THE EFFECT OF NEONATAL ADMINISTRATION OF OLEANOLIC ACID ON HEALTH OUTCOMES ASSOCIATED WITH DIET-INDUCED METABOLIC DYSFUNCTION IN RATS

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, School of Physiology, fulfilment of the requirements for the degree of Doctor of Philosophy (PhD).

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DECLARATION

I, **Trevor Tapiwa Nyakudya**, declare that the work contained in this thesis is my own, unaided work. This thesis is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. The work herein has not been submitted before for any degree or examination at any other University.

_____

Trevor Tapiwa Nyakudya

Signed on the_____ day of_____, 2018

I certify that all the experimental procedures used in this thesis were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2014/47/D) and the University of Johannesburg Research Ethics Committee (REC number: 01-02-2016).

CONFERENCE PROCEEDINGS AND PRESENTATIONS

The following presentations are offered in support of this thesis.

1) **Trevor T. Nyakudya**, Emmanuel Mukwevho, Kennedy Erlwanger. Short-term administration of metformin and oleanolic acid affect growth performance and surrogate markers of liver function in juvenile male and female Sprague Dawley rats on a high fructose diet. The 44th Annual Congress of the Physiological Society of Southern Africa. University of Cape Town, South Africa, 28-31 August 2016.

2) **Trevor T. Nyakudya**, Emmanuel Mukwevho, Pilani Nkomozepi, Kennedy Erlwanger. Early postnatal administration of oleanolic acid attenuates the development of non-alcoholic fatty liver disease in fructose-fed adult female Sprague Dawley rats. Experimental Biology Annual Conference. Chicago, Illinois, United States, 22-26 April 2017.

3) **Trevor T. Nyakudya**, Emmanuel Mukwevho, Kennedy Erlwanger. Neonatal administration of oleanolic acid ameliorates the development of health outcomes associated with metabolic dysfunction in fructose-fed adult rats. The 38th International Union of Physiological Sciences Congress. Rio de Janeiro, Brazil, 1-5 August 2017.

4) **Trevor T. Nyakudya**, Emmanuel Mukwevho, Kennedy Erlwanger. The protective effects of neonatal administration of oleanolic acid against the development of metabolic dysfunction in fructose-fed adult female rats. The 8th Cross Faculty Postgraduate Symposium (***First prize for best poster presentation in the Faculty of Health Sciences***). University of the Witwatersrand, Johannesburg, South Africa, 25-26 October 2017.

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ABSTRACT

The neonatal period is a critical window of developmental plasticity. Consumption of fructose-rich diets has been implicated in the increasing global prevalence of metabolic dysfunction (MD) and non-alcoholic liver disease (NAFLD). Interventions during periods of early ontogenic developmental plasticity can induce epigenetic changes which program metabolism for positive health benefits later in life. The phytochemical, oleanolic acid (OA) possesses anti-diabetic, anti-oxidant and anti-obesity effects. I investigated the potential protective effects of neonatal oral administration of OA on the subsequent development of health outcomes associated with fructose-induced MD and NAFLD in male and female rats.

The study was divided into two major experiments. In the first short-term experimental study, the potential of neonatal oral administration of OA to acutely protect against the development of fructose-induced oxidative damage, adverse general health outcomes and precocious maturation of the gastrointestinal tract (GIT) in suckling male and female rats was investigated. Male and female suckling rat pups (N=30) were randomly assigned to four groups and gavaged daily with 10 mℓ/kg body mass of: distilled water (DW) with 0.5% (v/v) dimethyl sulphoxide (vehicle control), oleanolic acid (OA; 60 mg/kg), high fructose solution (HF; 20% w/v), or OAHF for 7 days. On day 14, the pups were euthanised. Blood, liver and skeletal muscle samples were collected to determine clinical health profiles, hepatic lipid content and gene expression of anti-oxidant enzymes, superoxide dismutase (SOD2) and glutathione peroxidase (GPx1). Rats in all groups had a significant increase in body mass over the seven day treatment period (ANOVA; $P<0.05$). There were no significant differences in visceral organ masses, general clinical health profiles, liver lipid content and GIT morphometry across all treatment groups (ANOVA; $P>0.05$). Neonatal oral administration of fructose lowered the expression of genes for anti-oxidant enzymes (SOD2 and GPx1) which was prevented by OA (ANOVA; $P<0.05$). Findings from this study provide evidence that short-term neonatal oral administration of OA protects against fructose-induced oxidative damage with seemingly no adverse effects on health or the maturational and developmental changes of the gastrointestinal tract in suckling male and female pups.

In the second long-term experimental study, which was further subdivided into two studies, I investigated the potential protective effects of neonatal oral administration of OA on the

subsequent development of high fructose diet-induced a) metabolic dysfunction and b) NAFLD in male and female rats. Male and female suckling rats (N=112) were randomly assigned into four groups and gavaged daily with 10 m mℓ/kg body mass of: distilled water (DW) with 0.5% (v/v) dimethyl sulphoxide (vehicle control), oleanolic acid (OA; 60 mg/kg), high-fructose solution (HF; 20% w/v) and OAHF for 7 days. On day 21, the rats were weaned onto normal rat chow and plain drinking water up to day 55. From day 56, half of the rats in each treatment group were continued on plain water whilst the remainder were given a high fructose solution (20 % w/v) as drinking fluid *ad libitum* for eight weeks. On day 110 the rats were subjected to an oral glucose tolerance test (OGTT) and then euthanised on day 112. Fasting glucose, triglyceride levels and terminal body mass were measured before termination. Blood samples were collected to determine the effects of treatments on fasting levels of cholesterol, insulin, glucose, triglycerides, insulin resistance (HOMA-IR), glucose tolerance (area under the curve for OGTT), a surrogate biomarker of liver function, alanine amino transaminase (ALT) and non-tissue specific alkaline phosphatase (ALP). Body adiposity was determined by measuring visceral and epidydimal fat pad masses. Liver samples were used to measure hepatic lipid accumulation and hepatic histomorphometry. The livers were formalin fixed, paraffin embedded and sectioned at 3µm. The sections were stained with haematoxylin and eosin for assessment of inflammation and Masson's trichrome for visualisation of connective tissue and steatosis.

Male and female rats in all groups of the second experiment had a significant increase in body mass over the study period (ANOVA; $P<0.05$). In the general metabolic dysfunction study, rats which consumed fructose as neonates and then later as adults (HF+F) and those which consumed fructose only in adulthood (DW+F) had significant increases in terminal body mass (females only), visceral fat mass (males and females), serum triglycerides (females only), epididymal fat (males only), fasting plasma glucose (males and females), impaired glucose metabolism (females only), β-cell dysfunction and insulin resistance (males and females) compared to the other treatment groups ($P<0.05$). There were no differences in fasting cholesterol levels across all treatment groups in both male and female rats ($P>0.05$).

The sub-study on NAFLD revealed that fructose consumption in adulthood following neonatal fructose intake (HF+F) caused a 47-49% increase in hepatic lipid content of both

male and female rats (P<0.05). However, fructose administered in adulthood only (DW+F), caused a significant increase in liver lipid content in females only (P<0.05). NAFLD activity scores for steatosis were higher in male (HF+F) and female (DW+F and HF+F) rats compared to other treatment groups (P<0.05). Scores for inflammation were higher in female rats that received DW+F and HF+F (P<0.05) but not in male rats (P>0.05). NAFLD area fraction for fibrosis was 3 times higher in male and female rats that received a double hit neonatally and in adulthood (HF+F) and a late hit of fructose (DW+F) compared to the rats in the negative control group (P<0.05).

I have shown that administration of a high fructose diet had adverse effects on several health outcomes associated with MD and induced NAFLD. However, it was notable that the timing of the fructose intake in the life stage of rats had an impact on the development of MD and NAFLD phenotype. I also observed sex-specific differences in the metabolic response to dietary fructose, with females appearing to be more vulnerable to the development of MD and NAFLD. It is thus important to note that studies should not just focus on a single sex but should be comparative between the sexes. I have also demonstrated, for the first time, that neonatal oral administration of oleanolic acid protects against the subsequent development of fructose-induced health outcomes associated with metabolic dysfunction and NAFLD by reducing hepatic lipid storage, terminal liver masses and hepatic histomorphological changes associated with NAFLD. I conclude that neonatal interventional treatment with oleanolic acid during the critical window of developmental plasticity protected against the development of fructose diet-induced adverse health outcomes associated with MD and NAFLD in male and female Sprague Dawley rats. Therefore, OA is a phytochemical that exhibits potential in the prevention of neonatal programming of MD and NAFLD later in life. OA should be considered as a natural strategic prophylactic intervention during periods of developmental plasticity with a lot of potential in the fight against the scourge of metabolic disorders that have a significant negative impact on the health systems globally.

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DEDICATION

To my mother

Mrs Ena Mutembedza

1961-1999

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LIST OF ABBREVIATIONS

°C:	Degrees Celsius
♀:	Female rats
♂:	Male rats
AACE:	American Association of Clinical Endocrinologists
Acetyl Co-A:	Acetyl Coenzyme A
ACC:	Acetyl Coenzyme A carboxylase
ADA:	American Diabetes Association
ADP:	Adenosine diphosphate
AESC	Animal Ethics Screening Committee
ALB:	Albumin
ALP	Alkaline phosphatase
ALT:	Alanine amino transferase
AMP:	Adenosine monophosphate
AMPK:	Adenosine monophosphate activated protein kinase
AMY:	Amylase
ANOVA:	Analysis of variance
AOAC:	Association of Official Analytical Chemists
AST:	Aspartate amino transferase
ATP III:	Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults
ATP:	Adenosine triphosphate
AUC:	Area under the curve for the oral glucose tolerance test
bm:	Body mass
BMI:	Body mass index
BUN	Blood urea nitrogen
Ca:	Calcium
CAS:	Central animal services

CREA:	Creatinine
CT:	Computer tomography
CVDs:	Cardiovascular diseases
DHAP:	Dihydroxyacetone phosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNL:	*de novo* lipogenesis
DoHAD:	Developmental Origins of Adult Health and Disease
DW:	Distilled water
EGIR:	European Group for the Study of Insulin Resistance
ELISA:	Enzyme-linked immunosorbent assay
ERK:	Extracellular signal kinases
FFAs:	Free fatty acids
GGT:	Gamma glutamyl transferase
GIT:	Gastrointestinal tract
GLUT:	Glucose transporter
GPDH:	Glycerol-3-phosphate dehydrogenase
GPx1:	Glutathione peroxidase
HE:	Haematoxylin and eosin
h:	Hour(s)
HDL:	High density lipoprotein
HFCS55:	High fructose corn syrup
HF:	High fructose solution (20% w/v)
HOMA-IR:	Homeostatic model of insulin resistance
IDF:	International Diabetes Federation
IL-6:	Interleukin 6
IR:	Insulin resistance
kg:	Kilogram
JNK:	Jun N-terminal kinases

KHK:	Fructokinase
LDL:	Low density lipoprotein
LH	Luteinising hormone
LI:	Large intestines
LPL:	Lipoprotein lipase
MD:	Metabolic dysfunction
MetS:	Metabolic syndrome
min:	Minute(s)
mg:	Milligram(s)
mℓ:	Millilitre(s)
mm :	millimetre(s)
MRI :	Magnetic resonance imaging
mRNA:	Messenger ribonucleic acid
MT:	Masson's trichrome
NAFLD	Non-alcoholic fatty liver disease
NAS:	Non-alcoholic fatty liver disease activity score
NASH:	Non-alcoholic steatohepatitis
NCD:	Non-communicable disease
NCEP:	National Cholesterol Education Program
ncRNA:	non-coding ribonucleic acid
NFκ-B:	Nuclear factor kappa-B
NO:	Nitric oxide
Nrf2-GCL:	Nuclear factor erythroid-derived 2-like 2-Glutamate cysteine ligase
OA:	Oleanolic acid
OAHF:	Oleanolic acid and high fructose solution (20% w/v) treatment
OGTT:	Oral glucose tolerance test
PAI-1:	Plasminogen activator inhibitor-1
PD:	Post-natal day

PGC-1β :	Peroxisome proliferator-activated receptor-γ coactivator-1β
PHOS:	Phosphate
PKA:	Protein kinase A
PKC:	Protein kinase C
PPAR:	Peroxisome proliferator activated receptor
PPAR-α:	Peroxisome proliferator activated receptor-alpha
PPAR-γ:	Peroxisome proliferator activated receptor-gamma
REC:	Research ethics committee
RT-PCR:	Real-time polymerase chain reaction
ROS:	Reactive oxygen species
rTL:	Weight of organ relative to tibial length (g/cm or mg/mm)
SAT :	Subcutaneous adipose tissue
SD:	Standard deviation
SDR:	Sprague Dawley rat
SI:	Small intestines
SOD2:	Superoxide dismutase
SRC:	Standard rat chow
SREBP-1:	Sterol regulatory element binding protein-1
SSBs:	Sugar sweetened beverages
T2DM:	Type 2 diabetes mellitus
TBIL	Total bilirubin
TBM:	Terminal body mass
TCHOL:	Total cholesterol
TGs	Triglycerides
TNF:	Tumour necrosis factor
TPROT	Total protein
VAT:	Visceral adipose tissue
v/v:	volume/volume

VLDL:	Very low density lipoprotein
w/v:	weight/volume
WHO:	World Health Organisation
α:	Alpha
β:	Beta
γ:	Gamma

CHAPTER 1:

INTRODUCTION AND RATIONALE FOR THE STUDY

1.1 Organisation of the thesis

The current thesis has been written and presented in a divided block format consisting of seven chapters. **Chapter 1** is an introductory chapter for the study in which an insight into the problems associated with the global pandemic of metabolic syndrome (MetS) and obesity among adults is provided. This chapter is concluded by providing the justification, aims, specific objectives and hypotheses of this doctoral thesis.

Chapter 2 consist of a critical review of literature that is relevant to the current study. In this chapter, the different definitions of metabolic syndrome (MetS), its global prevalence and relevant statistics are discussed. The pathophysiology and risk factors involved in the pathogenesis and development of health outcomes associated with MetS such as lifestyle choices and genetic factors are deliberated. The role of fructose in the pathogenesis of obesity, insulin resistance and cardiovascular diseases (CVDs) is highlighted. The different strategies used in the management of MetS, the advantages and disadvantages of pharmaceutical agents and their possible adverse effects are discussed. The phytochemical oleanolic acid, which has a wide repertoire of biological activities, is introduced. The role of oleanolic acid (OA) administered in adult animals to ameliorate fructose-induced MetS is also discussed. The problems associated with the consumption of high fructose diets and the need for alternative and safe treatments of fructose-induced metabolic dysfunction are discussed. I also discuss the significance of neonatal programming in the pathogenesis of MetS in adulthood, special emphasis is placed on the neonatal period of development as a potential target for prophylactic and therapeutic approaches to improve health outcomes.

The actual study is divided into two main experiments, that is, the neonatal short-term study which was acute and the long-term (chronic) study which extended from the neonatal growth stage to the adult phase. The experimental details of the short-term neonatal study are described in Chapter 3, while the different aspects of the long-term or chronic study are described in Chapter 4 and Chapter 5.

Chapter 3 constitutes the first experimental study in which the potential short-term protective effect of neonatal oral administration of OA against fructose-induced oxidative damage, its effects on health and the developmental maturation of the gastrointestinal tract

(GIT) was investigated in suckling male and female rat pups. A brief introduction and justification for conducting the first experimental study are provided. A detailed description of the materials and methods used and findings are also presented. The relevance of the finding that neonatal oral OA administration protected against fructose-induced oxidative damage with no adverse effects on health or development of the GIT is discussed.

The second major experiment was further sub-divided into two. The first part of the study in Chapter 4 focuses on metabolic dysfunction (MD) while the second part in Chapter 5 focuses on the non-alcoholic fatty liver disease (NAFLD).

Chapter 4 consists of a brief introduction, materials and methods, results and discussion of the findings of the first part of the second experimental study. In this study, the potential long-term protective effect of neonatal oral administration of OA against the subsequent development of health outcomes associated with MD was investigated in fructose-fed male and female rats. The introduction focuses on the neonatal programming of MD and the possibility of targeting the neonatal period for prophylactic treatments against the development MD later in life. The materials and methods provide a detailed description of the experiments that were undertaken to measure several health outcomes which include: growth performance, GIT and visceral organ morphometry, glucose tolerance, insulin resistance and β-cell function, visceral and epididymal fat pad masses (body adiposity), serum triglyceride and cholesterol levels. The relevance of the result that fructose administration in neonates and during adulthood had adverse effects on several health outcomes associated with MD which were prevented by neonatal interventional treatment with OA is discussed.

In the final study, described in **Chapter 5**, the potential long-term protective effect of neonatal oral administration of OA against the subsequent development of non-alcoholic fatty liver disease (NAFLD) in fructose-fed male and female rats was investigated. Plasma levels of a surrogate enzyme marker of liver function, alanine amino transferase (ALT), a non-tissue specific enzyme, alkaline phosphatase (ALP), terminal relative liver masses and hepatic lipid content were determined. Histomorphometric techniques were used to evaluate the development of non-alcoholic fatty liver disease (NAFLD) by assessing steatosis,

inflammation and fibrosis. The chapter is concluded by discussing the significance of the major finding that administration of fructose neonatally and in adulthood caused the development of NAFLD, which was prevented by neonatal oral administration of OA. Chapter 5 constitutes a manuscript that has been accepted for publication in the peer-reviewed international accredited *Journal of Developmental Origins of Health and Disease* and part has also been published in *The FASEB Journal* as an abstract of the US Experimental Biology 2017 Annual Conference proceedings.

Chapter 6 provides an overall summary of the major findings, limitations and recommendations for new directions in research advances and proposed possible future studies.

As part of the Declaration, the main aim of the thesis was formulated by myself. Associate Professor Kennedy H Erlwanger and Professor Emmanuel Mukwevho assisted with the planning and execution of the study.

Chapter 7 consists of all the references that were used in the entire thesis.

The **Appendices** section consists of a set of appendices which includes copies of ethical clearance certificates, ethical modification and extension forms, soxhlet procedures, ELISA kit instructions, plagiarism declaration and Turn-it-in plagiarism similarity scores.

1.2 Introduction and justification for the study

Metabolic syndrome (MetS) is a prevalent, multifactorial and complex metabolic disease that is associated with an increase in the risk to develop type 2 diabetes mellitus (T2DM), obesity and major cardiovascular consequences such as hypertension, atherosclerosis and myocardial infarction (Grundy, 2016). Over the past several years, the prevalence of MetS and obesity has reached epidemic proportions in the Western countries and is on the rise in developing countries (Jaacks et al., 2016). The global increase in the incidence of MetS poses a major burden to the public health sector and has a tremendous negative impact on clinical, socio-economic, and patient-reported outcomes (Kelly et al., 2008).

There is increasing evidence from human clinical and experimental animal studies that the development of MetS and its complications is a result of complex interactions between environmental factors (dietary food sources), personal habits or choices (calorie intake and physical activity) and genetic factors (Hill and Peters, 1998, James et al., 2004, Corey and Kaplan, 2014). The increase in the global prevalence of MetS has also been attributed to excessive consumption of fructose, a sweetener that is used in several foods and beverages (Bray et al., 2004, Johnson et al., 2007).

Previous studies have shown that there is a link between nutrition during the neonatal period and the development of either positive health outcomes or adverse outcomes associated with MetS later in adult life (Brenseke et al., 2013, Low et al., 2017). The neonatal period is a critical window of developmental plasticity during which dietary manipulations can affect subsequent neonatal programming and genetic predisposition of offspring to metabolic disorders or good health in adult life (Tarry-Adkins and Ozanne, 2011, Wang, 2013). Epidemiological studies and experimental animal research have shown that the propensity to develop MetS later in life is increased when the early life development has been adversely affected. The pathogenesis of MetS involves epigenetic changes that alter gene expression as a result of an adaptation to environmental changes during neonatal development (Vickers, 2016b). Neonatal programming, therefore, offers a novel approach to investigate the mechanistic basis of the development of MetS later in life.

The emphasis of MetS management is on preventing the development or attenuation of the adverse effects of MetS outcomes. Current treatment and management options of MetS-associated health outcomes such as T2DM and CVDs involve the use of pharmacological agents that target the reduction of appetite, oxidative stress, inflammation, insulin resistance, modulation of insulin signalling and increase inenergy expenditure (Dal-Pan et al., 2010, Guo, 2014, Furukawa et al., 2017). However, prolonged treatment of MetS health outcomes using pharmacologic agents may have adverse side-effects. Moreover, the costs of such drugs are prohibitive and beyond the reach of many patients in developing countries of sub-Saharan Africa who are based in the underserved, resource-limited rural areas. Furthermore, most drugs are monotherapeutic, usually targeting only one aspect of the MetS and not the other

health outcomes, for instance, anti-hypertensive drugs will only protect against or ameliorate hypertension-related complications but not T2DM or obesity.

1.3 Justification of the study

In view of the pandemic increase in the prevalence of MetS and its associated complications, safe alternative preventive or therapeutic interventions that are administered, especially in the neonatal period are urgently needed, in order to reduce the negative impact of the adverse health effects of MetS and obesity (Roche et al., 2005). Due to the limitations associated with pharmaceutical drugs used for managing MetS-associated complications, there is a need to develop and adopt safer alternative or complementary approaches that would exert similar effects of calorie restriction and exercise or reduce appetite and energy storage. Any safe, natural approach in the treatment of MetS symptoms, especially those that can target the critical periods of developmental plasticity and can be used with or without modern medicine is worth exploring. There has been a move towards the use of natural, herbal medicines as alternatives to synthetic drugs for the treatment of chronic metabolic conditions. In fact, several rural-based and even urban-dwelling patients with chronic non-communicable diseases are opting for natural ethnomedicinal plant alternatives for their primary health care needs, due to their safety, easy accessibility and affordability. As a result, various phytotherapies are being investigated.

Medicinal plants are used to treat a wide range of diseases including MetS and its associated complications. Traditional treatments for health outcomes of MetS such as hypertension and T2DM are used throughout the world, either alone or as adjuncts to conventional pharmaceutical therapies (Duncan et al., 1999, Hosein Farzaei et al., 2016). Over 80% of the world's population in developing countries rely heavily on traditional ethnomedicinal plants for their primary healthcare needs (Sofowora, 1996, Helmstädter and Staiger, 2014). The therapeutic and pharmacological properties of several traditional medicinal plants that are in common use worldwide have been attributed to the presence of biologically active phytochemicals which may act individually, additively or synergistically to improve health.

Oleanolic acid (OA), is a biologically active pentacyclic triterpenoid compound commonly found in several plant species and fruits. OA was selected for this study as it has been shown

to possess several beneficial effects against the development of T2DM and MetS health outcomes without adverse side-effects. These beneficial pharmacological effects include hepatoprotection against diet-induced liver injury, anti-inflammatory, anti-diabetic, hypoglycaemic and anti-oxidant activities (Liu, 1995, Liu, 2005). However, the majority of the studies involving OA dietary supplementation have been performed in adult murine models (mice or rats) after the weaning period. There is a paucity of data from animal studies involving OA administration during the neonatal period which serves as an important developmental window of plasticity during which the predisposition to the development of metabolic disorders, such as the MetS in later life, could possibly be prevented. The neonatal murine model was selected for this study due to the altricial nature of rats which complete their development during the neonatal period making them a good model for studies on neonatal programming.

1.4 Aim and objectives

1.4.1 General aim

The current study was designed to investigate the potential short and long-term protective effect of neonatal oral administration of oleanolic acid against the subsequent development of dietary fructose-induced adverse short and long-term health outcomes associated with MetS in male and female rats using a neonatal programming rodent model.

1.4.2 Specific objectives

1.4.2.1 Short-term neonatal experimental study objectives

During the neonatal period of early lactation (post-natal day 1-14) the eyes of the rat pups are closed and their diet is restricted to milk. During this period, the rat pups are unable to forage and ingest extraneous material which alter metabolism and development of the gastrointestinal trat. This critical window of developmental plasticity characterised by rapid growth and physiological changes, is an opportune time for interventions which would not be influenced by other dietary factors. The specific objectives of the short-term neonatal

experimental study in the second week of post-natal life were therefore to determine the effects of neonatal oral administration of OA in fructose-fed male and female rats on:

a) Growth performance
b) Gastrointestinal tract and visceral organ morphometry.
c) Clinical health biomarkers
d) Intra-hepatic lipid accumulation and
e) Gene expression of anti-oxidant enzymes in muscle.

The second long-term experimental study was divided into two sub-studies, the first part (general metabolic dysfunction) and the second part (non-alcoholic fatty liver disease).

1.4.2.2 Long-term experimental study (general metabolism) objectives

Conditions presented during the critical neonatal period of development can lead to programmed permanent alterations of physiological systems such that the individual offspring is at an increased risk of developing metabolic disorders or positive health outcomes in adulthood.

The specific objectives of the first part of the second long-term experimental study were to determine the potential protective effects of neonatal oral administration of OA against the subsequent development of adverse health outcomes associated with metabolic dysfunction in fructose-fed neonatal male and female rats by assessing:

a) Growth performance
b) Gastrointestinal tract and accessory organ morphometry
c) Glucose tolerance
d) Insulin resistance and β-cell function
e) Visceral (male and female) and epididymal (males only) fat pad masses - body adiposity and
f) Fasting circulating metabolites.

1.4.2.3 Long-term experimental study (non-alcoholic fatty liver disease) objectives

The specific objectives of the second long-term experimental study were to determine the potential protective effects of neonatal oral administration of OA against the subsequent development of non-alcoholic fatty liver disease in fructose-fed neonatal male and female rats by assessing:

a) Terminal liver masses
b) Hepatic lipid accumulation
c) Histomorphometry and histopathology of hepatic tissue samples and
d) A surrogate biomarker of hepatic function (ALT) and a non-tissue specific enzyme (ALP).

1.5 Hypotheses

1.5.1 Short-term neonatal experimental study hypotheses

H_1: Neonatal oral administration of OA protects against fructose diet-induced down-regulation of anti-oxidant enzyme gene expression without adverse effects on health and precocious development of the GIT in suckling male and female rat pups.

H_0: Neonatal oral administration of OA does not protect against fructose diet-induced downregulation of anti-oxidant enzyme gene expression and has adverse effects on health and precocious development of the GIT in suckling male and female rat pups.

1.5.2 Long-term experimental study (general metabolism) hypotheses

H_1: Neonatal oral administration of OA protects against the subsequent development of metabolic dysfunction in fructose-fed male and female rats.

H_0: Neonatal oral administration of OA does not protect against the subsequent development of metabolic dysfunction in fructose-fed male and female rats.

1.5.3 Long-term experimental study (non-alcoholic fatty liver disease) hypotheses

H_1: Neonatal oral administration of OA protects against the development of non-alcoholic fatty liver disease in fructose-fed adult male and female rats.

H_0: Neonatal oral administration of OA does not protect against the development of non-alcoholic fatty liver disease in fructose-fed male and female rats.

In the following chapter, I will provide a detailed and critical review of literature that is pertinent and relevant to this study.

CHAPTER 2:

LITERATURE REVIEW

2.1 DEFINING METABOLIC SYNDROME

2.1.1 Definitions and diagnosis of metabolic syndrome

Metabolic syndrome (MetS) is defined as a cluster of interrelated adverse metabolic and cardiovascular disorders (CVDs) which are typically associated with a marked increase in the risk to develop central obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM), dyslipidaemia, hypertension, atherosclerosis and myocardial infarction (Spalding et al., 2009, Asrih and Jornayvaz, 2015, O'Neill and O'Driscoll, 2015, Furukawa et al., 2017). Metabolic syndrome is a concept that was first described by Kylin, a Swedish physician in the 1920s after noticing that hypertension, hyperuricaemia, hyperglycaemia and gout demonstrated a trend to occur together more frequently than would be possible just by coincidence in specific groups of individuals (Alberti et al., 2005, Kaur, 2014). Later in the 1960s, this cluster of cardiovascular risk factors was found to be related to hyperlipidaemia and central obesity (Avogaro et al., 1967).

In his Banting lecture in 1988, Gerald Reaven proposed the first generally accepted definition of the MetS and introduced the term "Syndrome X" in an effort to describe the interrelationship of cardiovascular risk factors such as obesity (or increase in triglyceride levels), hyperinsulinaemia (or glucose intolerance), and dyslipidaemia in patients who had a tendency to develop CVDs (Reaven, 1988). Reaven hypothesised a pathophysiological construct where the common characteristics of "Syndrome X" were IR and its compensatory hyperinsulinaemia which predisposes an apparently healthy individual to the development of hypertension, dyslipidaemia and T2DM (Reaven, 1988). According to Reaven, MetS is also associated with other metabolic disorders such as microalbuminuria, increased prothrombotic and anti-fibrinolytic factors (Björntorp, 1992, De Pergola and Pannacciulli, 2002). In order to describe this group of risk factors of metabolic origin, other researchers have used terms such as the deadly quartet syndrome (Kaplan, 1996), dysmetabolic syndrome (Groop and Orho-Melander, 2001) cardiovascular syndrome (Hjermann, 1992) and insulin resistance syndrome (Fletcher and Lamendola, 2004).

In order to provide a standard tool for defining MetS for researchers and clinicians, the World Health Organization (WHO) in 1998, coined the term "metabolic syndrome" and provided

the first complex and prescriptive definition of the metabolic disorder which they revised in 1999 (Alberti and Zimmet, 1998). The WHO defined MetS as "the presence of impaired glucose control and/or IR occurring together with at least any two of the following conditions: dyslipidaemia, high blood pressure, obesity or microalbuminuria" (Alberti and Zimmet, 1998). Following this attempt by the WHO to define MetS, numerous definitions of MetS have been proposed by different professional organisations.

The most widely acknowledged attempts to define the MetS after the WHO include proposals by: the European Group for the Study of Insulin Resistance (EGIR) (Balkau and Charles, 1999); the National Cholesterol Education Program (NCEP) (Schaefer et al., 1997); Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) (Expert Panel on Detection, 2001); the American Association of Clinical Endocrinologist (AACE) (Einhorn, 2003); the International Diabetes Federation (IDF) (Alberti et al., 2005) and the American Heart Association (AHA) (Grundy et al., 2005).

However, in 2009, the AHA; the World Health Federation; International Atherosclerosis Society; the IDF Task Force on Epidemiology and Prevention; the National Heart, Lung, and Blood Institute and the International Association for the Study of Obesity, released a joint statement regarding the clinical definition of MetS (O'Neill and O'Driscoll, 2015). In an effort to harmonise the definition of MetS by these professional boards, there was a consensus that the presence of IR and obesity were not pre-requisites for the diagnosis of MetS, but rather the occurrence of three out of the following five risk factors: increased waist circumference, high blood pressure, high triglyceride levels, low high-density lipoprotein-cholesterol (HDL-C) levels, high low-density lipoprotein (LDL) levels and persistent hyperglycaemia (O'Neill and O'Driscoll, 2015). The most commonly used definition criteria of MetS and its components from different international expert groups are summarised in Table 2.1. Although the definition and classification criteria of MetS differ from each of the proposed definitions, almost all of them include the same type of combination of central obesity, dyslipidaemia, elevated blood pressure, glucose intolerance, IR and other biochemical markers. MetS provides a practical tool for physicians and researchers to assess the risk of developing CVDs or T2DM (Kaur, 2014).

Table 2.1: Definitions of metabolic syndrome

Guideline	Criteria for diagnosis	Central obesity	Hypertension	Dyslipidaemia	Glucose/Insulin Other abnormalities
WHO (1999)	Presence of one of T2DM, impaired glucose tolerance or fasting glucose plus 2 other features	Waist: hip ratio > 0.9 (men); >0.85 (women) or BMI>30kg/m^2	≥140/90 mmHg	TGs≥1.7mmol/ℓ and HDLC ≤0.9mmol/ℓ (men); ≤1.0mmol/ℓ (women)	Microalbuminuria: urinary albumin excretion ≥20μg/min or ALB:CREA ≥30mg/g
EGIR (1999)	Insulin resistance plus 2 other features	*≥94cm (men) *≥80cm (women)	≥140/90 mmHg	TGs≥2.0mmol/ℓ and/or HDLC ≤0.9mmol/ℓ (men); ≤1.0mmol/ℓ (women)	Insulin resistance: Fasting insulin in top 25 percentile and plasma glucose>6.1mmol/ℓ
NCEP (2001)	Any three features	*>102 cm (men) *>88 cm (women)	≥130/85 mmHg	TGs≥1.7mmol/ℓ and/or HDLC ≤1.0mmol/ℓ (men); ≤1.3mmol/ℓ (women)	Plasma glucose ≥5.6mmol/ℓ
IDF (2005)	Central obesity plus 2 other features	*≥94cm (men) *≥80cm (women)	≥130/85 mmHg	TGs≥1.7mmol/ℓ and/or HDLC ≤1.0mmol/ℓ (men); ≤1.3mmol/ℓ (women)	Plasma glucose ≥5.6mmol/ℓ or previously diagnosed with T2DM

WHO = World Health Organization; EGIR = European Group for the Study of Insulin Resistance; NCEP = National Cholesterol Education Program; IDF = International Diabetes Federation; T2DM = type 2 diabetes mellitus; HDLC = high density lipoproteion cholesterol; TGs = triglycerides; ALB = albumin; CREA = creatinine. *Waist circumference. Table adapted and modified from Grundy et al. (2004).

As such MetS also strongly predicts the development of atherosclerotic CVDs (Isomaa et al., 2001), and T2DM (Lorenzo et al., 2003), although some studies argue that MetS does not predict cardiovascular events any better than the sum of its components (Eckel et al., 2010).

2.1.2 Prevalence and epidemiology of metabolic syndrome

Since the beginning of the millennium, the prevalence of MetS, obesity and T2DM has drastically increased and reached epidemic proportions (Florez et al., 2014). The dramatic rise in the prevalence of MetS over the past few decades has increased in parallel with the increasing prevalence of obesity (Després and Lemieux, 2006, Jaacks et al., 2016). Previously considered a problem in developed Western countries, MetS, obesity and T2DM prevalence is also on the rise in developing economies, particularly in urban areas where most people have access to and excessively consume high-energy foods and have adopted sedentary lifestyles (Lakka et al., 2003, O'Neill and O'Driscoll, 2015). The explosive increase in the global prevalence of metabolic disorders now pose a major burden and challenge to the public health sector in developing countries (Spalding et al., 2009). Presently, it is estimated that over 415 million people are affected by T2DM worldwide, and according to the IDF, this figure is projected to rise to over 642 million by 2040 (Rahelić, 2016).

Globally, there are over 1 billion overweight adults of which 400 million of these are obese, moreover there are 20 million children under 5-years old who are overweight (Cameron et al., 2004, WHO, 2013, WHO, 2016). Over a quarter of the world's adult population is estimated to have MetS, the prevalence of which is steadily increasing alongside that of obesity thus posing an escalating challenge to the public health sector in the 21st century (Kaur, 2014, Asrih and Jornayvaz, 2015). Studies have shown that the prevalence of MetS is higher in women than men (Hu et al., 2004). In several European countries and in the US the prevalence of MetS affects over a third of the adult population and the prevalence is higher in the elderly (Ford et al., 2002). Increasing longevity due to lifestyle prolongs the exposure of individuals to metabolic and cardiovascular risk factors which potentially increases the number of people that have MetS and its associated health outcomes such as obesity, T2DM and CVDs (Fontana and Partridge, 2015).

