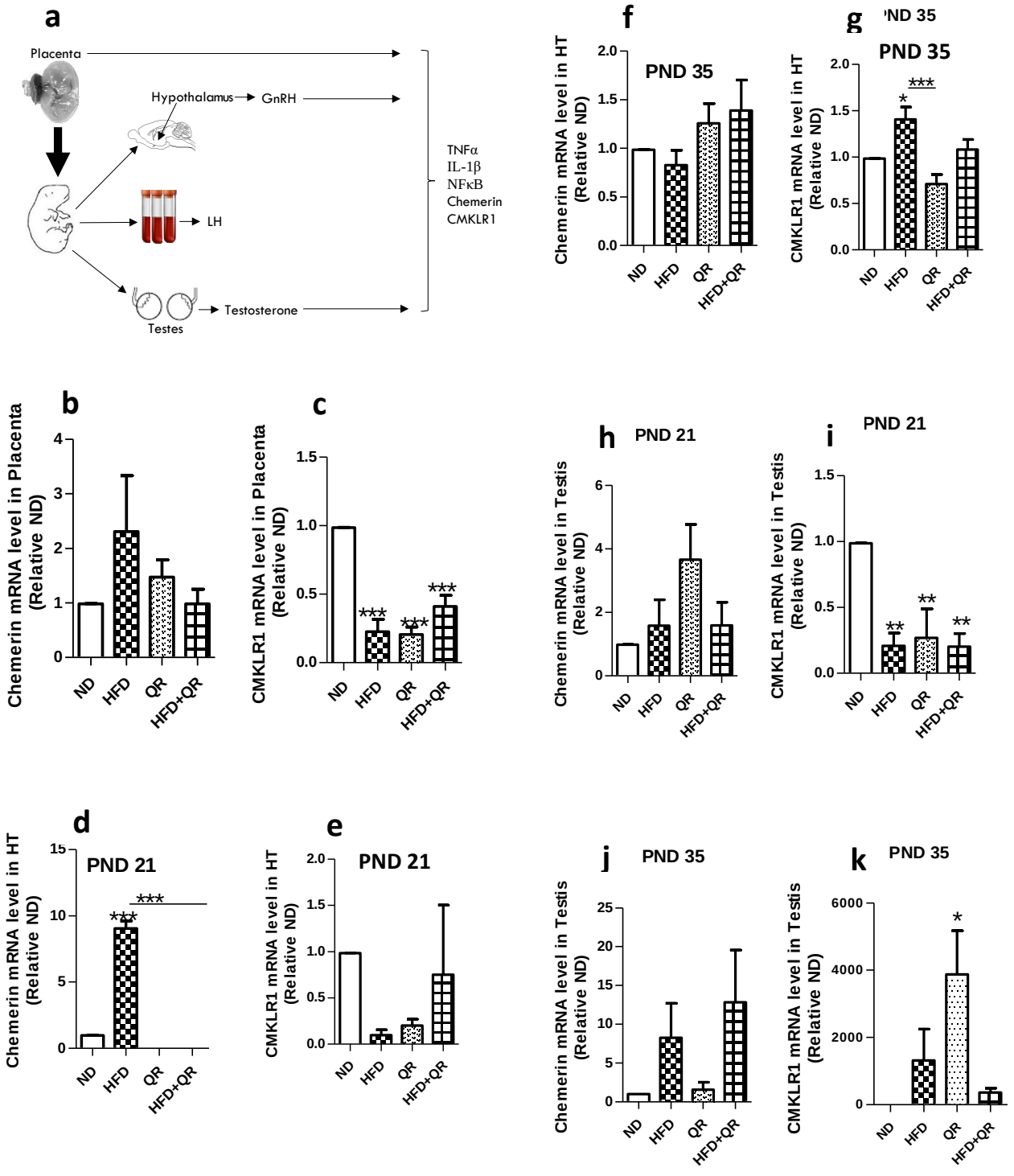


**Fig. 4.3:** Shows the mRNA expression of chemerin and its receptor CMKLR1 in the HT and testis of HFD naïve male offspring and placenta of HFD fed dams. The expression of chemerin

and CMKLR1 in the placenta was shown in fig.4.3b and fig.4.3c respectively. Expression of chemerin in the HT at PND 21 and PND35 is shown in fig.4.3d and fig.4.3f respectively while fig.4.3h and fig.4.3j shows the expression of chemerin in the testis at PND 35. CMKLR1 expression in the HT at PND 21 and PND 35 is shown in fig.4.3e and fig.4.3i**.** Expression of CMKLR1 in the testis at PND 21 and PND 35 was shown in fig.4.3i and fig.4.3k respectively. Values are expressed as mean ± SEM and level of significance set at $p<0.05$.

## DISCUSSION

In this present study, we investigated the impact of maternal HFD induced reproductive alterations in male offspring and further examined the potential benefit(s) of QR. We also investigated the possible role of certain adipocytes (chemerin and its receptor CMKLR1) and inflammatory markers in the regulation of male fertility. Studies have shown that alterations in maternal diet prior to or during gestation and lactation can lead to altered reproductive functions in their diet naïve offspring (35, 36).

We observed that TNF-α and IL-1β were significantly expressed in the placenta with no significant change in the expression of NFκB. This suggests the presence of inflammation in the placenta which could be due to HFD. In the offspring, there was a significant expression of TNF-α with no changes in the level of IL in the HT and testis at PND 21. Pro- inflammatory cytokines have been reported to be involved in the normal functioning of the male gonads and they are produced locally (24). It has been reported that, the excessive production of pro inflammatory cytokines such as TNF-α, IL-1 in the male reproductive system could be an indication of infertility (37). TNF-α inhibits the expression of steroidogenic enzyme in the Leydig cells through the activation of NFκB (38, 39) which in turn leads to the reduction in the secretion of testosterone (38). However, in this study, there was no significant change in the production of NFκB in the placenta and testes although testosterone was reduced which suggests that a different pathway aside activation of NFκB may be responsible for the reduction in testosterone.

Reproductive functions and fertility is controlled by a complex and highly coordinated neuroendocrine axis (14). Gonadotropins and androgens secreted by the hypothalamus and gonads respectively are controlled via the negative feedback loop and any alteration or poor regulation affects reproductive functions (40). From our study, we observed that there was a significant reduction in hypothalamic GnRH level at PND 21 and 28 in the male offspring. Our observations are consistent with a previous study by Rodriguez-Gonzalez et al (2014) which reported that HFD or maternal obesity potentially decreased testosterone and LH serum levels in the offspring and progressed with advancement in age (14). Similarly, the current study demonstrated a significant reduction in the LH levels of HFD offspring at PND 21. At the gonadal level, LH stimulates the Leydig cells to produce testosterone. It has been established that the pulsatile release of GnRH from the arcuate nucleus of the hypothalamus controls sperm formation by stimulating the release of pituitary LH regulated via the negative feedback loop modulated by testosterone (40, 41). Sex steroid induced negative feedback regulation of HPG functions (42-45) could possibly account for the observed decrease in GnRH and LH levels owing to increased testicular testosterone production at PND 21. In a study by Wang et al (2017), mice were treated with HFD and rutin to investigate the ameliorative effects of rutin on body weight and reproductive impairment. The authors found that rutin could protect against HFD induced reproductive impairment on epididymal cell structure and sperm motility (46). This is also in line with the result from our findings which indicated a potential for QR in restoring LH activity while maintaining testosterone levels.

Furthermore, chemerin was significantly expressed in the HT at PND 21 in the male offspring which could be a possible reason for the decreased level of GnRH at PND 21 whereas there was no significant change at PND 35. Surprisingly we observed an upregulation in the expression of CMKLR1in the HT at PND35 with a non- significant downregulation at PND 21. The exact reason for the inverse expression of the chemokine and its receptor is not fully understood. This could be a compensatory or protective mechanism where elevated levels of chemerin could trigger downregulation of its receptor as a protective mechanism although, our study indicated that testosterone was decreased at PND 35 and increased at PND 21. It is worthy of note that, chemerin and CMKLR1 are not uniformly expressed in the HT and its role

depends on the area where it is expressed (47). Although, it has been established that they are closely associated with the appetite control regions in the hypothalamus, It was reported that chemerin and CMKLR1 transcripts were found in the ependymal cells and tanycytes lining the third ventricle and that it also extends into the arcuate nucleus (48). Our study suggests that the upregulation of chemerin in the hypothalamus also regulates (reduces) the release of GnRH. Although, more studies are required to narrow down the expression of chemerin and its receptor to areas of the hypothalamus involved in regulation of reproductive functions in the male offspring.

Male fertility is dependent on a well-controlled hypothalamic pituitary-gonadal axis (fig.4.2a). Studies have shown that decline in testosterone level is also associated with aging (14, 49). In this study, we observed that at the transition phase to puberty (PND 28) and adulthood (PND 35) there was a decline in the serum level of testosterone in the HFD group when compared to the control group. Maternal HFD consumption or obesity and their male offspring fertility has been linked to alterations in testosterone level (35). Studies carried out in mice and human revealed that maternal HFD consumption or obesity stimulates the aromatization of testosterone to estradiol (E2) which consequently reduces the level of testosterone via impaired spermatogenesis (50), this finding also supports the reduced testosterone level observed in our study. In addition, Dupont et al (2014) carried out a study in rats to determine the concentration of testosterone in hyperlipidemic and hypercholesterolemic (HH) fed rabbit. They observed that serum free testosterone concentrations were decreased in the HH males which supports the link between maternal nutritional status and male fertility (14, 51).

Furthermore, we investigated the role of chemerin and its receptor CMKLR1 in the testis and HT of diet naïve male offspring. The link between HFD direct consumers (dams) and indirect consumers (offspring) was established by investigating the expression of chemerin and CMKLR1 receptor in the placenta (fig.4.3a). From our study, it appears that maternal HFD can cause an increase in the expression of chemerin accompanied by a decrease in the expression of its receptor. In a study carried out to investigate the putative role of chemerin in the placenta of rat and human throughout gestation, they observed an up regulation in the mRNA expression of chemerin in the placenta which is similar to what we observed in our study (20, 52). It has

been reported that chemerin acts via different signaling pathways and its effect could be time and dose dependent (17). Similar studies have also reported significantly increased levels of chemerin (20, 53, 54) in obese or diabetic conditions. In line with other studies, we also established that chemerin and its receptor CMKLR1 are expressed in the rat testis (19). Furthermore, we observed a non - significant up regulation/ expression of chemerin in the testis of the offspring at PND 21 and PND 35 in the HFD group which was accompanied by a decreased expression ($p<0.05$) of CMKLR1 at PND 21. The upregulation of chemerin could be the possible explanation for the observed decrease in testosterone level at PND 35. It has been established that chemerin function through its receptor- CMKLR1 in male steroidogenesis (4). In a study conducted by Li. L et al, (2014) using cultured rat Leydig cells, this was the only study till date that investigated the presence of chemerin and its receptors within the Leydig cells. They suggested that chemerin has a suppressive effect on testosterone synthesis (19, 55) and also established that chemerin is a peripherally acting molecule in the testis. From this study, there appears to be a relationship between the novel adipokine- chemerin and steroidogenesis although more studies is required to further elucidate its pathway of action along the HPG axis.