The prevalence of the health outcomes associated with MetS is expected to increase in the next few decades because the number of people over 65 years of age will almost double in the next few years (Wilson et al., 2005). Furthermore, over 20 million children under 5-years old are overweight and this childhood obesity and related metabolic disorders will translate to a further increase in adult obesity, predicted to reach 2.3 billion by 2020 and double by 2030 (WHO, 2016). In order to understand the described increase in the prevalence of MetS, it is crucial to know the risk factors involved in and contribute to the pathogenesis of MetS as highlighted in the following section.

2.2 PATHOPHYSIOLOGY AND RISK FACTORS FOR METABOLIC SYNDROME

2.2.1 Mechanisms underlying the development of metabolic syndrome

Epidemiological studies have established that there has been a recent increase in the prevalence of undesirable health outcomes associated with MetS in all ages including adolescents and young adults (Cameron et al., 2004, Dehghan et al., 2005). The development of health outcomes associated with MetS such as T2DM, dyslipidaemia, obesity and CVDs have primarily been attributed to the complex interaction of several factors that include genetic factors, age, ethnicity, urbanisation, physiological, behavioural and environmental influences (James et al., 2004, Vickers, 2011). The exact complete mechanisms underlying the pathogenesis and development of metabolic dysfunction are not fully understood (Eckel et al., 2004). It is a widely held view that the development of an obesogenic environment due to excessive consumption of high-calorific diets and a decrease in energy expenditure is the primary cause of obesity and related metabolic disorders, particularly T2DM and IR (Bakker et al., 2016, WHO, 2016). In the next section, I will discuss the role of several factors such as lifestyle choices and genetic factors that are associated with the development of MetS and its associated complications.

2.2.2 Lifestyle choices and the pathogenesis of metabolic disorders

The role of sedentary lifestyle habits and dietary choices in the development of MetS is well-established (Hu, 2003). Lifestyle choices such as poor dietary habits and a decrease in physical

activity have been implicated as the major contributing factors to the rise in the prevalence of health outcomes associated with MetS (Armitage et al., 2005). Although there is a general consensus, based on epidemiological studies, that a change in lifestyle induce weight loss and attenuates MetS health outcomes, the ideal diet and form of exercise for the treatment of MetS remains unclear. In the following sections, I discuss the role of the different lifestyle choices and their involvement in the pathogenesis and the aetiology of MetS.

2.2.2.1 Level of physical activity and exercise

Physical activity is defined as "planned and structured increase in energy expenditure by performing any repetitive bodily movements in order to improve or maintain physical fitness" (Caspersen et al., 1985). Physical activity can be structured (sporting activities, transport and leisure) or non-structured (housework or any movements of daily living) and as such not all physical activity is considered exercise (Wing, 1999). In developing countries, urbanisation due to an increase in the rural to urban migration has resulted in people gradually adopting sedentary lifestyles over the past several decades (Adediran et al., 2012). These sedentary lifestyles are characterised by physical inactivity and reduced exercise, which provides a putative explanation for the observed increase in the prevalence of metabolic disorders (Lakka et al., 2003). The decrease in physical activity and exercise coupled with everyday technologies that promote a reduction in the levels of physical activity has contributed to the development of poor aerobic fitness, MetS and obesity (Kautiainen et al., 2005). A Finnish study on 19-year old individuals has shown that daily television watching or viewing for more than 2 hours per day was associated with poor muscle fitness (Paalanne et al., 2009).

An increase in physical activity and fitness were shown to protect against the development of MetS through their positive effects on MetS health outcomes such as TG levels, CVDs, glucose and lipid metabolism (Xu et al., 2011). Large population-based epidemiological studies have demonstrated the beneficial effects of exercise and physical activity on health particularly, the improvement of cardio-metabolic factors (Lavie et al., 2015), reduction in the amount of adipose tissue (Rönn et al., 2013), insulin sensitivity, low levels of inflammatory cytokines and oxidative

stress markers (Fiuza-Luces et al., 2013). Aerobic exercise also promotes weight loss and body fat reduction in obese individuals (Kisner et al., 2017). The American College of Sports Medicine recommends 120-150 minutes of moderate exercise or physical activity per week in order to prevent excessive weight gain (Medicine, 2013). Aerobic exercise intervention has also been shown to significantly lower blood pressure (Lavie et al., 2015). A study conducted on male trainee military personnel showed that exercise resulted in weight and visceral fat mass reduction (Sporiš et al., 2014). Coupled with dietary interventions, exercise provides only a limited amount of additional weight loss compared to dietary interventions only (Jakicic et al., 2008). As such it is recommended to make use of exercise and dietary adjustments to minimise the risk of developing of MetS-associated symptoms.

2.2.2.2 Dietary factors and metabolic syndrome

The link between dietary choices and the risk of developing MetS is well-established (Khitan and Kim, 2013, Fontana and Partridge, 2015). Adoption of sedentary lifestyles that are characterised by consumption of high calorific diets has also been implicated in the development of MetS and obesity (Hu, 2003). In fact, dietary intake of high calorific processed foods has been associated with the development of individual health outcomes associated with MetS (Drewnowski, 2004). A high prevalence of MetS and obesity has been reported among individuals who consume a Western type of diet characterised by excessive intake of refined grains, processed foodstuffs, fried foods and red meat (Baxter et al., 2006). As a result of the importance of diet, dietary interventions have been used for treating or managing the various components of MetS (Esposito et al., 2004). Research on hypocalorific diets with high protein content or monounsaturated fatty acids, the intake of fruit, vegetables, cereals and fish was associated with a lower risk of developing MetS, improvement in lipid profiles and blood pressure (Sabaté and Wien, 2015). Calorie restriction and low-fat diets have also been shown to reduce the risk of developing clinical and biological markers linked to MetS (Riccardi and Rivellese, 2000). The global increase in the consumption of high-energy diets, especially those containing fructose as the main ingredient, has also been linked to the increased incidence of obesity and MetS (Rippe,

2014, Steinmann and Santer, 2016). The contribution of excessive fructose consumption to the development of MetS-associated risk factors is discussed in the following section.

2.2.3 The role of fructose in the development of metabolic disorders

Fructose or fruit sugar is a simple, sweet-tasting and ketogenic monosaccharide found naturally in honey, several fruits and vegetables (Zhang et al., 2014). Fructose is sweeter than other simple carbohydrates such as glucose or sucrose and can be absorbed from the intestines directly into the bloodstream (Bray et al., 2004). Fructose is manufactured industrially as high fructose corn syrup (HFCS-55) which consists of 55% fructose; 42% glucose and 3% high saccharide (White, 2009). HFCS-55 is derived from corn and during its manufacture, glucose from the corn-starch is chemically converted to fructose (Stanhope and Havel, 2009). Due to its sweetness and affordability, HCFS-55 is widely used commercially as the main sweetener in the manufacture of artificially-sweetened processed foodstuffs (such as baked goods, cereals, canned fruits, ice cream, jams, jellies and fruit juices) and sugar-sweetened beverages (SSBs) (Hanover and White, 1993, Tappy, 2017).

Following its introduction in the USA around the 1970s, intake of refined fructose has steadily been increasing among adolescents and young adults with dire metabolic consequences (Brinton, 2016). It is estimated that fructose consumption rose from an annual estimate of 8.1 kg/person at the beginning of the 19th century to about 65 kg/person in 2010 (Zheng et al., 2016). Due to the ubiquitous availability of fructose in processed foods and SSBs, the exact level of fructose consumption may be higher than is currently estimated (Goran et al., 2013). It is predicted that the trend in fructose consumption is going to continue to increase in future (Popkin and Hawkes, 2016).

The widespread increase in the dietary consumption of fructose over the past several decades, has contributed to the global obesity and MetS epidemic (Zheng et al., 2016). Fructose is regarded as a potent obesogenic agent as it disrupts the development and function of adipose tissue resulting in increased risk to develop obesity (Goran et al., 2013). Obesogens are

xenobiotic chemical substances that negatively affect lipid metabolism leading to the development of obesity (Trasande and Blumberg, 2018). Epidemiological and experimental studies have shown that excessive consumption of fructose contributes to the development of multiple endocrine and metabolic disturbances which often results in an increase in body mass, obesity, T2DM, hyperlipidaemia and hypertension (Kwon et al., 2008, Saygin et al., 2015, Tappy, 2017). Global ecological analyses studies have also shown that the widespread excessive consumption of fructose in processed foods has been associated with the increased risk to develop metabolic disorders thus implicating fructose as a major contributing factor in the development of health outcomes associated with MetS and the increase in their prevalence epidemic proportions (DiNicolantonio et al., 2015). In fact, the national prevalence of T2DM has been shown to be higher in countries that include fructose in their food supply than those that did not (Goran et al., 2013). A close parallel between the rise in the consumption of fructose and IR, T2DM, obesity, increase in LDL-C, TGs and CVDs have been demonstrated (Steinmann and Santer, 2016). Studies using murine models have also shown that the risk to develop health outcomes associated with MetS could be increased by the over-consumption of fructose (Vikas, 2008, Mamikutty et al., 2014, Lozano et al., 2016, Okoduwa et al., 2017). Moreover human and animal studies provide evidence that widespread excessive consumption of dietary fructose is closely linked to the development of cardio-metabolic disorders and associated co-morbidities (Johnson et al., 2007, Zheng et al., 2016).

The deleterious effects of excess fructose consumption on health outcomes associated with MetS during gestation and in adulthood are well researched (Zheng et al., 2016). There is evidence which points to the adverse effects of high-fructose diets administered during the critical periods of development (gestation, infancy and the neonatal period) (Goran et al., 2013, Zheng et al., 2016). Research has demonstrated that excessive consumption of fructose by mothers and their offspring during pregnancy, lactation and infancy can program the development of persistent metabolic disorders later in life due to early exposure to obesogens such as fructose (Zheng et al., 2016, Saad et al., 2016). This evidence highlights the role of foetal programming in the development of metabolic disorders later in life due to exposure to obesogens such as fructose.

There is however little information on the development of adverse MetS health outcomes resulting from the direct consumption of fructose in the neonatal period and the long-term effects in adult offspring following a secondary dietary fructose insult. Most studies focused on maternal fructose consumption during gestation and suckling, but few (Huynh et al., 2008, Ibrahim et al., 2017) have investigated the effect of giving a single neonatal hit or a double fructose hit in both the neonatal and adulthood phases.

2.2.3.1 Hepatic fructose metabolism

Fructose is absorbed in the intestine across the intestinal epithelium through GLUT5 and GLUT2 transporters at the apical side and through GLUT2 at the basolateral membrane (DeBosch et al., 2014, DiNicolantonio et al., 2015). After absorption, fructose enters the portal system to the liver for further metabolism (Bray et al., 2004). In the liver, fructose is primarily metabolised to fructose-1-phosphate, a reaction that is catalysed by fructokinase an enzyme whose activity is independent of insulin secretion from pancreatic β-cells (Kwon et al., 2008, Steinmann and Santer, 2016) (Figure 2.1). Fructose-1-phosphate is a precursor to the triglyceride (TG) molecule and supplies the body with 3-carbon molecules which are metabolised into glycerol and free fatty acids (FFAs) (Stanhope and Havel, 2008). The FFAs generated from fructose metabolism are taken up by visceral adipocytes resulting in the development of central obesity (Steinmann and Santer, 2016) and elevated plasma uric acid levels (Lin et al., 2013). Fructose has a higher lipogenic potential than glucose, often resulting in the rapid increase in plasma levels of FFAs compared to glucose. Glucose consumption triggers insulin secretion from β-cells and also causes leptin secretion which in turn suppresses appetite and food intake (Hedeskov, 1980). Unlike glucose, fructose does not stimulate insulin or leptin secretion thus by-passes the insulin-driven satiety system (Curry, 1989). Increased fructose consumption therefore results in less insulin being produced, which causes less leptin to be released and therefore removing the inhibition of food intake (Iizuka, 2017). Thus excessive fructose intake might result in unregulated hepatic fructose uptake and increased *de novo* lipogenesis (DNL), which contributes to increased body weight and the development of several MetS outcomes (Castro et al., 2011).

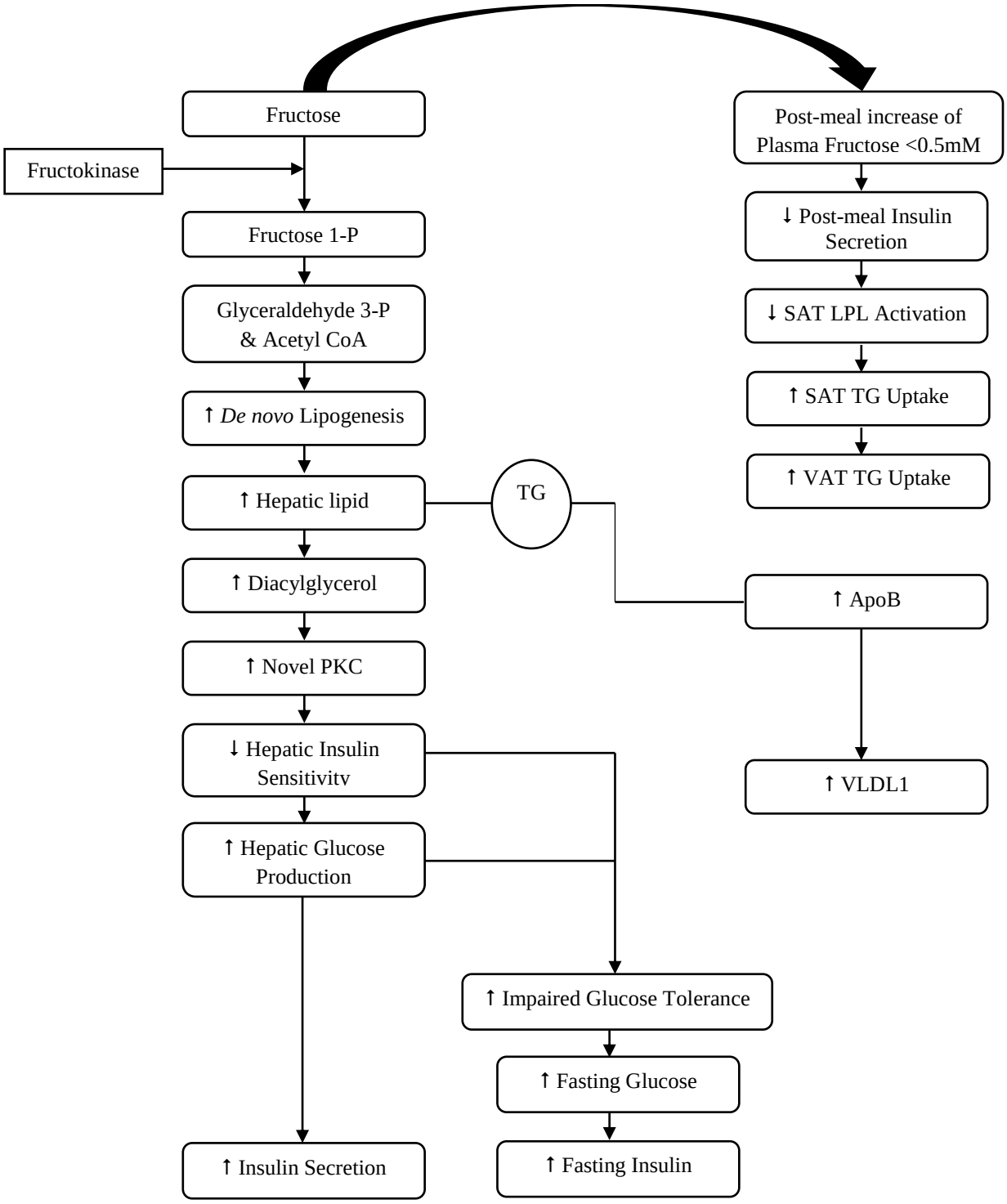

Figure 2.1: Major outcomes of fructose consumption on metabolism.

LPL = lipoprotein lipase; PKC = protein kinase C; TG = triglyceride; SAT = subcutaneous adipose tissue; VLDL = very low density lipoprotein; Apo B = apolipoprotein B; VAT = visceral adipose tissue. Adapted and modified from Stanhope and Havel (2009).

Excessive fructose consumption in rodent models resulted in the upregulation of hepatic GLUT5 gene expression, TNFα levels, hepatic lipid peroxidation and apoptotic activity (Basaranoglu et al., 2014). The lipogenic, pro-inflammatory and oxidative effects of excessive fructose consumption are strongly associated with uncontrolled ATP and phosphate depletion. Intracellular depletion of ATP and phosphate activates AMP deaminase leading to an increase in uric acid production from hepatic fructose metabolism Figure 2.2 (Khitan and Kim, 2013). Metabolism of glucose in the liver is regulated by phosphofructokinase which is inhibited by an increase in high-energy status and ATP. An increase in ATP would, therefore, decrease hepatic glucose intake and the generation of DNL substrates (Lanaspa et al., 2012). Unlike the hepatic metabolism of glucose, metabolism of fructose in the liver is not dependent on energy status and ATP (Stanhope et al., 2009). An increase in hepatic lipids from DNL increases ApoB and also increase the production of VLDL1 (Figure 2.1).

2.2.4 Genetic factors and molecular aspects in the pathogenesis of metabolic syndrome

There is no doubt that much of the rise in the prevalence of obesity and metabolic dysfunction can be attributed to environmental factors such as excessive consumption of energy-rich foods and adoption of sedentary lifestyles characterised by low physical activity. However familial studies have shown the genetic contribution and involvement in the pathogenesis of health outcomes associated with MetS (Roche et al., 2005). The development of MetS outcomes in families and related individuals or offspring suggests that the metabolic risk factors may be genetically transmitted from parents to offspring (Bayol et al., 2008). Moreover, several genome-wide studies have demonstrated the presence of a genetic basis for the development of the individual components of metabolic dysfunction (Neel, 1962, Laker et al., 2013, Navarro et al., 2015a). Epigenetic changes, which also play an important role in the development of MetS risk factors will be discussed in detail in section 2.5. According to the thrifty gene hypothesis, genetic selection favours energy-saving genotypes in an environment where there is a shortage of food supply (McCance et al., 1994). Individuals with such energy-saving genotypes tend to store energy as fat instead of glycogen for use during periods of starvation (Speakman, 2006). If such

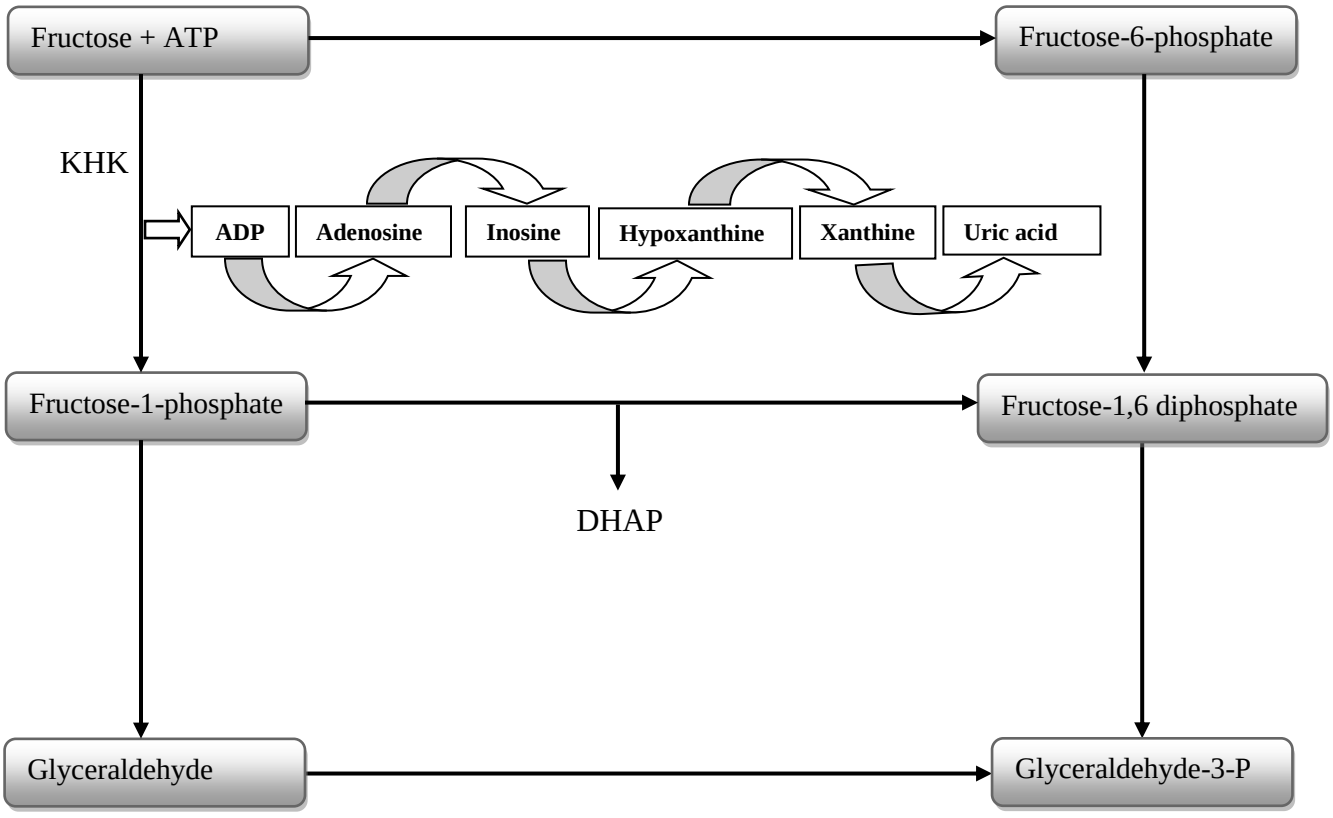

Figure 2.2: Uric acid production in hepatic metabolism of fructose.

ADP = adenosine diphosphate; ATP = adenosine triphosphate; DHAP = Dihydroxyacetone phosphate; KHK = fructokinase. Fructose-1-phosphate is further metabolized by aldolase B and trio kinase to glyceraldehyde-3-phosphate. Adapted and modified from (Khitan and Kim, 2013).

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genotypes are exposed to an abundant food supply later on in adulthood, individuals will be predisposed to the development of MetS (Neel, 1999).

Another possible explanation of the genetic contribution to MetS outcomes is the thrifty phenotype hypothesis (Groop, 2000). This hypothesis postulates that intra-uterine malnutrition and poor foetal or early post-natal nutrition leads to a low birth-weight and increases the vulnerability to develop MetS later in adult life (Groop, 2000). The risk of developing MetS associated with low birth-weight increases in families with MetS, suggesting that low birth weight could be a phenotype for the thrifty gene (Hales and Barker, 2001). Several candidate genes have been linked to the development of MetS. There are however no genetic tests available to diagnose MetS due to the complex interplay between the genes and the environment. The genetics of MetS involves several genes that individually have weak effects but may interact with each other synergistically and interact with environmental factors (diet, physical activity, alcohol consumption and smoking) in the pathogenesis of the MetS (Gluckman et al., 2015).

2.2.5 Age, hormonal changes and other factors

The prevalence of MetS appears to increase with age and it is more frequent in males than in females (Ervin, 2009). Other behavioural factors such as smoking (Lee et al., 2005), sleep deprivation (Van Cauter et al., 2008) and mental stress have also been associated with the development of MetS-health outcomes.

Individual and ethnic variations also contribute to the clinical manifestation of MetS (Grundy, 2005). A large epidemiological study in the US found that the prevalence of MetS and its associated complications was lower in African-American men than their white counterparts and lower in white women than in African-American women (Ford et al., 2010).

Having discussed the risk factors involved in the pathogenesis of MetS in this section, it is important to discuss the clinical signs and indicators of MetS as I have done in the next section.

2.3 CLINICAL MANIFESTATIONS OF METABOLIC SYNDROME

2.3.1 Visceral obesity, dyslipidaemia and abdominal adiposity

Visceral or central obesity is defined by the World Health Organisation (WHO) as "the excessive accumulation and increase in the mass of visceral adipose tissue which may or may not adversely affect health" (Rodríguez-Ortiz et al., 2016). In individuals with visceral obesity, approximately 10% of the total body fat is visceral fat in the peritoneal cavity, while 25-50% of the total abdominal fat mass is visceral fat (Miles and Jensen, 2005). Over the past few decades, the prevalence of obesity and overweight individuals in all ages including adolescents and young adults has almost doubled in the developed Western countries (WHO, 2016). The rise in the prevalence of obesity has been attributed to several lifestyle factors and changes in socio-economic environments. Sedentary lifestyles are characterised by excessive consumption of hyper-energetic diets and a decrease in physical activity all of which are at the centre of these observed trends (Björntorp, 1992, Hu, 2003).

Obesity, physical inactivity and low cardiorespiratory fitness usually occur simultaneously and, more often than not, they are associated with each other (Lakka et al., 2003). In obese individuals, the excessive accumulation of visceral fat (visceral obesity) is a major cause of morbidity and mortality in developed , and more recently developing countries (Drewnowski, 2004, Drewnowski and Darmon, 2005, Furukawa et al., 2017). Prospective studies have indicated that abdominal obesity accurately predicts the development of health outcomes associated with MetS and CVDs (Després and Lemieux, 2006). An increase in body weight of 1 kg increases the risk of CVD mortality by between 1-1.5% with 22 kg/m^2, as a starting BMI (Romero-Corral et al., 2006).

Adipose tissue is an active endocrine organ that secretes specific hormones and pro-inflammatory adipokines or adipocytokines such as leptin, TNF-α, IL-6, resistin and adiponectin (Galic et al., 2010). Adipokines are cell signalling proteins that are produced by adipocytes in adipose tissue and are responsible for mediating inflammation and IR (Pessin and Kwon, 2013,

Jung and Choi, 2014, Fasshauer and Blüher, 2015). In addition to its ability to store excess energy, adipose tissue is also an active metabolic organ that secretes free fatty acids (FFAs) and a variety of bioactive molecules (Galic et al., 2010, Mulder, 2017). Previous studies have shown that with an increase in abdominal adiposity (hyperplasia and hypertrophy) there is a parallel increase in circulating levels of FFAs and an increase in the production of pro-inflammatory cytokines such as IL-6, plasminogen activator inhibitor-1 (PAI-1) and TNF-α (Fantuzzi, 2005). The increase in the pro-inflammatory adipokine production by hypertrophying adipose tissue has been reported to reduce the production of anti-inflammatory adipokines (Shoelson et al., 2006). This causes imbalance in the production of pro- and anti-inflammatory adipokines by hypertrophying adipose tissue and results in the development of a low-grade inflammatory state which may cause insulin resistance (IR) through the impairment of insulin signal transduction mechanisms (Xu et al., 2003).

Dyslipidaemia in obese and overweight individuals exhibiting health outcomes associated with MetS is often characterised by increased levels of TGs and LDL-C and low levels of HDLC (Taskinen and Borén, 2015). Excess visceral adipose tissue supplies adipokines and FFAs directly to the hepatic portal circulation thus affecting hepatic metabolism of glucose and lipids (Toledo-Corral et al., 2015). However, subcutaneous fat secretes adipokines and FFAs to the systemic circulation and may not have a major bearing on hepatic lipid and carbohydrate metabolism. Normally there is a balance between lipolysis and TG synthesis in adipose tissue. However, an increase in visceral adipose tissue mass leads to a corresponding increase in lipolysis, which in turn results in an increase in the influx of adipose tissue-derived FFAs and lipogenic products into the liver (Tessari et al., 2009). TG accumulation in the liver may cause a decrease in hepatic insulin sensitivity thus further contributing to dyslipidaemia (Than and Newsome, 2015). The influx of FFAs into the liver leads to an increase in the production of triglyceride-rich lipoproteins such as apoB (Stanhope et al., 2009). ApoB is a precursor for cholesterol and triglyceride-containing lipoproteins transported from the liver to the site of use. Overproduction of hepatic VLDL has been implicated as an underlying factor in the development of metabolic dyslipidaemia (Grundy, 2016).

It is essential to regularly assess the amount of visceral body fat since its excessive accumulation may result in adverse health outcomes. The obese status of an individual has always traditionally been estimated by the measurements of body mass index (BMI), waist circumference, waist-to-hip ratio and skinfold thickness (Rao and Patra, 2016). These methods are easy, affordable and non-invasive. Based on BMI measurements, an overweight individual is defined as a BMI of 25-29.9 kg/m^2 while an obese individual has a BMI of >30 kg/m^2 (WHO, 2013). The waist circumference is regarded as a more practical and better measure of intra-abdominal fat accumulation than waist-to-hip ratio and BMI (Rao and Patra, 2016). Imaging methods such as magnetic resonance (MRI) and computer tomography (CT) are non-invasive and have been utilised as reliable and gold standard for measuring adipose tissue (Uppot et al., 2007). These methods are however expensive and time-consuming and as such may not be used where research in a large population are involved (Ross et al., 1993). Due to the limitations associated with MRI, CT and the anthropometric measurements, bioimpedance analysis has been widely used to assess body composition (Kyle et al., 2004). In addition to the development of impaired lipid metabolism, impaired glucose metabolism is also considered as an important manifestation of MetS.

2.3.2 Impaired glucose homeostasis

2.3.2.1 Insulin resistance

Insulin resistance (IR) is defined as "insufficient insulin action in the liver, skeletal muscle and adipose tissue" which result in fasting hyperinsulinaemia to maintain euglycaemia (Gustafson et al., 2015, Valle et al., 2016). Insulin resistance is considered as a common link between obesity (excess visceral fat) and the development of several health outcomes associated with MetS (Reaven, 1988, Lebovitz, 2001). Insulin plays a central role in the control of energy balance in the body by acting on the brain via specific central receptors (Brüning et al., 2000, Xu et al., 2017). The brain responds more to foods that contain glucose than fructose, as such excessive consumption of high fructose diets does not stimulate insulin secretion from the β-cells of the pancreas (Elliott et al., 2002). This causes a decrease in the levels of insulin which in turn

increases the risk of developing obesity and excessive body mass gain (Elliott et al., 2002). The development of MetS risk factors is common in overweight and obese individuals, as excess visceral fat accumulation increase the vulnerability of developing IR (Gustafson et al., 2015). Recent evidence places the development of obesity at the beginning of the process that leads to metabolic decline, before the development of IR, T2DM and CVDs (Figure 2.3).

The development of IR however suppresses the gluconeogenic and lipogenic effect of insulin, leading to increased lipolysis in adipose tissue. The elevated FFAs from lipolysis in adipose tissue further contribute to the development of IR by suppressing insulin signalling (Toledo-Corral et al., 2015). An overload of FFA production from lipolysis will also cause ectopic lipid formation in the liver through *de novo* lipogenesis (DNL) causing steatosis and progression to non-alcoholic fatty liver disease (NAFLD). Non-alcoholic fatty liver disease pathogenesis will be discussed in detail in section 2.4. The FFA overload due to IR also has adverse metabolic consequences in the pancreas and peripheral tissues such as skeletal muscles where FFAs decrease muscle tissue sensitivity to insulin (Figure 2.4) (Chow et al., 2017). In order to maintain euglycaemia and compensate for the decrease in insulin sensitivity and the insulin resistant state due to excess FFAs, more insulin is secreted resulting in hyperinsulinaemia (Fletcher and Lamendola, 2004). Failure to produce adequate insulin causes decreased cellular glucose uptake resulting in persistent hyperglycaemia and the development of T2DM as discussed in the next section.

2.3.3.2 Development of type 2 diabetes mellitus

The prevalence of type 2 diabetes mellitus (T2DM) and MetS have been on the rise since the middle of the 20th century. Empirical evidence seem to suggest that the trend is continuing to increase as we enter the 21st century. T2DM and MetS pose a threat to health care systems in both developed and developing countries (Alberti and Zimmet, 1998, ADA, 2014b). Diabetes is now considered as the most common non-communicable disease worldwide and the fourth leading cause of death in developed countries (WHO, 2016). The global prevalence of T2DM among male and female adults (20-79 years) has been estimated at 6.4% affecting 285 million

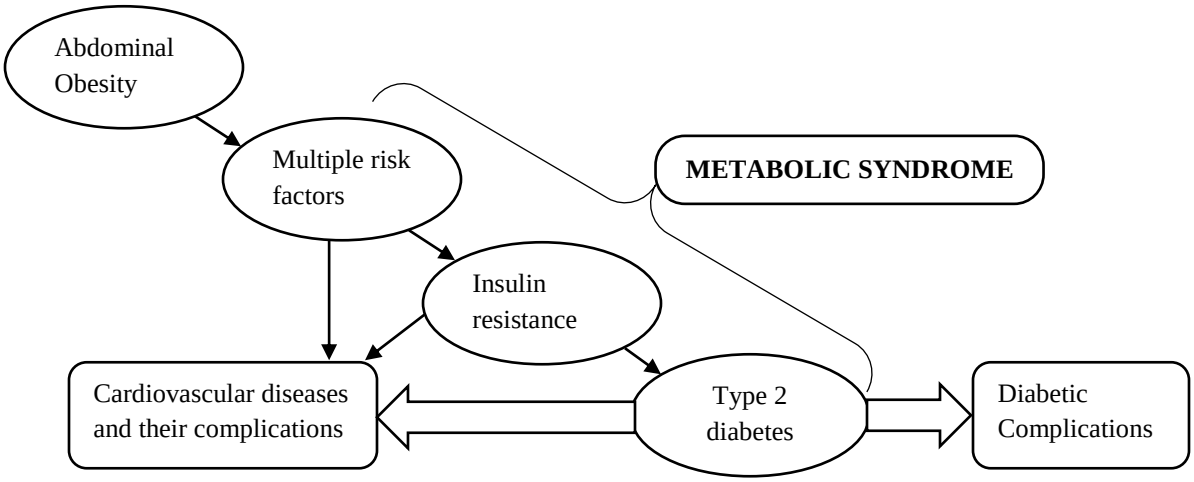
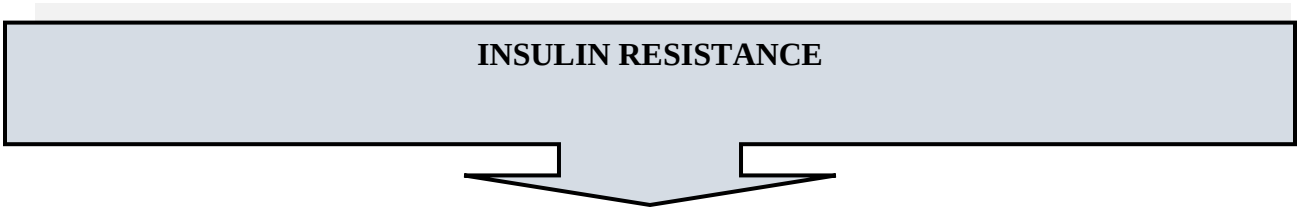


Figure 2.3: Progression and outcomes of the metabolic syndrome.

Adapted and modified from Grundy (2016).



SKELETAL MUSCLE	LIVER	PANCREAS	VASCULAR SYSTEM
Reduced insulin effect Reduced glucose uptake	Increased glucose production Dyslipidaemia (↓HDL, ↑TGs, small dense LDL Inflammation	Increased insulin secretion to compensate for hyperglycaemia β-cell exhaustion in response to chronic hyperglycaemia	Endothelial dysfunction (↓NO) Elevated blood pressure Impaired thrombolysis

Figure 2.4: Adverse effects of insulin resistance in liver, pancreas, skeletal muscles and the vascular system.