In conclusion, from this current study we were able to establish that chemerin, CMKLR1, TNF-α, Il-1β, and aging affect steroidogenesis of HFD naïve male offspring, although the pathway linking them to male infertility has not been fully established. Whereas, QR supplementation had little or no significant effect on steroidogenesis in the offspring.

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# CHAPTER FIVE

In this article, HFD-induced oxidative and inflammatory changes in the brain linked to the regulatory roles of GLP-1 incretin hormone was examined. This is in a manuscript format titled: "**Biochemical changes in the brain of rats prenatally exposed to high-fat diet".**

**Authors names:**

Toluwalope E. Adeyemi†℗, Dikeledi M. Kekana*℗, Anand Nadar†, Duyilemi C. Ajonijebu*

**Authors Affiliation:**

†Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

*Department of Physiology, School of Biomolecular and Chemical Sciences, Faculty of Science, Nelson Mandela University, Port Elizabeth, South Africa.

**Corresponding Author:** Duyilemi C. Ajonijebu*****

**E-mail:** aduyilemi@yahoo.com

**℗ These authors contributed equally to this work and are regarded as co-first authors.**

**ABSTRACT**

The mechanistic link between maternal high-fat feeding and postnatally induced sex-dependent neurochemical alterations in diet-naïve offspring's brain is not completely understood. In this study, we examined the molecular underpinnings of quercetin-3-O-rutinoside (QR) and glucagon-like peptide 1 (GLP-1) regulatory effects on high-fat diet (HFD) induced neuroinflammatory and/or biochemical changes in direct consumers and diet-naïve descendants. Pregnant rats (previously fed normal diet (ND) or 45% HFD) were maintained on supplemented chow (plus 150 mg/kg QR administered orally) – ND/QR, HFD/QR throughout gestation. Subsequently, the animals were sacrificed, and brain samples were processed for expression of TNF-α, GLP-1, MDA and GSH/NO content. The data show that chronic consumption of HFD by dams failed to alter significantly the brain TNF-α levels in dams and offspring at postnatal day (PND) 21. However, a non-significant increase in TNF-α levels that appears suppressed by QR was observed in the female offspring rats of HFD-fed dams at PND 28. Surprisingly, HFD-fed dams exhibited non-significant increase in brain MDA levels accompanied by time and sex-dependent changes in lipid peroxidation in their progenies. NO and GSH levels were not directly affected by maternal HFD treatment, whereas brain GSH concentration increased significantly in the female offspring of HFD-fed dams at PND 28. Moreover, GLP-1 expression significantly increased in dams that received QR supplemented chow, but not in the offspring. In conclusion, the current findings indicate that maternal consumption of 45% HFD only produced biochemical changes in the brain which can potentially trigger neuroinflammatory changes in the progenies.

**Keywords:** High-fat diet; neuroinflammation; sex differences; quercetin-3-o rutinoside; glucagon-like peptide-1; sensitization.

## Introduction

Several animal studies have demonstrated that prolonged feeding of high-fat diet (HFD) to normal rats can be used to induce obese phenotypes (Graf et al 2016, Srinivasan et al 2006, Vuong et al 2017) like what is obtainable in the western societies. This dietary practice may have immediate and long-term consequences such as altered energy homeostasis, generalized metabolic syndrome, dysregulated neuro-energetic circuitry and chronic inflammatory responses (Buettner et al 2007, Kang et al 2014, White et al 2009). In addition, there is accumulating evidence that consumption of HFD may not directly activate central inflammatory pathways but mostly initiate sensitization process that provokes amplified proinflammatory cytokine signaling (mostly TNF-α and IL-1β) and production of chemokines, reactive oxygen species (ROS) and secondary messengers (prostaglandins and nitric oxide) by the central innate immune cells (Norden et al 2016). Amongst the HFD-induced changes, increased oxidative and inflammatory signaling have been widely implicated in several disease pathologies (White et al 2009).

Srinivasan and colleagues have established the relationship between maternal HFD consumption and altered fetal programming with severe consequences on offspring vulnerability to developing metabolic syndrome-like phenotype (Srinivasan et al 2006). Similar correlations also exist between prenatal exposure to HFD and postnatal development of neurobehavioral deficits and/or psychiatric changes. For instance, White et al (2009) reported that progenies of dams that were fed HFD for about 20 weeks performed poorly on memory function test and further manifested significant increases in inducible nitric oxide synthase (iNOS) and markers of inflammation (IL-6, Iba-1 and GFAP) (White et al 2009). Importantly, this implies that maternal environmental insult or nutritional drift can adversely affect neurodevelopment during and after pregnancy and subsequent offspring behaviour. Since most reported studies engaged both dams and their offspring in the HFD treatment plan, it therefore remains unclear the impact of maternal HFD consumption on neurodevelopmental changes in diet-naïve offspring post-weaning.

Moreover, it has been demonstrated that quercetin-3-O- rutinoside (QR) has neuroprotective effects on the brain. Its administration caused diminished ischemic neural apoptosis by increasing endogenous defence enzymes thus attenuating lipid peroxidation (Bhandary et al 2012). It has also been found to be useful in hypoxic conditions by reducing the levels of oxidative stress (Pu et al 2007). Although less information exists in the literature on the efficiency of QR as a potent antioxidant or suitable anti-inflammatory candidate against HFD-induced neural damage, previous studies have shown that QR potentially reduced inflammation in rats' brain with sporadic dementia of Alzheimer type by decreasing microglial expression of proinflammatory cytokines TNF-α and IL-1β (Ganeshpurkar & Saluja 2017, Javed et al 2012). In addition, there is accumulating evidence that brain glucagon-like peptide-1 (GLP-1) is involved in nutrient metabolism and homeostasis and further regulate inflammatory processes in the brain (Bae & Song 2017, Daniele et al 2015, Kim et al 2017). Of interest is the study by Gaballah et al (2017) which demonstrated that co-treatments of quercetin and GLP-1 analogue liraglutide enhanced significant improvements in HFD-induced biochemical, oxidative and histopathological changes in obese rats (Gaballah et al 2017). Another study by Scudiero and Verderame (2017) also underlined the importance of estrogen receptors (ERα and ERβ) in the regulation of HFD-induced modulation in brain energetic metabolism (Scudiero & Verderame 2017). Despite these significant advances, our understanding of the molecular underpinnings of GLP-1 neuroprotective effects against sex differences in postnatally induced oxidative or neuroinflammatory responses to maternal HFD exposure remains incomplete. Also, the complementary roles of QR and enhanced GLP-1 signaling effects on HFD-induced alterations in brain homeostasis requires further investigation.

This study was undertaken to investigate the impact of HFD consumption on the brain chemistry of direct consumers and diet-naïve progenies with core interest in sex differences associated with oxidative and pro-inflammatory changes. We further investigated the potential benefits of QR and GLP-1 regulatory effects on HFD-induced central inflammation and metabolic changes.

## Materials and Methods

### Ethics Statement

All animal procedures were approved by the University of KwaZulu-Natal's Animal ethics committee (approval number AREC/005/018D) in accordance with National Institute of Health (NIH) guidelines for the care and use of laboratory animals. Approval for the retrieval and usage of animal tissues was granted by the Nelson Mandela University Animal Ethics Committee (approval number A19-SCI-PHS-001).

### Rats, Dietary Treatments and Sacrifice

Adult Sprague Dawley rats (Females, *N=56*, Males, *N=28*) weighing 180-200g were purchased from and housed within the animal holding facility of Biomedical Resource Unit, BRU (University of KwaZulu-Natal, South Africa). Post weight randomization, the female rats were divided into two groups (n=28/group) and fed either normal diet (ND; BRU) or 45% HFD (diet sourced from Prof. D. Katerere's Laboratory at Tshwane University of Technology, Pretoria) for eight weeks. Thereafter, the female rats were mated with their male counterparts fed ND. Post conception, the pregnant rats were further divided into four groups of which two groups continued with either ND or HFD whereas the remaining groups were fed either ND+150 mg/kg quercetin-3-o-rutinoside (QR) or HFD+150 mg/kg QR. Please note that QR was administered orally to the animals. After birth, the dams continued with their respective diets and sacrificed via inhalation of Isofor in an air-tight chamber along with half of the offspring at postnatal day (PND) 21. The remaining half of the offspring were weaned, continued ND, and later sacrificed at PND 28. At both killing time points, the brain samples were harvested using appropriate bone forceps and stored at -80$^{o}$C in bio-freezer. The preserved brain tissues were transferred to Physiology laboratory at Nelson Mandela University (South Africa) for further biochemical and molecular processing.

### Enzyme-Linked Immunosorbent Assay (ELISA) Analysis

Proinflammatory cytokine TNF-α and incretin-like GLP-1 levels in tissue supernatants were determined using sandwich ELISA kits (Elabscience Biotechnology, USA) after whole brain

homogenization with bullet blender (Next Advance, USA). Briefly, samples were added to the flat bottom 96-well ELISA plates pre-coated with appropriate capturing antibodies specific to proteins of interest. Appropriate biotinylated secondary antibodies specific for rat TNF-α/GLP-1 and avidin-horseradish peroxidase conjugate were added followed by incubation and several washes. Spectrophotometrically, absorbance was determined using microtiter plate reader (Multi-scan GP, Thermo Fischer Scientific, UK).

**Determination of TBARS in Brain Samples**

Lipid peroxidation is an indicator of degenerative process commonly associated with endo-cyclization of oxygenated fatty acids with potential neurotoxic activity (Montine et al 2002). In this study, malondialdehyde (MDA) an intermediate product of lipoperoxidation (Hipkiss et al 1997) was determined by measuring its biochemical equivalent thiobarbituric-acid-reacting substances (TBARS) in rats brain as previously described (Berkiks et al 2019). Brain tissue was homogenised with 0.2% phosphoric acid, centrifuged at 1000 rpm for 10 mins and the supernatant (200 µL) was pipetted into test tubes while the following reagents were added: 500 µL 2% phosphoric acid, 200 µL 7% phosphoric acid, 400 µL BHT/TBA solution, 100 µL 1M HCL. The mixture was vortexed, heated at 100$^{o}$C for 15 mins and allowed to cool at room temperature. 2ml butanol was later added to all samples and vortexed again. After vortexing, TBARS were determined by measuring absorbance at 532nm and 600nm. The concentration was calculated using the formula:

$$\text{Concentration} = \frac{A_{532} - A_{600}}{1.56}$$

A= Absorbance.

**Reduced Glutathione (GSH) and Nitric Oxide (NO) Content in the Brain**

Assessment of total GSH content in the brain samples was based on oxidation of sulfhydryl reagent 0.5mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) to form 5'- thio-2-nitroenzoic acid (TNB) (Ellman 1959). At first, sample proteins were precipitated with 10% trichloroacetic acid (TCA). Thereafter, 0.5 mM DTNB and 0.2 M sodium phosphate buffer (contains 0.2 mM

mono-and-dibasic sodium phosphate solutions) were added to the supernatants of tissue homogenates and incubated for 15 mins. Total glutathione was assayed at 415 nm absorbance. NO levels were estimated using Griess technique based on two-step diazotization reaction resulting in formation of chromophoric azo-derivative (Bryan & Grisham 2007). Equal volumes of tissue homogenate and Griess reagent were mixed and incubated in the dark for 30 min at room temperature. Absorbance was measured at 548 nm.

**Statistical Analysis**

Data were analysed using GraphPad Prism software (version 5.0). Shapiro–Wilk normality test was used to determine if the data was normally distributed or not. Where distribution was normal, data were analysed using one-way analysis of variance (ANOVA) for TNF-α, MDA, GSH, NO, and GLP-1 in the brain tissues of dams. Two-way ANOVA was used to analyse results for TNF-α, MDA, GSH, NO, and GLP-1 in the offspring at PND 21 and 28. Comparisons between groups were performed by Bonferroni post-hoc test and student t-test. All data are reported as mean ± SEM, while $P<0.05$ was considered statistically significant

**Results**

**Maternal HFD consumption altered postnatal neuroinflammatory response in diet-naïve descendants.**

To investigate the impact of HFD on inflammatory response changes in the brain, we used ELISA assay technique to determine the concentration of proinflammatory cytokine TNF-α in the whole brain of rats after 8 weeks of HFD consumption. One-Way ANOVA indicates significant effects of HFD on maternal brain health ($F_{(3,23)} = 3.228$; $p=0.0443$, Fig. 5.1b). However, Bonferroni *post hoc* test indicates no significant differences in the brain TNF-α levels within the treatment groups ($p>0.05$, Fig. 5.1b). We further examined the brains of diet-naïve offspring rats for possible inflammatory response changes at PND 21 and 28 via TNF-α expression and sought to establish whether the postnatally induced changes are sex-dependent or not. The data indicated no significant main effects of maternal HFD consumption ($F_{(3,24)} = 0.7962$; $p=0.5080$) and sex differences ($F_{(1,24)} = 0.4639$; $p=0.5023$) on TNF-α production in the

brains of PND 21 male and female offspring (Fig. 5.1c). In contrast, two-way ANOVA demonstrated significant effects of prenatal HFD exposure on central induction of proinflammatory TNF-α in PND 28 offspring ($F_{(3,16)}$ = 3.688; p=0.0343, Fig. 5.1d). The data also show that the postnatally induced brain TNF-α was significantly suppressed by QR only in the female offspring (p<0.05, Fig. 5.1d).

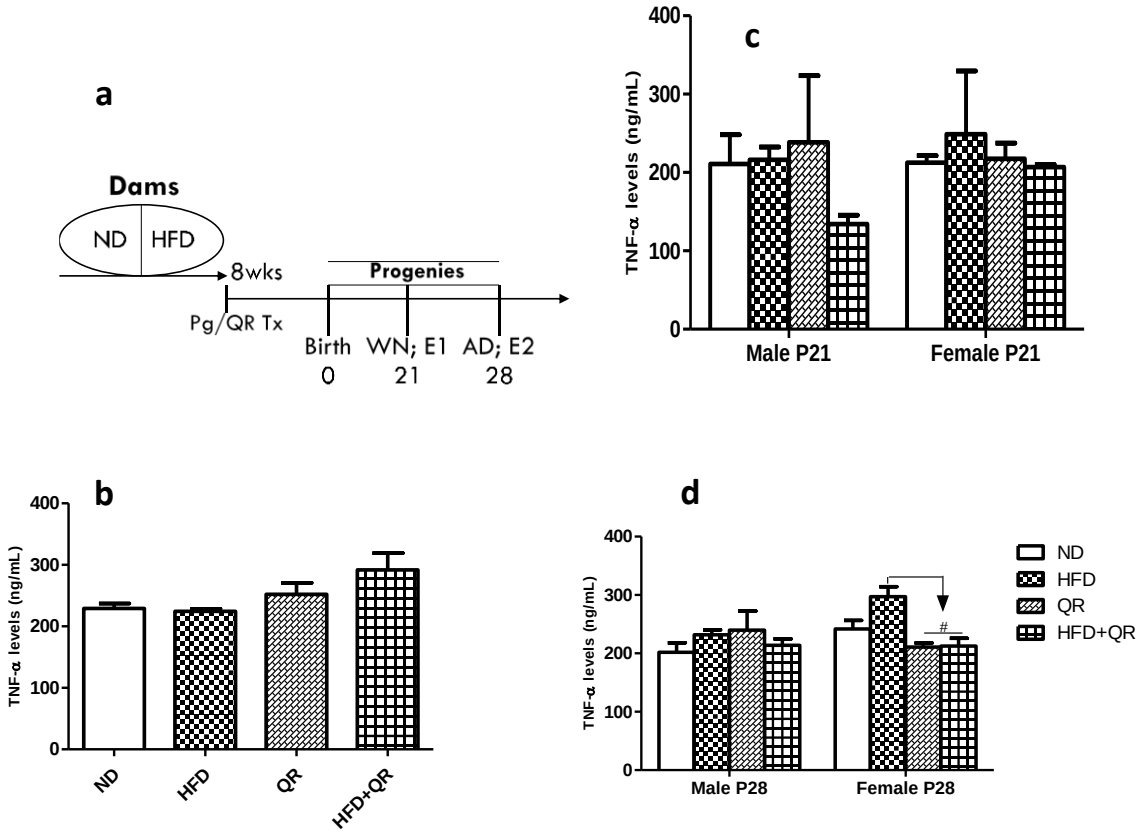


**Fig 5.1:** The figures above show (a) timeline illustrating the treatment protocols; Pg=pregnancy, Tx=treatment/supplement, WN=weaning, E=euthanasia, AD=adolescence, (b) TNF-α concentration in dams' brain after 8 weeks HFD consumption, (c-d) postnatally induced sex-dependent TNF-α production in the offspring's brain at PND 21 and 28. Data shown represents mean ± SEM; n=6 per group. **P<0.05* compared to ND; One-way or two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

**Postnatally induced lipo-peroxidative changes are time dependent.**

To explore the neuroinflammatory changes associated with HFD consumption, lipid peroxidation parameters were further analysed in the brain samples of both dams and offspring via assessment of MDA concentration using TBARS as previously described (Berkiks et al., 2019). The data indicate non-significant increase or decrease in MDA concentration in the brains of HFD-fed or QR treated dams, respectively ($p>0.05$, Fig. 5.2a). Overall, one-way ANOVA demonstrate no significant impact of either treatment on lipid peroxidation in the neural tissues. ($p>0.05$, Fig. 5.2a). Surprisingly, two-Way ANOVA demonstrated significant effects of maternal HFD consumption on central MDA concentration in PND21 and 28 offspring ($p<0.05$, Fig. 5.2b, 5.2c) and significant differences between the genders ($p<0.001$, Fig. 5.2b, 5.2c) at these time points. PND21 female offspring displayed significantly higher levels of lipid peroxidation than their male counterparts ($p=0.0280$), while postnatally induced lipid peroxidation was significantly increased by quercetin only in the female offspring ($p<0.001$, Fig. 5.2b). In contrast, lipid peroxidation was significantly higher in male than female offspring at PND 28 ($p=0.0089$, Fig. 5.2c).

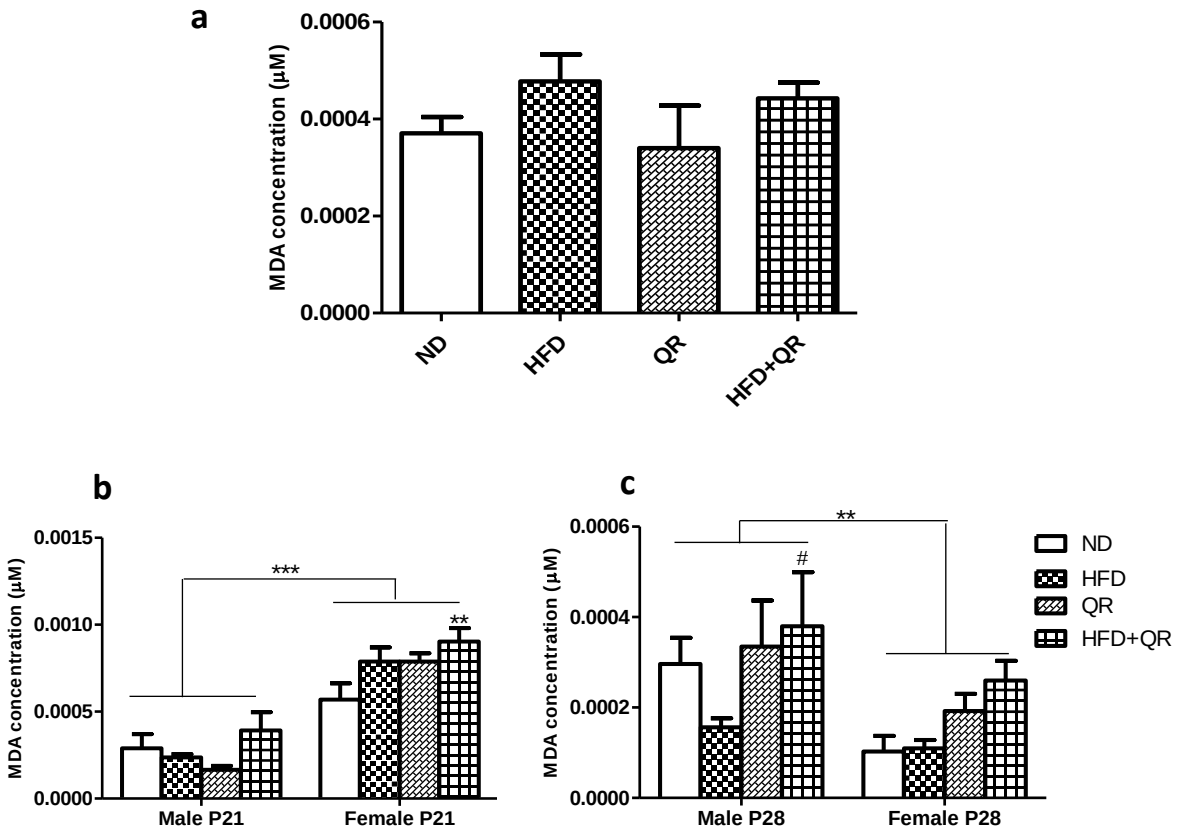


**Fig. 5.2:** Lipid peroxidation changes in the brain (a) impact of HFD consumption on MDA concentration in dam's brain, (b-c) Sex-dependent changes at PND 21 and 28. Data shown represents mean ± SEM; n=6 per group. *$P<0.05$* compared to ND; One-way or two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