HDL = high density lipoprotein; LDL = low density lipoprotein; TGs = triglycerides; NO = nitric oxide.

adults in 2010 and is expected to rise to 7.7% affecting 439 million adults by 2030 (Chan, 2017). In the years between 2010-2030, it is predicted that there will be a 69% increase in the number of adults with T2DM in developing and 20% in developed nations (Guariguata et al., 2014).

Diabetes is characterised by IR and a decrease in insulin production and secretion by pancreatic β-cells. The pancreatic β-cell dysfunction contributes significantly to the pathogenesis of T2DM (Weir and Bonner-Weir, 2004). Insulin is required for the insulin-dependent cellular glucose uptake by hepatic and adipose tissue (Samuel and Shulman, 2016). A decrease in the capacity to secrete insulin by pancreatic β-cell causes impaired glucose metabolism, persistent hyperglycaemia and glucose intolerance (Bastard et al., 2006). Unlike IR or insulin insensitivity which occurs early and is partially associated with obesity, pancreatic β-cell function declines gradually with time, even when glucose levels are still considered normal before the onset of hyperglycaemia and T2DM (Weir and Bonner-Weir, 2004, Robinson and van Soeren, 2004). These risk factors are associated with low life expectancy and a decrease in the quality of life for the affected patients.

In the next section I will discuss the cardiovascular consequences of MetS

2.3.3 Cardiovascular outcomes and metabolic syndrome

The relationship between MetS and CVDs such as hypertension is complex and has been a subject of debate for several years. Considerable evidence now exist from studies which show that the risk to develop CVDs increase as MetS outcomes such as glucose intolerance and IR progresses (Ginsberg, 2000). Hyperglycaemia in patients with T2DM is a risk factor for diabetic CVD complications such as coronary heart disease, peripheral vascular disease and cerebrovascular disease (Johnson et al., 2007). In fact, hyperglycaemia, impaired glucose metabolism and IR can be used as an independent predictor of CVDs such as atherosclerosis (Pyörälä et al., 2000). Development of T2DM can lead to the pathogenesis of several macrovascular and microvascular changes which include atherosclerosis, nephropathy and retinopathy (Camer et al., 2014). The main components of T2DM include elevated plasma

glucose, atherogenic dyslipidaemia, pro-inflammatory state, vascular dysfunction, pro-thrombotic state (Fletcher and Lamendola, 2004). The increased insulin levels increase sympathetic nerve activity and cause arteriolar dilation in skeletal muscles via the stimulation of nitric oxide (NO) production in endothelial cells (Esler et al., 2001). However, with the progression of IR, the vasodilatory effect induced by insulin is abolished resulting in vasoconstriction and an increase in total peripheral resistance and blood pressure (Masuo et al., 2010).

The risk of developing coronary artery disease, stroke and peripheral arterial disease is four times higher in patients with T2DM and MetS than in those without (Thiruvoipati et al., 2015). Excess adipose tissue in obese individuals also secrete several vasoactive peptides and adipokines which may impair the vasodilatory effect of insulin resulting in the development of hypertension (Andersson et al., 2016). High-density lipoprotein cholesterol (HDLC) has anti-atherosclerotic and anti-inflammatory properties, however, low HDLC levels due to MetS progression is associated with an increased risk to develop CVDs and stroke (Grundy, 2016). Therefore elevated levels of apoB containing lipoproteins induced by dyslipidaemia could cause hypertension by mechanisms partially related to obesity and IR (Pringle and Butler, 2016, Olga et al., 2017). The disorders in lipid and glucose metabolism associated with IR also causes hypertension by inducing vascular damage, especially damage to the arteriolar endothelium, thus impairing NO release and causing endothelial dysfunction (Cubbon et al., 2016). The impaired glucose and lipid metabolism which affect cardiovascular function is also known to negatively impact hepatic physiology, with the development of steatosis, inflammation and fibrosis which all contribute to the development of NAFLD as described in the next section of the review of the literature.

2.4. NON-ALCOHOL FATTY LIVER DISEASE

2.4.1 Characteristics of non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is defined as "the presence of macrovascular steatosis in the presence of less than 20 g of alcohol ingestion per day" (Tolman and Dalpiaz, 2007). NAFLD encompasses a wide spectrum of hepato-clinicopathological conditions that ranges from hepatic steatosis to hepatic necroinflammation or steatohepatitis (NASH) which can progress to hepatic fibrosis or cirrhosis and eventually hepatic carcinoma or hepatocellular cancer (Day and James, 1998, Basaranoglu et al., 2014). NASH is regarded as the most severe form of NAFLD and is accompanied by lipid peroxidation and the generation of free radicals which in turn trigger an inflammatory response and activation of hepatic stellate cells resulting in fibrosis (Castro et al., 2011, Dietrich and Hellerbrand, 2014). NAFLD develops in the absence of excessive alcohol consumption, steatogenic medication or underlying steatogenic pathologies (Basaranoglu et al., 2014). The steatosis that accompanies the pathogenesis of NAFLD leads to hepatic fat build up and an increase in total liver mass of over 5% (Collier, 2007, Basaranoglu et al., 2014, Li and Hsieh, 2014). Hepatic lipid accumulation of between 5-10% is considered as fatty liver disease (Younossi et al., 2011). In both human clinical and experimental animal studies, the progression of NAFLD is closely linked to the development of health outcomes associated with MetS, as such NAFLD is considered to be the "hepatic manifestation" of MetS (Lonardo et al., 2015, Asrih and Jornayvaz, 2015).

2.4.2 Prevalence of non-alcoholic fatty liver disease

NAFLD has a high global prevalence which is increasing (Al Rifai et al., 2015). According to Bellentani et al. (2010), the recent increase in the global prevalence of NAFLD ranges from 6.3-33% with an average of 20% of the population, depending on the diagnostic tool used. When other co-morbidities are considered, even higher prevalence is reported. The prevalence of NAFLD in obese diabetic individuals can be as high as 70-90% (Castro et al., 2011). The increase in the prevalence of NAFLD has been identified as a major health problem leading to an

increase in mortality and morbidity (Cusi et al., 2017). The prevalence of NAFLD parallels the increase in the prevalence of MetS and obesity and the burden of NAFLD is predicted to increase (Zarghani et al., 2016).

2.4.3 Aetiology and pathophysiology of non-alcoholic fatty liver disease

Several factors have been implicated in the rise of the NAFLD pandemic. The main risk factors that contribute to the development of NAFLD include, but are not limited to the adoption of sedentary lifestyles, obesity, insulin resistance, genetics and altered dietary patterns such as excessive consumption of Western high-calorific diets that are high in fat and carbohydrate content, particularly fructose (Castro et al., 2011, Lim et al., 2010, Softic et al., 2016). There are several dietary elements with added fructose and these include fructose-sweetened carbonated beverages, cakes, candy, processed foods and artificial sweeteners (Softic et al., 2016, Tappy and Lê, 2010).

Several metabolic pathways are involved in the pathogenesis of NAFLD and these include but are not limited to; i) an increase in extra-hepatic mobilisation of fatty acids from visceral adipose tissue due to lipolysis causing an influx of triglycerides into the liver, ii) increased FFAs supply to the liver due to high dietary fat intake iii) impaired hepatic β-oxidation of FFAs from mitochondrial dysfunction iv) increased hepatic DNL and v) decreased export of FFAs due to reduced synthesis and secretion of VLDL (Musso et al., 2009, Cusi et al., 2017).

Day and James (1998) proposed a “two-hit hypothesis” in an effort to explain the development of NAFLD. According to this hypothesis, the pathogenesis of NAFLD requires a “double hit”. The “first hit” results in the accumulation of hepatic lipids. The accumulation hepatic lipids is primarily due to IR. Insulin resistance triggers the secretion of adipokines from adipocytes, changes the rate of production and transport of triglycerides (TGs) by hepatocytes (Hashimoto et al., 2013). Insulin resistance also increases lipolysis in adipocytes which in turn causes the release of FFAs thus exposing the liver to excess FFAs (Castro et al., 2011). In the “first hit” of

NAFLD pathogenesis, there is also DNL which increases intra-hepatic TG levels resulting in steatosis (Castro et al., 2011).

The progression of hepatic steatosis to NAFLD in the second "hit" causes excessive stimulation of mitochondrial β-oxidation of FFAs resulting in impaired mitochondrial function (Tessari et al., 2009). The resultant mitochondrial dysfunction causes the production of ROS from the excessive flux of electrons in the electron transport chain (Ramalho-Santos et al., 2008). The oxidative stress will trigger the release of pro-inflammatory cytokines (TNF-α and IL-6), migration of white blood cells and production of collagen from hepatic stellate cells causing fibrosis and lipid peroxidation (Hotamisligil, 2006, Rector et al., 2008). Pro-inflammatory cytokines produced in response to oxidative stress will also contribute to the development of peripheral and hepatic IR, an increase in lipolysis and more FFA influx into the liver which causes further hepatocellular damage (Naik and Dixit, 2011).

Epidemiological studies have linked the recent increase in the prevalence of obesity and NAFLD with excessive fructose consumption (Lim et al., 2010). Dietary fructose intake also increases hepatic fructose transporter-5 (GLUT-5) gene expression and lipid peroxidation (Douard and Ferraris, 2008). Fructose is an important hepatic substrate for the synthesis of FFAs during DNL (Castro et al., 2011). Hepatic metabolism of fructose causes the generation of uric acid with transient ATP depletion from the phosphorylation of fructose (Yang et al., 2017). The depletion of ATP and production of uric acid are responsible for the lipogenic and pro-inflammatory effect of fructose (Shih et al., 2015). In light of the aetiology and pathophysiology of NAFLD, it is important to correctly diagnose it before effective treatment and management strategies can be implemented. These NAFLD diagnostic criteria are discussed in the following section.

2.4.4 Diagnosis of non-alcoholic fatty liver disease

The diagnosis of NAFLD is dependent on the presence of hepatic lipid droplets using a variety of biochemical, imaging and histomorphological analyses (Hashimoto et al., 2013). The most commonly used surrogate biomarkers of liver function in NAFLD are the elevation of plasma

alanine amino transferase (ALT), aspartate amino transferase (AST) and gamma-glutamyl transferase (GGT). These markers of liver damage are strongly associated with fat accumulation in the liver and are considered as good predictors of liver function (Dodurka and Kraft, 1995). However, the gold standard method for the diagnosis of NAFLD is liver biopsy (Marchesini et al., 2003). Based on histomorphometric techniques and universally accepted scoring systems, steatosis in NAFLD is considered as fat being more than 5% of the liver mass (Kleiner et al., 2005, Torruellas et al., 2014). Due to the invasive nature of biopsies, other non-invasive techniques such as ultrasonography, computed tomography and magnetic resonance imaging (MRI) are preferred (Saadeh et al., 2002, Oliva and Saini, 2004). The disadvantages of the new non-invasive imaging techniques are that, unlike the biopsies, they fail to detect inflammation and the early stages of fibrosis (Mishra and Younossi, 2007). Following the correct diagnosis of NAFLD, several treatment strategies described in the next section may be used.

2.4.5 Treatment and management of non-alcoholic fatty liver disease

Studies have shown that intervention and management criteria that emphasises on lifestyle modification coupled with cognitive behavioural therapy results in a significant change in health markers of NAFLD (surrogate biomarkers of liver function and steatosis) (Roberts et al., 2017). Lifestyle changes include reducing excessive calorific intake and an increase in physical activity or exercise. More details on the role of lifestyle modification and management of NAFLD and MetS are provided in section 2.6. Other therapeutic methods used to manage NAFLD involve the use of pharmacological drugs. Examples of such drugs include sibutramine, which is used to reduce appetite and increase energy expenditure (Day and James, 1998). Orlistat, a drug that inhibits enteric lipase, causing fat malabsorption is used to reduce body weight (Hanefeld and Sachse, 2002). Some hypoglycaemic and insulin-sensitising drugs are also used to manage NAFLD. These include the use of thiazolidinediones such as pioglitazone which attenuates hepatic metabolic dysfunction by normalising plasma liver enzyme levels steatosis and hepatic inflammation (Scheen, 2001). Metformin, a biguanide that stimulates mitochondrial β-oxidation

and suppresses lipogenic genes, reduces the risk to develop IR by promoting the use of FFAs as an energy source (Tolman and Dalpiaz, 2007).

Since NAFLD is closely associated with MetS health outcomes such as dyslipidaemia, the treatment of dyslipidaemia may be considered as indirectly treating NAFLD. Anti-hyperlipidaemia medication has been used as a treatment option for NAFLD and coupled with lifestyle changes results in significant improvement in hepatic steatosis and liver function (Chatrath et al., 2012). Fibrates such as fenofibrate and statins such as atorvastatin are known to reduce hypertriglyceridaemia and positively affect hepatic lipid metabolism and content (Maki et al., 2016). Fibric acid derivatives such as gemfibrozil have also been used to treat NAFLD due to their lipid-lowering effects (Basaranoglu et al., 1999, Antonopoulos et al., 2006)

NAFLD progression is also characterised by inflammation, oxidative stress and damage, as such the use of anti-oxidants may also protect against hepatocellular damage by ROS. Vitamin E is one such anti-oxidant that is effective as a potential therapeutic nutraceutical for the management of NAFLD (Maki et al., 2016). Extreme therapeutic approaches include bariatric surgery which is known to improve MetS and reduce fat accumulation in the hepatocytes (Angrisani et al., 2017).

Recently, it has become increasingly clear that susceptibility to develop health outcomes associated with MetS and NAFLD is strongly affected by exposure to an adverse early life developmental environment during pregnancy and postnatal life (Zheng et al., 2016). The following section provides a detailed description of the role of programming in the development of metabolic syndrome.

2.5 PROGRAMMING OF METABOLIC SYNDROME

While it is clear that lifestyle choices and behaviours contribute to the MetS epidemic, accumulating epidemiological and empirical evidence from animal studies suggests that the nutritional status during critical stages of development in early life can “program” individuals to develop the metabolic syndrome later in life (Alfaradhi and Ozanne, 2011, Saad et al., 2016). In

the following sections, I will review the recent literature from epidemiological and animal studies that have established a link between the peri-conceptual, foetal and neonatal phases of life and the subsequent development of adult obesity and MetS associated complications.

2.5.1 Pre-conceptual and maternal programming of metabolic disorders

Maternal obesity is increasingly prevalent and may affect the long-term health of the child. The surge in the prevalence of overweight and obesity in women of childbearing age continue to increase globally, presenting a worrying picture of transgenerational transmission of several susceptibility genes to their offspring (Rinaudo and Wang, 2012). Maternal obesity among women of reproductive age plays a direct role in the transmission of obesogenic and diabetogenic traits from one generation to the next (Tain et al., 2015). Extensive epidemiological data provide an association between maternal obesity and nutrition during gestation and offspring obesity. A number of animal models have been established in order to uncover the underlying mechanisms contributing to the programming of physiological systems (Goran et al., 2013).

Maternal environment especially nutrition during pregnancy and the early post-natal period is a critical factor that may alter the development of metabolic systems, which may also predispose the offspring to the development of cardio-metabolic diseases such as obesity, T2DM and CVDs later in adulthood (Li et al., 2011, Penfold and Ozanne, 2015). A growing number of clinical and experimental studies suggest that maternal obesity and excessive consumption of high-energy diets can program the offspring physiological system, predisposing them to MetS in adulthood (Vickers, 2011). This occurs as a result of the irreversible transmission of several susceptibility genes and obesogenic or diabetogenic traits by obese mothers to their children, resulting in cyclical transgenerational transmission of obesity (Agius et al., 2013). Moreover, the epigenetic modification of metabolic genes, some of which may be transmitted through gametes, may also result in the epigenetic modification of the genes in the foetal genome due to the *in utero* environment presented and influenced by the mother's obesity (Kitsiou-Tzeli et al., 2017).

Experimental animal studies have shown that exposure of dams to a high fructose diet during gestation and lactation promoted offspring obesity through several mechanisms that include but not limited to: i) direct effects of dietary fructose on the development of adipose tissue ii) disruptive effects of fructose on the hypothalamic regulating pathways of energy balance iii) disruptive effects of fructose on the neuro-endocrine signalling between the adipose tissue and the hypothalamus (Figure 2.5) (Goran et al., 2013, Saad et al., 2016). Despite the widespread consumption of fructose-containing foods and sugar-sweetened beverages and the rising incidence of maternal obesity, little is known about the long-term effects of direct neonatal fructose consumption on the development of metabolic health of the offspring later on in adulthood. Although the mechanisms are not yet fully elucidated, this programming is generally considered an irreversible change in developmental trajectory (Vickers, 2011).

2.5.2 *In utero* programming of metabolic disorders

There is no doubt that much of the rise in the prevalence of metabolic disorders can be attributed to lifestyle factors such as the excess consumption of energy-dense foods and a decrease in physical activity or exercise. However, the 'foetal origins' hypothesis, first proposed by Barker (2001), states that metabolic disorders that manifest in adulthood may originate during an individual's foetal development, due to changes in the uterine environment that the individual is subjected to (Neitzke et al., 2011). This has led to the proposition of the developmental origins of health and disease (DoHAD) hypotheses, which suggests that the environmental factors and conditions presented during critical stages of development in early life (pre-conceptual, prenatal, gestation and the early post-natal period) can lead to "programmed" permanent alterations in the state of health or disease later in adult life (Ramírez-Espinosa et al., 2011, Gluckman et al., 2015).

According to the DoHAD model, exposure of the developing foetus to sub-optimal conditions during uterine life results in permanent programming of several developmental processes in expectation or anticipation of a similar environment condition later in life (Armitage et al., 2005, McArdle et al., 2006). The foetus adapts its physiological development to adverse environmental

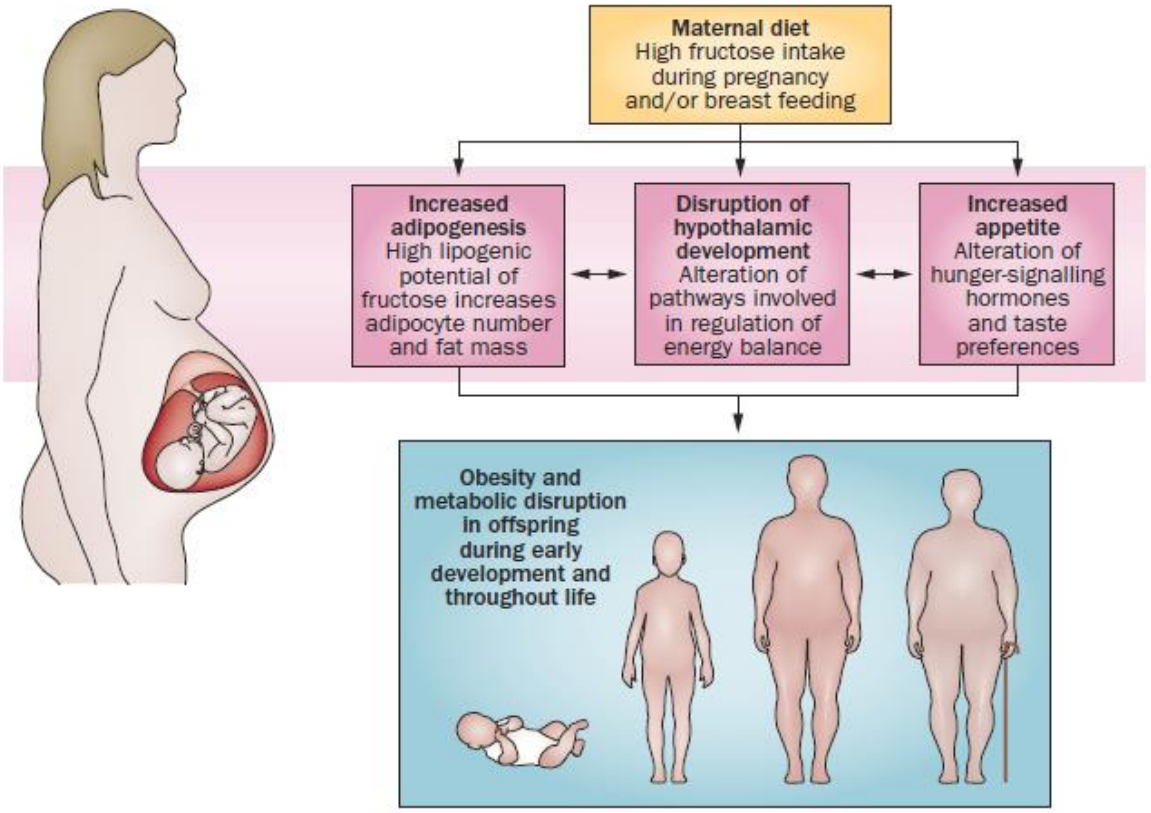


Figure 2.5: The link between obesity and fructose exposure during critical developmental periods.

Original figure adapted from Goran et al. (2013).

cues *in utero* with permanent readjustments in homeostatic systems to maximise its immediate chances of survival postnatally (Heindel et al., 2015). These adaptations may include resetting of set-points of metabolic homeostasis and endocrine systems and the down-regulation of growth that is commonly reflected in an altered birth phenotype. However, these adaptations, known as predictive adaptive responses, may be disadvantageous in postnatal life and may lead to an increased risk of chronic non-communicable disease in adulthood if there is a mismatch between the in utero and postnatal nutritional environments (Vickers et al., 2007). For instance, poor foetal nutrition *in utero* drives metabolic adaptations to maximise the chances of survival in conditions of on-going nutritional deprivation (Hales and Barker, 2001, Barker, 2001).

Such adaptations would be beneficial if there are continued poor nutritional conditions post-natally, however if the conditions during the early post-natal period are ideal and providing plenty nutrition, the mismatched offspring phenotype may not be able to deal with the altered environment resulting in the predisposition to the development of a wide range of metabolic diseases (Simmons, 2005). More recently, the "predictive adaptive response (PARs)" hypothesis proposes that the degree of mismatch between the pre- and post-natal environments is a major determinant of the subsequent development of disease (Gluckman et al., 2008). Thus, it is thought that whilst these changes in foetal physiology may be beneficial for short-term survival *in utero,* they may be maladaptive in post natal life, contributing to poor health outcomes when offspring are exposed to catch-up growth, diet-induced obesity and other metabolic factors (Gluckman et al., 2015).

Murine experimental models have proved useful in the investigation of the potential mechanisms involved in 'foetal programming' (Cottrell and Ozanne, 2008). Rats and mice treated with a protein-deficient diet during pregnancy produced offspring that had low-birth weight and developed metabolic derangements with age (Low et al., 2017). Some rodent studies have shown that feeding mothers a high fructose diet during gestation resulted in the birth of offspring that displayed a phenotype similar to that human MetS and had an increased risk of developing obesity in adult life (Saad et al., 2016). Some of the observed deleterious changes in metabolic-

endocrine function in offspring born to fructose-fed dams include disturbance of the hypothalamic circuitry controlling appetite, accumulation of visceral adipose tissue, hyperleptinaemia and impaired insulin sensitivity (Ghezzi et al., 2011, Clayton et al., 2015, Rodríguez et al., 2016). The above-mentioned experimental studies provide a clear insight into the importance of the nutritional status during early life in the development of metabolic disorders later in adult life.

2.5.3 Neonatal programming of metabolic syndrome

In addition to maternal nutritional status during gestation, the early postnatal period is also a critical "window" of plasticity in which the development of the offspring can be influenced (Vickers, 2011). As such developmental programming is not limited to the *in utero* environment alone, but continues even in the post-natal period where there is continuous growth and rapid developments of physiological systems (Wang, 2013). Animal studies have shown that providing offspring with a high carbohydrate formula milk diet in the neonatal period led to the development of obesity in adult offspring, which was transmitted to the next generation even after females received a standard chow diet post weaning (Srinivasan and Patel, 2008). The effect of fructose administration during critical developmental periods was also examined by feeding lactating rats either tap water or a 10% (w/v) fructose solution and it was shown that offspring of fructose-fed rats had increased body mass, decreased hypothalamic sensitivity to exogenous leptin, increased food intake, IR and increased adipose tissue deposition (Alzamendi et al., 2010). Furthermore, in some studies in which 10-week old pups whose dams were fed a high fat and high-fructose diets during gestation and lactation showed that these rats had a higher preference of high-energy foods and had more body fat, higher glucose and TG levels (Ghusain-Choueir, 1995; Bayol et al 2008). Extensive epidemiological and experimental evidence indicates that a sub-optimal environment during the neonatal development in both humans and animals may program offspring susceptibility to the development of chronic diseases such as obesity and diabetes later in life (Spencer, 2012, Wang, 2013). This evidence demonstrates the

significance of the neonatal period in predisposing offspring to MetS and related metabolic disorders.

Several studies highlighted the importance of maternal diets during gestation and lactation in the predisposition of offspring to the development of undesirable metabolic disorders such as the MetS in later life. Few studies, however (Ibrahim et al., 2017) have investigated the effects of directly feeding or treating neonatal rats on adult health. This study involves the treatment of neonatal rats with fructose and oleanolic acid in the neonatal period in an effort to investigate the potential protective effect of OA against the subsequent development of metabolic disorders later in adulthood.

Rats follow an altricial mode of development, as such, the rat pups are born relatively 'premature' when compared to precocial species and consequently they complete their post-conceptual maturation during the neonatal period (Scheiber et al., 2017). The early neonatal period in rats is equivalent to the last trimester of human gestational development (Sengupta, 2013). The neonatal period of rats is a critical period of developmental plasticity which can be used as a good model of neonatal programming as it can be targeted for the manipulation and triggering of epigenetic changes, resulting in long-term effects on health outcomes associated with MetS. The role of epigenetic changes in the development of MetS is highlighted in the next section.

2.5.4 The role of epigenetic changes in the development of metabolic syndrome

Epigenetics is defined as "somatically heritable states of gene expression resulting from changes in chromatin structure without alterations in the DNA sequence" (Masuyama and Hiramatsu, 2012). These changes include DNA methylation, histone modifications as well as chromatin remodelling (Qiu, 2006). Studies have shown that nutrient intake during critical periods of development can affect the epigenetic phenomena by modifying DNA through methylation and histone modification (Choi and Friso, 2010). DNA methylation and histone modification alter how critical genes associated with the development of physiological and pathological processes,

including foetal and neonatal metabolic programming are expressed (Choi and Friso, 2010). Lately, epigenetics has been accepted as a plausible explanation for the development of several metabolic disorders such as T2DM, obesity and inflammation (Choi and Friso, 2010). A study on mice that were exposed to a high-fat diet *in utero* showed that they developed a T2DM phenotype that was transmitted to their offspring (Gniuli et al., 2008). Another study on rats showed that the offspring of high-fat diet -induced obese dams had higher body masses, developed hypertension and hyperglycaemia (Samuelsson et al., 2008). Taken together, the abovementioned studies provide considerable evidence that exposure to a nutritional insult early in life might lead to the development of MetS-associated health outcomes through epigenetic modifications of the genes encoding metabolic regulatory hormones and enzymes in the offspring (Masuyama and Hiramatsu, 2012).

Having highlighted the pathophysiology, risk factors and clinical manifestations of MetS and NAFLD as well as the role of programming and the epigenetic phenomena in the development of these metabolic disorders later in adult life, in the following sections, I now focus on the strategies that are used in management of the health outcomes associated with MetS.

2.6 MANAGEMENT OF METABOLIC SYNDROME AND ITS RISK FACTORS

2.6.1 Aim of the management of metabolic syndrome

Given that MetS and related co-morbidities now pose an excessive burden to healthcare systems, increasing interest in their management has become a priority. Preventive or therapeutic interventional strategies for MetS and its associated complications are urgently needed due to the pandemic increase in the prevalence of MetS. According to the ATPIII, management of MetS has two objectives namely to i) reducing the underlying causes of MetS and its components and ii) identification of MetS risk factors that require treatment (Grundy et al., 2005). With the rise in the prevalence of MetS, the care for patients with MetS is required in order to ameliorate the harmful effects caused by lifestyle choices (diet, exercise and smoking) and genetic factors. There are two general approaches that are used to treat MetS. Therapeutic lifestyle changes make

up first-line therapy for MetS, with increased emphasis on diet, physical activity and weight reduction (Keck et al., 2016). Current clinical studies have shown that the use of lifestyle modifications in the management of MetS and obesity is effective in preventing the development of adverse MetS-health outcomes.

The second approach involves the use of pharmacological drugs to directly treat risk factors of the MetS (Maksimov et al., 2016). In the following sections, I will provide more details on the different management strategies for MetS that are currently in use.

2.6.2 Management of underlying metabolic risk factors: Lifestyle choices

The primary therapy for the treatment of the MetS and its complications involves various lifestyle adjustments such as weight loss, diet modifications and physical activity, which aim to alleviate the underlying risk factors (Grundy et al., 2005, Kaur, 2014).

2.6.2.1 Physical activity and exercise

The National Cholesterol Education Programme (NCEP) Adult Treatment Panel III guidelines for the treatment of MetS recommended an increase in physical activity as a first-line treatment of MetS (Expert Panel on Detection, 2001). Physical activity coupled with diet and weight control are regarded as the cornerstones in the prevention and treatment of MetS (Lakka and Laaksonen, 2007). Epidemiological studies have shown that an increase in physical activity and exercise promotes the development of desirable parameters such as improved BMI, lower blood pressure, triglyceride levels and HDLC (Blumenthal et al., 2000, Balducci et al., 2010). A randomised controlled trial has also indicated that regular aerobic exercise training confers positive health benefits such as good cardiorespiratory fitness and attenuates the development of individual components of metabolic and cardiovascular risk factors related to the MetS (Ades and Savage, 2014).

2.6.2.2 Dietary choices

In addition to exercise, dietary modifications have been used in the management of MetS and can also be beneficial for many of the components of the metabolic and cardiovascular risk factors. The favourable effects of diet on the components of MetS are stronger when combined with exercise, as supported by recent evidence from randomised controlled trials in which physical activity combined with diet reduced BMI and blood pressure (Johns et al., 2014). In a study involving obese male volunteers, dietary counselling administered by the physician reduced the risk of developing MetS and T2DM (Bo et al., 2007). Other interventional studies suggest that diets with low saturated fat, high fibre, high fruit and vegetables and low glycaemic indices decrease the risk for diabetes and CVDs (Sylvetsky et al., 2017). The abovementioned findings provide evidence that diet may be used as an effective strategy for lowering the risk of developing MetS and its associated complications. However, a dietary approach has to be used together with increased physical activity or exercise and weight loss.

2.6.2.3 Weight loss

Reduction of excessive body weight is considered as one of the key elements in preventing and treating of the MetS risk factors. Epidemiological and clinical prospective studies and clinical trials have shown that a reduction of body weight by between 5-10 % significantly decreases the risk of developing blood pressure, improve blood lipid profile, body adiposity, insulin sensitivity and glucose tolerance (Watts et al., 2005). Weight loss can be achieved through an increase in aerobic exercise and reducing the excessive consumption of high-energy diets or a combination of both (Fiuza-Luces et al., 2013, Lavie et al., 2015).

2.6.3 Management of metabolic risk factors: Conventional pharmaceutical treatments

For patients whose MetS complications are not attenuated by lifestyle modifications, the use of pharmacological agents is recommended. Drug therapy for the MetS focuses on improving the individual risk factors such as adiposity, dyslipidaemia, hypertension and hyperglycaemia (Grundy et al., 2005). Diabetes is a metabolic and endocrine disorder, characterised by persistent

hyperglycaemia, glucose intolerance and IR (Alberti and Zimmet, 1998, ADA, 2014c). Extensive research has confirmed that inflammation is also implicated in the pathogenesis of diabetes and its co-morbidities (Shoelson et al., 2006, Valle et al., 2016). Diabetic patients exhibit features of an inflammatory process that is characterised by the presence of pro-inflammatory cytokines, immune cell infiltration and impaired metabolic function (Hotamisligil, 2006). Several anti-diabetic drugs are often prescribed to diabetic patients in order to decrease the adverse effects of diabetes (Gothai et al., 2016). Metformin administered in a group of volunteers with MetS, coupled with moderate aerobic exercise reduced the incidence of MetS by 41% (Smith et al., 2007).

The prolonged use of pharmacological agents in the treatment or management of MetS health outcomes is not always successful as a result of the adverse side-effects. Existing drugs that are currently used for T2DM have limited efficacy and are associated with secondary complications such as CVDs, renal failure, hepatic damage, dizziness, mental disorders to name but a few (Athyros et al., 2006).

2.6.4 Medicinal plants and the management of metabolic syndrome

Owing to the adverse side-effects associated with the prolonged use of conventional pharmacological agents, identifying novel medicinal agents, especially those that are derived from natural products offer alternative possibilities for future development of successful anti-diabetic therapies (Camer et al., 2014). Several medicinal plants have been used since time immemorial to manage and prevent the development of T2DM and associated conditions (Bailey and Day, 1989, Sofowora, 1996, WHO, 2001, Xi et al., 2008). Several pharmaceutical drugs that have been approved for clinical trials and used within the last few decades were derived from plants. Examples of such drugs that have been in long-term use include artemisin, taxoids and camptothecins (De Smet, 1997, Saklani and Kutty, 2008).

Phytochemical compounds have been shown to confer some protection against the pathology of T2DM through the attenuation of inflammatory and oxidative stress mediators (Gothai et al.,

2016). There has been a drive towards the use of safe and natural herbal medicines as potential alternatives to synthetic drugs with fewer side-effects (Patel et al., 2012). The therapeutic value of natural medicinal plants that are commonly used can be attributed to the presence of a variety of biologically active phytochemical compounds which may act individually, additively or synergistically to improve health (Raskin et al., 2002).

The natural phytochemical compounds are currently in higher demand than the synthetic medicines due to their easy accessibility, affordability, availability, efficacy and fewer side effects (Phillipson, 2001, Nasri and Shirzad, 2013). Studies on some of these phytochemical compounds have shown that they possess anti-diabetic, anti-oxidant, anti-obesity and anti-inflammatory properties among other beneficial properties (Dembinska-Kiec et al., 2008). The most common biologically active phytochemicals that are found in medicinal plants used for the treatment of T2DM and other MetS risk factors include flavonoids, saponins, tannins, glucosinolates, carotenoids, phytates, myrcelin, glucosides, pectins and phyto-oestrogens (Tiwari and Rao, 2002, Leiherer et al., 2013). The potential of these bioactive phytochemicals to improve health lies in their ability to lower blood pressure, reduce oxidative stress and inflammation, improve lipid profiles and enhance glucose metabolism (Chang et al., 2013).

The role of phytochemical compounds as protective dietary supplements has become very important especially with favourable effects on the prevalence of several chronic disorders and health outcomes associated with MetS (Liu, 2003, Dillard and German, 2000). Nutritional and pharmacological interventions during the critical period of development may prevent or reverse the metabolic consequences of developmental programming. Due to their safety profile, the therapeutic potential of several phytochemicals that occur naturally in fruits, vegetables and ethnomedicinal plants are under investigation. Oleanolic acid is one such phytochemical that was selected in this study to investigate its potential protective effect against the subsequent development of MetS in adulthood when administered in the neonatal period. In the next section I will discuss the several beneficial pharmacological properties of oleanolic acid.