**Glutathione and Nitric oxide levels**

GSH represents one of the intracellular antioxidant systems employed to scavenge and convert ROS to non-reactive forms. In the current study, antioxidant capacity of endogenous brain GSH in dams previously exposed to HFD and QR treatments and impact on their progenies were assessed. ANOVA demonstrated no significant impact of HFD consumption on GSH concentration in the dams' brain ($p>0.05$, Fig. 5.3a). However, there were non-significant decrease or increases in brain GSH levels in HFD-fed or QR treated dams, respectively

(p>0.05, Fig. 5.3a). Our data also indicate no significant main effects of maternal HFD consumption and sex differences on brain GSH antioxidant levels of PND 21 male and female offspring (p>0.05, Fig. 5.3b). However, further data analysis show that female offspring of HFD-fed dams expressed higher concentration of total brain GSH compared to their male counterparts at PND 28 (p=0.0403, Fig. 5.3c). To investigate the impact of maternal HFD consumption on NO-mediated cerebral blood flow and regulation of neuronal metabolic status, NO production in dams' brain was assayed using Griess reagent. Data analysis revealed significant main effects of treatments on NO production in the dams' brain ($F_{(3,23)}$ = 1.694; p=0.0017), while Bonferroni *post hoc* comparison test only demonstrated a non-significantly increased NO concentration in HFD+QR group compared to ND (p>0.05, Fig. 5.3d). We further assessed NO activity in the brains of the progenies subject to sexual dimorphic effects. Our findings show that there were only significant effects of maternal treatment on NO concentration in the brains of PND21 offspring ($F_{(3,40)}$ = 3.242; p=0.0319, Fig. 5.3e), but no gender effect (p>0.05, Fig. 5.3e). Both the diet and gender had no significant impact on the offspring NO activities at PND28 (p>0.05, Fig. 5.3f).

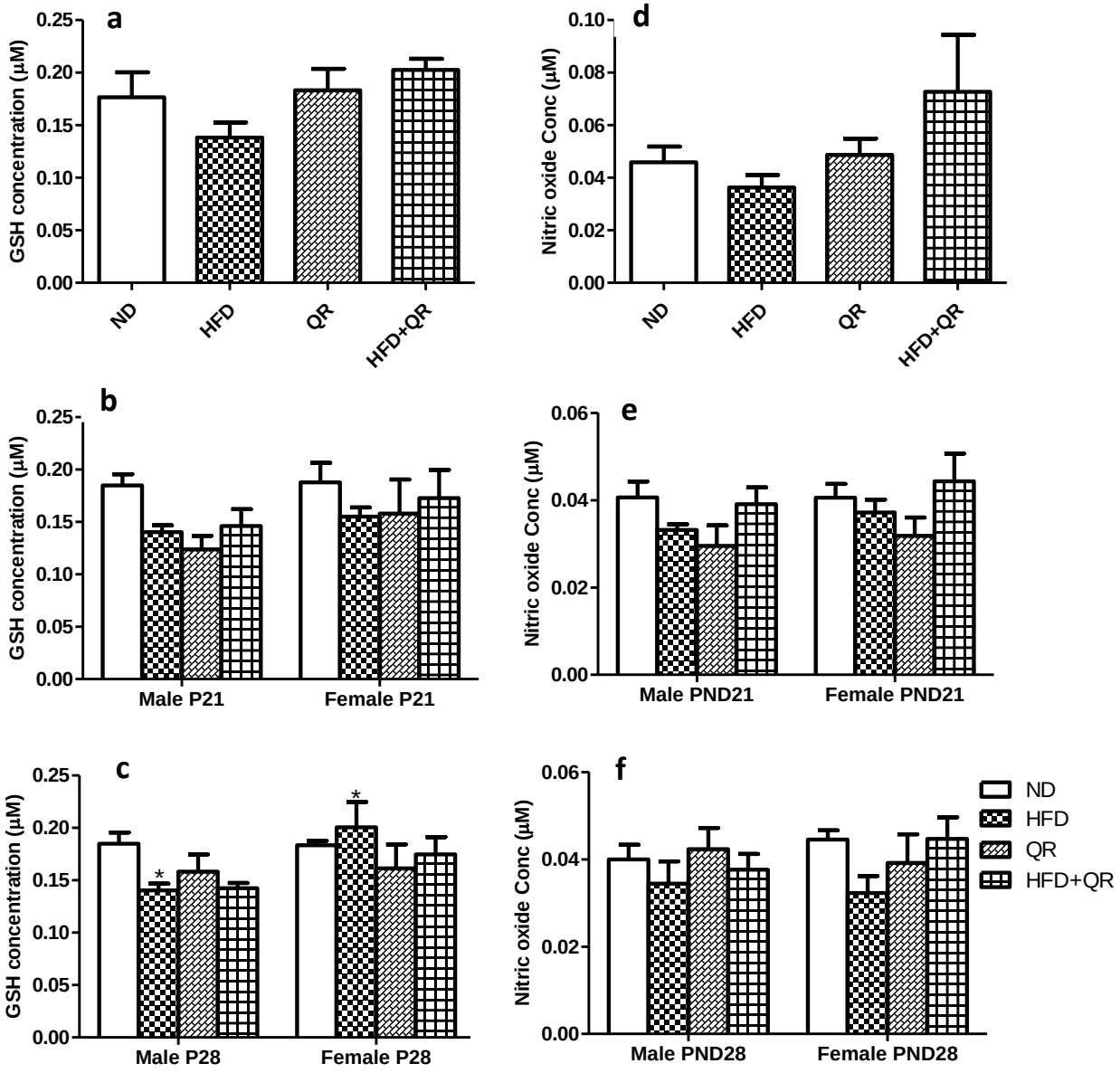


**Fig. 5.3:** Endogenous brain GSH and NO levels in HFD-fed dams and their progenies. (a-c) brain GSH content in dams, PND 21 and 28 offspring respectively, (d-e) brain NO levels in dams and sex-dependent NO activity in PND 21 and 28 offspring respectively. Data shown represents mean ± SEM; n=6 per group. **P<0.05* compared to ND; One-way or two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

**Quercetin-3-o-rutinoside potentiates brain GLP-1 signalling in HFD-fed dams.**

We next investigated the impact of HFD consumption on brain glucose metabolism via expression of incretin hormone GLP-1. Our findings show that there were significant effects of HFD alone on GLP-1 in the maternal brain, whereas ANOVA indicated mild significant difference between ND and HFD+QR ($p<0.05$, Fig. 5.4a). Moreover, neither HFD or QR alone caused any changes ($p>0.05$, Fig. 5.4a). Also, the impact of maternal exposure to HFD and sexual dimorphism on brain GLP-1 signalling in the offspring was further assessed. Two-way ANOVA indicates no significant main effects of maternal HFD consumption and sex differences on GLP-1 expression in the brains of PND 21 ($p>0.05$, Fig. 5.4b, 5.6b) and 28 ($p>0.05$, Fig. 5.4c) male and female offspring.

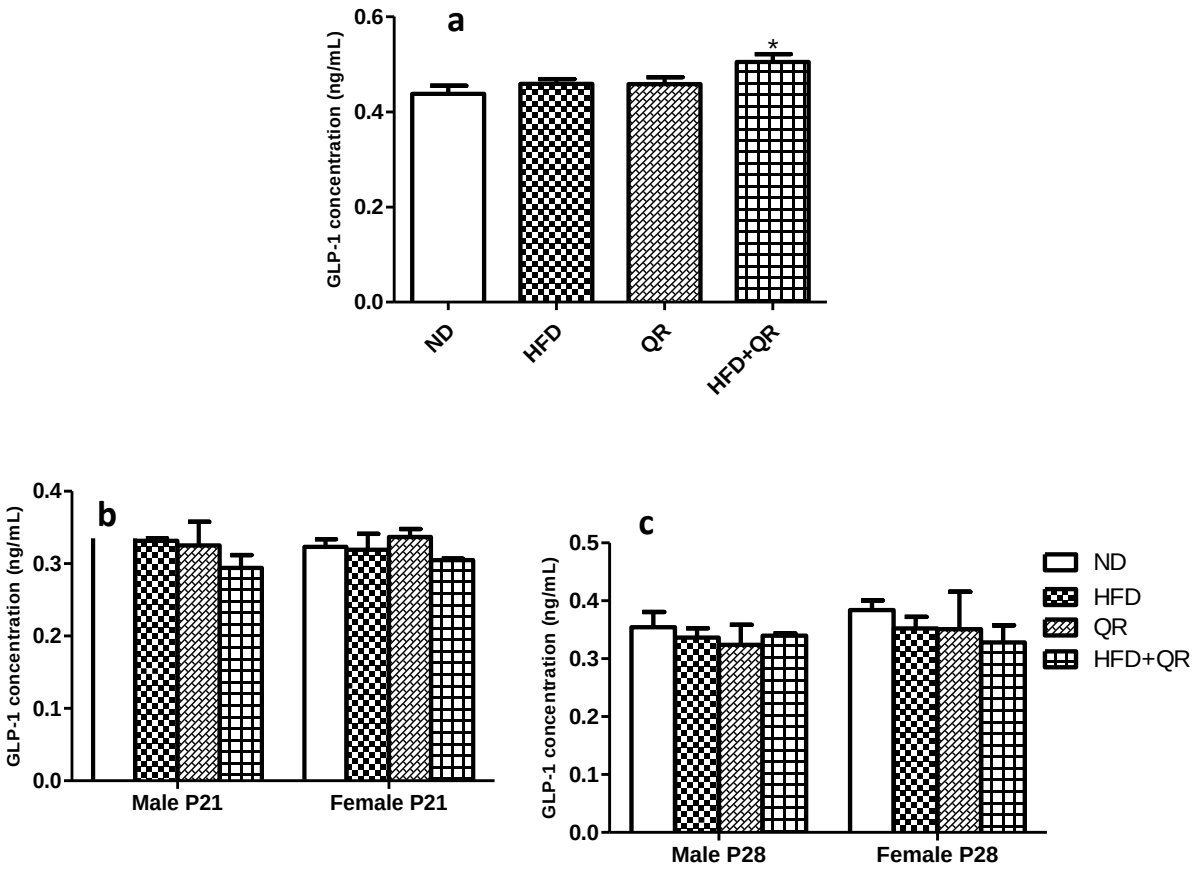

**Fig 5.4:** The above graphs show (a) endogenous expression of brain GLP-1 in dams following HFD consumption, (b-c) sex-dependent changes in GLP-1 concentration in the brains of diet-naïve offspring at PND 21 and 28. Data shown represents mean ± SEM; n=6 per group. *$P<0.05$ compared to ND; One-way or two-way ANOVA, followed by Bonferroni *post hoc* comparison test.

**Discussion**

In this study, we investigated the impact of maternal HFD consumption on neurodevelopmental changes and postnatal alterations in the brain chemistry of diet-naïve descendants. We further sought to unravel the pathophysiology associated with HFD-induced neuroinflammation as well as the therapeutic roles of QR.

There is growing evidence that nutritional challenge imposed by exposure to HFD may indirectly induce central inflammatory responses primed by structural and phenotypic alterations in central innate immune cells and exaggerated cytokine production (Sobesky et al 2014). TNF-α is one of the important proinflammatory cytokines released in large amounts by CNS microglia and exerts both homeostatic and pathophysiological roles (Baranowska-Bik et al 2008, Taipa et al 2018). In the current study, we observed that adult female rats that consumed HFD for eleven weeks did not exhibit significant change in brain TNF-α expression. Our findings are not in line with a previous study by Zhang et al (2004) who reported that consumption of HFD elicited neural inflammatory responses marked by elevated NF-κB in the cerebral cortex of male Sprague-Dawley rats (Zhang et al 2005). In our study, it appears that lack of TNF-α expression in the dams may be attributed to duration of HFD consumption not long enough to induce obese phenotypes and significant central immune responses. These observations agree with previous studies that demonstrated that consumption of HFD by young adult rodents consequentially provoke an overt circulatory and peripheral low-grade inflammation (Cano et al 2009, Xu et al 2002) whereas the ability to directly induce central inflammation strongly depends on manifestation of diabetes-like symptoms usually associated with an extreme prolonged HFD consumption (Jeon et al 2012). Despite the insignificant changes in TNF-α expression in the dams, our data further indicated the possibility that

maternal HFD exposure served as a trigger that primed the induction of low-grade neuroinflammatory changes in the diet-naïve female offspring rats particularly at PND 28, even without prior immune challenge. Also, Scudiero and Verderame (2017) have previously reported that the estrogen-17β sex hormone regulates brain bioenergetics and demonstrated that expression of its active receptor subunits (ERα and ERβ) are affected by HFD in a time-dependent manner (Scudiero & Verderame 2017). Since other studies have widely documented the neuroprotective roles of estrogen in various disease pathologies (Behl 2002, Bryant & Dorsa 2010, Suzuki et al 2009), it may be assumed that the observed decrease in brain TNF-α in the female offspring of dams treated with QR in the present study may be attributed to the hormonal changes that occurred during transit to adolescence which appears independent of the energetic metabolism.

Recent demonstration by Maciejczyk et al (2018) showed that chronic consumption of HFD induced insulin resistance in most tissues, resulting in redox imbalance, and enhanced neural oxidative damage followed by alternations in enzymatic and non-enzymatic brain antioxidants (Maciejczyk et al 2018). We observed that HFD consumption did not cause significant changes in central lipid peroxidation, GSH and NO concentrations in the dams. This further confirms that little or insignificant neural damage is associated with consumption of 45% HFD for approximately eleven weeks. Interestingly, our findings further indicate sex-dependent postnatally induced differential changes in brain MDA concentration. It is still unclear the reason for this wide variations particularly in the female rats, but we suggest that the alternate changes in MDA levels may be caused by either 'primed sensitization' (Sobesky et al 2014) associated with maternal diet experience or developmental and hormonal changes occurring during transition to adulthood. For instance, a previous research reported that serum estradiol in mice showed a marked increase between PND 26 and PND 29 (Ahima et al 1997) and thus may account for the overall decrease in brain MDA in our female rats at PND 28 compared to increase at PND 21.

In a study conducted by Kim et al (2017), it was shown that GLP-1 receptor (GLP-1R) agonist exendin-4 reduced ROS accumulation and lipid peroxidation thus preventing DNA oxidative damage and protected the neural tissues against ischemia and induced neuroinflammation (Kim

et al 2017). In the current study, increased expression of GLP-1 in the brains of HFD+QR dams could be an indication of activated oxygen and glucose delivery to the brain in response to underlying metabolic changes. Even though we expected that QR will exert neuroprotective effects against HFD-induced oxidative challenges by activating the neuropeptidergic circuits, but in this case, it appears that endogenous GLP-1 expression is further influenced by QR treatment which possibly activate mechanisms for mitigating the underpinned biochemical changes. According to a study by Zhang et al (2017), GLP-1 has been shown to promote regulation of cholesterol metabolism and has the ability to ameliorate lipotoxic oxidative stress. Moreover, the observed mild changes in GLP-1 concentration in the brain of HFD+QR fed dams may suggest complementary effects of QR on GLP-1 signaling without influence by HFD treatment.

In conclusion, the results of this investigation clearly show that consumption of 45% HFD for eleven weeks is not sufficient to produce a valid model of diet-induced neuroinflammation or brain oxidative stress. Despite lack of detectable changes in CNS pro-inflammatory markers, there was evidence for biochemical changes in the brain. QR is a well-researched anti-inflammatory agent but its beneficial roles strongly depend on its dosage. In this study, we could not fully establish the neuroprotective roles of QR since only a single dose of 150mg/kg was considered and may not account for the protective mechanisms of GLP-1 against HFD-induced biochemical changes. Therefore, future studies are required to ascertain the exact dose of QR that is beneficial to neural cells, epigenetic mechanisms associated with pathophysiology of HFD and its transgenerational impact.

**Author contribution:** T.E., A., and D.C participated in the research design. T.E performed the animal experiment, while D.M and T.E carried out the molecular/ biochemical assays. Data analysis and interpretation was done by D.C and D.M, whereas D.M., D.C., and T.E participated in the manuscript writing.

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# CHAPTER SIX

# SYNTHESIS AND CONCLUSION

## 6.1 Synthesis

Over the last few decades, there has been a global decline in fertility among couples with a concurrent increase in obesity (1, 2) which suggests a possible link between obesity and infertility (3, 4). Several factors contribute to the etiology of obesity (5) and one of the major factor is the consumption of an energy dense food such as a high fat diet . It is worthy of note that programming of health and diseases in offspring has been reported to be mostly due to environmental factors (during perinatal life) rather than genetic factors (6). Therefore, maternal nutrition prior to or during gestation and lactation may be a harbinger to the reproductive health of their diet naïve offspring to illuminate on the mechanisms involved and possible intervention at critical periods of development. This study was carried out to investigate the impact of maternal high-fat diet on metabolic and reproductive profile with a major focus on the hypothalamic-pituitary axis at different critical periods of development in their diet naïve offspring and gonadal axis in the male offspring. Studies have shown that oxidative stress could be the link between adverse fetal growth and later health alterations (7, 8). Therefore, we sought to examine the putative role of Quercetin-3-O-rutinoside (a potent antioxidant) on fetal and maternal transcriptional, biochemical changes and offspring reproductive profile. The regulatory role of adipokines and cytokines on sex steroids in diet naïve male offspring was also investigated.

This study was carried out using the following objectives:

4. To validate an existing rodent model of HFD-induced metabolic dysregulation using 45% high-fat and investigate whether maternal consumption of HFD throughout gestation could impact on the health of their progenies.
5. To examine changes in plasma lipid content (TC, HDL, LDL and TG), tissue oxidative and inflammatory changes and possible alterations in the expression of chemerin and CMKLR1 genes in the placenta, hypothalamus, and testes of diet-naïve offspring rats, influenced by maternal HFD consumption.

6. To investigate whether QR supplementation could possibly reverse the HFD-induced metabolic alterations and changes in reproductive hormone profile (GnRH, LH, testosterone) of offspring rats that were prenatally exposed to maternal HFD.

### 6.1.1 The relationship between Maternal High Fat diet and offspring fertility

A comprehensive review of the literature on developmental programming of the reproductive health of offspring was conducted to elucidate the association between maternal consumption of a high fat diet (HFD) and reproductive health of their offspring. This was discussed extensively in chapter 2 where we identified some progress and shortcomings in the study of the etiology of fertility. Some of the shortcomings in the conventional experimental approach to the study of developmental programming is the critical periods of development which includes the transition from childhood to early adulthood. This necessitated the study of reproductive and metabolic changes that occurs as development progresses from weanling-postnatal day (PND) 21, PND 28 to early adulthood PND 35 in rat models of high fat diet throughout the study. We also review the putative role of the major organ of the fetal placental unit (placenta) cannot be overemphasised. It forms the major means of transfer of nutrient and other substances from the mother to the developing child. In this study, we examined all biochemical and transcriptional markers in the placenta which provides the link between the dams and offspring. From the review, we also discovered that there is a dearth of knowledge on how maternal high fat diet affects the diet naïve male offspring. In view of this, the effect of maternal high fat diet on the hypothalamic pituitary gonadal axis was examined in the male offspring (chapter four).

### 6.1.2 High fat diet induced transgenerational oxidative stress

Oxidative stress has been implicated in the development programming and epigenetic alterations. To therefore build on previous knowledge, chapter three (objectives 1 and 2) forms an extensive study on the transgenerational impact of HFD on oxidative stress and lipid profile of Sprague dawley rats fed 45% HFD eights weeks prior to gestation and throughout lactation.

As an intervention, their diet was supplemented with Quercetin-3-O-rutinoside throughout lactation. The lipid profile of high fat diet dams was examined eight weeks after HFD, the placenta and liver of dams and offspring (at PND 21, PND 28 and PND 35) were harvested for biochemical analysis. We also monitored the food and water intake of the dams for eight weeks prior mating. We found out that:

1. High fat diet fed rats consumed more water daily with no marked changes in food intake compared to their counterparts fed the control diet.
2. After eight weeks of HFD consumption, there was no marked difference in body weight, body mass index (BMI), and glucose tolerance in the HFD fed rats.
3. Hypertriglyceridemia, increased Low-density lipoprotein (LDL) level, a decrease in plasma cholesterol, and High-density lipoprotein (HDL) level in rats fed HFD for eight weeks.
4. However, we observed certain phenotypic changes in the HFD naïve male and female offspring. They had delayed fur appearance with reduced body weight compared to the offspring of the normal diet fed dams.
5. Lipid peroxidation noted in the placenta and liver of HFD fed dams and in the liver of PND 21 male offspring of HFD dams. There was also evidence of hepatic nitrosative stress in the female offspring at PND 28.
6. Oxidative stress parameters (reduced glutathione, superoxide dismutase, catalase, and total antioxidant capacity) showed evidence that HFD may lead to persistent hepatic oxidative damage in diet naïve offspring and treatment with QR has insignificant ameliorative effect which could probably be due to the duration of treatment and dosage.