2.7 OLEANOLIC ACID

Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) is a biologically active pentacyclic triterpenoid phytochemical and a common aglycone of many saponins that exists widely in several plant species belonging to the *Oleaceae* family such as olives, (*Olea europaea*) (Sánchez-Quesada et al., 2015, Lin et al., 2016). Oleanolic acid (OA) is also found in foodstuffs such as clover flower, olive leaves, virgin olive oil, mistletoe sprouts and fruits (apples and dates) (Yoshikawa and Matsuda, 2000, Camer et al., 2014, Rodriguez-Rodriguez, 2015) and some commonly used medicinal plants (*Crataegus pinnafitida* and *Eclipta alba*) (Liu, 2005, Jäger et al., 2009).

2.7.1 Pharmacological properties of oleanolic acid

Oleanolic acid possesses several potential pharmacological properties that exhibit a wide range of therapeutic activities without adverse side effects (Castellano et al., 2013b, Ayeleso et al., 2017). Oleanolic acid was selected for the present study due to these pharmacological activities which include hepatoprotection against chemical or fructose-induced liver injury (Liu et al., 1995a, Nyakudya et al., 2017), anti-inflammation (Singh et al., 1992, Nkeh-Chungag et al., 2015) anti-diabetic (Wang et al., 2011, Zeng et al., 2012, Castellano et al., 2013b), anti-oxidant activities (Yin and Chan, 2007, Tsai and Yin, 2008, Tsai and Yin, 2012, Tsao and Yin, 2015) and anti-glycosilative effects (Xi et al., 2008, Wang et al., 2010b). In the following sub-sections I will review each of the abovementioned beneficial properties of OA in detail.

2.7.1.1 Anti-oxidant properties of oleanolic acid

Oxidative stress caused by reactive oxygen species (ROS) plays an important role in the processes involved in the pathophysiology of chronic inflammation associated with the pathogenesis of MetS. The intracellular redox homeostasis is maintained by balancing the production of ROS with their removal through intracellular anti-oxidant defence systems (Chen and Kunsch, 2004). Activation of cytoprotective anti-oxidant genes can suppress oxidative stress associated with MetS. Therapeutic approaches that reduce oxidative stress contribute to the

improvement of glucose metabolism and prevention of the metabolic complications of MetS and T2DM. Currently, there are four types of chemical synthetic drugs, (sulphonylureas, biguanides, α-glucosidase inhibitors and euglycaemic agents) that possess anti-oxidant properties and are being used clinically for the treatment and management of T2DM (Wang et al., 2011). In previous studies, OA has been shown to possess anti-oxidant properties through enhancing cellular anti-oxidant defences (Wang et al., 2010a). A study using a rodent model showed that OA ameliorated tert-butyl hydroperoxide (tBHP)-induced oxidative injury through stimulating the cellular production of anti-oxidants and the upregulation of the expression of genes for key anti-oxidant enzymes (Wang et al., 2010b). OA also activates c-Jun N-terminal kinases (JNK) and extracellular signal kinases (ERKs) which are also involved in the anti-oxidant activity of OA (Wang et al., 2010b).

2.7.1.2 Anti-inflammatory properties of oleanolic acid

According to the American Diabetes Association (ADA), diabetes is a metabolic and endocrine disorder that is characterised by hyperglycaemia and glucose intolerance (Alberti and Zimmet, 1998, ADA, 2014a). The pathogenesis of these metabolic disorders is closely linked with inflammatory processes (Xu et al., 2003, Shoelson et al., 2006). The inflammatory processes in diabetic patients are mediated by pro-inflammatory cytokines which cause immune cell infiltration and metabolic dysfunction (Hotamisligil, 2006). OA administered in adulthood has been shown to possess anti-inflammatory properties through its modulation of inflammatory processes (Singh et al., 1992, Tsai and Yin, 2008, Tsai and Yin, 2012, Tsao and Yin, 2015).

2.7.1.3 Anti-diabetic and hypoglycaemic properties of oleanolic acid

OA and other related triterpenoid compounds have been shown to possess hypoglycaemic effects in rodent studies (Gao et al., 2009, Gao et al., 2007). The anti-diabetic and hypoglycaemic effects of OA have been attributed to its ability to improve insulin signalling by enhancing insulin receptors and promoting glucose uptake into peripheral tissues through the upregulation of GLUT-4 transporters (Castellano et al., 2013b, Camer et al., 2014). OA also exhibits anti-

diabetic effects by reducing intestinal glucose absorption, increasing insulin sensitivity, improving lipid metabolism, decreasing endogenous glucose production (hepatic gluconeogenesis) and promoting body weight loss (Camer et al., 2014). Studies in adult animals have shown that OA administered in adult animal models possess beneficial effects against the development of diabetes and MetS by preserving β-cell functionality, improving insulin sensitivity and enhancing insulin secretion by pancreatic β-cell (Teodoro et al., 2008, Castellano et al., 2013b, Wang et al., 2013). OA has also been shown to attenuate fructose-induced hyperglycaemia through modulating enzymes involved in carbohydrate digestion, insulin secretion and signalling (Liu et al., 2013, Wang et al., 2013).

2.7.1.4 Hypolipidaemic properties of oleanolic acid

A study conducted in insulin resistant and obese rats showed that administration of OA prevented the excessive accumulation of fat and TGs, possibly through the inhibition of mitochondrial oxidative stress via activation of Nrf2–GCLc signal (Wang et al., 2013). In some rodent studies, OA has been shown to improve lipid metabolism by downregulating the expression of lipogenic genes (de Melo et al., 2010, Chen et al., 2017). OA treatment has also been shown to possess therapeutic effects on high cholesterol diet-induced hyperlipidaemia by inhibiting the intestinal absorption and storage of cholesterol in a rodent model (Liu et al., 2007).

2.7.1.5 Effect of oleanolic acid on body weight

In addition to its glucose-lowering and hypolipidaemic effects, OA also causes body weight loss when administered in obese and diabetic adult rats (Wang et al., 2011). In another experimental study, oral administration of triterpenoid compounds (OA and ursolic acid) decreased body mass gain in HFD-induced obesity in rats (Camer et al., 2014). Based on the findings from the current study, it is possible that OA administered in the neonatal period of development programmed for normal body masses in fructose-fed female rats.

2.7.1.6 Hepatoprotective effect of oleanolic acid

Consumption of high fructose-containing beverages has been linked to metabolic disorders, and contributed to the development of non-alcoholic fatty liver disease (NAFLD) in human and animal models (Ouyang et al., 2008, Lim et al., 2010, Mock et al., 2017). The earliest stage in the development of NAFLD is the excessive accumulation of TGs in the liver (hepatic steatosis) in the absence of excessive alcohol consumption (Zarghani et al., 2016). If left untreated, NAFLD can progress to non-alcoholic steatohepatitis (NASH), which is characterised by hepatocyte injury, hepatic inflammation and collagen deposition or fibrosis (Rinella, 2015). One of the complications of the pathogenesis of NAFLD and hepatocellular damage is the hepatocellular enzyme leakage, as indicated by an increase in plasma enzyme activity of aspartate amino transferase (AST) and alanine amino transferase (ALT) (Camer et al., 2014). The hepatoprotective effect of OA has been demonstrated by its ability to reduce the increase in the activity of AST and ALT induced by the progression of NAFLD (Dufour et al., 2000, Ozer et al., 2008). OA has also protected against chemical-induced hepatic oxidative stress-induced liver damage by increasing the activities of anti-oxidant enzymes SOD2 and GPx1 (Liu et al., 1995b). An increase in the activity of these anti-oxidant enzymes results in the reduction of free radicals and lipid peroxidation. The anti-oxidant effect of OA therefore is beneficial in the treatment and prevention of oxidative stress-induced hepatic damage. OA administration is also known to increase gene expression of glyoxalase 1, an enzyme which metabolises methylglyoxal (Wang et al., 2010b). Methylglyoxal is important in the polyol pathway which is involved in the development of diabetic complications and liver damage (Seo et al., 2014). Reducing the levels of methylglyoxal attenuates liver injury and the progression of T2DM (Cederbaum et al., 2009).

Medicinal plants have widely been used either alone or as adjuvants for conventional treatment of a wide range of diseases including MetS, but few of these plants have been evaluated for their potential to prevent the development of MetS. The majority of studies that demonstrated the anti-oxidant, hypolipidaemic, anti-inflammatory, hepatoprotective and anti-diabetic properties of OA were performed in murine models after the weaning period. There is currently a paucity of

information from animal studies involving the oral administration of OA during the neonatal period which serves as an important window of developmental plasticity during which the predisposition to the development of MetS in later in adult life could possibly be prevented. OA administration in adulthood has proven to be effective in the treatment and management of MetS and its associated complications. OA is a good candidate for prophylactic therapy, especially when administered during the critical periods of developmental plasticity. It is, therefore, one of the objectives of this study to investigate the potential protective effect of neonatal oral administration of OA against the development of health outcomes associated with high fructose diet-induced MetS in adult male and female rats.

In the next chapter, I will provide a detailed description of the first experimental study in which I investigated the potential protective effects of short-term neonatal oral administration of OA against the development of fructose-induced oxidative damage, development of adverse health outcomes and maturation of the gastrointestinal tract in suckling male and female rat pups. A brief introduction and justification for conducting this short-term experimental study are provided. This is followed by a detailed description of the materials and methods used, the findings obtained and discussion of the results.

CHAPTER 3:

THE EFFECT OF NEONATAL ORAL ADMINISTRATION OF OLEANOLIC ACID ON THE DEVELOPMENT OF FRUCTOSE-INDUCED OXIDATIVE DAMAGE, ADVERSE HEALTH OUTCOMES AND MATURATION OF THE GASTROINTESTINAL TRACT IN FRUCTOSE-FED SUCKLING MALE AND FEMALE RATS

3.1. INTRODUCTION

Metabolic syndrome (MetS) is a prevalent, multifactorial and complex disease that is associated with a marked increase in the risk to develop metabolic disorders and major cardiovascular consequences (Khitan and Kim, 2013, O'Neill and O'Driscoll, 2015). The rise in the global prevalence of MetS has been attributed to the adoption of sedentary lifestyles that are characterised by low physical activity and the consumption of high-energy diets, especially those that contain fructose (Maarman et al., 2016, Tappy, 2017). For a detailed description of the contribution of lifestyle choices, particularly the role of dietary fructose, to the pathogenesis and prevalence of MetS, refer to section 2.2.

The excessive consumption of fructose causes the development of obesity through mechanisms that I described in section 2.2.3. Obesity is regarded as the main causative factor in the development of health outcomes associated with MetS (Furukawa et al., 2017). The accumulation of fat in obesity causes systemic oxidative stress via the production of reactive oxygen species (ROS) from the accumulating adipose tissue (Higdon and Frei, 2003, Rani et al., 2016). The overproduction of ROS by adipocytes contributes to the development of metabolic disorders by decreasing the expression of anti-oxidant enzymes (Sankhla et al., 2012). Oxidative stress, a result of the inability of the anti-oxidant cellular defence mechanisms to reduce ROS, also causes dysregulation of adipocytokines, increases the levels of pro-inflammatory cytokines and oxidative damage by altering mitochondrial bioenergetics (Fernández-Sánchez et al., 2011, Savini et al., 2013). Studies performed in adult animal models have shown that the activation of cytoprotective anti-oxidant genes can suppress the development of oxidative stress associated with MetS (Chen and Kunsch, 2004, Furukawa et al., 2017). Therapeutic approaches that reduce oxidative stress will, therefore, contribute to the improvement of glycaemic control and prevention of metabolic complications of MetS and T2DM (Pall and Levine, 2015).

Recent epidemiological and experimental animal studies have established a link between the peri-conceptual, foetal and early infant phases of life (Vickers, 2016a) and the subsequent development of adult obesity and MetS (Alfaradhi and Ozanne, 2011, Vickers, 2016b, Wang et

al., 2017). Rats have an altricial mode of development post-conception and the rat pups are incapable of foraging for their own food during the neonatal period (Scheiber et al., 2017). Rat pups have a brief and accelerated neonatal period compared to precocial animals. Rat pups develop rapidly during the neonatal period and attain sexual maturity by 6 weeks of age (Sengupta, 2013). In the early neonatal period of rat pups (post-natal day 1-13), their eyes are closed and they subsist entirely on their mother's milk (Sengupta, 2013).

From around post-natal day 14 up to weaning, the pups' eyes are open and there is developmental re-organisation of ingestive behaviour as the pups start to independently select and ingest solid foods in addition to their mother's milk (Quinn, 2005). Weaning of the pups is a milestone that represents the transition from dependence on the mother's milk to nutritional independence from milk and is characterised by the ingestion of more solid foods (Pérez-Cano et al., 2012). In the early post-weaning period, there is a gradual progression to adult function as the pups start to reliably ingest solid foods.

The early neonatal period (first couple of days) in rats can thus be compared to the last gestational trimester of *in utero* human development (Clancy et al., 2001, Quinn, 2005, Sengupta, 2013). This makes the neonatal period of rat development a critical window of plasticity in which the development of the physiological systems of the pups can be influenced (Vickers, 2011). Animal and epidemiological studies have shown that dietary and pharmacological manipulations during the perinatal suckling period have long-lasting and sometimes irreversible effects in adulthood (Lucas, 1998, McArdle et al., 2006, Pico et al., 2007, Gluckman et al., 2015). This means that developmental programming in rats is not only limited to the *in utero* environment but continues even in the early post-natal period (lactation) where there is continuous growth, rapid development and maturation of various physiological systems (Wang, 2013).

The effect of diet and nutrition, during the neonatal period, on neonatal growth and physiology is important not only because this is a critical stage of developmental plasticity, but also because it potentially has long-lasting positive or negative effects on health in adulthood. Any ingested

dietary material comes into contact with the gastrointestinal tract (GIT) first for digestion and absorption. The GIT is a source of several peptides and hormones that are involved in regulating GIT function and general metabolism (Date et al., 2000, Ahlman and Nilsson, 2001). Dietary or nutritional manipulations during the neonatal period may therefore cause long-term irreversible positive or negative effects on the development of the GIT and its metabolic function. Some studies in which bioactive phytochemicals in medicinal plant extracts were administered during the suckling period have shown that phytochemicals had a trophic effect on the GIT and caused precocious maturation of the GIT (Beya et al., 2012). Due to the limited data from epidemiological and human interventional trials in early life, the role of dietary manipulations on the development of the neonatal GIT using neonatal animal models holds the key to understanding the nutritional interaction during this important developmental period. As a result of the rapid growth, development and sensitivity of the GIT during the neonatal period, the neonatal suckling rat is an important model for GIT development in early life.

Existing standard treatment of MetS involves the use of pharmaceutical and dietary phytochemical agents that target specific biochemical pathways involved in the metabolism of nutrients (Dik, 2013, Lane, 2016). Phytochemicals have been used alone as nutraceuticals or in combination with standard treatments in the management of MetS (Wang et al., 2015). For this study, I selected oleanolic acid (OA) due to its proven beneficial pharmacological properties such as anti-diabetic, hypoglycaemic and anti-oxidant activities, as well as its ready availability in foodstuffs such as virgin olive oil, fruits and medicinal plants (Jäger et al., 2009). A detailed description of the pharmacological properties of OA is provided in section 2.7. The anti-diabetic effects of OA observed in adult animal studies can be attributed to its ability to preserve β-cell functionality, improving insulin sensitivity and attenuating fructose-induced hyperglycaemia (Liu et al., 2013, Castellano et al., 2013b).

OA has been shown to exhibit its anti-oxidant properties through enhancing the expression and activity of anti-oxidant enzymes such as glutathione peroxidase (GPx1) and superoxide dismutase (SOD2) (Liu, 1995, Wang et al., 2010a). An increase in the activity of the anti-oxidant

enzymes reduces free radicals and lipid peroxidation (Camer et al., 2014). A study conducted in insulin-resistant adult rats showed that OA administration prevented mitochondrial oxidative stress via the activation of Nuclear factor erythroid-derived 2-like 2- Glutamate cysteine ligase (Nrf2-GCLc) signal (Camer et al., 2014, Chen et al., 2015). The anti-oxidant effect of OA is therefore beneficial in the treatment and prevention of metabolic disorders induced by oxidative stress, especially when they are administered in the neonatal period.

Previous studies on the beneficial pharmacological effects of OA on MetS have been done in adult rats and none have been done in neonatal rats, especially in the early post-natal period, which is considered as the critical phase of development during which epigenetic changes are likely to cause metabolic changes that exert lifelong effects into adulthood. Moreover, despite the widespread beneficial properties of OA, there seems to be limited knowledge on whether its administration in the neonatal phase could protect against oxidative damage, development of negative health outcomes and the and precocious maturation of the GIT induced by the administration of fructose in the neonatal period.

3.1.1 Aim

The current study sought to investigate the potential protective effect of neonatal oral administration of OA against fructose-induced oxidative damage, the development of adverse health outcomes and precocious maturation of the GIT in suckling fructose-fed male and female pups.

3.1.2 Specific objectives

The specific objectives of the short-term neonatal experimental study were to determine the potential protective effects of neonatal oral administration of OA against the development of fructose diet-induced oxidative damage, negative health outcomes and precocious maturation of the GIT in suckling male and female pups by assessing:

a) Growth performance – terminal body mass, indices of linear growth as determined by the length of the tibiae and femurs

b) Gastrointestinal tract and visceral organ morphometry
c) Measurement of 12 biochemical health profile markers in plasma
d) Intra-hepatic lipid accumulation
e) Gene expression of anti-oxidant enzymes – determined through RNA extraction, cDNA synthesis, real-time quantitative PCR of superoxide dismutase (SOD1) and glutathione peroxidase (GPx1) genes in skeletal muscle samples.

3.2. MATERIALS AND METHODS

3.2.1 Ethical clearance and study site

The study was conducted according to the International Standards of Care and Use of Animals in Research, and approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC ethical clearance number: 2014/47/D; **Appendix 2-3**) and the University of Johannesburg Research Ethics Committee (REC number: 01-02-2016; **Appendix 5**). The study was conducted in the multi-purpose animal unit of the Central Animal Services at the University of the Witwatersrand, South Africa.

3.2.2 Experimental animals and housing

The experiments were performed on 7-day old litters of five nursing Sprague Dawley (*Rattus norvegicus*) dams each with between 8-12 (average 10) rat pups (N=30), supplied by the Central Animal Services, University of the Witwatersrand. Each dam and its respective litter were housed in the same acrylic cages with stainless steel mesh lids. Wood shavings were used as bedding and changed twice a week. The room temperature was maintained at 25 ± 2ºC. Dams and the rat pups were placed on a 12-h light and dark cycle (with lights on at 07:00 am). There was adequate ventilation of the room at all times. The rat pups were marked on their tails with different coloured codes using non-toxic ink containing permanent markers for easy identification. The dams did not receive any experimental treatment but were provided with normal commercial rat chow (Epol®, Johannesburg, South Africa) and water *ad libitum* throughout the suckling period. During the 7-day experimental period, dams were allowed to

freely nurse until euthanasia of the rat pups on post-natal day (PD) 14. The dams were also weighed twice a week as part of the routine health monitoring and were returned to stock immediately after euthanasia of their rat pups.

3.2.3 Study design and dietary treatments

The 5-day old rat pups were weighed on the PD5 following parturition and given a day for acclimatisation before receiving treatments. On PD6 the rat pups were randomly allocated into four treatment groups (Figure 3.2), each consisting of a minimum of seven mixed male and female rat pups. Rat pups in each litter were assigned to different groups to avoid dam-effect bias. The pups were weighed daily to adjust treatment dosage per body mass and received the following treatments: **Group 1**: Control (DW) - distilled water with 0.5% (v/v) dimethyl sulphoxide (DMSO) which was used as a vehicle control; **Group 2**: Oleanolic acid (OA) – Oleanolic acid (60 mg/kg body mass) which was used to investigate the metabolic effects of OA; **Group 3**: High fructose solution (HF) – 20% (w/v) fructose solution which was used to induce metabolic dysfunction; **Group 4**: Oleanolic acid and high fructose diet (OAHF) – a combination of oleanolic acid (60 mg/kg body mass) and 20% (w/v) high fructose solution which was used to investigate whether OA would prevent the metabolic dysfunction caused by fructose.

All treatments were administered once daily, for seven days (PD7 to PD13), at a volume of 10 mℓ/kg body mass via orogastric gavage. After administration of treatments, all the rat pups were observed for 20 min for unusual behavioural changes and clinical signs of toxicity of the treatments throughout the course of the experimental treatments.

3.2.5 Terminal procedure

3.2.5.1 Sample collection

At the end of the 7-day experimental treatment period (on PD 14), the pups were euthanised by an intraperitoneal injection of sodium pentobarbital (200 mg/kg body mass; Euthanaze ®, Centaur Laboratories, Johannesburg, South Africa). For ethical reasons and because of their

young age, the rat pups were not fasted prior to termination. The thorax was opened and a 1-mℓ syringe with a 21-gauge needle was used to collect blood via cardiac puncture. The blood was placed into heparin-coated tubes. The tubes were gently inverted for 30 seconds to mix the anti-coagulant with the blood and then centrifuged at 3500 × *g* at 4°C for 15 min. The plasma was separated, frozen and stored in microtubes in a freezer at -20°C for the determination of clinical biochemistry and general health profiles. The triceps muscle samples were dissected out, snap frozen in liquid nitrogen and stored at -80°C in cryovial tubes until further molecular analyses.

3.2.5.2 Determination of visceral organ morphometry

Following blood sample collection, the abdomen was cut via a midline incision. The stomach, caeca, liver, kidneys, small and large intestines were carefully dissected out. The luminal contents of stomach, caeca, small and large intestines were emptied by gently squeezing them out after which the gastrointestinal viscera were weighed on a digital analytical balance (Presica 310M®; Precision Instruments, Switzerland). Gross morphometric measurements of the small and large intestines were determined using a ruler by placing each on a straight line with minimum stretching on a dissecting board.

3.2.6 Measurement of growth performance

3.2.6.1 Body mass determination

The pups were weighed daily to determine the effects of the different treatments on growth pattern. The difference between the initial body mass and the body mass at termination was taken as the total body mass gain over the 7-day treatment period.

3.2.6.2 Determination of indices of linear bone growth

The left hind leg was severed from each of the carcasses, cleaned off of all the flesh and then femora and tibiae were separated. The bones were dried to constant weight in an oven (Salvis®, Salvis Laboratory, Switzerland) at 50°C for 6 days and then weighed using a balance (Precisa

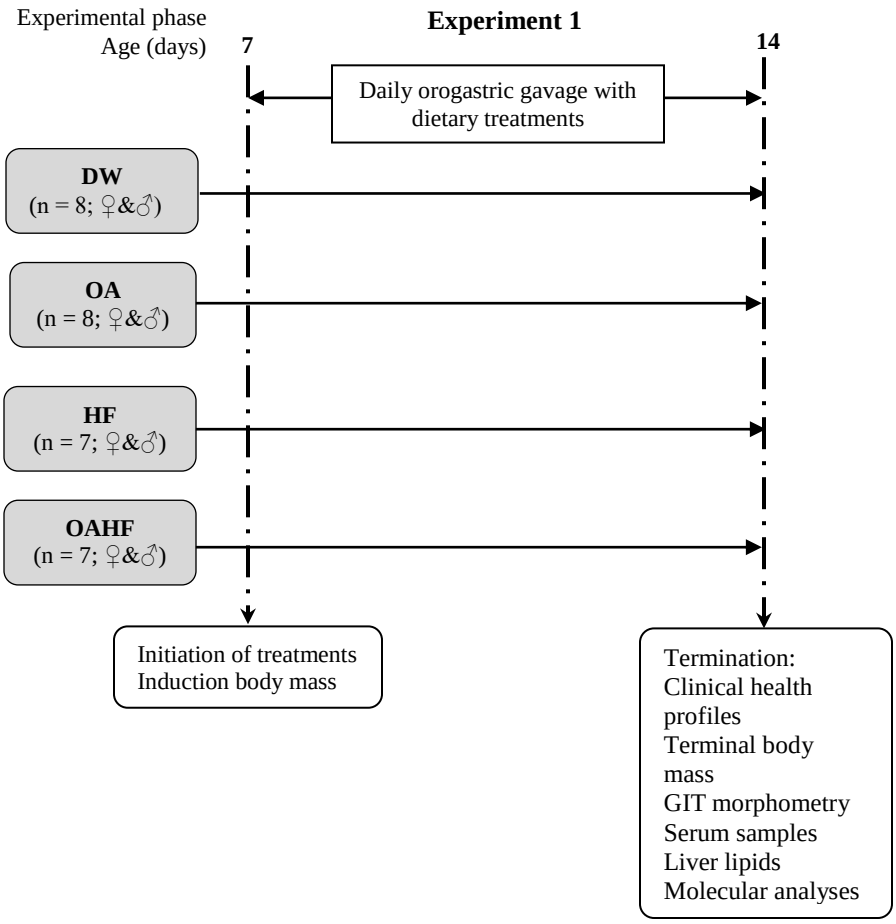


Figure 3.1: Flow diagram showing the experimental groups, stages of development, sequence and timing of interventions and measurements for the first experimental study.

DW = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSO in the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg body mass) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg body mass) and 20% (w/v) fructose solution in the neonatal period (n = 7); GIT = gastrointestinal tract; ♀ = female rats; ♂ = male rats.

310M, Precisa Instruments, Switzerland). The lengths of the femora (measured between the distal femoral articular surface to the greater trochanter) and tibiae (measured between tibia head medial malleolus) were measured by a vernier callipers (Hi-impact, Dejuca, Johannesburg, South Africa) and were used as indices of linear growth in the pups.

Tibial and femoral estimates of bone densities (Seedor index) were calculated as follows:

Seedor index ($mg.mm^{-1}$) = mass of bone (mg) /length of bone (mm) (Seedor et al., 2005).

3.2.7. Determination of biochemical health profile markers

The effect of OA on the general health status of the rat pups was determined by measuring twelve biochemical parameters. Using the plasma samples collected at termination, general biochemical profiles (cholesterol, glucose, phosphate, calcium total bilirubin), a surrogate marker of hepatic function (alanine amino transferase), a non-tissue specific enzyme (alkaline phosphatase), renal function (blood urea nitrogen and creatinine) and protein profiles (total protein, albumin, and globulin) were measured using a calibrated automatic biochemical analyser (IDEXX VetTest®, Clinical Chemistry Analyser, IDEXX Laboratories Inc, Westbrook, Maine, USA) as per manufacturer instructions. Briefly, stored plasma samples were thawed at room temperature. The samples were then gently inverted to mix the contents and placed into the analyser which automatically drew up 150 µℓ of the plasma. The analyser then loaded 10 µℓ of plasma onto each of the 12 pre-loaded disks after which each sample was then analysed and printouts provided. The results from the measurement of enzyme markers were reported as units per litre (U/ℓ).

3.2.8. Determination of hepatic lipid content

The liver samples from individual rats from each of the four different treatment groups were pooled together per group. An accredited laboratory (South African National Accreditation System) of the Agricultural Research Council (ARC) in Pretoria determined the intra-hepatic

lipid content. The Soxhlet method AOAC (See **Appendix 6** for detailed description of the methodology) was used to determine the hepatic lipid content. Briefly, the liver samples were free-dried (lyophilised) in a freeze dryer (Model BK-FD12, SP Scientific, New York, USA) and ground into a fine powder. The ground sample (2.5 g) was placed in an extraction thimble and extracted using 60 mℓ of petroleum ether using the Tecator Soxtec System HT 1043 extraction unit (Gemini BV Laboratories, Apeldoorn, Nederland). The extracted lipid was determined gravimetrically. Hepatic lipid content determination was performed in triplicate for each treatment group.

3.2.9 Determination of gene expression for the antioxidant enzymes

3.2.9.1 Extraction of RNA from triceps muscle tissue

Triceps muscle samples (200 mg) were crushed and weighed into falcon tubes. The samples were kept on dry ice until the homogenisation procedure was performed. TRIzol (1 mℓ) was added and samples were then homogenised on ice. Chloroform (200 µℓ) was added to the homogenate and vortexed vigorously and then incubated on ice for 15 min. The homogenate was then centrifuged at 12000 × *g* for 15 min at 4ºC. The aqueous phase was transferred into a falcon tube and RNA precipitated with 0.5 mℓ isopropanol by gentle mixing. The mixture was then incubated for further 10 min on ice and then centrifuged 12000 × *g* for 10 min at 4ºC. The supernatant was discarded leaving the pellet to which 1 mℓ of 70% ethanol was added then centrifuged at 7500 × *g* for 10 min at 4ºC and the supernatant was discarded. The residue was air dried and the RNA pellet resuspended in RNase free water. "RNA was quantified using the Qubit 4 fluorometer (Thermofisher, Johannesburg, South Africa) following the manufacturer's instructions."

3.2.9.2 Determination of RNA integrity

A 1% agarose gel was prepared to assess the integrity of the RNA extracted from the triceps muscle tissue. To an empty glass bottle, 15 g of agarose was weighed followed by the addition of 150 mℓ Tris base, acetic acid and EDTA (TAE) buffer. The reagents were mixed thoroughly and

heated for 1 min in the microwave to dissolve the agarose. Thereafter, the reagents were allowed to cool to at least 50ºC before the addition of 1.5 µℓ of ethidium bromide into the gel and mixed. The gel was then poured into a casting tray that was sealed at both ends. The comb was placed in the gel casting tray and molten agarose solution was poured into the casting tray. The gel was then allowed to cool and solidify. Thereafter the gel was placed in the electrophoresis chamber where 4 µℓ of RNA sample from each of the different treatment groups. Bromophenol dye (6 µℓ) was loaded into the gel wells and TAE buffer poured into the chamber up to 2-3 mm over the gel. The gel was run at 80V for 60 min after which the gel was placed in a UV box and photographed (Figure 3.3).

3.2.9.3 First-strand cDNA synthesis

The synthesis of cDNA was performed using a cDNA synthesis kit (Superscript VILO cDNA, Thermofisher Scientific, Johannesburg, South Africa). Briefly, 4 µℓ 5X VILO reaction mixture and 2 µℓ 10X Superscript enzyme mix, were added to a falcon tube. Immediately after, 1.92 µℓ, 1.34 µℓ, 1.07 µℓ and 1.1 µℓ of DW, OA, HF and OAHF RNA samples respectively were added to each falcon tube. The Falcon tube contents were then made up to 20 µℓ with DEPC-treated water and the reaction mixture was gently mixed and incubated at 25ºC for 10 min. Thereafter, the tube contents were further incubated at 42ºC for 60 min and the reaction was terminated at 85ºC for 5 min.

3.2.9.4 Real-time quantitative polymerase chain reaction (PCR) assay

The real-time quantitative PCR gene expression of SOD2 and GPx1 was performed based on Applied Biosystems Thermo Fisher Scientific protocol. The cDNA (2 µℓ) was used for PCR amplification. Specific primers to the targeted gene regions (SOD2 and GPx) were designed to amplify and determine the expression level of targeted genes (Table 3.1). Actin was used as a reference gene. A PCR reaction mixture containing 5 µℓ PowerUp^{TM} SYBR Green Master Mix (2X), 1 µℓ each of forward primer and reverse primer, 2 µℓ cDNA template, 1 µℓ RNase-free

water was prepared. The reagents were mixed thoroughly and centrifuged briefly to spin down the contents and remove air bubbles. The reaction mixtures were transferred to each well of an

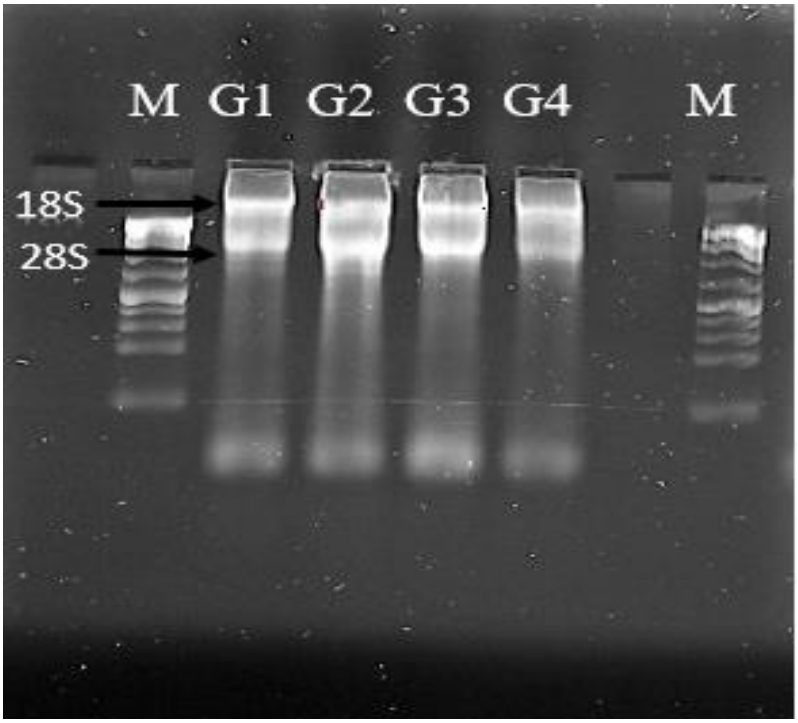


Figure 3.2: Agarose gel showing the integrity of RNA from the different treatment groups.

G1 RNA band is for DW = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSO in the neonatal phase (n = 8).

G2 RNA band is for OA = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg body mass) in the neonatal phase (n = 8).

G3 RNA band is for HF = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7).

G4 RNA band is for OAHF = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg body mass) and (20% w/v) fructose solution in the neonatal period (n = 7).

M bands are for the Ambion millennium markers. The 18s and 28s (black arrows) RNA bands confirm the intactness of the extracted RNA.

Table 3.1: List of primers used for real-time qPCR analysis

Gene	Primer sequence (5´-3´)	Amplicon length
SOD2	Forward – GTGAACAACCTGAACGCCAC (20)	149
	Reverse – CCTACAGGCCCCCAAAACAT(20)	
GPx1	Forward – CAGTCCACCGTGTATGCCTT (20)	104
	Reverse – GTAAAGAGCGGGTGAGCCTT (20)	

SOD2 = superoxide dismutase; GPx1 = glutathione peroxidase; qPCR = quantitative polymerase chain reaction.

optical plate which was sealed with an optical adhesive cover. The reaction plate was then placed in the PCR thermocycler which was programmed to the following settings: UDG activation (2 min at 50ºC), initial denaturation (2 min at 95ºC); 40 cycles of denaturation (15s at 95ºC); 40 cycles annealing and extension (1 min at 60ºC).

3.2.10. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analysed using GraphPad Prism for Windows Version 7.0 (GraphPad Software Inc., San Diego, USA). Samples from male and female rats were pooled together for all the variables that were measured as there were no significant sex differences across all treatment groups. A two-way repeated measures analysis of variance (ANOVA), with Bonferroni *post-hoc* test, was used to analyse body mass changes with day as a within-subjects factor and treatment as a between-subjects factor. A one-way ANOVA with Bonferroni *post-hoc* test was used to compare the means for all the other parameters measured. The level of significance acceptable was $P \leq 0.05$.

3.3. RESULTS

3.3.1. The effect of neonatal oral administration of oleanolic acid on growth performance in suckling male and female rats

3.3.1.1 Growth rate and body mass gain

The rat pups in all treatment groups exhibited a significant increase ($P<0.05$; Figure 3.4) in body mass over the seven day treatment period (PD7) to PD14). There were no significant differences in the induction and terminal body masses across all the treatment groups ($P>0.05$; Figure 3.5).

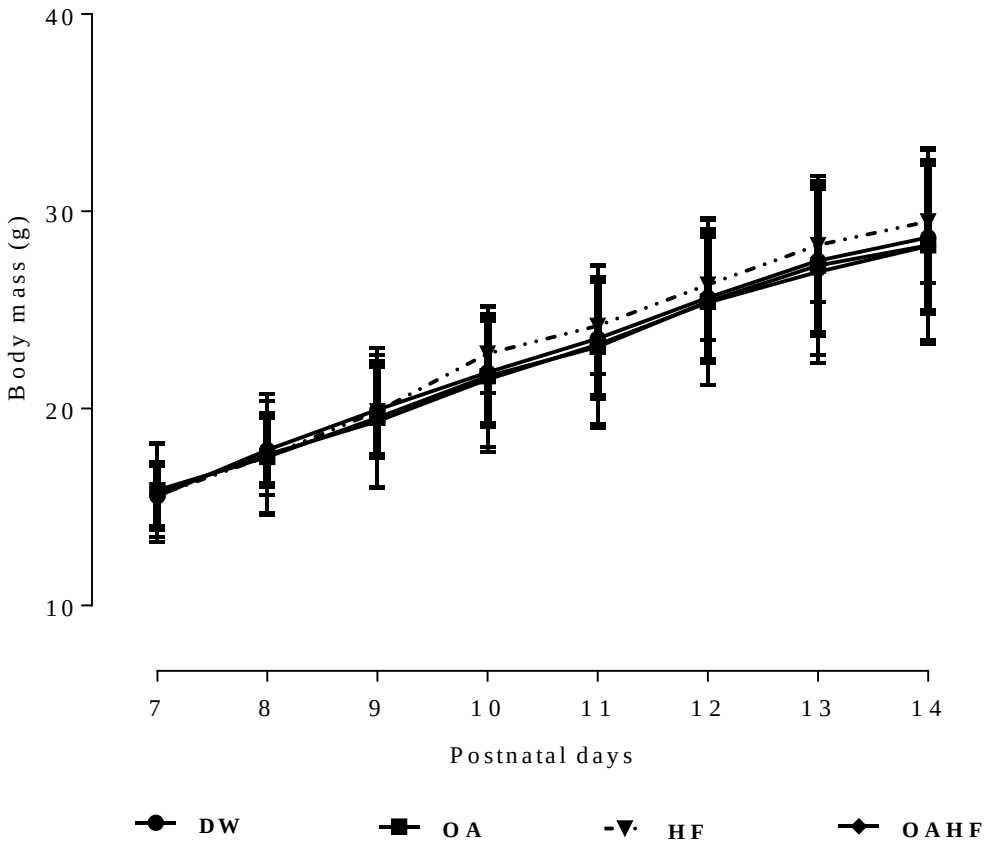


Figure 3.3: The effect of neonatal oral administration of oleanolic acid on the growth rates of suckling male and female pups.

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7).

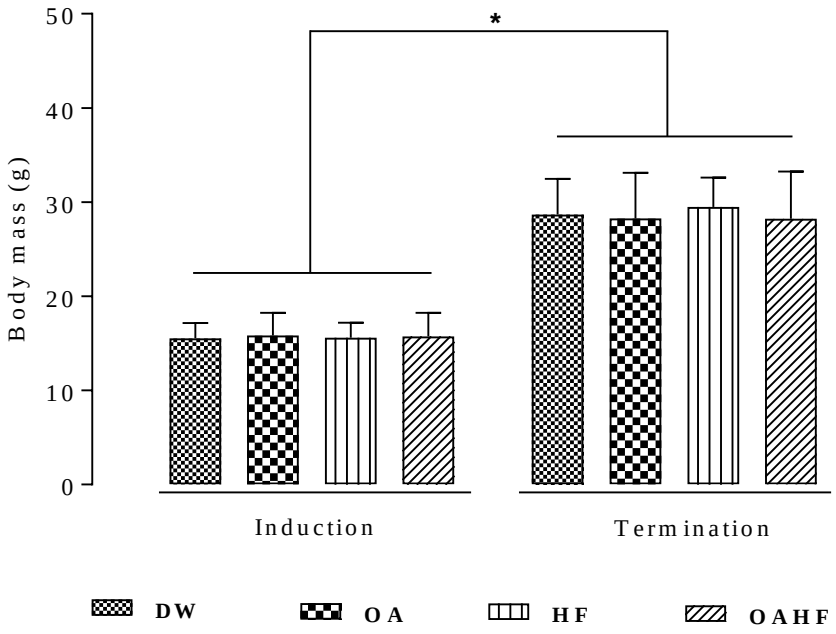


Figure 3.4: The effects of neonatal oral administration of oleanolic acid on the induction (post-natal day 7) and terminal (post-natal day 14) body mass of suckling male and female pups.

Data presented as mean ± standard deviation. *Significant increase in body mass from induction to termination for all treatment groups (P<0.05). **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSO in the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7).

3.3.1.2 Linear growth

There were no significant differences in the femoral and tibial bone masses, lengths and bone Seedor indices, across the different treatment groups ($P>0.05$; Table 3.2).

Table 3.2: The effect of neonatal oral administration of oleanolic acid on tibial and femoral masses, lengths and Seedor indices in suckling male and female pups.

	DW	**OA**	**HF**	**OAHF**
Tibia				
Mass (mg)	38.50 ± 1.4	35.9 ± 2.0	37.6 ± 1.1	34 ± 2.8
Length (mm)	15.2 ± 0.7	15 ± 0.8	17.1 ±0 .9	14.8 ± 0.9
¥Seedor index (mg/mm)	2.54 ± 0.13	2.4 ± 0.13	2.2 ± 0.08	2.30 ± 0.14
Femur				
Mass (mg)	37.8 ± 5.8	33.5 ± 4.5	37.6 ± 8.5	34.1 ± 5.5
Length (mm)	11.4 ± 0.9	11.6 ± 0.7	11.9 ± 1.7	11.1 ± 1.5
Seedor index (mg/mm)	3.32 ± 0.45	2.88 ± 0.31	3.174 ± 0.43	3.07 ± 0.39

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7). ¥Seedor index = bone density in mg/mm.

3.3.2 The effect of neonatal oral administration of oleanolic acid on the gastrointestinal tract (GIT) and visceral organ morphometry in suckling male and female pups

There were no significant differences in the relative masses of the small and large intestines, absolute and relative masses of the caecum, stomach and kidneys across the different treatment groups ($P>0.05$; Table 3.3).

Table 3.3: The effect of neonatal oral administration of oleanolic acid on the lengths, absolute and relative weights of visceral organs in suckling male and female pups.

	DW	**OA**	**HF**	**OAHF**
SI (cm)	54.44 ± 9.23	46.75 ± 16.48	52.24 ± 5.06	53.34 ± 8.59
SI (g)	0.74 ± 0.19	0.74 ± 0.22	0.84 ± 0.27	0.93 ± 0.32
SI ¥rTL	0.49 ± 0.14	0.45 ± 0.15	0.49 ± 0.14	0.62 ± 0.19
LI (cm)	7.09 ± 0.79	6.63 ± 2.72	7.64 ± 0.56	7.47 ± 0.55
LI (g)	0.12 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	0.13 ± 0.03
LI rTL	0.08 ± 0.01	0.08 ± 0.02	0.08 ± 0.00	0.09 ± 0.01
Liver (g)	0.90 ± 0.17	0.94 ± 0.17	0.94 ± 0.16	1.01 ± 0.21
Liver rTL	0.60 ± 0.13	0.63 ± 0.13	0.55 ± 0.10	0.68 ± 0.13
Caecum (g)	0.08 ± 0.02	0.06 ± 0.03	0.07 ± 0.01	0.07 ± 0.02
Caecum rTL	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.00	0.05 ± 0.01
Stomach (g)	0.21 ± 0.02	0.21 ± 0.05	0.20 ± 0.03	0.20 ± 0.01
Stomach rTL	0.14 ± 0.02	0.14 ± 0.03	0.12 ± 0.02	0.13 ± 0.01
Kidneys (g)	0.38 ± 0.04	0.27 ± 0.04	0.38 ± 0.03	0.32 ± 0.14
Kidneys rTL	0.25 ± 0.03	0.27 ± 0.04	0.23 ± 0.03	0.22 ± 0.10

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7); LI = large intestine; SI = small intestine; ¥rTL = weight of organ masses expressed relative to tibial length (g/cm).

3.3.3 The effect of neonatal oral administration of oleanolic acid on the general clinical health profiles in suckling male and female pups

3.3.3.1 Measurement of biomarkers of hepatic function and hepatic lipid storage

There were no significant differences on the surrogate marker of hepatic function (alanine amino transferase), and a non-tissue specific alkaline phosphatase and hepatic lipid content across all the treatment groups ($P>0.05$; Table 3.4).

Table 3.4: The effect of neonatal oral administration of oleanolic acid on a biomarker of hepatic function (alanine amino transferase), a non-tissue specific alkaline phosphatase and hepatic lipid storage in suckling male and female pups.

Parameter	**DW**	**OA**	**HF**	**OAHF**
ALT (U/ℓ)	35 ± 5.9	45 ± 14.1	51.8 ± 15.5	43.9 ± 7.2
ALP (U/ℓ)	299.1 ± 63.8	309.3 ± 58.5	394.3 ± 70.3	269.4 ± 62.7
#Hepatic lipid content (%)	2.8 ± 0.02	2.7 ± 0.02	3.2 ± 0.01	3.2 ± 0.01

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7). ALT = alanine amino transferase; ALP = alkaline phosphatase; #Hepatic lipid content expressed as a percentage of liver mass.

3.3.3.2 Biomarkers of renal function and general clinical biochemistry

There were no significant differences on the surrogate markers of renal function (blood urea nitrogen and creatinine) and general clinical biochemistry (phosphate, calcium, total protein, albumin, globulin, glucose and cholesterol) across all the treatment groups ($P>0.05$; Table 3.5).

Table 3.5: The effects of neonatal oral administration of oleanolic acid on biomarkers of renal function and general clinical biochemistry in suckling male and female pups.

Parameter	DW	OA	HF	OAHF
BUN (mmol/ℓ)	4.6 ± 0.7	4.2 ± 0.9	5.2 ± 0.6	4.3 ± 0.5
CREA (µmol/ℓ)	16.9 ± 3.2	18 ± 0	10.3 ± 3.4	14.4 ± 7.1
TBIL (µmol/ℓ)	4 ± 2.7	5.5 ± 3.3	8 ± 3.8	4.12 ± 2.1
PHOS (mmol/ℓ)	2.8 ± 0.2	3.17 ± 0.5	2.9 ± 0.3	3.2 ± 0.3
Ca (mmol/ℓ)	2.7 ± 0.4	2.0 ± 0.9	2.3 ± 0.6	2.5 ± 0.7
TPROT (g/ℓ)	38.1 ± 4.1	41.3 ± 3.7	41.4 ± 5.2	40 ± 3.0
ALB (g/ℓ)	21.1 ± 2.9	19.5 ± 2.4	20.17 ± 2.7	19.3 ± 1.6
GLOB (g/ℓ)	17 ± 4.5	21.7 ± 2.0	21.3 ± 1.2	20.4 ± 2.9

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSO in the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7). BUN = blood urea nitrogen; TBIL = total bilirubin; ALB = albumin; CREA = creatinine; PHOS = phosphate; Ca = calcium; TPROT = total protein; GLOB = globulin.

3.3.3.3 General clinical biochemistry (metabolic substrates)

There were no significant differences in the concentrations of circulating metabolic substrates (cholesterol and glucose) across all treatment groups ($P>0.05$; Table 3.6).

Table 3.6: The effects of neonatal oral administration of oleanolic acid on the concentrations of circulating metabolic substrates in suckling male and female pups.

Parameter	**DW**	**OA**	**HF**	**OAHF**
Glucose (mmol/ℓ)	7.8 ± 1.3	7.6 ± 1.0	8.1 ± 0.3	8.5 ± 1.7
Cholesterol (mmol/ℓ)	4.3 ± 0.4	4.3 ± 0.5	4.3 ± 0.5	4.4 ± 0.8

Data presented as mean ± standard deviation. **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7).

3.3.4 The effect of neonatal oral administration of oleanolic acid on gene expression of anti-oxidant enzymes in suckling male and female pups

3.3.4.1 Superoxide dismutase gene expression

Neonatal fructose administration significantly downregulated the expression of superoxide dismutase (SOD2) by 25% compared to the control group ($P<0.05$; Figure 3.6). However neonatal administration of OA prevented the fructose-induced downregulation in rats that received a combination of OA and a high fructose solution (OAHF) and upregulated the expression of the SOD2 gene by 30% (OA) compared to the control group ($P<0.05$; Figure 3.6).

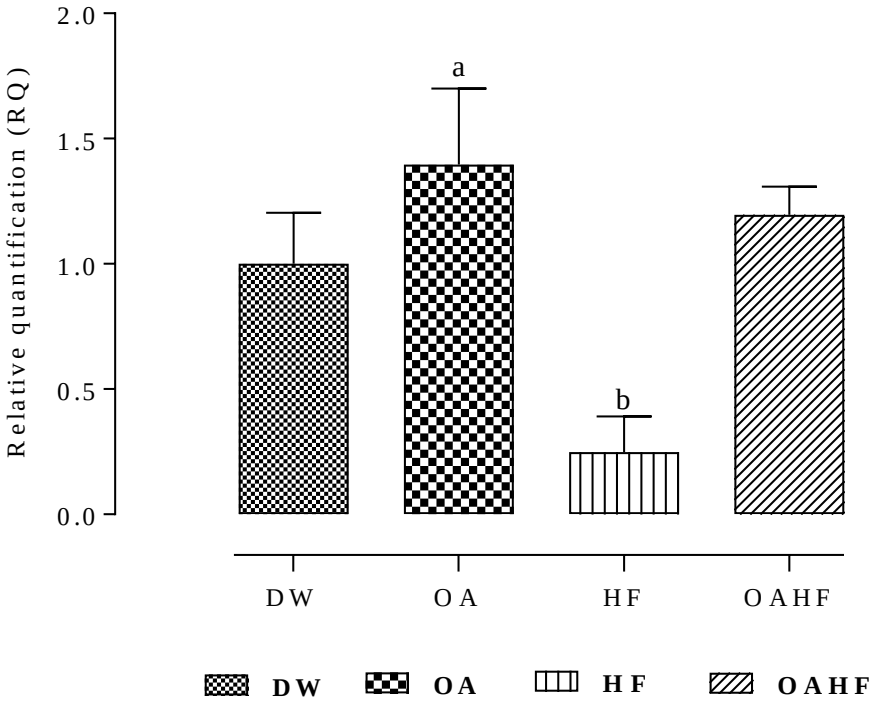


Figure 3.5: The effect of neonatal oral administration of oleanolic acid on the expression of superoxide dismutase (SOD) gene in suckling male and female pups.

[a]Significant increase in the expression of SOD2 compared to the control group (P<0.05), [b]Significant decrease in the expression of SOD2 compared to the control group (P<0.05). **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7). Expression of β-actin was used as an internal control.

3.3.4.2 Glutathione peroxidase gene expression

Neonatal fructose administration caused a 25% decrease in the expression of glutathione peroxidase (GPx1) compared to the control group ($P<0.05$; Figure 3.7). However neonatal administration of OA prevented the fructose-induced downregulation of the expression of GPx gene by 30% (OA) compared to the control group ($P<0.05$; Figure 3.7).

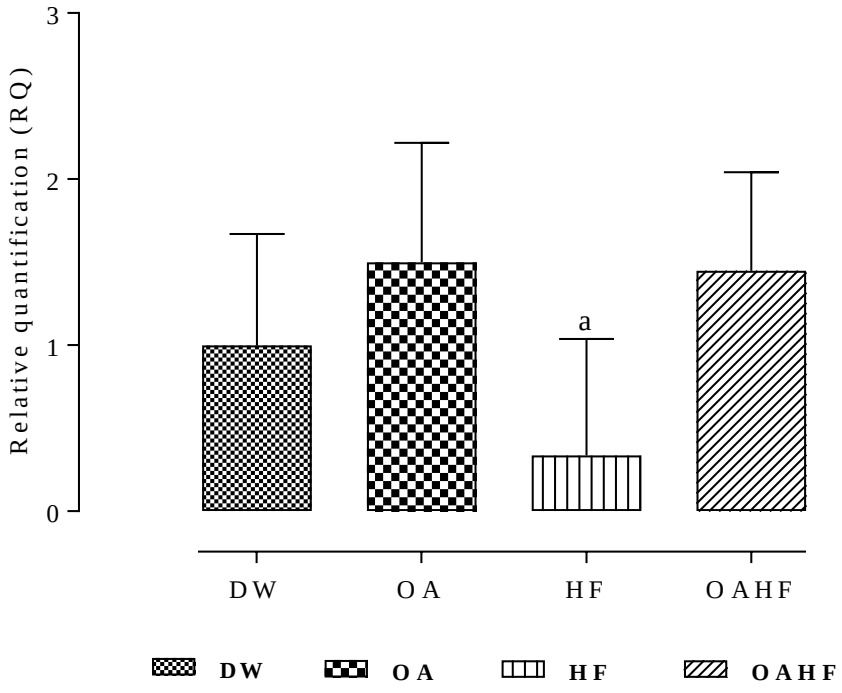


Figure 3.6: The effect of neonatal oral administration of oleanolic acid on the expression of glutathione peroxide (GPx1) gene in suckling male and female pups.

[a]Significant decrease in the expression of GPx1 compared to the control group ($P<0.05$). **DW** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) DMSOin the neonatal phase (n = 8); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 8); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 7); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period (n = 7). Expression of β-actin was used as an internal control.

3.4 DISCUSSION

This study was designed to investigate the potential protective effect of neonatal (7 days) oral administration oleanolic acid against fructose-induced oxidative damage, development of negative health outcomes and precocious maturation of the GIT in suckling male and female pups. We showed that short-term neonatal administration of OA protected against fructose-induced oxidative damage, had no adverse effects on health and did not cause precocious development of the GIT in suckling rats. The prophylactic use of OA in the fight against metabolic syndrome during the critical developmental period prevents oxidative damage, had no apparent adverse effects on health and did not induce precocious development of the GIT in suckling rats.

3.4.1 The effect of neonatal oral administration of oleanolic acid on growth performance in suckling male and female pups

Findings from this study showed that administering OA neonatally via orogastric gavage did not have negative effects on the growth of male and female suckling rats over the 7-day experimental treatment period. Although not statistically significant, high fructose diet (HF) had a moderate growth promoting effect over the same treatment period. Several studies have shown that nutritional (Benevenga et al., 1995) and pharmacological (Maltin et al., 1986) manipulation during the early neonatal phase of development have an effect on growth rate and pattern of the rats. The growth performance in neonatal animals can have a bearing on physiological systems in adulthood (Hack et al., 2002, Singhal et al., 2004). Low birth weight in humans and poor nutrition during the neonatal period affects growth performance and has been associated with the development of chronic illnesses such as hypertension, T2DM, and obesity later in adult life (Barker et al., 1993, Curhan et al., 1996).

Body mass changes have previously been used as a measure of growth in rodent studies, but due to fluctuations in factors such as the hydration status of animals and food intake, body mass is deemed to be an unreliable index of growth (Yin et al., 1982). Consequently, the use of tibial

length as a reliable indicator of linear growth is recommended (Sundström et al., 2014). Tibiae from rat pups treated with OA and HF had similar lengths compared to the rest of the treatment groups, including the control. This finding further confirms (as shown by the body masses) the non-toxic effects of OA on linear growth in male and female suckling pups. To further assess growth performance, plasma samples could have been used to measure insulin-like growth factor-1 (IGF-1), a hormone that plays an important role in coordinating balanced growth among multiple tissues and organs (Yakar et al., 2002). However, due to the size of the animals at termination, the volume of blood samples collected were inadequate to perform hormonal assays in addition to the blood biochemical assays which were undertaken.

3.4.2 The effect of neonatal oral administration of oleanolic acid on the morphometry of the abdominal viscera in male and female suckling pups

Our results also show that neonatal oral administration of OA did not have any apparent effects on the morphometry of the GIT and accessory structures of the GIT, suggesting that administration of OA does not induce precocious development of the GIT and may not have adverse effects on gut health in neonates. Our results on suckling pups rats concur with findings from other studies that did not show any significant changes in GIT morphometry following dietary treatment during the neonatal period (Beya et al., 2012). Determination of the morphological characteristics of the developing GIT in neonates has been used as reliable criteria for assessing the effects of dietary treatments on the physiology of neonatal animals (Guilloteau et al., 2010). The first port of call for all ingested food is the gastrointestinal tract (GIT), an organ system whose primary function is to digest, produce metabolic regulatory hormones and peptides, extract and absorb nutrients from ingested food among other functions. The GIT is also under direct exposure to the food that we ingest, as such any variations in dietary intake may affect its functionality (Salminen et al., 1998). Ingested food triggers the release of regulatory hormones and peptides from enteroendocrine cells of the GIT resulting in the modification of GIT function. Unlike the precocious GIT of man which is normally functional at birth, the altricial rat GIT is relatively undeveloped at birth and all of the functional development occurs in

the early post-natal period (Henning, 1987). The GIT of the rat is functionally immature for the first 2 weeks of life, this is followed by rapid development and extensive changes in week 3 (Downes and Burns, 2008). Thus the targeted intervention was during the second week of life to explore the impact of the OA on GIT growth. Maturation of the rat GIT occurs by the replacement of cells rather than modification of existing cells and the mucosal mass of the rat becomes fairly constant after about 40 days (Jean, 1993). Previous studies have shown that phytochemical consumption during the neonatal period promotes the increase in the growth of the GIT (Botermans and Pierzynowski, 1999) and caecum (Erlwanger and Cooper, 2008, Beya et al., 2012).

As a result, dietary changes introduced during suckling, a period of developmental plasticity, could be a potential cause of several diseases, dysfunction of the GIT or positive health outcomes later in adult life (McCance and Huether, 2015). In fact, research has indicated that the alteration in the dietary composition in the early post-natal period has a causal role in metabolic and digestive development in the intestines (Henning, 1981, Khan et al., 2005, Armitage et al., 2005).

3.4.3 The effect of neonatal oral administration of oleanolic acid on the general clinical health profiles in suckling male and female pups

3.4.3.1 Surrogate markers of liver function

Findings from the current study show that neonatal fructose and OA administration did not affect circulating serum levels of the biomarkers of liver function and possibly did not cause adverse hepatocellular changes. Previous studies in adult rats have shown that OA ameliorates hepatic injury and lowers the levels of liver function enzymes after feeding fructose in a dose-dependent manner (LIU et al., 1994, Liu et al., 1995a). The liver plays an important role in the metabolism of nutrients such as carbohydrates, lipids and proteins (Rouiller, 2013, Oosterveer and Schoonjans, 2014, Chai et al., 2015). The liver also detoxifies harmful chemicals and drugs (Chai et al., 2015). Excessive exposure of the liver to dietary and pharmacologic toxic substances may cause hepatocellular damage, particularly the structural integrity of the parenchymal

hepatocytes which may affect the hepatic physiology (Leise et al., 2014). It is possible that the failure to develop impaired hepatic function following neonatal administration of high fructose solution may be attributed to the absence of GLUT5 fructose transporters whose expression increases post-weaning (Shu et al., 1997).

In the absence of terminal histology of liver samples, as was the case in this study due to the lack of adequate samples, it is recommended to measure serum or plasma concentrations of surrogate biomarkers of liver function for animal experimental research (Vella et al., 2012). Surrogate biomarkers of liver function include TP, ALP, AST, ALT and TBIL among others (Vella et al., 2012). TP gives an estimate of both ALB and GLOB and can also be used to interpret the functional integrity of the liver. Serum ALB indicates the nutritional status and the liver's synthetic ability, as such any changes in ALB may reflect hepatobiliary irregularities (Thapa and Walia, 2007).

The commonly measured biomarker of hepatocellular damage which was measured in this study was ALT (Moseley, 1996). ALT is a cytosolic enzyme that is released into the blood after the damage to hepatocytes (Scheig, 1996). Unlike ALP, a non-tissue specific enzyme which is produced by several sources such as bone and the uterus (Limdi and Hyde, 2003), ALT is a reliable measure of the extent of liver damage and the potential hepatotoxicity of pharmaceutical drugs or dietary components (Heemskerk et al., 2009). The lack of significant increases in the circulating levels of ALT in suckling rats that received OA suggests that OA had no hepatotoxic effects on liver function and can thus be used safely in the neonatal period.

3.4.3.2 Renal function and general clinical biochemistry

We also assessed renal function in the pups by measuring the serum concentrations of creatinine, BUN, phosphate, calcium and albumin. Our results showed that short-term neonatal oral administration of OA neither altered renal function nor affected the general clinical health of the suckling male and female pups. Kidneys are important in the homeostatic regulation of body fluids osmolarity, acid-base balance and blood pressure. They also work together with the liver

to detoxify and excrete metabolic waste by-products. Creatinine and urea are the most reliable clinical estimates of glomerular filtration rate (GFR), a standard index of renal function (Carvounis et al., 2002). Increased serum concentration of urea and creatinine reflect considerable damage to the kidneys particularly renal tubular function and filtration at the glomerular filtration membrane (Brisco et al., 2013). The lack of significant changes in plasma urea and creatinine in OA-treated rat pups suggest that neonatal oral administration of OA has no adverse effects on renal function.

3.4.3.3 Hepatic lipid storage and biomarkers of metabolic function

There were no differences observed in hepatic lipid content in male and female suckling pups administered with OA, suggesting that neonatal OA administration had no apparent effects on hepatic lipid content in the suckling rats. In addition to detoxifying xenobiotic substances, the liver plays a major role in the metabolism of various nutrients (Corey and Kaplan, 2014). The liver is the primary organ in lipogenesis, gluconeogenesis and cholesterol metabolism (Bechmann et al., 2012, Rui, 2014). Metabolic syndrome induces a change in hepatic lipid and carbohydrate metabolism which ultimately causes accumulation and storage of lipids in the liver (Corey and Kaplan, 2014). This leads to hepatocellular changes associated with non-alcoholic liver disease (NAFLD) (Basaranoglu et al., 2014). The lipids that are stored in the liver come from circulating free fatty acids that are derived from the dysregulation of peripheral lipolysis (Bechmann et al., 2012).

The non-fasting plasma glucose and cholesterol levels following the 7-day treatment period were not different across all the treatment groups. This possibly suggests that short-term administration of OA did not negatively affect hepatic glucose and cholesterol metabolism.

3.4.4 The effect of neonatal oral administration of oleanolic acid against fructose-induced oxidative damage in suckling male and female pups

In the current study, the gene expression of anti-oxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx1) was determined. We showed that neonatal oral administration

of fructose downregulated the expression of SOD2 and GPx1 by 25%, which was prevented by neonatal administration of OA. Superoxide dismutase (SOD) and glutathione peroxidase (GPx1) are important anti-oxidant enzymes which constitute cellular defence mechanisms that protect cellular components against free radical-induced injury caused by reactive oxygen species (ROS) (Pigeolet et al., 1990, Schieber and Chandel, 2014, Poprac et al., 2017). Excessive fructose consumption negatively affects the cellular anti-oxidant capacity through its effects on the generation of ROS which causes a decrease in the levels and activities of GPx and SOD2 (Abdel-Kawi et al., 2016). Previous studies in which expression of genes for anti-oxidant enzymes was assessed, showed that the anti-oxidant capacity in fructose-fed rats was decreased (Girard et al., 2006). Fructose increases the influx of triglycerides into hepatocytes causing overproduction of ROS through the β-oxidation of free fatty acids (Furukawa et al., 2017). The elevation of pro-oxidant species causes damage to cellular membranes and DNA (Thannickal and Fanburg, 2000). The observed downregulation of anti-oxidant enzyme genes in fructose-fed rats in this study could be attributed to the effect of fructose on the generation of ROS through mitochondrial β-oxidation (Botezelli et al., 2012).

Neonatal OA treatment prevented the fructose-induced decrease in anti-oxidant genes (GPx1 and SOD). A previous study in an insulin resistant adult rat model of genetic hypertension showed that OA possesses anti-oxidant activities (Sloboda et al., 2014). In an *in vitro* anti-oxidant activity-assessing model, OA acted as a free radical-scavenger through direct chemical reactions and a biological molecule, which may have enhanced the anti-oxidant defences (Wang et al., 2010a). The protective effect of OA against fructose-induced oxidative damage observed in this study could be attributed to the anti-oxidant effect of OA reported previously in animal models (Gao et al., 2009, Wang et al., 2013).These findings suggest that OA improves hepatic insulin resistance through inhibition of mitochondrial ROS, hypolipidaemic and anti-inflammatory effects (Wang et al., 2013).

3.5 CONCLUSION

Findings from this study provide evidence that short-term neonatal oral administration of OA protects against fructose-induced oxidative damage with seemingly no adverse effects on health or the maturational and developmental changes of the gastrointestinal tract in suckling male and female pups. The prophylactic use of OA in the fight against metabolic syndrome during the critical developmental period does not seem to carry health risks. Although the neonatal experimental model did not seem to work for MetS risk factors since I did not observe any major gross changes, it does not rule out epigenetic changes with long-term implications on MetS risk factors later on in adulthood. This provides justification for performing the next experiment which involves investigating the potential protective effect of neonatal administration of oleanolic acid against the subsequent development of fructose-induced metabolic dysfunction (Chapter 4) and non-alcoholic fatty liver disease (Chapter 5) in adult male and female rats.

CHAPTER 4:

THE EFFECT OF NEONATAL ADMINISTRATION OF OLEANOLIC ACID ON THE SUBSEQUENT DEVELOPMENT OF FRUCTOSE-INDUCED METABOLIC DYSFUNCTION IN ADULT MALE AND FEMALE RATS

4.1 INTRODUCTION

Metabolic syndrome (MetS), obesity and type 2 diabetes (T2DM) have drastically increased to epidemic proportions worldwide in the last few decades (Jaacks et al., 2016). I have provided detailed information on the definitions of MetS, its diagnosis, prevalence and epidemiology, risk factors and clinical manifestations in sections 2.1, 2.2 and 2.3 respectively. Recent human epidemiological and rodent experimental studies have shown the developmental origins of metabolic disorders and associated diseases by establishing a link between the peri-conceptual, fetal or early infant phases of life and the subsequent development of adult obesity and the MetS (Alfaradhi and Ozanne, 2011, Zheng et al., 2016, Kitsiou-Tzeli and Tzetis, 2017). The neonatal period has been identified as a critical window of developmental plasticity during which the nutritional status of the offspring can affect their subsequent development and confer epigenetic predisposition of the offspring to positive health outcomes or metabolic disorders later in life (Godfrey et al., 2010, Couvreur et al., 2011, Gluckman et al., 2015, Smith and Ryckman, 2015).

A growing number of clinical and experimental studies have shown that nutritional manipulations or stressful events during the neonatal period can influence epigenetic regulation of gene expression by changing the timing and direction of DNA methylation, histone modification and transcription of non-coding ribonucleic acid (ncRNA) (Desai et al., 2013, Low et al., 2017, Seki et al., 2017). The epigenetic phenomenon by which nutritional, hormonal, pharmacological and other stressful events acting in the critical periods of development, such as gestation and the neonatal period modify the development of certain physiological functions is known as neonatal programming (Ozanne and Hales, 2002, de Moura and Cottini, 2005).

The vulnerability to the development of health outcomes associated with metabolic disorders such as T2DM and obesity is determined by exposure to adverse early life nutritional environments during gestation and the early postnatal period (Zheng et al., 2016). Maternal nutritional status during pregnancy and that of the offspring in the early neonatal period are also important in the development of metabolic disorders in the offspring (Rando and Simmons, 2015). The diet-induced epigenetic changes triggered in the neonatal period usually only become

apparent later in life as either positive health outcomes or manifestations of metabolic disorders following a second hit or challenge (Heindel et al., 2015). Fructose consumption by the mother and/or offspring during the perinatal period has been associated with the development of metabolic disorders of the offspring later on in adulthood (Ghezzi et al., 2011, Clayton et al., 2015). The metabolic developmental plasticity associated with the neonatal period makes it an important window of opportunity that can be targeted by pharmacological or dietary manipulations for prophylactic treatments that may result in the programming for positive health outcomes for the rest of the individual's life.

In view of the increasing global prevalence of diet-induced metabolic disorders and the possibility of targeting the neonatal period for prophylactic treatments, alternative therapeutic interventions that can be administered in the neonatal period are needed to ameliorate the impact of the adverse health effects of obesity and MetS (Roche et al., 2005). Most strategies used in the management of MetS emphasise the use of pharmacological agents to manage or reduce the risk of developing health outcomes associated with obesity and metabolic dysfunction by targeting energy expenditure in adulthood (Kuete, 2017). However few studies have focussed on the neonatal period as a potential target for epigenetic modifications for the prevention of these metabolic conditions. Moreover prolonged pharmacological treatment of obesity and MetS may have adverse side-effects and reduces the quality of life for patients (Nathan et al., 2009, Wang et al., 2011). The lack of patient compliance is also identified as having a negative outcome on the use of chronic medication (Hamine et al., 2015).

Medicinal plants and herbs are used to prevent, treat and manage a wide range of diseases including metabolic disorders and their associated complications (Kuete, 2017). The therapeutic properties of medicinal plants can be attributed to the presence of phytochemicals, which may act individually, additively or synergistically to improve health (Gurib-Fakim, 2006). Oleanolic acid is a bioactive pentacyclic triterpenoid phytochemical that is widely distributed in several plant species (Sánchez-Quesada et al., 2015, Lin et al., 2016), foodstuffs (Yoshikawa and Matsuda, 2000, Rodriguez-Rodriguez, 2015) and some commonly used medicinal plants (Jäger

et al., 2009, Liu, 2005). Oleanolic acid possesses several potential pharmacological activities that exhibit several therapeutic activities without the adverse side-effects of the commonly used synthetic pharmacological agents (Castellano et al., 2013). Oleanolic acid was used for the present study due to these previously described pharmacological activities which include hepatoprotection (Liu et al., 1995a, Nyakudya et al., 2017), anti-inflammatory (Singh et al., 1992, Nkeh-Chungag et al., 2015) anti-diabetic action (Gao et al., 2009, Wang et al., 2011, Zeng et al., 2012, Castellano et al., 2013b, Castellano et al., 2016), anti-oxidant activities (Yin and Chan, 2007, Tsai and Yin, 2008, Tsai and Yin, 2012, Tsao and Yin, 2015) and anti-glycosilative effects (Xi et al., 2008, Wang et al., 2010b). The abovementioned beneficial pharmacological properties of OA are described in detail in section 2.7.

Findings from the first experimental phase of the current study showed that OA prevented fructose-induced oxidative damage without adverse effects in suckling rats. Most of these studies targeted the gestational period of development and adulthood (Moore, 2010, Beck et al., 2012). However, until now, few if any studies have been undertaken to investigate the effect of neonatal administration of fructose administration on the subsequent development of abnormal glucose metabolism (Ibrahim et al., 2017). There is limited research on the long-term effects of high fructose administration during the neonatal period and the subsequent development of metabolic dysfunction later in life.