Overall, we concluded that mater HFD can induce persistent hepatic oxidative damage in their diet naïve offspring with minimal effect of 150mg/kg QR.

### 6.1.3 The link between Maternal HFD and HPG axis of Offspring.

To achieve objective 3 (chapter four), we first assessed the hormonal profile of both male and female diet naïve offspring. Using the Enzyme linked immunosorbent assay method, we quantified the levels hypothalamic expression of gonadotropin releasing hormone (GnRH), serum levels of Luteinising hormone (LH) in both male and female offspring at PND 21, 28 and 35 and testicular testosterone levels in the male offspring. We observed that the male offspring appeared more vulnerable to the impact of maternal HFD exposure during intrauterine life. Previous studies have shown that oestrogen seems to have a protective effect (9, 10) and the etiology of male infertility is not fully understood as its cause seems idiopathic (11). To further probe into the possible etiology of male infertility and its association with adipokines and cytokines, Real time qualitative polymerase chain reaction method was used to quantify the tissue expression of the novel adipokine chemerin and its receptor CMKLR1, pro inflammatory cytokine IL-1β, TNF-α and NFκB in the hypothalamus and testis of the male offspring at childhood and early adulthood. Our findings revealed that:

1. GnRH and LH levels were reduced in both male (PND 21 and PND 28) and female (PND 21) offspring. While testosterone levels were reduced at transition from adolescence to adulthood.
2. CMKLR1 receptor was significant downregulated in the testis at PND 35 and downregulation of chemerin which further establish its link in male steroidogenesis.
3. Pro inflammatory cytokines were significantly expressed in the placenta, testis and hypothalamus. High levels of pro inflammatory cytokines in the male gonad is an indication of infertility (12).
4. Surprisingly, QR treatment seems to further exacerbate the effect of HFD on HPG axis.

From objective 3 , we can therefore conclude that HFD has a significant effect on sex steroids, local adipokines and cytokines with minimal ameliorative effects of QR treatment.

### 6.1.4 Biochemical changes in the brain of rats prenatally exposed to high-fat diet

We also further examined the metabolic and pro inflammatory status of the whole brain in chapter five. This forms part of a collaborative study with Physiology Department, Faculty of Science, Nelson Mandela University. Here we assessed the molecular underpinnings of HFD induced neuroinflammatory (TNF-α) and biochemical changes (reduced glutathione, Superoxide dismutase, catalase and malondialdehyde) in direct consumers (dams) and diet naïve offspring. Glucagon like peptide-1 (GLP-1) expression was increased in direct consumers which is indicative of increased oxygen delivery to the brain due to oxidative damage. There a time and gender dependent lipid peroxidation in diet naïve offspring accompanied by a non-significant increase in TNF-α level. Though the TNF-α in direct consumers was not significantly affected the diet naïve PND 28 female offspring were affected which could be due to primed sensitization during intrauterine life. Even through there is evidence of GLP-1 induced bioenergetic changes.

## 6.2 Conclusion

In conclusion, this study investigates the etiology of developmental programming of reproductive functions in diet naïve offspring. We were able to establish that maternal high fat diet can induce persistent oxidative stress in their diet naïve offspring which is a major marker of epigenetic alterations. The expression of inflammatory cytokines in the brain, hypothalamic and testicular tissues further supports the link between inflammation, oxidative stress and reduced steroidogenesis at adulthood. There was evidence of GLP-1 induced bioenergetic activities in the brain tissues of diet naïve offspring. The presence of increased pro inflammatory cytokines in the testis is anti-fertility (12). Furthermore, we also observed that maternal HFD can program the infertility in their offspring especially the male offspring through the regulation of the release of gonadotropins and androgens along the HPG axis. In addition, this study also shows that there might be a relationship between the novel chemerin, CMKLR1, and TNF-α in the regulation of male steroidogenesis. Surprisingly, treatment with

QR had little or no significant effect on the molecular and biochemical underpinnings of maternal HFD induced biochemical and reproductive profile of their diet naïve offspring.

This current study holds promising answer to understanding some of the idiopathic causes of male infertility. However, there were some limitations. One major limitation is that we did not do a comparative analysis of QR treatment using different dosage. The duration of exposure to HFD prior gestation could have been longer with a higher of HFD. We therefore recommend that future study could:

1. Use a higher dose of QR and treat the animals on HFD for longer period before gestation. A higher percentage of HFD may present more metabolic and reproductive alterations in their offspring.
2. Access the impact of maternal HFD on the sperm quality (sperm motility, sperm viability, sperm count) and integrity.
3. Further examine the impact of maternal HFD on diet naïve male offspring at cellular (Sertoli and Leydig cells) level for an in-depth understanding of its spermatogenic and steroidogenic impact.
4. Investigate the impact of paternal HFD on reproductive and biochemical profile of their diet naïve offspring.

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# APPENDIX I (a)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 April 2018

**Ms Toluwalope Esther Adeyemi (217081454)**
**School of Laboratory Medicine & Medical Sciences**
**Westville Campus**

Dear Ms Adeyemi,

**Protocol reference number: AREC/005/018D**
**Project title:** Impact of Omega 3 Alpha Linolenic Acid on Biochemical and Reproductive Profiles of Sprague Dawley rats prenatally exposed to Metabolic Disorder

**Full Approval – Research Application**

With regards to your revised applications received on 14 March 2018 and 04 April 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

**CONDITIONS:**

1. Only 56 female and 28 male animals can be used for mating purposes with max 7 animals per group.
2. Ms Adeyemi can only start with the animal work as soon as the Basic Animal Handling course has been completed and proof of attendance was submitted to the AREC Office once obtained.

**Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.**

**Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.**

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

**The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 April 2019.**

**Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.**

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

.....

**Prof S Islam, PhD**
**Chair: Animal Research Ethics Committee**

**/ms**
Cc Supervisor: Dr Anand Nadar Cc Registrar: Mr Simon Mokoena Cc NSPCA: Ms Anita Engelbrecht Cc BRU – Dr L Bester

**Animal Research Ethics Committee (AREC)**
**Ms Mariette Snyman (Administrator)**
**Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Telephone:** +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
**Website:** http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

**APPENDIX I(b)**

AMEND APPROVAL - T ADEYEMI (REF: AREC/005/018D)

Yahoo/Archive

**Mariette Snyman** <snymanm@ukzn.ac.za> Fri, May 25, 2018 at 11:54 AM
**To:** adeyemi.tolulope@yahoo.com
**Cc:** Anand Nadar, Linda Bester, Ritta Radebe

Dear Ms Adeyemi,

Your request for an amendment (attached) received on 18 May 2018 has been APPROVED.

Please note that only two amendments are allowed during the course of the study, otherwise a new applications needs to be submitted.

Kind regards,

Mariette

Mariette Snyman
Research Office
*AREC Administrator*
University of KwaZulu-Natal
Govan Mbeki Building
Westville Campus

# APPENDIX I(c)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

**Ms Toluwalope Esther Adeyemi (217081454)**
**School of Laboratory Medicine & Medical Sciences**
**Westville Campus**

Dear Ms Adeyemi,

**Protocol reference number: AREC/005/018D**
**Project title:** Impact of Quercetin-3-O-Rutinoside on biochemical and reproductive profile of Sprague Dawley rats prenatally exposed to high fat diet.

**Full Approval – Renewal Application**

With regards to your renewal application received on 19 July 2019. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

**Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.**

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

**The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 25 September 2020.**

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

**Dr Sanil D Singh, PhD**
**Chair: Animal Research Ethics Committee**

**/kr**

cc Supervisor: Dr Anand Nadar
cc BRU Manager: Dr Jaca

**Animal Research Ethics Committee (AREC)**
**Ms Mariette Snyman (Administrator)**
**Westville Campus, Govan Mbeki Building**
**Postal Address:** Private Bag X54001, Durban 4000
**Telephone:** +27 (0) 31 260 8350 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
**Website:** http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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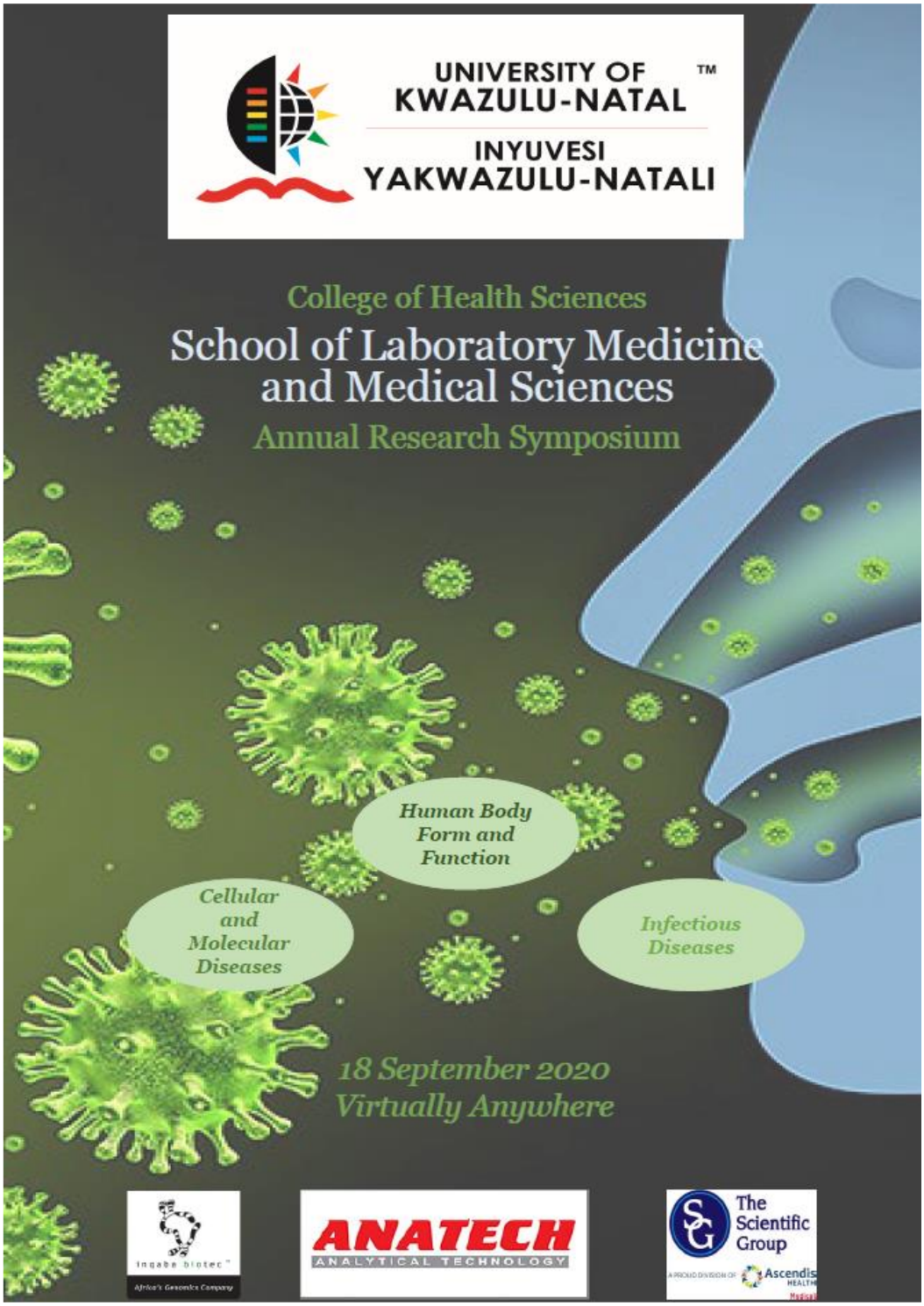
**APPENDIX II**


UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI
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# PRIMED SENSITIZATION TO LOW-GRADE NEUROINFLAMMATORY CHANGES IN RATS PRENATALLY EXPOSED TO HIGH- FAT DIET

**Adeyemi T.E†, Kekana D.M*, Nadar A.†, Ajonijebu D.C***

†Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

*Department of Physiology, School of Biomolecular and Chemical Sciences, Faculty of Science, Nelson Mandela University, Port Elizabeth, South Africa

**ABSTRACT BODY:**

The mechanistic link between maternal high-fat feeding and postnatally induced sex-dependent neurochemical alterations in diet-naïve offspring's brain is not completely understood. In this study, we examined the molecular underpinnings of quercetin-3-o rutinoside (QR) and glucagon-like peptide 1 (GLP-1) regulatory effects on high-fat diet (HFD) induced neuroinflammatory and/or biochemical changes in direct consumers and diet-naïve descendants. Pregnant rats (previously fed normal diet (ND) or 45% HFD) were maintained on supplemented chow (plus 150mg/kg QR) – ND/QR, HFD/QR throughout gestation. Subsequently, the animals were sacrificed, and brain samples were processed for expression of TNF-α, GLP-1, MDA and GSH/NO content. The data show that chronic consumption of HFD by dams failed to alter significantly the brain TNF-α levels in dams and offspring at postnatal day (PND) 21. However, a non-significant increase in TNF-α levels that appears suppressed by QR was observed in the female offspring rats of HFD-fed dams at PND 28. Surprisingly, HFD-fed dams exhibited non-significant increase in brain MDA levels accompanied by time and sex-dependent changes in lipid peroxidation in their progenies. NO and GSH levels were not directly affected by maternal HFD treatment, whereas brain GSH concentration increased significantly in the female offspring of HFD-fed dams at PND 28. Moreover, GLP-1 expression significantly increased in dams that received QR supplemented chow, but not in the offspring. In conclusion, the current findings indicate that maternal consumption of 45% HFD only produced low-grade inflammatory and biochemical changes in the brain which can potentially trigger neuroinflammatory changes in the progenies.

Research Theme: NEUROINFLAMMATION AND DEVELOPMENTAL PROGRAMMING

Ethics Number: AREC/005.018D

Please tick the appropriate box

| | |
|---|---|
| X | Poster Presentation |
| | Oral Presentation |

## APPENDIX III(a)


Dear TOLUWALOPE ADEYEMI
Your abstract titled: **"Maternal High fat consumption may induce a post-natal oxidative stress challenge in the offspring with severe metabolic and reproductive consequences. "** has been accepted for presentation at the PSSA 2019 conference taking place at ICC East-London from 18 -21 August 2019

Presentation Method: Wyndham (Oral competition for PhD & MSc students)

The approved program indicating your own time slot will be available on the website at www.pssa2019.com in due course.

Please note the following important details:

1. Please register ASAP (by 2nd August 2018). Please refer to the website for online registration and payment and email pssa2019@wsu.ac.za with your proof.

2. We urge you to book your accommodation immediately as all accommodation is booked on a first-come-first-served basis and will be subject to availability. Accommodation details are available on the PSSA2019 website.

3. Should you for any reason not want to participate in the conference, or if you want to withdraw your abstract/s kindly e-mail the conference organizers immediately or before the 2nd August 2018 at pssa2019@wsu.ac.za.

We look forward to welcoming you at the conference!

[redacted]

Prof C Sewani-Rusike (PSSA2019 Conference Chair)

**APPENDIX III(b)**

# 47TH CONFERENCE OF THE PHYSIOLOGY SOCIETY OF SOUTHERN AFRICA

## 18 - 21 AUGUST 2019
## ICC EAST-LONDON

THEME:

**Recent advances in Physiological sciences: Synergism in health and disease**

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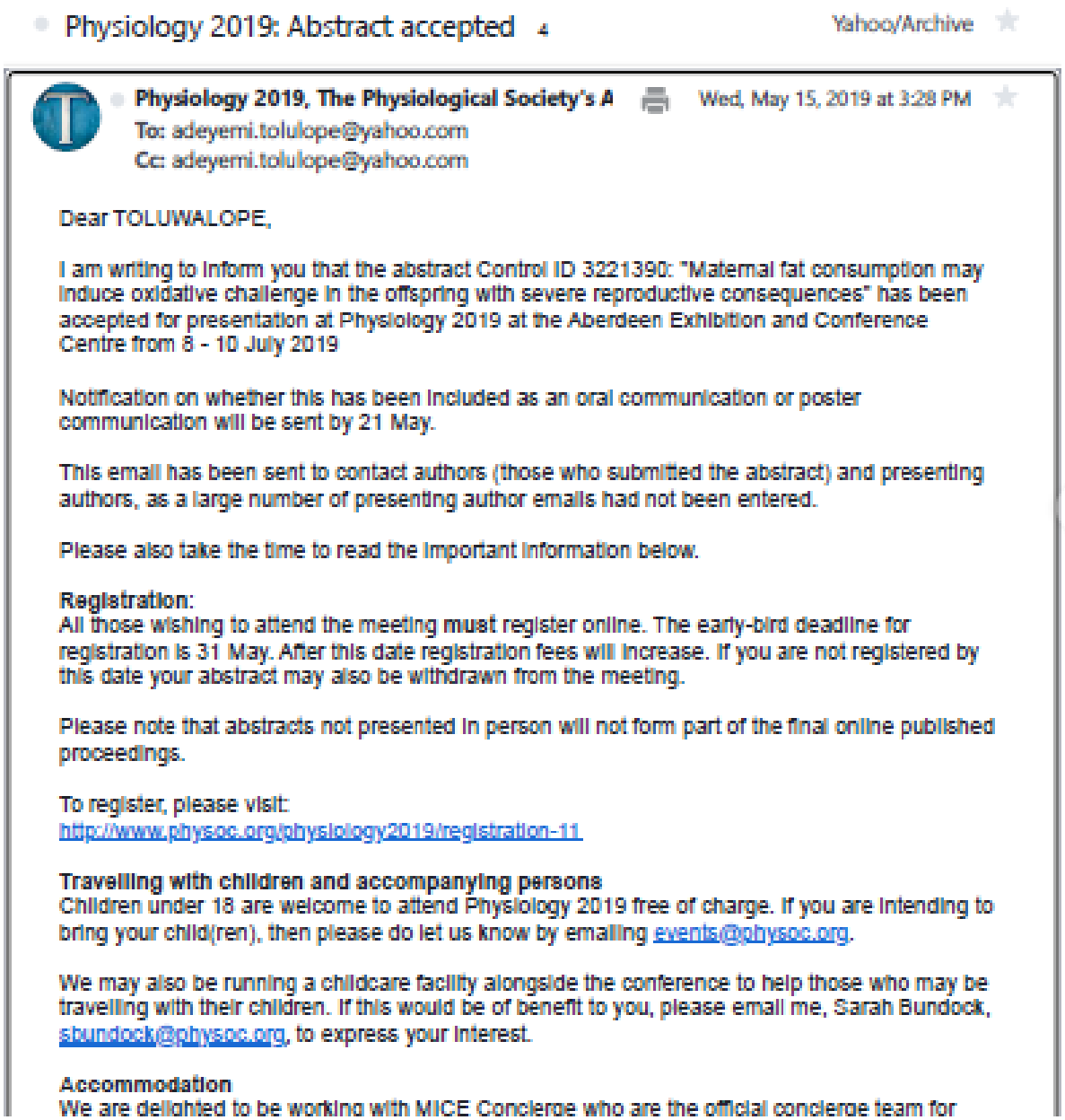
| | |
|---|---|
| 1415-1430 | MATERNAL HIGH FAT CONSUMPTION MAY INDUCE A POST-NATAL OXIDATIVE STRESS CHALLENGE IN THE OFFSPRING WITH SEVERE METABOLIC AND REPRODUCTIVE CONSEQUENCES.<br>Adeyemi T. E - University of KwaZulu-Natal |
| 1430-1445 | NEUROSTEROIDOGENESIS AND ITS ROLE IN NEUROINFLAMMATION: IN SEARCH OF THE ELUSIVE<br>Powrie, Y.S.L - Stellenbosch University, |
| 1445-1500 ✓ | RELOOKING AT SODIUM ORTHOVANADATE: AN INHIBITOR OF THE SERINE/THREONINE PHOSPHATASE, PP2A.<br>White, C - Stellenbosch University |
| 1500-1515 | THE EFFECTS OF PLANT-DERIVED OLEANOLIC ACID (OA) ON SELECTED EARLY DERANGEMENTS ASSOCIATED WITH VASCULAR AND RENAL COMPLICATIONS ON HIGH-FAT HIGH-CARBOHYDRATE (HFHC) DIET-INDUCED PRE-DIABETIC SPRAGUE DAWLEY (SD)RATS<br>Gamede, M.,- University of KwaZulu-Natal |
| 15.30 – 1545 | COFFEE AND VIEWING OF POSTERS |
| 1545 – 1700 | Johnny Van Der Walt poster competition |
| 1900 – 2100 | Conference Gala Dinner and Competition Awards |

**WEDNESDAY 21ST AUGUST**

| TIME | TITLES |
|---|---|
| 0800 – 0830 | Onsite Registration |
| 0830 – 1030 | **Plenary CHAIR:DR MARY PIPEDI - TSHEKISO** |
| 0830-0915 | MODELING APPROACHES IN INFANTILE HAEMANGIOMA: IMPLICATIONS FOR NOVEL THERAPEUTIC STRATEGIES<br>Dr PL Mabeta<br>University of Pretoria |
| 0915-1030 | THE RELEVANCE OF INTELLECTUAL PROPERTY PROTECTION IN THE HEALTH SECTOR<br>Dr K. Faul,<br>National Intellectual Property Management Office |

**APPENDIX IV(a)**

**Abstract accepted for presentation at Physoc (Physiological Society) Aberdeen UK 2019**



Physiology 2019: Abstract accepted 4 Yahoo/Archive

**Physiology 2019, The Physiological Society's A** Wed, May 15, 2019 at 3:28 PM
To: adeyemi.tolulope@yahoo.com
Cc: adeyemi.tolulope@yahoo.com

Dear TOLUWALOPE,

I am writing to inform you that the abstract Control ID 3221390: "Maternal fat consumption may induce oxidative challenge in the offspring with severe reproductive consequences" has been accepted for presentation at Physiology 2019 at the Aberdeen Exhibition and Conference Centre from 8 - 10 July 2019

Notification on whether this has been included as an oral communication or poster communication will be sent by 21 May.