In as much as OA has been shown to possess several therapeutic properties in the management of diet-induced metabolic disorders in adulthood, its potential protective effect against the development of health outcomes associated with fructose-induced metabolic syndrome later in life when administered in the neonatal period, a critical window of developmental plasticity needs to be investigated. Rats are altricial species consequently the neonatal period provides a window for ontogenic plasticity and represents a viable interventional phase for neonatal programming.

4.1.1 Aim

This study was therefore designed to investigate whether the administration of OA during the neonatal period would prevent the development of fructose-induced metabolic disorders (excluding the liver) in fructose-fed male and female rats.

4.1.2 Specific objectives

The specific objectives of this long-term experimental study were to determine the potential protective effect of neonatal oral administration of OA against the subsequent development of fructose diet-induced health outcomes associated with metabolic dysfunction in fructose-fed (with different intervention times) male and female rats by assessing:

a) Growth performance – terminal body mass, Seedor indices of linear growth, tibial and femoral lengths and masses and bone radiography.
b) Gastrointestinal tract and visceral organ morphometry – measurement of visceral organ masses relative to tibial length.
c) Glucose tolerance - fasting plasma glucose, oral glucose tolerance test, and area under the curve for the oral glucose tolerance test
d) Insulin resistance and β-cell function – fasting plasma insulin and the homeostatic model of insulin resistance (HOMA-IR).
e) Body adiposity and visceral fat deposition – visceral fat pad masses (male and female rats) and epididymal fat masses (male rats only).
f) Circulating metabolites – fasting plasma triglyceride and total cholesterol levels.

4.2 MATERIALS AND METHODS

4.2.1 Ethical clearance for the study

The study, conducted in the Central Animal Services (CAS) unit at the Faculty of Health Sciences, University of the Witwatersrand, was done in accordance with the International Standards of Care and use of Animals in Research and was approved by the Animal Ethics

Screening Committee (AESC) of the University of the Witwatersrand, Johannesburg, South Africa (AESC clearance number: 2014/47/D; **Appendix 2-4**) and the University of Johannesburg Research Ethics Committee (REC number: 01-02-2016; **Appendix 5**). Sample and tissue assays were done in laboratories of the School of Physiology, University of the Witwatersrand, Department of Human Anatomy and Physiology, University of Johannesburg and the Agricultural Research Council, Irene Analytical Services, Pretoria.

4.2.2 Housing and animal husbandry

Sprague Dawley (*Rattus norvegicus*) dams, each with between 8-12 pups were used in the study. The rats were supplied by the CAS, University of the Witwatersrand and housed in a conventional animal room in the rodent section of the CAS. Each dam and its respective litter were housed in acrylic cages in which wood shavings were used as bedding. The bedding was changed once a week. The ambient environmental room temperature was maintained at 25 ± 2ºC and a 12-h light and dark cycle followed (with lights on at 07:00 am). Adequate positive pressure ventilation of the room was maintained at all times. The dams were supplied with standard rat chow (SRC) (Epol®, South Africa, Johannesburg) and water *ad libitum* throughout the suckling period. During the 7-day experimental period, the dams were allowed to freely nurse until weaning of the pups on post-natal day (PD) 21. The dams were returned to stock after weaning of their offspring. The weaned rats were housed individually as described for the dams above.

4.2.3 Chemicals and reagents used

The double-antibody enzyme-linked immunosorbent assay (ELISA) kits used to quantify insulin (ElabScience®, Wuhan, China) were purchased from Biocom Biotech, South Africa and used according to the manufacturer instructions. The dosages of oleanolic acid (Sigma, Johannesburg, South Africa) used in this study were similar to those used by Bachhav et al. (2015). The commercial fructose (Huletts®, Fructose Concentrated Sweetness, Johannesburg, South Africa) used in this study was purchased from Dischem Pharmacies, Johannesburg, South Africa. The 20% fructose solution used in the neonatal and adult phases was prepared by adding 200 g

fructose to a litre of warm water. The dosage of fructose (20% w/v) used for this study was prepared based on a previous metabolic study on murine models (Mamikutty et al., 2014). In the second phase of the study, red and blue food colouring (Robertsons Retailer Brands (Pty) Ltd, Johannesburg, South Africa) were added to the fructose solution (red colourant) and ordinary tap water (blue colourant) to help with identification of the fluids to be given to the animals receiving the fructose and tap water. The glucose solution that was used for the oral glucose tolerance test was prepared by dissolving 50 g of D-glucose (Merck Chemicals (Pty) Ltd, Johannesburg, South Africa) in 100 mℓ of distilled water.

4.2.4 Study design and dietary treatments

This was an interventional comparative study in which 112 male and female Sprague Dawley pups from 10 nursing dams were used. The study design is summarised in Figure 4.1 below. The study was divided into two experimental interventional phases. The first phase of the study was between post-natal day (PD) 7 to post-natal day 13. During the first phase, the first nutritional intervention was introduced in order to induce neonatal metabolic programming. The litter from each of the 10 nursing dams were randomly assigned to the four neonatal treatment groups resulting in each treatment group consisting of 26-31 male and female pups. This random allocation of littermates to each of the different treatment groups was done to avoid dam-effect bias. For easy identification, a colour-coded numbering system was used. The pups were marked on their tails with non-toxic ink-based permanent marker pens.

The pups were weighed daily to monitor the health of the animals and adjust treatment amounts to maintain a constant dosage per body mass and they received the following treatments:

Group 1: Distilled water (DW) – distilled water with (0.5% v/v) dimethyl sulphoxide (DMSO) which was used as a vehicle control to dissolve the OA (n = 26; 13 males and 13 females);

Group 2: Oleanolic acid (OA) – oleanolic acid (60 mg/kg body mass) reconstituted in (0.5% v/v) DMSO (n = 31; 15 males and 16 females);

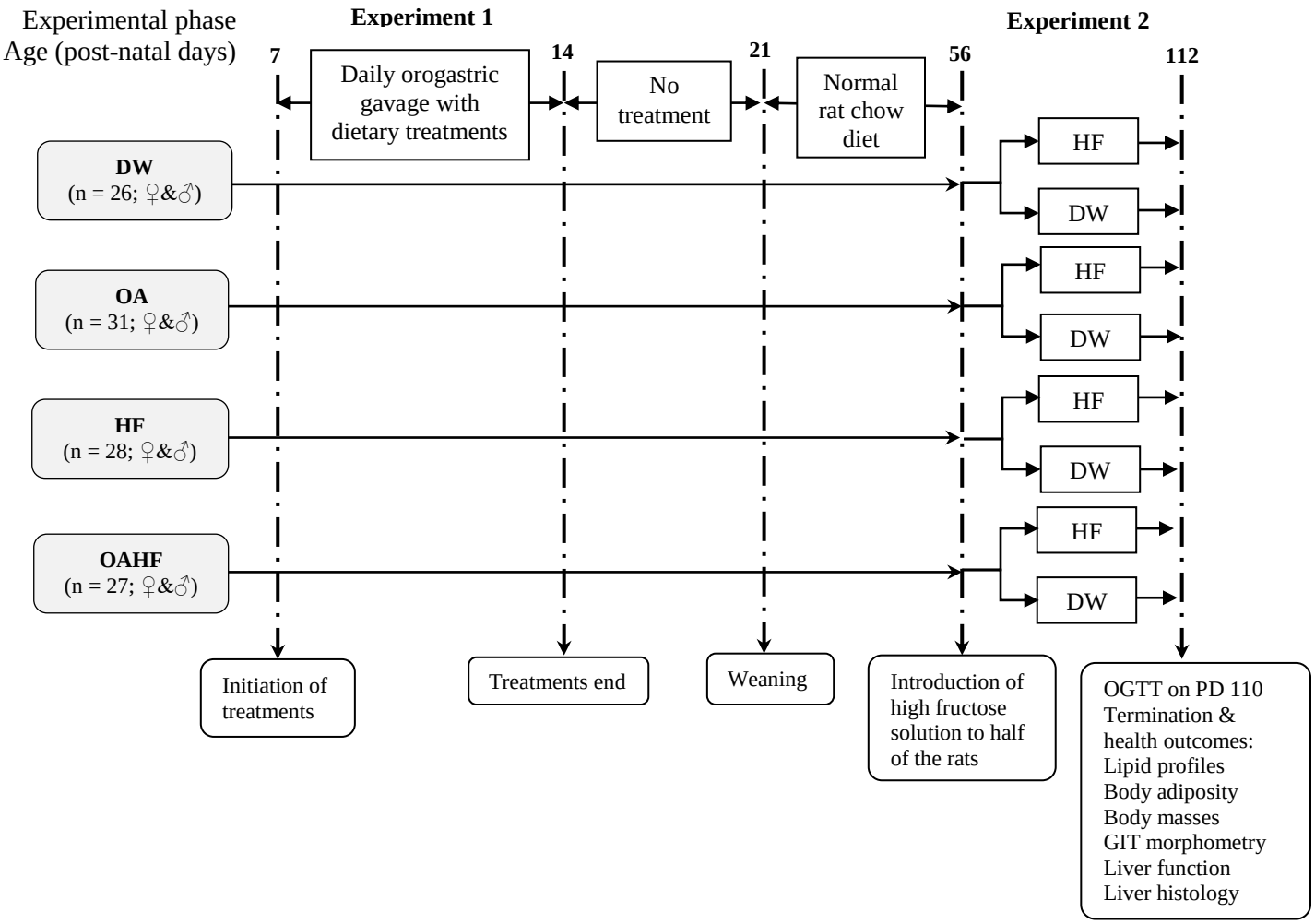


Figure 4.1: Schematic flow diagram showing the experimental groups, stages of development, sequence and timing of interventions and measurements for the second experimental study**.**

DW = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) dimethyl sulphoxide in the neonatal phase (n = 26; ♀&♂); **OA** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) in the neonatal phase (n = 31; ♀&♂); **HF** = gavaged daily with 10 mℓ/kg of 20% (w/v) fructose solution in the neonatal phase (n = 28; ♀&♂); **OAHF** = gavaged daily with 10 mℓ/kg body mass of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in

the neonatal period (n = 27; ♀&♂); OGGT = oral glucose tolerance test; GIT = gastrointestinal tract; PD = post-natal day; ♀ = female rats; ♂ = male rats.

Group 3: High fructose solution (HF) – 20% (w/v) fructose solution made up in (0.5% v/v) DMSO (n = 28; 13 males and 15 females);

Group 4: Oleanolic acid and high fructose solution (OAHF) – oleanolic acid (60 mg/kg body mass) and 20% (w/v) fructose solution constituted in (0.5% v/v) DMSO (n = 27; 14 males and 13 females).

All treatments in the first short-term experiment were administered once daily in the morning (between 09:00-10:00), for seven consecutive days (PD7 to PD13), at a volume of 10 mℓ/kg body mass via orogastric gavage using a 20G orogastric tube attached to a 1 mℓ syringe. After administration of treatments, all the pups were observed for 20 minutes for unusual behavioural changes.

Following the daily treatments during PD7-PD13, the rats were allowed to continue to suckle until they were weaned on PD21 after which they had *ad libitum* access to SRC and plain drinking tap water until PD55 which is generally recognised as post-pubertal and the commencement of adulthood in rats. In the second experimental interventional phase, a second dietary intervention was introduced PD56 up to PD112 (adult phase). During this adulthood period, PD56 to PD112, all rats received *ad libitum* access to SRC, however, half (n = 56) the number of male and female rats in each group received plain drinking water whilst the other half (n = 56) received 20% fructose (w/v) as drinking fluid (Figure 4.1). Each treatment group thus had 6-8 male or female pups. The fructose solution was given in the long-term study, as a secondary dietary insult in order to induce the development of health outcomes associated with metabolic dysfunction (Miller and Adeli, 2008, Mock et al., 2017). The following groups were formed following the introduction of a secondary dietary insult in adulthood:

Group 1: DW + W = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) dimethyl sulphoxide in the neonatal period followed by *ad libitum* access to plain drinking water post-weaning and throughout adulthood (n = 14; 7 males and 7 females);

Group 2: DW + F = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) dimethyl sulphoxide in the neonatal period followed by *ad libitum* access to 20% (w/v) fructose solution as drinking fluid in adulthood (n = 12; 6 males and 6 females);

Group 3: OA + W = gavaged daily with 10 mℓ/kg of 60 mg/kg body mass oleanolic acid in the neonatal period followed by *ad libitum* access to plain drinking water post-weaning and throughout adulthood (n = 16; 8 males and 8 females);

Group 4: OA + F = gavaged with 10 mℓ/kg of 60 mg/kg body mass oleanolic acid in the neonatal period followed by *ad libitum* access to 20% (w/v) fructose as drinking fluid in adulthood (n = 15; 7 males and 8 females);

Group 5: HF + W = gavaged daily with 10 mℓ/kg body mass 20% (w/v) fructose solution in the neonatal period followed by *ad libitum* access to plain drinking water post-weaning and throughout adulthood (n = 13; 6 males and 7 females);

Group 6: HF + F = gavaged daily with 10 mℓ/kg body mass 20% (w/v) fructose solution in the neonatal period followed by *ad libitum* access to 20% (w/v) fructose as drinking fluid in adulthood (n = 15; 7 males and 8 females);

Group 7: OAHF + W = gavaged daily with 10 mℓ/kg body mass of a combination of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period followed by *ad libitum* access to plain drinking water post-weaning and throughout adulthood (n = 13; 7 males and 6 females);

Group 8: OAHF + F = gavaged daily with 10 mℓ/kg body mass of a combination of oleanolic acid (60 mg/kg) and 20% (w/v) fructose solution in the neonatal period followed by *ad libitum* access to 20% (w/v) fructose solution as drinking fluid in adulthood (n = 14; 7 males and 7 females).

In the current study, rats were subjected to either a single or double hit according to the single or double hit hypothesis (Tamashiro and Moran, 2010). The single hit was either an early neonatal administration of fructose (20% w/v) or the provision of fructose in drinking water (20% w/v) later in life. The double hit was characterised by dietary interventions with fructose at two growth stages; a fructose hit neonatally in early-life (first-hit) which presumably would predispose the rats to the onset of metabolic derangements followed by another dietary fructose intervention in later life (the second-hit in adulthood).

In the rats that did not receive the early 'hit' with fructose in the neonatal period, the fructose in adulthood represented a single first hit in adulthood. For the rats which received an early hit as neonates, the fructose intervention in adulthood represented a late second hit. Thus the latter group of rats had a double hit of fructose in their lifespan. Consequently in terms of fructose intake, the rats either had only an early (neonatal) hit with fructose or a late (in adulthood) hit with fructose or a double hit (both neonatally and then later on as adults).

Drinking water and fructose solution were changed twice a week, the drinking bottles were washed, changed, and then fresh solutions were prepared and provided.

4.2.5 Oral glucose tolerance test

At the end of the 16 week study period (PD110), an oral glucose tolerance test (OGTT) was performed after an overnight fast. Briefly, fasting blood glucose was determined and then each rat was administered with a dose of 2 g/kg body mass of sterile 50% (w/v) D-glucose solution (Sigma, Johannesburg, South Africa) via orogastric gavage (Huhn et al., 2016). Thereafter blood glucose concentrations were measured at T = 15, 30, 60, 120 and 180 min using a calibrated glucometer (Contour Plus® Glucometer (Bayer (Pty) Ltd, Johannesburg, South Africa). The blood samples were collected from the tail vein. The incremental changes in blood glucose after administration of the glucose load were expressed as an area under the curve (AUC) from the time when the fasting blood was drawn (T = 0) until 180 min post-load blood sampling (Kwon et al., 2008).

4.2.6 Terminal procedures

After the OGTT, the rats were placed back onto their respective dietary treatments for a further 48 hours. Thereafter, the animals were fasted overnight and their fasting blood glucose levels measured the following morning, using the calibrated glucometer Contour Plus® glucometer (Bayer (Pty) Ltd, Johannesburg, South Africa). The rats were then euthanased by an overdose of intra-peritoneally injected sodium pentobarbital (200 mg/kg body mass; Eutha-naze®, Bayer Corporation, Johannesburg, South Africa).

4.2.6.1 Blood and adipose tissue sample collection

Following euthanasia of the rats, blood was collected via cardiac puncture using 21G needles and 10 mℓ syringes and transferred into heparinised blood collection tubes (BD Vacutainer® Systems, Meylan Cedex, France). The blood samples were centrifuged for 15 min at 5000 × *g* at 20°C (Sorvall RT® 6000B centrifuge, Rockville, USA). The collected plasma samples were stored at -20˚C for the analysis of insulin and cholesterol.

The abdomen was opened via a midline incision. The visceral (and epididymal in males) fat pads were dissected out and weighed.

4.2.6.2 Determination of gastrointestinal tract and visceral organ morphometry

The liver, stomach, pancreas, small and large intestines, visceral fat, epididymal fat, heart and kidney were carefully dissected out. The stomach, caecum and intestinal contents were gently emptied and the gastrointestinal tract and other viscera were weighed on an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). With minimum stretching, the lengths of the small and large intestines were measured using a ruler attached to a dissection board.

4.2.6.3 Determination of circulating cholesterol and triglyceride concentrations

The plasma concentrations of triglycerides were measured using a calibrated Accutrend® Triglyceride Plus Cholesterol Meter (Roche, Mannheim, Germany) in accordance with the

manufacturer's instructions. Plasma cholesterol was measured using a calibrated automatic biochemical analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc.,Westbrook, Maine, USA) as per the manufacturer's instructions. Briefly, stored plasma samples were thawed and allowed to reach room temperature. The samples were then gently inverted to mix the contents and placed into the analyser which automatically drew up 40 µℓ of the plasma. The analyser then loaded 10 µℓ of plasma onto each of the pre-loaded cholesterol disks after which each sample was then analysed and printouts provided.

4.2.7 Measurement of plasma insulin and calculation of the homeostatic model assessment of insulin resistance (HOMA-IR)

Plasma levels of insulin were measured using an enzyme-linked immunosorbent assay (ELISA) kit for rats (Elabscience ®, Rat INS ELISA kit, Wuhan, China) following the manufacturer's instructions (**Appendix 6**). Insulin resistance was calculated by means of the homeostatic model assessment index (HOMA-IR) (Valle et al., 2016). Insulin resistance was calculated by means of the homeostatic model assessment index (HOMA-IR) using the relationship between the fasting blood glucose and insulin levels, according to the following formula:

HOMA-IR = Insulin (µU/ℓ) × Blood glucose (mM)/22.5 (Valle et al., 2016). Plasma levels of insulin were converted from ng/g to µU/ℓ for the calculation of HOMA-IR.

4.2.8 Measurement of growth performance

4.2.8.1 Body mass measurements

In the neonatal phase, the pups were weighed daily (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa) for seven days (PD7 to PD14) to monitor growth performance and for the maintenance of a constant dosage of the different experimental treatments. After weaning and in adulthood the body masses of the rats were measured twice a week to monitor the health and growth performance.

4.2.8.2 Determination of indices of linear bone growth

The femoral attachment of left hind leg to the pelvis was excised from each of the carcasses, defleshed and disarticulated from the tibia. The bones were dried to constant weight in an oven (Salvis®, Salvis Lab, Switzerland) at 50°C for 5 days and then weighed. The fibula was removed from the tibia after which the tibial and femur lengths were measured with a pair of vernier callipers (Hi-impact, Dejuca, South Africa). The length of the tibia which is less prone to acute variation compared to body mass was used to calculate the relative masses of visceral and epidydimal fat.

4.2.8.3 Determination of bone density

Seedor index was then calculated using the formula:

Seedor index (Bone density) = mass of bone (mg) /length of bone (mm) (Seedor et al., 2005)

4.2.8.4 Bone radiographic images

Radiographic images were taken at the Central Animal Services using a Fuji film X-ray machine (Industrial X-ray Film FR, Tokyo, Japan) to further assess bone density of the tibiae and femora. The bones were placed on the photographic plate at a distance of 1 m from the X-ray camera. The settings of the X-ray machine were adjusted to 4.8 kV, 0.71 mA per plate for both male and female rats.

4.2.9 Statistical analyses

Data were expressed as mean ± standard deviation and analysed using GraphPad Prism for Windows Version 7.0 (GraphPad Software Inc., San Diego, USA). The total area under the curve (AUC) for the OGTT was calculated by the trapezoidal method to assess glucose tolerance (Huhn et al., 2016). A two-way repeated measures analysis of variance (ANOVA), with Bonferroni *post-hoc* test, was used to analyse terminal body mass with day as a within-subjects factor and treatment as a between-subjects factor. A one-way ANOVA with Bonferroni *post-hoc*

test was used to compare the means for all the other parameters measured from different treatment groups. The level of significance acceptable was $P \leq 0.05$.

4.3 RESULTS

4.3.1 Effect of neonatal oral administration of oleanolic acid on weaning and terminal body masses of fructose-fed male and female rats

4.3.1.1 Body mass measurements

In male rats, induction body masses were similar across all experimental treatment groups ($P>0.05$; Figure 4.1). Body masses of male rats from all experimental treatment groups increased significantly from induction to weaning and from weaning up to termination ($P<0.05$; Figure 4.1). There were no significant differences in terminal body masses from all experimental treatment groups ($P>0.05$; Figure 4.1).

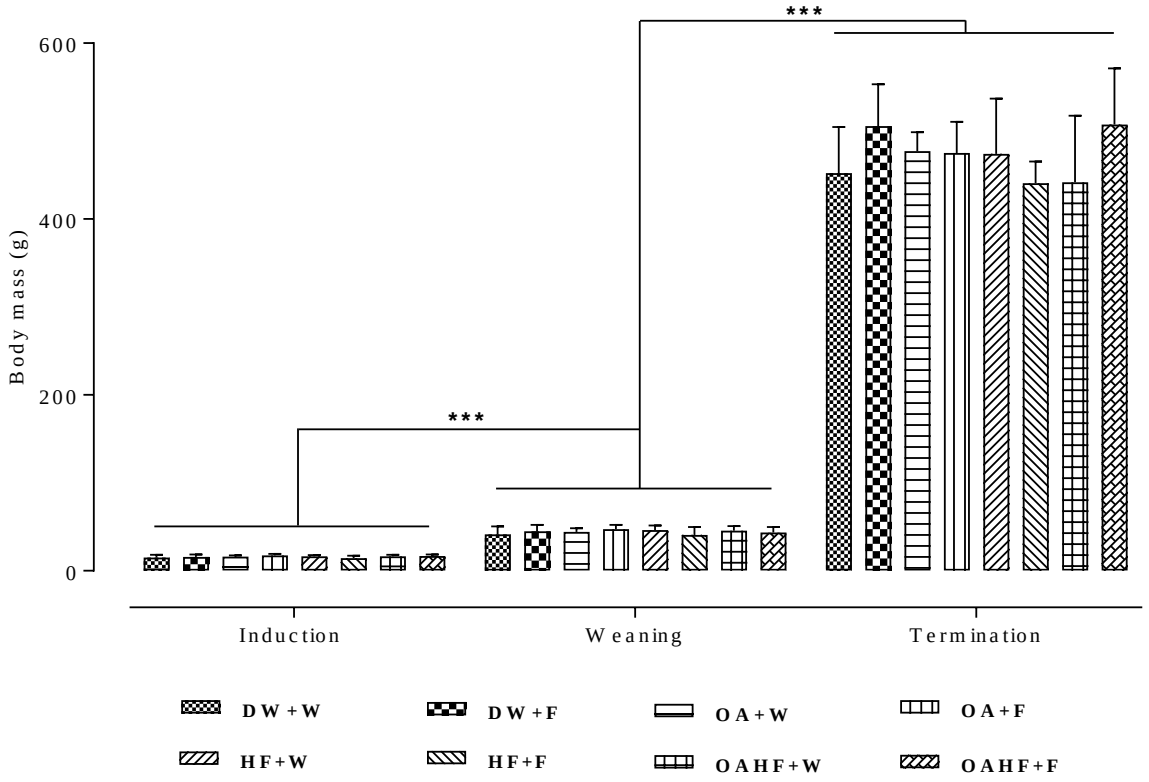


Figure 4.1: The effect of neonatal oral administration of oleanolic acid or fructose on weaning and terminal body masses of male rats fed a high fructose diet, as neonates and, or in adulthood.

All data presented as mean ± standard deviation. ***Significant increase in body mass from induction to weaning and from weaning to termination (P<0.005). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood

(n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

In female rats, induction body masses were also similar across all experimental treatment groups (P>0.05; Figure 4.2). Body masses of female rats from all treatment groups increased significantly from induction to weaning and from weaning up to termination (P<0.05; Figure 4.2). A double hit of fructose, first early in the neonatal period and then later in adulthood (HF+F) resulted in a significant increase in terminal body mass compared to other treatment groups (P<0.05; Figure 4.2). Rats neonatally administered OA and subjected to the double hit of fructose (OAHF+F) did not show the significant increase in terminal body mass observed in rats receiving the double hit (HF+F) of fructose (P<0.05; Figure 4.2). Thus OA prevented the increase in terminal mass due to a double hit with fructose.

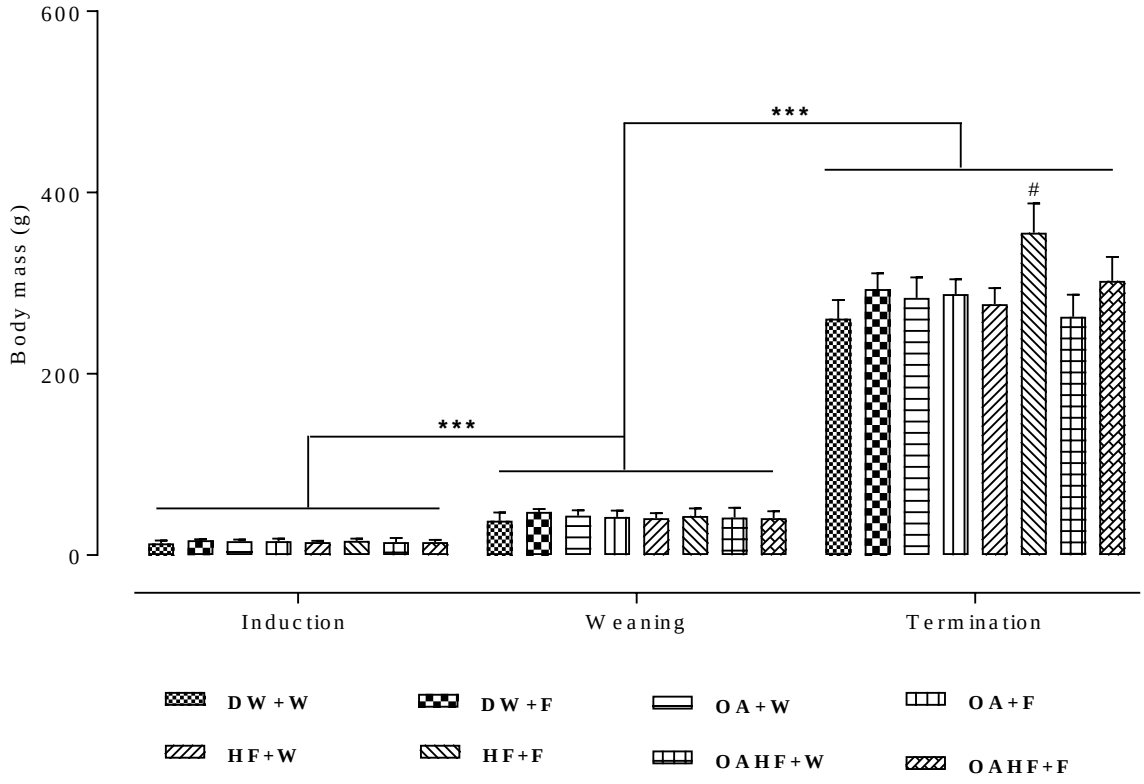


Figure 4.2: The effect of neonatal oral administration of oleanolic acid or fructose on weaning and terminal body masses of female rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. ***Significant increase in body mass from induction to weaning and from weaning to termination (P<0.005); #Significant increase in terminal body mass for the HF+F treatment group (P<0.005). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and

high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

4.3.1.2 Linear growth

There was a significant increase in tibial masses of male rats receiving a single hit of fructose late in adulthood (DW+F) ($P<0.05$; Table 4.1). The tibial lengths, femoral lengths and masses, and all the bone densities were similar across all experimental treatment groups ($P>0.05$; Table 4.1). Neonatal oral administration of OA prevented the fructose-induced increase in tibial masses ($P<0.05$; Table 4.1).

Table 4.1: The effect of neonatal oral administration of oleanolic acid or fructose on tibial and femoral masses, lengths and Seedor indices in male rats fed a high fructose diet.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Tibia mass (mg)	596.4±36.7	[a]647.2±50.6	617.4±51.6	589±42.2	632.3±50.3	567.6±44.3	601.9±39.8	622±23.5
Tibia length (mm)	42.2±1.1	43.4±1.2	43.4±0.8	42.7±1.1	43.9±2.3	40.7±2.7	43.3±1.3	42.7±1.3
[¥]Seedor index (Tibia)	14.1±0.7	14.9±0.9	14.2±1.0	13.8±0.8	14.4±1.3	14.0±1.7	13.9±0.7	14.6±0.4
Femur mass (mg)	751.3±66.4	820±58.3	818.8±60.1	784.3±36.5	808.6±48.1	740±66.3	784.3±52.2	808.6±37.6
Femur length (mm)	37.9±0.7	38.7±0.5	38.7±0.5	38.0±0.6	39.0±1.8	37.5±0.9	38.3±0.3	37.8±1.3
Seedor index (Femur)	20.2±1.3	21.2±1.4	21.5±1.3	20.6±0.9	20.8±1.5	19.7±1.4	20.5±1.3	21.4±0.8

All data presented as mean ± standard deviation. [a]Significant increase in tibial mass compared to other treatment groups (P<0.005); **DW + W** = gavaged daily with 10 mℓ/kg body mass of distilled water with 0.5% (v/v) dimethyl sulphoxide in the neonatal period followed by *ad libitum* access to plain drinking water post-weaning and throughout adulthood (n = 7); **DW + W** = distilled water

neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). ¥Seedor index = bone density in mg/mm.

In female rats, there was a significant increase in the tibial masses of rats that received a single hit of fructose late in adulthood (DW+F) and those that received a double hit of fructose early in the neonatal period and late in adulthood compared to the other treatment groups (HF+F) (P<0.05; Table 4.2). Neonatal oral administration of OA prevented the increase in tibial masses caused by fructose administration (P<0.05; Table 4.2). Tibial and femoral lengths and bone densities were not significantly different across all experimental treatment groups (P<0.05).

Table 4.2: The effect of neonatal oral administration of oleanolic acid or fructose on tibial and femoral masses, lengths and Seedor indices in female rats fed a high fructose diet.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Tibia mass (mg)	413.4±20.3	[a]487.5±65.3	465.4±24.7	479.6±44.3	461±25.55	475.2±29.4	447.7±29.6	[b]482.3±31.3
Tibia length (mm)	38.6±0.7	39.6±0.7	39.2±1.0	38.6±0.9	39.3±0.6	39.2±0.9	39.1±0.6	39.5±1.2
[¥]Seedor index (Tibia)	10.7±0.5	12.3±1.7	11.9±0.4	12.4±1.1	11.7±0.7	12.1±0.5	11.5±0.6	12.2±0.6
Femur mass (mg)	562.9±22.9	606.3±27.2	610±48.4	596.3±35.4	595±29.8	623.3±52.4	576.7±43.7	630±39.4
Femur length (mm)	37.9±0.7	38.7±0.5	38.7±0.5	38.0±0.6	39.0±1.8	37.5±0.9	38.3±0.3	37.8±1.3
Seedor index (Femur)	16.7±0.6	17.5±0.8	17.4±1.3	17.7±0.8	17.3±0.8	18.1±1.3	16.9±1.1	18.3±0.8

All data presented as mean ± standard deviation. [ab]Significant increase in tibial and femoral masses compared to other treatment groups ($P<0.05$).

DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). ¥Seedor index = bone density in mg/mm.

4.3.1.3 Radiographic images

In adult male rats, there were no observable morphological differences between the tibiae across different experimental treatment groups (Figure 4.3).

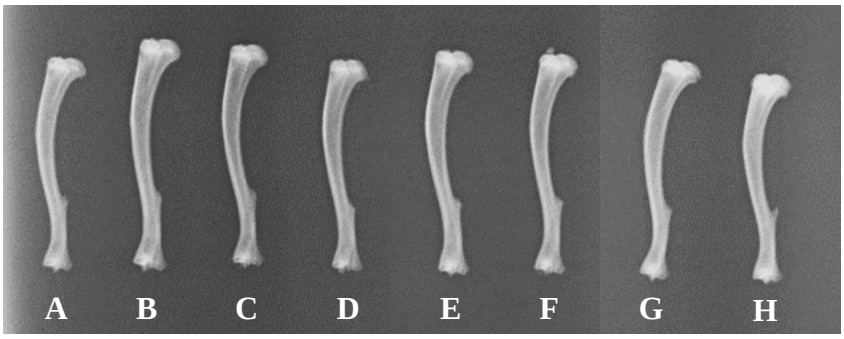


Figure 4.3: Radiographic images showing the tibiae from a representative male rat from each of the experimental groups.

A) DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **B) DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

In adult female rats, there were no observable morphological differences between the tibiae across different experimental treatment groups (Figure 4.4).

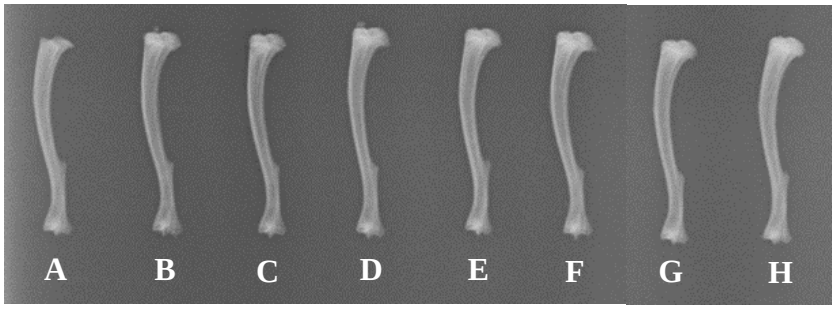


Figure 4.4: Radiographic images showing the tibiae from a representative female rat from each of the experimental groups.

A) DW + W = distilled water neonatally + plain drinking water post weaning and throughout adulthood (n = 7); **B) DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

4.3.2 The effect of neonatal oral administration oleanolic acid on the morphometry of the gastrointestinal tract and visceral organs in fructose-fed male and female rats

4.3.2.1 Gastrointestinal tract organs and pancreas

In adult male rats, there were no significant differences in pancreatic masses, small and large intestine masses and lengths of male rats across experimental treatment groups (P>0.05; Table 4.3).

Table 4.3: The effect of neonatal oral administration of oleanolic acid or fructose on the absolute (g) and relative to tibia length (g/cm) masses, lengths of gastrointestinal tract organs and pancreas of male rats fed a high fructose diet.

Organ	**Treatment group**							
	DW+W	**DMW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
SI (cm)	137.3±10.7	140.3±6.8	142.1±2.9	138.3±10.5	136.9±13.0	137.1±5.76	138.6±9.6	141.3±11
SI (g)	8.3±0.9	8.7±1.0	8.9±0.8	8.5±1.2	8.3±1.1	7.9±0.5	8.2±0.9	8.8±1.5
SI (rTL)	2.0±0.2	1.9±0.2	2.0±0.2	2.0±0.3	1.9±0.3	1.9±0.2	1.9±0.2	2.1±0.4
LI (cm)	24.9±1.5	24.2±2.8	24.6±2.3	23.9±1.9	25.7±2.3	20.3±9.2	24.3±1.8	24.7±2.1
LI (g)	2.2±0.4	2.2±0.2	2.3±0.3	1.8±0.3	2.3±0.3	1.9±0.3	2.1±0.4	2.0±0.4
LI (rTL)	0.5±0.1	0.5±0.1	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1
PANCR (g)	1.5±0.2	1.5±0.2	1.4±0.3	1.5±0.1	1.4±0.3	1.2±0.3	1.5±0.3	1.3±0.2
PANCR (rTL)	0.4±0.1	0.4±0.0	0.3±0.1	0.4±0	0.3±0.1	0.3±0.1	0.4±0.1	0.3±0.1

All data presented as mean ± standard deviation. All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution

as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of organ relative to tibial length (g/cm); SI = small intestine; LI = large intestine; PANCR = pancreas.

In adult female rats, there were no significant differences in pancreatic masses, small and large intestine masses and lengths across treatment groups (P>0.05; Table 4.4).

Table 4.4: The effect of neonatal oral administration of oleanolic acid or fructose on the absolute (g) and relative to tibia length (g/cm) masses, lengths of gastrointestinal tract organs and pancreas of female rats fed a high fructose diet.

Organ	**Treatment group**							
	DW+W	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
SI (cm)	122.3±14.0	130±25.9	131.9±9.9	125.4±4.8	126.1±9.3	125.8±6.0	123.3±6.7	126.1±6.9
SI (g)	5.7±0.7	6.3±0.8	6.7±1.1	6.3±0.5	6.1±0.7	6.5±0.9	5.9±0.6	6.7±0.6
SI (rTL)	1.5±0.2	1.6±0.2	1.7±0.3	1.6±0.1	1.6±0.2	1.5±0.5	1.5±0.2	1.5±0.5
LI (cm)	21.7±1.5	20.1±2.2	22.35±3.0	20.5±1.5	23.0±1.8	21.3±1.3	22.0±2.1	22.0±2.3
LI (g)	1.5±0.2	1.4±0.3	1.7±0.4	1.4±0.2	1.7±0.2	1.6±0.3	1.7±0.2	1.4±0.3
LI (rTL)	0.4±0.0	0.4±0.1	0.4±0.1	0.4±0.0	0.4±0.1	0.4±0.1	0.4±0.1	0.3±0.1
PANCR (g)	1.0±0.2	1.2±0.2	1.1±0.2	1.0±0.2	1.1±0.2	1.1±0.1	1.1±0.1	1.2±0.4
PANCR (rTL)	0.3±0.1	0.3±0.0	0.2±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.3±0.0	0.3±0.1

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF +**

W = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of organ relative to tibial length (g/cm); SI = small intestine; LI = large intestine; PANCR = pancreas.

4.3.2.2 Visceral organs

In adult male rats, there were no significant differences in the heart masses ($P>0.05$; Table 4.5). The absolute and relative kidney masses of rats that received OA in the neonatal period and plain water in adulthood (OA+W) and OA neonatally and a single hit of fructose in adulthood (OA+F) were significantly lower compared to rats from the other treatment groups ($P<0.05$; Table 4.5).

Table 4.5: The effect of neonatal oral administration of oleanolic acid or fructose on the masses of visceral organs in male rats fed a high fructose diet.

Organ	**Treatment group**							
	DW+W	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Heart (g)	1.4±0.1	1.6±0.3	1.5±0.2	1.5±0.1	1.4±0.2	1.4±0.1	1.4±0.1	1.5±0.3
Heart (rTL)	0.3±0.0	0.4±0.1	0.3±0.0	0.4±0.0	0.3±0.1	0.3±0.1	0.3±0.0	0.4±0.1
Kidneys (g)	2.6±0.4	2.6±0.3	[a]1.4±0.1	[b]1.7±0.2	2.7±0.3	2.4±0.2	2.7±0.2	2.7±0.4
Kidneys (rTL)	0.6±0.1	0.6±0.1	[c]0.3±0.0	[d]0.4±0.1	0.6±0.1	0.6±0.1	0.6±0.0	0.6±0.1

All data presented as mean ± standard deviation. [abcd]Significantly lower absolute and relative kidney masses ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of organ relative to tibial length (g/cm).

In adult female rats, there were no significant differences in the masses and lengths (where applicable) of visceral organs across all treatment groups ($P>0.05$; Table 4.6).

Table 4.6: The effect of neonatal oral administration of oleanolic acid or fructose on the absolute (g) and relative to tibia length (g/cm) masses of visceral organs in female rats fed a high fructose diet.

Organ	Treatment group							
	DW+W	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Heart (g)	1.1±0.3	0.9±0.3	1.0±0.1	0.9±0.1	1.0±0.1	1.1±0.2	0.9±0.1	1.0±0.1
Heart (rTL)	0.3±0.1	0.2±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.1	0.2±0.0	0.2±0.1
Kidneys (g)	1.4±0.1	1.7±0.2	1.3±0.1	1.6±0.1	1.6±0.2	1.6±0.2	1.6±0.3	1.7±0.2
Kidneys (rTL)	0.4±0.0	0.4±0.1	0.3±0.0	0.4±0.0	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of organ relative to tibial length (g/cm).

4.3.3 The effect of neonatal oral administration of oleanolic acid on glycaemic control in fructose-fed male and female rats

4.3.3.1 Oral glucose tolerance test (OGTT)

The pre-gavage fasting blood glucose concentration was significantly higher in male rats that received a double hit of fructose early in the neonatal period and in adulthood ($P<0.05$; Figure 4.5)
The blood glucose concentrations of rats administered with DW+F, HF+W and HF+F peaked at T = 15 post-gavage and were significantly greater ($P<0.05$) than the basal concentration (T = 0) and returned to a basal concentration 180 minutes post-gavage. The blood glucose concentration of rats administered with DW+W, OA+W, OA+W, OAHF+W and OAHF+F peaked at T = 30 post-gavage and was significantly greater ($P<0.05$; Figure 4.5) than the basal concentration (T = 0) and returned to a basal concentration 180 minutes post-gavage (Figure 4.5). There were no significant differences in peak blood glucose concentrations across treatment groups ($P>0.05$). However, the group receiving a double hit of fructose in the neonatal phase and late in adulthood had significantly higher peak glucose levels ($P<0.05$; Figure 4.5).

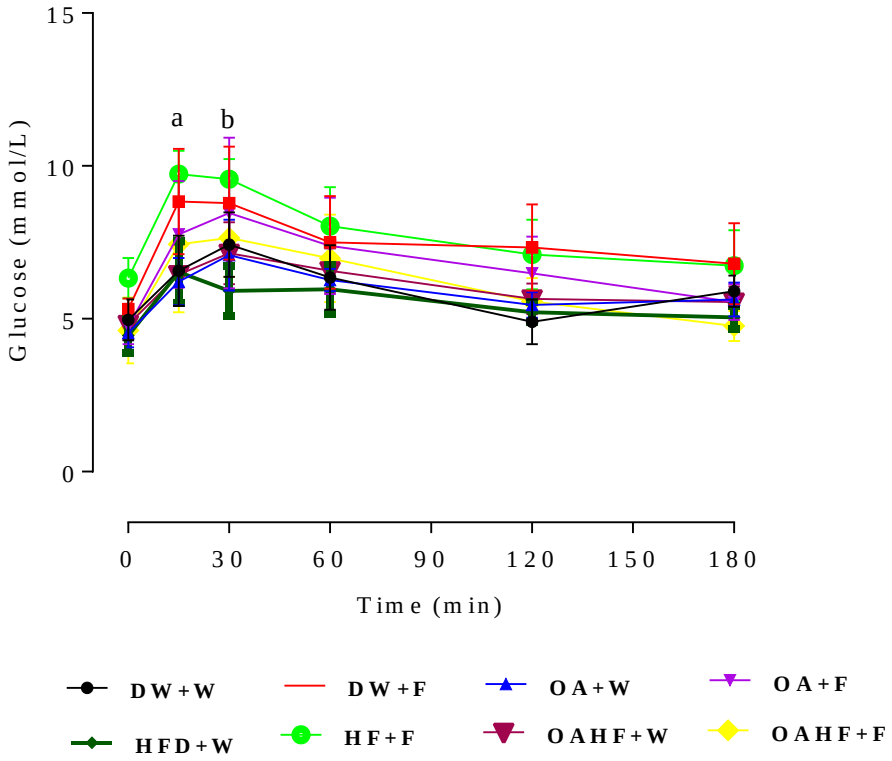


Figure 4.5: The effect of neonatal oral administration of oleanolic acid or fructose on glucose tolerance in male rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [ab]Significant differences in blood glucose concentrations at T = 15 and 30 vs blood glucose at T = 0 (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood

(n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

In adult female rats, the pre-gavage glucose concentration was significantly higher in rats that received a double hit of fructose early in the neonatal period and late in adulthood ($P<0.05$; Figure 4.6).The blood glucose concentrations of rats that received DW+W, DW+F, OA+F, HF+F, HF+W, OA+W, OAHF+W and OAHF+F peaked at T = 15 post-gavage and were significantly greater ($P<0.05$; Figure 4.6) than the basal concentration (T = 0) and returned to a basal concentration 180 minutes post-gavage. Blood glucose concentrations of rats that received OA+W and HF+F peaked at T = 30 post-gavage and was significantly greater ($P< 0.05$; Figure 4.6) than the basal concentration (T = 0) and returned to a basal concentration 180 minutes post-gavage (Figure 4.6). There were no significant differences in the peak blood glucose concentrations ($P>0.05$; Figure 4.6).

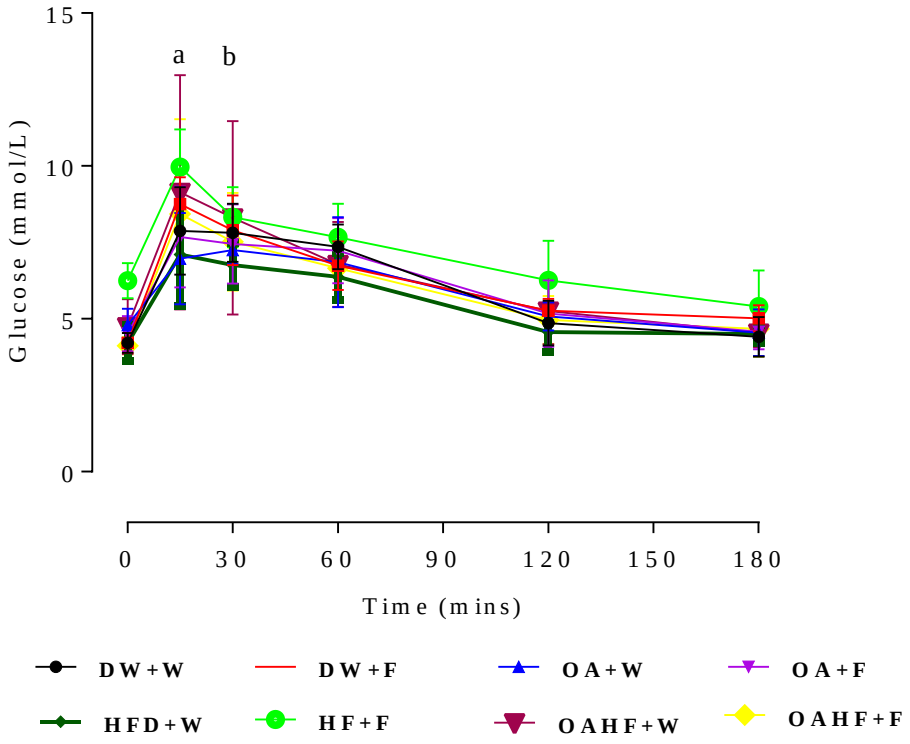


Figure 4.6: The effect of neonatal oral administration of oleanolic acid or fructose on glucose tolerance in female rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [ab]Significant differences in blood glucose concentrations at T = 15 and 30 vs blood glucose at T = 0 (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood

(n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

There was no significant difference in the total area under the curve (AUC) of oral glucose tolerance test (OGTT) for male rats across all experimental treatment groups (P>0.05; Figure 4.7).

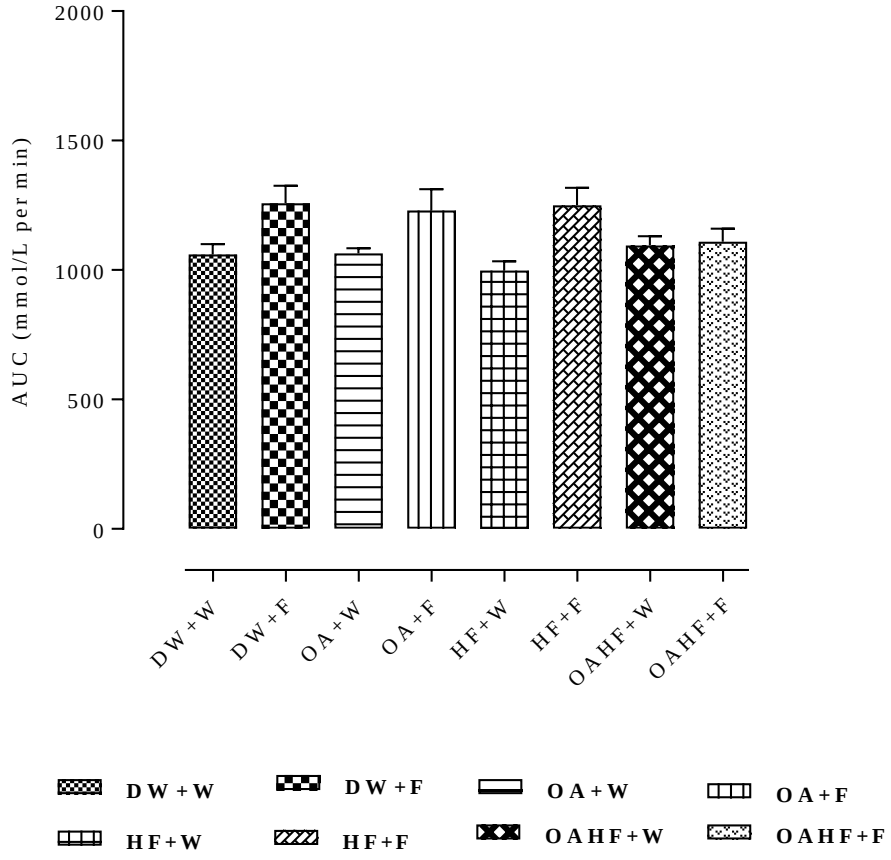


Figure 4.7: The effect of neonatal oral administration of oleanolic acid or fructose on the total area under the curve of the oral glucose tolerance test of male rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high

fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

In female rats, fructose consumption either late in adulthood (DW+F) or as a double hit early in the neonatal period and late in adulthood (HF+F) resulted in up to 37% increase in the total AUC compared with other experimental treatment groups ($P<0.05$; Figure 4.8). Neonatal administration of OA prevented the increase in AUC induced by the late single hit of fructose (OA+F) and a double hit (OAHF+F) fructose effects ($P<0.05$; Figure 4.8). There were no significant differences in the total AUC in rats that received neonatal OA and the control group that did not receive fructose (DW+W) ($P>0.05$).

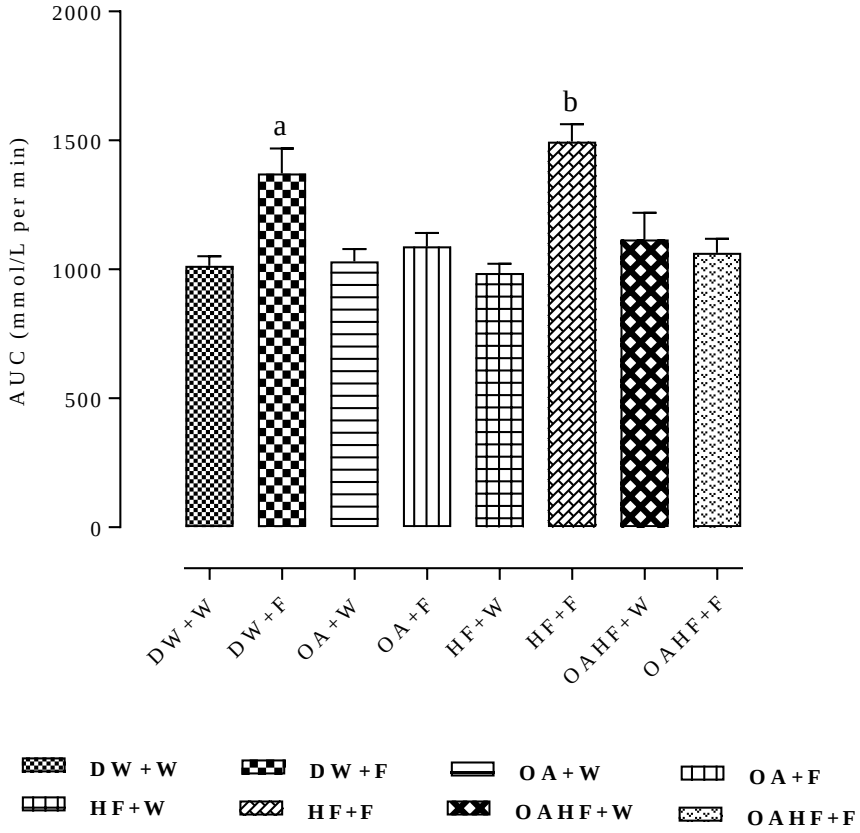


Figure 4.8: The effect of neonatal oral administration of oleanolic acid or fructose on the total area under the curve of the oral glucose tolerance test of female rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [ab]Significant differences in blood glucose concentrations between OA + F and DW+F ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout

adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

4.3.3.2 Fasting blood glucose, insulin homeostatic model assessment of insulin resistance (HOMA-IR)

In male rats, fructose consumption either late in adulthood (DW+F) or a double hit (HF+F) resulted in an increase in fasting levels of glucose and the HOMA-IR compared to other treatment groups ($P<0.05$; Table 4.7). However, there were no significant differences in fasting levels of insulin in male rats across all treatment groups ($P>0.05$; Table 4.7). Oral administration of OA in the neonatal period prevented the increase in fasting glucose and HOMA-IR observed as a result of the double hit with fructose ($P>0.001$; Table 4.7).

Table 4.7: The effect of neonatal oral administration of oleanolic acid or fructose on fasting blood glucose and plasma insulin concentration and HOMA-IR index in male rats fed a high fructose diet.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Glucose (mmol/ℓ)	4.9±0.7	5.3±0.3	4.5±0.5	4.7±0.6	4.5±0.4	[a]6.3±0.7	4.8±0.5	4.5±1.1
[¥]Insulin (ng/mℓ)	45.3±10.5	53.2±10.2	44.6±9.4	51.4±8.7	39.8±5.2	56.9±7.9	41.9±10.2	46.5±16.5
HOMA-IR	0.24±0.06	[b]0.33±0.06	0.23±0.05	0.27±0.07	0.18±0.03	[c]0.35±0.12	0.22±0.05	0.22±0.07

All data presented as mean ± standard deviation. [abc]Significant increase in glucose and HOMA-IR for groups receiving fructose late (DW+F) and a double hit neonatally and in adulthood (HF+F) compared with other treatment groups ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). HOMA-IR = homeostatic model

assessment of insulin resistance; ¥Insulin concentration was converted to μU/ℓ for the calculation of HOMA-IR.

In female rats, fructose consumption either late in adulthood (DW+F) or as a double hit early in the neonatal period and late in adulthood (HF+F) resulted in an increase in fasting levels of glucose and HOMA-IR compared with other experimental treatment groups ($P<0.05$; Table 4.8). Neonatal oral OA administration with the double hit fructose (OAHF+F) prevented the effects on glucose levels and HOMA-IR in the rats that had the double hit of fructose without any other intervention (HF+F) ($P<0.05$; Table 4.8). There were no significant differences in the fasting levels of insulin across all treatment groups in female rats ($P>0.05$; Table 4.8).

Table 4.8: The effect of neonatal oral administration of oleanolic acid or fructose on fasting blood glucose and plasma insulin concentration and HOMA-IR index in female rats fed a high fructose diet.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Glucose (mmol/ℓ)	4.2±0.3	4.6±0.8	4.8±0.5	4.5±0.6	4.2±0.6	[a]6.2±0.7	4.7±0.9	4.1±0.3
[¥]Insulin (ng/mℓ)	42.2±9.5	45.1±13.1	41.6±12.6	48.1±11.2	43.8±6.3	55.7.0±11.5	46.4±5.2	46.3±6.4
HOMA-IR	0.19±0.05	0.30±0.08	0.22±0.06	0.22±0.07	0.20±0.03	[b]0.38±0.07	0.24±0.05	0.22±0.04

All data presented as mean ± standard deviation. [ab]Significant increase in glucose and HOMA-IR for groups receiving fructose late (DW+F) and a double hit neonatally and in adulthood (HF+F) compared with other treatment groups ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). HOMA-IR = homeostatic model

assessment of insulin resistance; ¥Insulin concentration was converted to μU/ℓ for the calculation of HOMA-IR.

4.3.4 The effect of neonatal oral administration of oleanolic acid on visceral and epididymal fat pad masses in fructose-fed rats

4.3.4.1 Visceral and epididymal fat pad masses

Male rats that received fructose either late in adulthood, with or without neonatal OA (DW+F and OA+F) or as a double hit neonatally and late in adulthood, (HF+F) had up to 40% increase in the mass of the relative epididymal fat pads ($P<0.05$; Figure 4.9A). A double hit with fructose (early in the neonatal period and late in adulthood) (HF+F) caused up to 40% increase in relative visceral fat mass (adjusted to relative tibial length) compared to the other experimental treatment groups ($P<0.05$; Figure 4.9B). Rats which received neonatal oral administration of OA and the double hit of fructose (OAHF+F) had significantly lower measures of adiposity than the rats which had the double hit of fructose without any other treatment intervention (HF+F) ($P<0.05$; Figure 4.9A and Figure 4.9B), but there was no significant difference when compared to the rats that had the late hit with fructose (OA+F) ($P>0.05$; Figure 4.9A and Figure 4.9B).

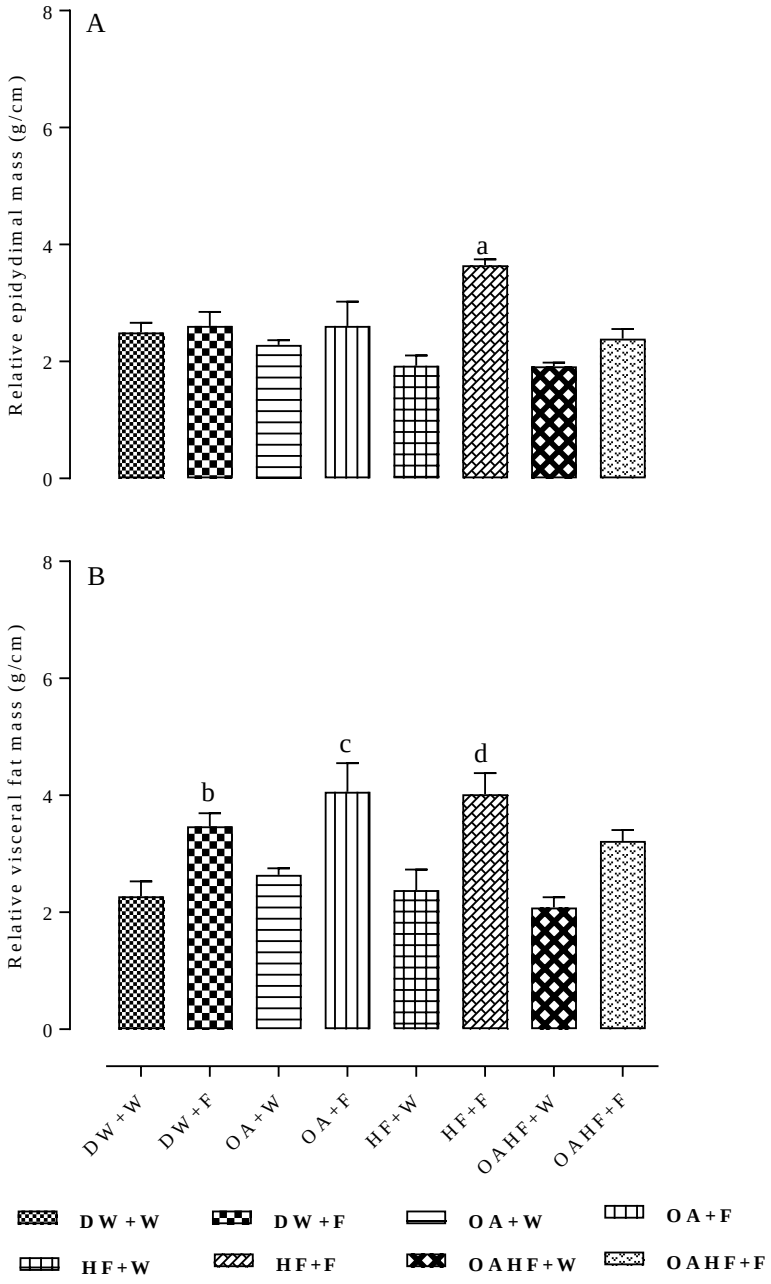


Figure 4.9: The effect of neonatal oral administration of oleanolic acid or fructose on relative (to tibial length) epididymal (A) and relative visceral (B) fat mass in male rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [abcd]Significant increase in visceral and epididymal fat pad (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of fat pad relative to tibial length (g/cm).

In female rats, fructose consumption either late in adulthood (DW+F) or as a double hit early neonatally and late in adulthood (HF+F) resulted in up to 26% and 65% respectively increase in relative visceral fat masses compared to the other experimental groups ($P<0.05$; Figure 4.10). Neonatal oral OA administration prevented the late single hit (OA+F group) and the double hit (OAHF+F group) fructose effects on visceral fat mass ($P<0.05$; Figure 4.10). No significant differences were observed between the group that received neonatal OA and the control group which did not receive fructose (DW+W) ($P>0.05$; Figure 4.10).

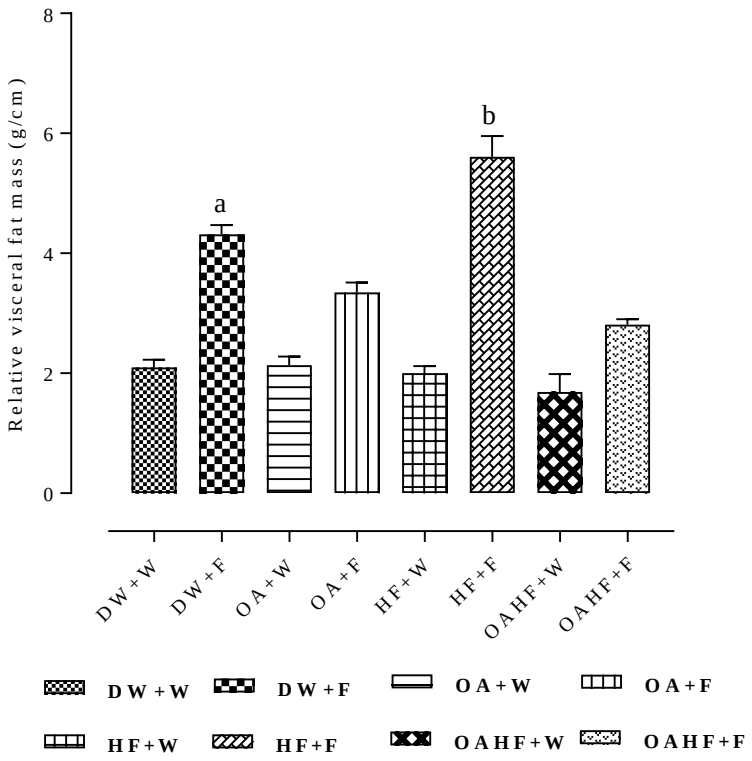


Figure 4.10: The effect of neonatal oral administration of oleanolic acid or fructose on visceral fat mass in female rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [ab]Significant increase in relative visceral fat masses (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain

drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). rTL = weight of fat pad relative to tibial length (g/cm).

4.3.5 The effect of neonatal oral administration of oleanolic acid on the concentration circulating cholesterol and triglycerides in fructose-fed male and female rats

There were no significant differences in fasting triglyceride and cholesterol levels of the male rats across all experimental treatment groups (P>0.05; Table 4.11).

Table 4.9: The effect of neonatal oral administration of oleanolic acid or fructose on circulating triglycerides and cholesterol in male rats fed a high fructose diet.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
TG (mmol/ℓ)	1.5±0.2	2.2±0.7	1.5±0.3	1.9±0.4	1.5±0.1	2.1±0.5	1.7±0.7	2.1±0.7
CHOL (mmol/ℓ)	3.7±0.7	3.9±0.4	3.7±0.7	4.1±0.5	3.2±0.7	3.4±0.4	3.8±0.6	3.2±0.9

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). TG = triglycerides; CHOL = cholesterol.

The levels of circulating triglycerides in female rats that received only a late fructose hit (DW+F) and those that received the double hit with fructose (in the neonatal period and in adulthood, HF+F) significantly increased by up to 65% than rats from the other experimental treatment groups ($P<0.05$; Table 4.12). Oral administration of OA in the neonatal period prevented the increase in the levels of triglycerides observed as a result of either a late single hit or a double hit with fructose (OA+F vs DW+F; OAHF+F vs HF+F respectively; $P<0.05$; Table 1.4B).

Table 4.10: The effect of neonatal oral administration of oleanolic acid or fructose on circulating triglycerides and cholesterol in female rats fed a high fructose diet in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
TG (mmol/ℓ)	1.5±0.3	[a]2.8±0.6	1.5±0.2	1.7±0.3	1.56±0.2	[b]2.7±0.4	1.4±0.3	1.6±0.4
CHOL (mmol/ℓ)	2.9±1.3	4.1±1.0	3.1±0.4	3.9±0.8	3.2±0.4	4.0±0.6	2.6±1.7	3.2±1.1

All data presented as mean ± standard deviation. [ab]Significant increase in fasting triglyceride levels (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).TG = triglycerides; CHOL = cholesterol.

4.4 DISCUSSION

In this study, I sought to investigate the potential protective effect of neonatal oral administration of oleanolic acid (OA) against the subsequent development of health outcomes associated with metabolic dysfunction induced by the consumption of fructose in different stages of life and the consequent outcomes in adulthood of male and female rats. Fructose caused metabolic derangements in the male and female rats, however, this was affected by the timing of the fructose intervention(s). I also noted sex differences in responses to the high fructose diets. I have shown that a double hit of fructose wherein it was administered in the neonatal period followed by a secondary dietary insult in adulthood resulted in the development of several negative health outcomes associated with metabolic dysfunction, namely the significant increases in terminal body mass (females only), visceral fat mass (males and females), serum triglycerides (females only), epididymal fat (males only), fasting plasma glucose (males and females), impaired glucose metabolism (females only), β-cell dysfunction and insulin resistance (males and females). The single late fructose hit in adulthood resulted in impaired glucose metabolism, increased visceral fat pad masses and levels of triglycerides in female but not male rats. Oral administration of OA in the neonatal period successfully attenuated the manifestation of fructose-induced metabolic disorders in both male and female rats.

4.4.1 The effect of neonatal oral administration of oleanolic acid on growth performance in fructose-fed male and female rats

The results of this current study showed an increase in body masses across treatment groups from weaning and termination in male and female rats. There were no differences in body masses of male rats across all treatment groups at weaning and termination. However, in female rats, administration of a double hit of fructose neonatally and administered as a secondary dietary insult in adulthood caused an increase in terminal body mass which was not observed in male rats and was prevented by neonatal administration of OA. Due to the obesogenic nature of fructose, the neonatal treatment with fructose and its consumption in adulthood as a secondary dietary insult could be the main culprit in the observed increase in terminal body mass in female rats that

received a double hit. This study produced results which corroborate previous findings in human and animal studies which showed that neonatal consumption of fructose resulted in abnormal body weight gain (Stanhope and Havel, 2009, Bocarsly et al., 2010). While increased body mass alone may not represent obesity, the co-existence of other obesogenic variables such as accumulation of visceral fat, increased levels of triglycerides (TG) as I have shown, provides confirmation of the obese status especially in the female rats (Bocarsly et al., 2010).

Due to factors highlighted in section 3.4.1, body mass changes may not be an accurate measure of linear growth, hence the use of tibial masses is strongly recommended. The lack of significant differences in the tibial length and masses and Seedor indices for male and female rats support the result that different treatments had no adverse effects on the growth performance of the rats. Neonatal oral administration of OA prevented the fructose-induced increase in terminal body mass observed in female rats, suggesting that neonatal interventions with OA do not negatively affect growth. OA may be used in the neonatal period against excessive body mass gain in adulthood without adverse effects on their growth performance. The specific molecular mechanism(s) through which OA prevents fructose-induced body mass increases requires further investigation.

4.4.2 The effect of neonatal oral administration oleanolic acid on visceral and epididymal fat masses in fructose-fed male and female rats

Findings from this study showed that consumption of fructose in adulthood only and when consumed as a double hit (neonatally and in adulthood) resulted in visceral fat (relative to tibial length) accumulation in both male and female rats. However, it was the double hit of fructose that caused a 40% increase in epididymal fat mass in male rats compared to the control and the other treatment groups. These findings suggest that the single late and double fructose hits may have programmed the accumulation of epididymal fat in males as well as visceral fat accumulation in both male and female rats. The total adipose tissue accumulation plays an important role in the development of metabolic disorders such as IR, T2DM and CVDs (Wajchenberg, 2000). However, there are some fat depots that are more associated with the development of metabolic

risk factors than others (Bjørndal et al., 2011). A human study has shown that in men, the excessive accretion of omental and mesenteric adipose tissue, both part of visceral adipose tissue (VAT), is strongly associated with CVDs and T2DM (Hoffstedt et al., 1997). In rodents however, gonadal VAT surrounding testis (epididymal fat) and ovaries is regarded as one of the largest depots that contribute to the development of metabolic disorders in rodents on a high-energy cafeteria diet (Bjørndal et al., 2011). It is therefore possible that the difference in the epididymal fat and the rest of the visceral fat responses observed in male rats that received a double hit of fructose could be due to epididymal fat being more vulnerable to accumulate than the other visceral fat depots in response to high-fructose diets. Based on recent neonatal programming studies (Simpson et al., 2016, Beckford et al., 2017) and results from this study, I hypothesise that neonatal fructose consumption may have programmed adipose tissue development during the critical period which resulted in the observed visceral (male and female rats) and epididymal fat (male rats) accumulation later in adulthood. The neonatal programming of visceral fat accumulation and lipid metabolism induced by fructose may have caused the development and manifestation of other MetS-associated outcomes in adulthood upon the introduction of a secondary dietary fructose insult as we have already shown with glucose metabolism in this study.