This email has been sent to contact authors (those who submitted the abstract) and presenting authors, as a large number of presenting author emails had not been entered.

Please also take the time to read the important information below.

**Registration:**
All those wishing to attend the meeting **must** register online. The early-bird deadline for registration is 31 May. After this date registration fees will increase. If you are not registered by this date your abstract may also be withdrawn from the meeting.

Please note that abstracts not presented in person will not form part of the final online published proceedings.

To register, please visit:
http://www.physoc.org/physiology2019/registration-11

**Travelling with children and accompanying persons**
Children under 18 are welcome to attend Physiology 2019 free of charge. If you are intending to bring your child(ren), then please do let us know by emailing events@physoc.org.

We may also be running a childcare facility alongside the conference to help those who may be travelling with their children. If this would be of benefit to you, please email me, Sarah Bundock, sbundock@physoc.org, to express your interest.

**Accommodation**
We are delighted to be working with MICE Concierge who are the official concierge team for

**APPENDIX IV (b)**

PC181

## Maternal fat consumption may induce oxidative challenge in the offspring with severe reproductive consequences

T.E. ADEYEMI, M. CHANNA and A. NADAR

*DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF KWAZULU NATAL, DURBAN SOUTH AFRICA, Durban, South Africa*

High fat diets(HFD) is associated with oxidative stress and cellular damage accompanied by changes in some metabolic functions. However, little is known about maternal fat consumption on endogenous modulation of antioxidant network and reproductive profiles of progenies. In the present study, we investigated the effects of Quercetin-3-O-rutinoside (QR) on maternal high fat diet (HFD)-induced oxidative stress and hormonal functions in offspring. The research was approved by the Animal Research Ethics Committee, University of KwaZulu-Natal.
Fifty-six female Sprague Dawley rats (180-200g) were divided into 2 groups (*n*=28/group), fed either normal diet (ND) or 45% HFD for 8 weeks and then mated. Post conception, rats were further divided into 4 groups each, of which 2 groups continued with ND or HFD while the remaining groups received either QR (150mg/kg) or HFD+QR. The rats were sacrificed in batches with the first set on gestation day (GD) 19, while the second group was allowed to litter naturally, and pups sacrificed at postnatal day (PND) 21, 28 and 35 respectively. We monitored changes in body weight, performed the oral glucose tolerance test and measured hormone

*490P*

levels including hypothalamic GnRH, plasma LH and testosterone using ELISA. Catalase, superoxide dismutase, reduced glutathione, nitric oxide and malondialdehyde in the placenta and liver of dams and offspring were quantified. Results were expressed as mean ± standard deviation and analyzed using ANOVA while the significance level was set at $P<0.05$.
HFD-fed rats showed increases in body weight but not sensitive to oral glucose. Our data also showed significant increases in liver MDA levels in placenta of HFD-fed dams and liver of HFD offspring at PND 21. SOD activity was decreased in the liver and placenta of all treatment groups of G19 dams, whereas HFD-treated postpartum day (PPD) 21 dams exhibited significant increases in both SOD and catalase enzymatic activities in the liver. Moreover, GSH concentration was increased in QR offspring at D21, decreased in HFD-treated groups at PND 28, and increased in all three groups at PND 35. Liver NO level was also increased in HFD GD19 dams and significant decreases in placenta and liver of HFD+QR PPD21 dams. Hormonal analysis further showed significant decrease in hypothalamic GnRH of HFD offspring at PND 21 and 28. We observed an initial rise in GnRH concentration in QR at PND 21 which later returned to low levels in the offspring throughout the study period. Also, plasma LH was decreased by either HFD or QR at PND21, as well as a significant decrease in testosterone levels in HFD offspring at PND 28 which appears reversed by QR. The results indicate that maternal nutritional drift may induce an oxidative stress challenge in the offspring, leading to hypothalamic-pituitary-gonadal axis dysregulation with a possibility of severe reproductive and metabolic consequences.
**Keywords:** Developmental programming, Nutrition, Quercetin-3-O-rutinoside

Zambrano, E., et al., *Fetal programming of sexual development and reproductive function.* Molecular and cellular endocrinology, 2014. 382(1): p. 538-549.

Jahan, S., et al., *Ameliorative effects of rutin against metabolic, biochemical and hormonal disturbances in polycystic ovary syndrome in rats.* Journal of ovarian research, 2016. 9(1): p. 86.

Niture, N.T., A.A. Ansari, and S.R. Naik, *Anti-hyperglycemic activity of rutin in streptozotocin-induced diabetic rats: an effect mediated through cytokines, antioxidants and lipid biomarkers.* 2014

Liang, C., K. DeCourcy, and M.R. Prater, *High–saturated-fat diet induces gestational diabetes and placental vasculopathy in C57BL/6 mice.* Metabolism, 2010. 59(7): p. 943-950.

Charan, J. and N. Kantharia, *How to calculate sample size in animal studies?* Journal of pharmacology & pharmacotherapeutics, 2013. 4(4): p. 303.

college of Health Sciences University of Kwazulu Natal, Durban South Africa

*Where applicable, the authors confirm that the experiments described here conform with the ethical requirements.*

*491P*

## APPENDIX V

HELIYON-D-20-07205R3 Status Update 7 Yahoo/HELIYON

me **Mcgee, Harry P. (ELS-LOW)** <h.mcgee@cell.com> Wed, Mar 10 at 4:45 PM
**To:** adeyemi.tolulope@yahoo.com

Dear Dr Adeyemi,

I hope this email finds you well.

I am emailing regarding your submission currently under consideration at Heliyon, 'Postnatally induced metabolic and oxidative changes associated with maternal high-fat consumption were mildly affected by Quercetin-3-O-rutinoside treatment in rats'.

Recently you inquired about the status of your submission, I am happy to inform you that the handling editor of your submission, and the section editor of Heliyon Food Science and Nutrition, have both made an 'Accept' decision for your submission. This means your paper has now been passed back to me so that I can assess it for final editorial changes we require prior to publication. I hope to do this as soon as possible, at which point I will make a decision for your submission.

Thank you very much for your patience, and please do not hesitate to contact me if you have any questions or concerns.

Best wishes,

Harry McGee

Heliyon|Editorial Assistant

h.mcgee@cell.com

# APPENDIX VI

Life Sciences 271 (2021) 119179

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

## Evaluation of maternal high-fat diet and Quercetin-3-O-rutinoside treatment on the reproductive profile of diet naïve male offspring

Toluwalope E. Adeyemi *, Mahendra L. Channa, Anand Nadar

*Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa*

ARTICLE INFO

*Keywords:*
Male fertility
Developmental programming
High-fat diet
Sex hormones
Pro-inflammatory cytokines
Adipokines

ABSTRACT

*Background:* Male infertility and reproductive dysfunctions have become major global health problems. Although several causative factors have been attributed to this challenge, of importance are alterations in maternal-foetal environment, diet-induced transcriptional changes and dysregulation in chemical signaling via hypothalamic-gonadal axis.

*Aim:* The present study investigated the impact of maternal high-fat diet (HFD) consumption and the putative role of Quercetin-3-O-rutinoside on reproductive functions of male offspring rats at critical developmental stages with a quest to unravel the underpinned molecular changes.

*Materials and methods:* Fifty-six pregnant rats (previously fed normal diet ND) or 45% HFD) were maintained on supplemented chow (150 mg/kg QR) – ND/QR, HFD/QR throughout gestation. Subsequently, dams ($n = 7$) and offspring ($n = 6$) were sacrificed at post-natal day (PND) 21, 28 and 35, respectively, and the blood, placenta, hypothalamus (HT), and testicular samples were processed for molecular analysis of Gonadotropin-releasing hormone (GnRH), Luteinizing hormone (LH), testosterone, chemerin, chemokine-like receptor 1 (CMKLR1), tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β) and nuclear factor kappa B (NF-κB).

*Key findings:* We observed a significant decrease in GnRH level in the HFD group at PND21 and PND28 in male offspring and treatment with QR significantly reduced GnRH. There was a significant reduction in LH levels in the HFD group at PND 21 in the male offspring accompanied by a significant decrease in testosterone level at PND 28 and PND35 which appears to be age dependent. In the HT, Chemerin and CMKLR1 was significantly upregulated in the HFD group at PND 21 and PND 35 respectively while CMKLR1 was significantly down-regulated in the HFD group of the placenta and testis at PND 21. TNF-α, IL-1β and NF-κB were also expressed in the placenta, HT and testis at PND 21.

*Significance:* Male fertility is affected by maternal HFD consumption while chemerin, CMKLR1 and TNF-α, may play a significant role in male steroidogenesis. Treatment with QR had little or no ameliorative effect on HFD induced alterations in male reproductive functions.

### 1. Introduction

Multiple lines of inquiry indicate that obesity can adversely impact reproductive functions associated with cases of subfertility and infertility [1–3]. Obesity impacts fertility through various pathways, some of which include adipokines [4], sex steroids [5], leptin and localized or systemic oxidative stress [2]. Since the mid-1900s, there has been a progressive inverse relationship between sperm count and a global increase in obesity. A clinical study conducted by Lui Y. and Ding Z. (2017) showed that obese or overweight men had reduced sperm quality, including sperm concentration, sperm motility, decreased acrosome reaction, increased DNA damage, and lower embryo implantation rates compared to men with normal body mass index (BMI) [2]. It is worthy of note that male infertility accounts for 70% of infertile cases owing to progressive decrease in sperm count over the last 40 years in western countries [6,7]. Although the primary reason for this decline in male fertility has not been fully established but it has been attributed to the effect of perinatal life environmental modifications which could be direct or through epigenetic modifications [8]. Extensive research conducted by Skakkebaek et al. (2015) indicated that issues related to male

* Corresponding author.
*E-mail addresses:* 217081454@stu.ukzn.ac.za (T.E. Adeyemi), channam@ukzn.ac.za (M.L. Channa), nadara@ukzn.ac.za (A. Nadar).

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