The significantly heavier visceral fat pad masses observed in this study in fructose-fed female and male rats, corroborate previous findings which also reported an increase in visceral fat deposition and a corresponding increase in body mass following excessive consumption of fructose in adulthood (Huynh et al., 2008). Despite the lack of differences in terminal body masses of the male rats that received either a double hit of fructose or a single late hit, as observed in female rats, there were differences in visceral (both males and females) and epididymal (males only) fat mass. Findings from previous studies also suggest that excessive consumption of fructose increases the levels of plasma triglycerides which may accelerate the accretion of body fat and causing visceral obesity and body mass gain (Stanhope and Havel, 2008, Stanhope and Havel, 2009), a trend that I have observed in the current study, especially in female rats that received a double hit or a single late fructose hit.

Although male and female rats receiving a single late and a double fructose hit exhibited increased visceral fat mass relative to tibial length compared with all other treatment groups, this increase was higher and pronounced in female (26% single hit and 65% double hit) than in male (40% double hit) rats. This finding suggests that female offspring were vulnerable to greater fructose-induced adiposity than their male counterparts, a finding that is in line with the greater body mass increase in fructose-fed females (double hit), also previously reported by Bayol et al. (2008). Human studies have shown that the development of adipose depots differs depending on sex due to hormonal differences, with women exhibiting a tendency to accumulate greater total body fat than men (Chusyd et al., 2016). It is thus likely that androgens and female hormones, which control several metabolic pathways (Kalyani and Dobs, 2007) and are involved in the pathogenesis of metabolic disorders (Muller et al., 2005) may have contributed to the observed sexual dimorphic differences in obesity observed in this study. Androgens are known to promote cellular glucose uptake and energy utilisation in skeletal muscles and liver (Navarro et al., 2015b) thus reducing the tendency to accumulate total body fat in men.

The excessive accumulation of epididymal fat in male rats is linked to the development of infertility (Ghanayem et al., 2010). Dietary obesogens such as fructose have been shown to cause adipogenesis and epididymal fat accumulation resulting in the disruption of testicular physiology and metabolism (Cardoso et al., 2017). This, in turn, causes male infertility that can be passed on to future generations through epigenetic modifications passed by the male gametes (Cardoso et al., 2017). Human studies have also shown that over-consumption of high-energy diets adversely affects the male reproductive axis and consequently affects the testicular physiology with serious negative reproductive outcomes such as infertility (Rato et al., 2014). Given the similarities in the biology of the epididymal fat in rodents and humans, it is possible that the male rats that had more epididymal fat from consuming a double hit of fructose diet may have an increased risk to develop infertility while those that received OA neonatally may be protected against the development of infertility a finding that may be extrapolated into a human population. Although important similarities exist between epididymal fat physiology of rodents and humans, minor differences exist which warrants careful consideration and interpretation of results from rodent

studies for extrapolation into humans (Bjørndal et al., 2011, Chusyd et al., 2016). Further studies on the effect of fructose on testicular function and the potential protective role of OA using the current model are recommended.

It is interesting to note that neonatal oral administration of OA protected against fructose diet-induced increase in epididymal fat mass in males and visceral fat pad masses caused by either a single late hit or a double fructose hit in both male and female rats. Previous animal studies have shown that OA administration in adulthood exhibited hepatic lipid-lowering effects by decreasing hepatic expression of peroxisome proliferator-activated receptor-γ coactivator-1β (PGC-1β), an important regulator in maintaining hepatic lipid homeostasis, and its associated downstream target genes (Chen et al., 2017). However further studies should be done using the current animal model to elucidate the molecular mechanisms underlying the observed visceral and epididymal fat lowering effect of OA administered during the neonatal period.

4.4.3 The effect of neonatal oral administration of oleanolic acid on morphometry of the gastrointestinal and visceral organs in fructose-fed male and female rats

My findings did not show any clear trends in the absolute and relative masses and lengths of small and large intestine across the treatment groups in both male and female rats. This finding suggests that neonatal treatment with either OA or fructose did not compromise or promote the development of GIT organs (small and large intestine). Except for the groups that received OA+W and OA+F (which had significantly lower absolute and relative kidney masses), all treatment groups in males and females had similar visceral organ (heart, kidneys and testis) masses. The lack of differences in visceral organ masses in both male and female rats following neonatal treatment with OA and fructose suggests that the neonatal oral administration of OA did not cause atrophy or hypertrophy of visceral organs.

Given the lack of adverse effects on the GIT and visceral organs, we propose that OA administered neonatally may be used for the prophylactic treatment of MetS and its associated

risk factors without adversely affecting the growth and development of the GIT and visceral organs.

4.4.4 The effect of neonatal oral administration of oleanolic acid on glucose tolerance in fructose-fed male and female rats.

Both a late single hit and a double hit of 20% w/v fructose solution as drinking fluid caused glucose intolerance (total area under the curve of the OGTT) in the female, but not in male rats. The late single fructose hit resulted in the development of IR or β-cell dysfunction (HOMA-IR) in male but not in female rats. However, the double fructose hit caused IR or β-cell dysfunction and hyperglycaemia in both male and female rats. None of the treatments induced an increase in the levels of insulin in both male and female rats.

The oral glucose tolerance test (OGTT) is an important clinical tool for the characterisation of metabolic phenotype (Ayala et al., 2010). It is used to diagnose impaired glucose tolerance and as a standardised test of carbohydrate metabolism by assessing the ability to dispose of an oral glucose load over time (Ernsberger and Koletsky, 2012, Ayala et al., 2010). Sustained hyperglycaemia (>120min) in plasma glucose constitutes impaired glucose tolerance and can be used together with fasting hyperglycaemia to diagnose patients with T2DM (Kwon et al., 2008).

My findings on glucose tolerance suggest that a double fructose hit adversely affected glucose metabolism by impairing the ability to tolerate a glucose load in female rats and caused IR and possibly pancreatic β-cell dysfunction in both male and female rats. Insulin resistance reported in this study for male and female rats that received a double hit of fructose also corroborates earlier findings by Huynh et al. (2008) who also demonstrated the manifestation of IR following a double hit of fructose neonatally and in adulthood. However, results on the levels of insulin in fructose-fed rats from the current study are at variance with the same study by Huynh et al. (2008) who reported the development of hyperinsulinaemia in rats that received 10% fructose in the neonatal period and 65% fructose diet in adulthood. The observed variance in levels of insulin can be explained in part by the differences in the quantity and method of fructose administration in adulthood. In the current study, adult rats received 20% w/v fructose solution as

a secondary dietary insult, in contrast to Huynh et al. (2008) who gave 65% w/w in the feed which possibly provided more calories resulting in hyperinsulinaemia which we did not observe. Although hyperglycaemia alone does not indicate whether there is an insufficiency of insulin secretion (Ernsberger and Koletsky, 2012), it is possible that its development observed in this study following a double hit of fructose in both male and female rats coupled with impaired glucose tolerance and IR suggests that the fructose that was administered in the neonatal period may have been effective in programming the neonatal rats for the development of hyperglycaemia, impaired glucose tolerance and IR later in adulthood after exposure to a secondary dietary insult.

The development of hyperglycaemia, glucose intolerance and insulin resistance that was induced by either a single late and double fructose hit was prevented by the neonatal oral administration of OA in male and female rats. These findings expand on previous findings that reported the anti-diabetic effects of plant-derived OA in adult rats (Gao et al., 2007). Diabetes is characterised by persistent hyperglycaemia, poor glycaemic control and IR (Valle et al., 2016). Reactive oxygen species (ROS) have been suspected to play a role in the progression from normal glucose metabolism to impaired glucose tolerance and development of IR (Wang et al., 2011). In the first experimental phase of this study (Chapter 3) I showed that OA protects against fructose-induced oxidative damage by preventing the fructose-induced downregulation of the expression of GPx and SOD genes in suckling rats. By upregulating the expression of anti-oxidant enzymes, GPx and SOD, OA promotes anti-oxidant cellular defences which play a role in its glucose-lowering effects (Wang et al., 2011). Previous studies have demonstrated the anti-oxidant and anti-glycative role of OA, as such it is possible that the protective role of OA against the development of dysregulation of glucose metabolism observed in this study following a late single hit or a double fructose hit, could be due to the ability of OA to scavenge for free radicals and enhancing anti-oxidant cellular defence system (Wang et al., 2013). The observed improvement of glucose tolerance and IR by OA also suggests that OA may be promoting insulin signal transduction mechanisms and inhibiting poor handling of glucose caused by oxidative stress and IR (Wang et al., 2011).

OA also protects against oxidative stress-induced IR (Wang et al., 2011). Mechanistic studies have shown that triterpenoid compounds such as OA act as hypoglycaemic and anti-obesity agents mainly through (i) reducing the intestinal absorption of glucose; (ii) decreasing endogenous glucose production such as hepatic gluconeogenesis; (iii) increasing insulin sensitivity; (iv) improving lipid metabolism; and (v) promoting body weight loss (Camer et al., 2014). In addition to these promising beneficial effects, it is believed that OA and associated triterpenoid protect against diabetes-related co-morbidities due to their anti-atherogenic, anti-inflammatory, and anti-oxidant properties (Wang et al., 2013).

4.4.5 The effect of neonatal oral administration of oleanolic acid on circulating triglycerides and cholesterol in fructose-fed male and female rats

The late single fructose hit and a double hit of fructose caused an increase in the level of triglycerides (TGs) in female but not in male rats. Contrary to my findings, a study in adult male and female rats has shown that excessive consumption of fructose causes an increase in body weight that is accompanied by an increase in VAT and elevated circulating levels of TGs in rats (Bocarsly et al., 2010). The observed hypertriglyceridaemia in female rats following administration of a late single hit and a double fructose hit could be explained in part by the fructose-mediated neonatal programming of lipogenic genes (Herman and Samuel, 2016, Saad et al., 2016) and the increased vulnerability to develop hypertriglyceridaemia in fructose-fed female rats. Although male rats that received a late single fructose hit and a double hit of fructose had increased visceral fat accretion, they neither had increased terminal body weights nor TG levels. Regional differences in adipose tissue distribution which is affected by sex hormones may have resulted in the observed sexual dimorphic differences in circulating TG levels. Fructose is predominantly metabolised in the liver and due to its high lipogenic potential, its excessive consumption is likely to increase the metabolic burden on the liver resulting in the development of hepatic steatosis through *de novo* lipogenesis (DNL) (Bocarsly et al., 2010). The observed increase in the levels of TGs in fructose-fed female animals could also be due to the upregulation of hepatic DNL and secretion of excess hepatic lipids which contributes to the plasma pool of TGs (Basaranoglu et al., 2014).

Fructose consumption as either a late single hit or a double hit did not cause an increase in the levels of cholesterol levels across all treatment groups in both male and female rats. The manifestation of fructose-induced metabolic disorders in adulthood also include hypercholesterolaemia, especially when the high fructose diet is fortified by fats (Okoduwa et al., 2017). In the absence of supplemented fat or very high fructose diets, it is uncommon to induce hypercholesterolaemia in rats, this finding which is corroborated by our findings (Huang et al., 2004, Seneff et al., 2011).

4.5 CONCLUSION

I have shown that fructose administration had adverse effects on several health outcomes associated with metabolic dysfunction. The timing (late or double hit) of the administration of fructose had an effect on the development of metabolic dysfunction. I also observed sex-specific differences in the metabolic response to dietary fructose, showing the significance of considering sex effects in metabolic studies. I conclude that neonatal interventional treatment with oleanolic acid during the critical window of developmental plasticity protected against the development of fructose diet-induced health outcomes associated with metabolic dysfunction in male and female Sprague Dawley rats. Following studies in higher animals, OA should be considered as a natural strategic prophylactic intervention with a lot of potential in the fight against the scourge of metabolic disorders that are impacting significantly on the health systems globally.

In the first part of the second long-term experiment, I have demonstrated the protective effect of administering OA neonatally against the development of fructose-induced health outcomes associated with MetS in male and female rats. In the following chapter which constitutes the second part of the long-term experiment, I will focus my investigation on the potential protective effect of neonatal oral administration of OA against the development of non-alcoholic fatty liver disease (NAFLD). NAFLD is considered as hepatic manifestation of MetS and it is important to investigate the effect of fructose feeding on its development and the potential role of OA to protect against its development.

CHAPTER 5:

THE EFFECT OF NEONATAL ORAL ADMINISTRATION OF OLEANOLIC ACID ON THE SUBSEQUENT DEVELOPMENT OF HIGH FRUCTOSE DIET-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE IN RATS

5.1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the accumulation of lipids in hepatocytes in the absence of alcohol abuse, steatogenic medication or underlying pathological conditions that cause steatosis (Basaranoglu et al., 2014). For a detailed description of the definition, prevalence, pathogenesis, diagnosis and treatment of NAFLD refer to section 2.4. The development of metabolic disorders such as NAFLD in adulthood has been known to be programmable in the different phases of ontogeny such as gestation and in the neonatal phase of development which are characterized by phenotypic plasticity (Vickers, 2011, Li et al., 2015). Studies have shown that increased consumption of fructose during the critical periods of developmental plasticity predisposes offspring to the development of metabolic disorders later on in adult life (Mortensen et al., 2014, Tain et al., 2015, Rodríguez et al., 2016).

Rats are an altricial species and several of the developmental changes occurring during the early neonatal phase correspond to those occurring in the third trimester of precocious species including humans (Clancy et al., 2001). Consequently, the neonatal rat represents an experimental model for studying developmental changes that are associated with the human third trimester of pregnancy (Clancy et al., 2001). Thus for my study, I chose to use neonatal rats as an experimental model for developmental metabolic plasticity.

Current therapeutic options for MetS and NAFLD include lifestyle changes such as a reduction in dietary calorific intake, and an increase in physical activity (Adams and Angulo, 2006). Traditional pharmacological agents that are currently being used to treat and manage metabolic disorders and NAFLD include insulin-sensitizing drugs like metformin, anti-oxidants such as Vitamin E and lipid-lowering drugs like fenofibrate (Adams and Angulo, 2006, Harrison, 2006). This treatment approach often administered to adults, amongst other mechanisms is directed at weight loss and increasing energy expenditure so that less energy is stored in the adipose tissue (Harrison, 2006, Tolman and Dalpiaz, 2007). These conventional synthetic medicines are often associated with a number of side effects including lactic acidosis, liver toxicity and hypoglycaemia (Adams and Angulo, 2006). There is need to develop alternative treatments or

prophylactic interventions, preferably using affordable natural products that form part of our normal diets, having fewer side effects, and safer than conventional medicines. Ideally, the alternative pharmacological interventions should be administered during the critical phases of development to provide long-term health benefits.

Oleanolic acid (OA), a naturally-occurring triterpenoid (Castellano et al., 2013a) was selected for this study due to its several pharmacological benefits (Camer et al., 2014, Lin et al., 2016). Previous studies on murine models have shown that OA treatment in adulthood significantly reduces diet-induced hepatic lipid accumulation and confers hepatic protection through the modulation of hepatic SREB-1c-mediated expression of genes responsible for DNL (Liu et al., 2013). Most studies on the beneficial pharmacological effects of OA have been performed in adult animals but none have been performed in the neonatal phase.

The neonatal period selected for the experimental treatments in the current study is a critical window of developmental phenotypic plasticity in which development and programming of several phenotypes such as good health profiles and metabolic disorders that are observed later in adulthood occur (Wang et al., 2012, Wang, 2013).

5.1.1 Aim

The current study was designed to investigate the potential hepatoprotective effect of OA administered in the neonatal phase on the development of fructose-induced hepatic manifestations of metabolic dysfunction in a murine experimental model.

5.1.2 Specific objectives

The specific objectives of this part of the second long-term experimental study were to determine the potential protective effect of neonatal oral administration of OA against the subsequent development of fructose-diet induced non-alcoholic fatty liver disease in male and female rats by assessing:

a) Terminal liver masses – relative to tibial length

b) Hepatic lipid accumulation – determined by measuring hepatic content using the Soxhlet method.
c) Histomorphometry of the liver tissues samples – for the determination of steatosis, inflammation and fibrosis
d) Surrogate markers of liver function – measurement of serum enzyme markers of liver function such as alanine amino transferase (ALT) and the non-tissue specific alkaline phosphatase (ALP).

5.2 MATERIALS AND METHODS

5.2.1 Ethical clearance for the study

All animal experimental procedures were performed according to the protocols approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC ethical clearance number: 2014/47/D; **Appendix 2-3**) and the University of Johannesburg Research Ethics Committee (REC number: 01-02-2016; **Appendix 5**). Sample and liver tissue assays were done in appropriate laboratories of the School of Physiology, University of the Witwatersrand and the Department of Biomedical Technology, University of Johannesburg.

5.2.2 Housing and animal husbandry

Sprague Dawley (*Rattus norvegicus*) dams and their respective litter were housed according to the conditions described in section 4.2.2.

5.2.3 Study design and experimental dietary treatments

This was an interventional comparative study in which 112 male and female neonatal rats from 13 nursing dams were used. Figure 4.1 shows a schematic representation of the study design, showing the experimental groups, stages of development, sequence and timing of interventions and health outcomes that were measured. The study design for this study is described in detail in section 4.2.4.

5.2.4 Terminal procedures

The rats were then euthanased on PD112 and blood samples collected as described in section 4.2.6. The abdominal cavity was opened via a midline incision and the liver was carefully dissected out. The liver samples were weighed (Presica 310M®; Precision Instruments, Switzerland) and masses recorded. Thereafter a section of each liver sample was dissected from the right medial lobe and preserved in 10% phosphate buffered formalin for histological analyses. The remainder of the liver sample was stored at -20ºC for determination of liver lipid content.

5.2.5 Measurement of surrogate markers of liver function

Using the plasma samples collected at termination, surrogate markers of hepatic function, alkaline phosphatase (ALP) and alanine amino transferase (ALT) were measured by standard enzymatic techniques using a calibrated automatic biochemical analyser as described in section 3.2.7. The results from the measurement of enzyme markers were reported as units per litre (U/ℓ). The biochemical analyser automatically loaded and analysed 10 µℓ of serum sample onto each of the 2 pre-loaded disks for ALT and ALP before results were displayed and printed out.

5.2.6 Preparation and processing of liver tissue for histological examination

Following fixation, the liver tissue samples processed using the automatic tissue processor (Microm STP 120 ThermoScientific, Massachusetts, USA), embedded in paraffin wax, and sectioned at 3µm thickness using a rotary microtome and then mounted on glass slides. The tissue sections were then either stained with haematoxylin and eosin (HE) to assess hepatocellular changes or Masson's trichrome (MT) stain to assess fibrosis according to standard protocols as described by Bancroft and Gamble (2008). Photomicrographs of the stained sections were acquired using a Leica ICC50 HD video camera linked to a Leica DM 500 microscope. The photomicrographs were analyzed using the ImageJ (Abràmoff et al., 2004). Composite images were prepared with CorelDraw X3 Software (Version 13, Corel Corporation, Ottawa, Canada). No pixelation adjustments of the captured photomicrographs were undertaken except for adjustment of contrast and brightness.

5.2.7 Histomorphometry and histological examination of the liver samples

The haematoxylin and eosin-stained (HE) stained sections of the liver were semi-quantitatively scored for steatosis and inflammation according to the NASH Clinical Research Network (NASH-CRN) and Kleiner et al. (2005). Steatosis was determined by analyzing hepatocellular vesicular steatosis, based on the total area affected, grading was done as follows: Grade 0 = <5% steatosis; Grade 1 = 5–33% steatosis; Grade 2 = 33–66% steatosis; Grade 3 = >66% steatosis per camera field of the parenchyma. Inflammation was scored by counting the number of inflammatory cell aggregates in the liver parenchyma (Liang et al., 2014) and graded as follows: Grade 0 = none or no foci of inflammation per camera field; Grade 1 = fewer than 2 foci per camera field; Grade 2 = 2-4 foci per camera field; Grade 3 = >4 foci per camera field at ×20.

Fibrosis was quantitatively assessed from photomicrographs of the portal areas of MT-stained sections at ×40 using ImageJ (Schneider et al., 2012). Briefly, the images were converted to 8-bit scale, a threshold set manually and area fraction covered by connective tissue was quantified. To avoid sampling errors, liver samples were obtained from the right medial lobe and all the histological features were semi-quantitatively and quantitatively assessed by a histologist (PN) who was blinded to the animal treatments.

5.2.8 Determination of hepatic lipid content

Liver samples from each of the four different treatment groups for male and female rats were pooled together and sent to the Agricultural Research Council (ARC), for the determination of intra-hepatic lipid content as described in section 3.2.8 and **Appendix 6**. Liver lipids for each experimental group were determined in triplicate.

5.2.9 Statistical analyses

Results were presented as mean ± standard deviation (SD) and analysed using GraphPad Prism for Windows Version 7.0 (GraphPad Software Inc., San Diego, USA). For parametric data, the differences between groups were statistically analysed by one-way ANOVA followed by

Bonferroni's *post-hoc* test were used. The Kruskal-Wallis test was used to analyse non-alcoholic fatty liver disease scores for steatosis and inflammation followed by Dunn's *post-hoc* test. $P \leq 0.05$ was considered significant.

5.3 RESULTS

5.3.1 The effect of neonatal oral administration of oleanolic acid on liver masses (absolute and relative to tibia length) and hepatic lipid content in fructose-fed adult male and female rats

In male rats, consumption of fructose either late in adulthood (DW+F) or as a double hit early in the neonatal period and late in adulthood (HF+F) resulted in up to 19% increase in liver masses compared to other treatment groups ($P<0.05$; Table 5.1). Oral administration of OA in the neonatal period prevented the late single hit (OA+F) and double hit (OAHF+F) effects of fructose on the gain in liver mass ($P<0.05$). There were no significant differences in liver masses between neonatal administration of OA and the control group which did not receive any fructose throughout its lifespan (DW+W) ($P>0.05$).

Table 5.1: The effect of neonatal oral administration of oleanolic acid or fructose on the absolute (g) and relative (g/cm tibia) liver masses of male rats fed a high fructose diet in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Liver (g)	11.7±1.8	13.5±1.2	11.0±0.7	12.5±1.4	13.5±1.4	[b]17.2±1.8	12.1±1.3	12.8±2.1
Liver (g/cm tibia)	2.8±0.4	[a]3.6±0.3	2.5±0.2	2.9±0.4	3.1±0.3	[b]3.7±1.6	2.4±1.1	3.0±0.6

All data presented as mean ± standard deviation. [ab]Significant increase in absolute and relative liver masses ($P<0.05$) compared to the other groups. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). Liver masses expressed relative to tibia length.

Although the absolute liver mass was significantly higher ($P<0.05$; Table 5.2) for female rats that received a double hit of fructose (HF+F), when adjusted to relative tibial length, the relative liver mass was not significantly different compared to other treatment groups ($P>0.05$; Table 5.2).

Table 5.2: The effect of neonatal oral administration of oleanolic acid or fructose on the absolute (g) and relative (g/cm tibia) liver masses of female rats fed a high fructose diet in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Liver (g)	6.1±0.5	7.8±0.4	6.2±0.6	6.6±0.8	8.4±0.5	[a]9.2±0.7	6.2±0.6	6.7±0.6
Liver (g/cm)	1.6±0.1	2.0±0.1	1.6±0.1	1.7±0.2	2.2±0.1	2.1±0.7	1.6±0.2	1.6±0.5

All data presented as mean ± standard deviation. [a]Significant increase in absolute and relative liver masses ($P<0.05$) compared to the other groups. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post- weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). Liver masses expressed relative to tibial length (g/cm**).**

In male rats, only the double hit (HF+F) of fructose (early in the neonatal period and late in adulthood) resulted in a significant accumulation (47% increase) in hepatic lipid content compared to other treatment groups ($P<0.05$; Figure 5.1). Oral administration of OA in the neonatal period prevented the increased hepatic lipid content observed as a result of the double hit with fructose (OAHF + HF vs HF +F; $P<0.001$; Figure 5.1).

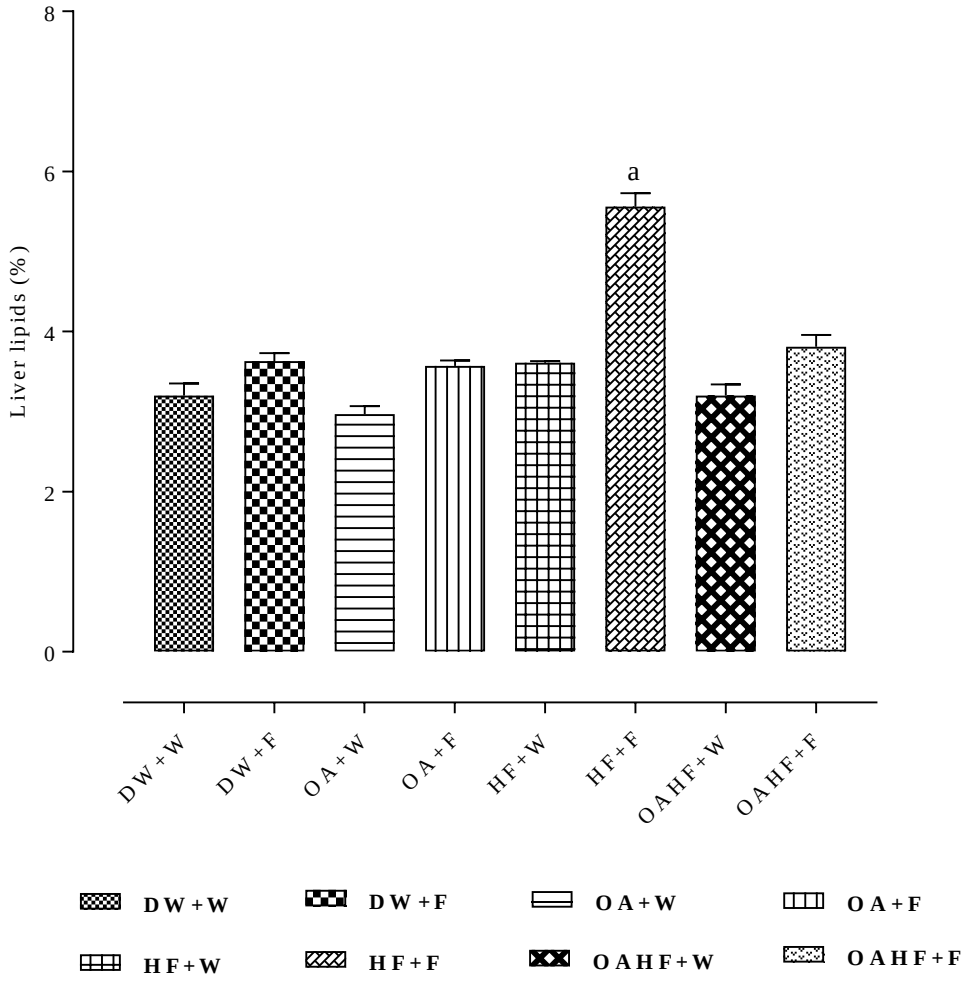


Figure 5.1: The effect of neonatal oral administration of oleanolic acid or fructose on hepatic lipid storage in male rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [a]Significant increase in hepatic lipid content in rats that received a double hit of fructose neonatally and in adulthood (HF+F) than in other treatment groups (P<0.05). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n =

6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

In females, hepatic lipid accumulation was not restricted to the double hit with fructose. The liver lipid content for female rats that received a late fructose hit (DW+F) and those that received a double hit neonatally and in adulthood (HF+F) was up to 49% higher than female rats from the other treatment groups ($P<0.05$; Figure 5.2). Oral administration of OA in the neonatal period prevented the increased hepatic lipid content observed as a result of the late single hit and double hit with fructose (OA + F vs DW + F and OAHF + F vs HF +F; $P>0.001$; Figure 5.2).

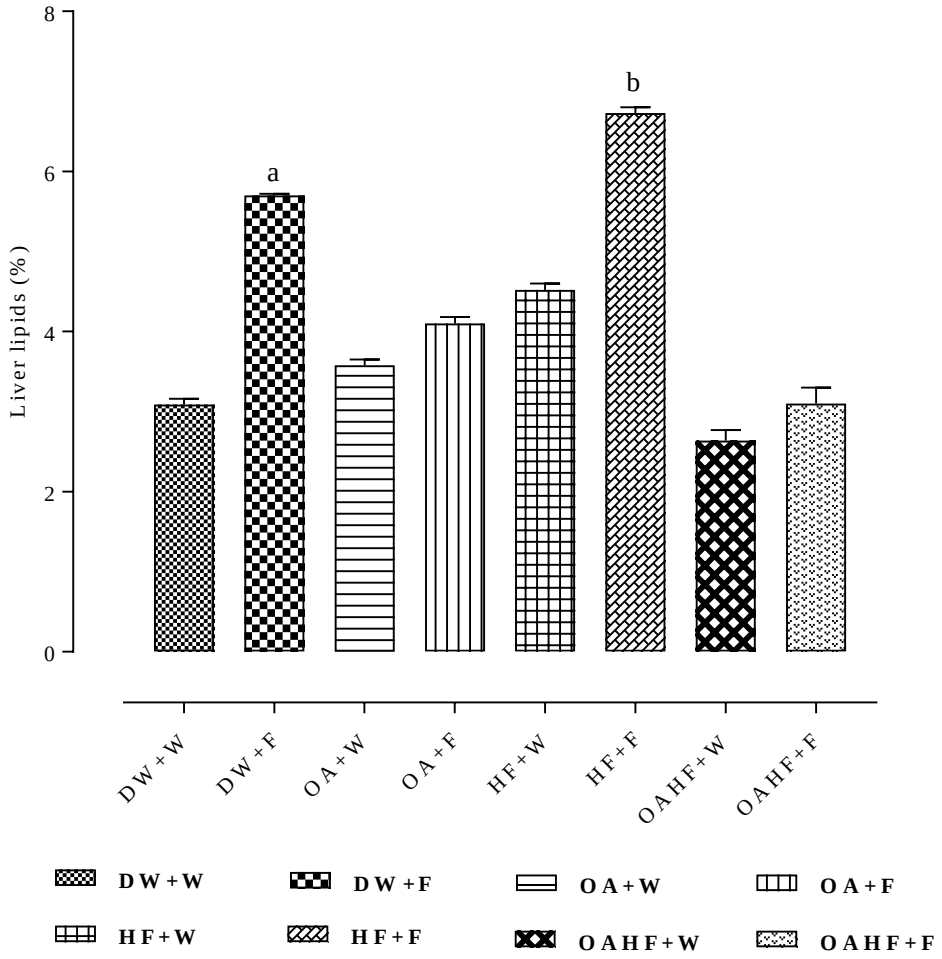


Figure 5.2: The effect of neonatal oral administration of oleanolic acid or fructose on hepatic lipid storage in adult female rats fed a high fructose diet as neonates and, or in adulthood.

All data presented as mean ± standard deviation. [ab]Significant increase ($P<0.05$) in hepatic lipid content in rats that received only fructose late (DW + F) and those that received fructose as neonates and as adults (HF+F) than the control rats that did not receive any fructose throughout the study period (DW + W). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain

drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

5.3.2 The effect of neonatal oral administration of oleanolic acid on hepatic histomorphometry of fructose-fed adult male and female rats

In male rats, steatosis scores were greater in the rats that received a double hit of fructose neonatally and in adulthood (HF+F) compared to all the other experimental groups (Kruskal Wallis; Table 5.3). In addition, both macro and microvesicular steatosis that was periportally distributed was observed in the HF+F group (Figure 5.3F). However, there were no significant differences in the scores for hepatic inflammation across all treatment groups ($P>0.05$). Fibrosis area fraction was up to 3 times higher in the rats that only received a late fructose hit (DW+F) and a double fructose hit (neonatally and in adulthood; HF+F) compared to other treatment groups ($P<0.05$; Table 5.3). Fibrosis in these two groups (DW+F and HF+F) was mainly periportal (Figure 5.4B and 5.4F). Neonatal oral administration of OA prevented the development of steatosis and fibrosis induced by a late (DW+F) and a double hit of fructose (HF+F).

Table 5.3: The effect of neonatal oral administration of oleanolic acid or fructose on **s**teatosis, fibrosis and inflammation scores in male rats fed a high fructose diet as neonates and, or later in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Steatosis	0.3±0.6	1.7±0.6	0±0	0.7±0.6	0.7±0.6	2.3±0.6	0.3±0.6	1±0
Inflammation	1.3±0.6	0.3±0.6	0.3±0.6	0.3±0.6	1.7±0.6	0.3±0.6	0.7±0.6	1.3±0.6
Fibrosis (Area fraction)	1.2±1.2	[a]10.4±1.8	0.9±0.9	3.9±0.4	2.6±0.8	[b]12.2±3.1	1.1±0.9	3.6±1.0

All data presented as mean ± standard deviation. [ab]Significant increase in hepatic fibrosis area fraction in rats that received DW + F and HF+F than rats from other experimental treatment groups ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

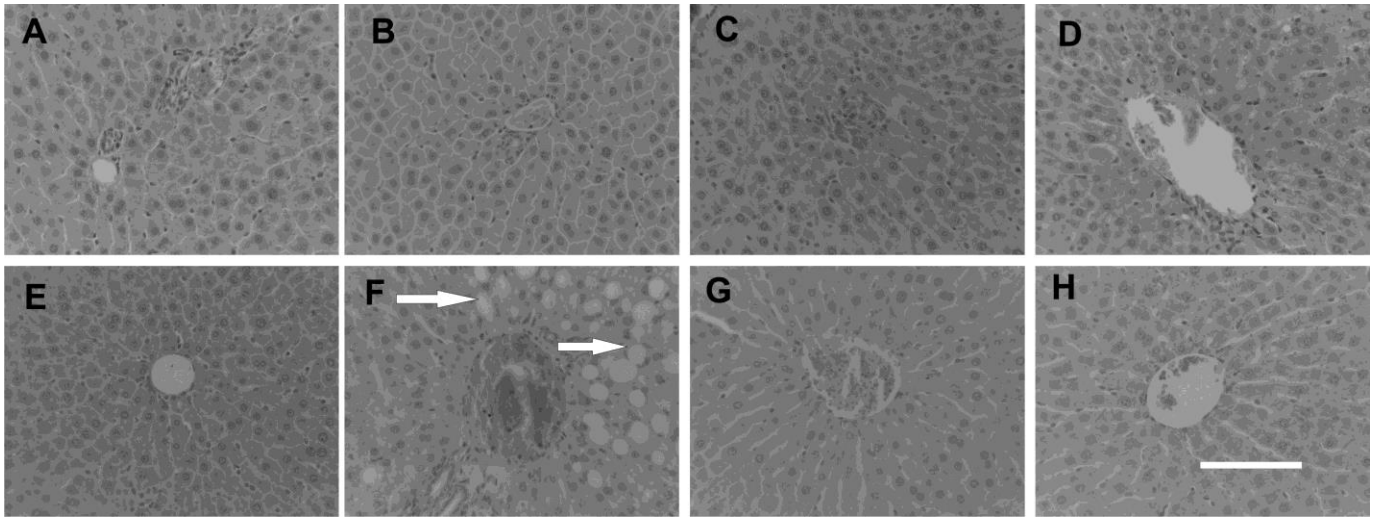


Figure 5.3: Photomicrographs showing histopathological features after haematoxylin and eosin staining of liver cross-sections from a representative male rat from each experimental treatment group.

A) DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **B) DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). The open arrows indicate steatosis. Scale bar = 30 μm in the HE stain sections.

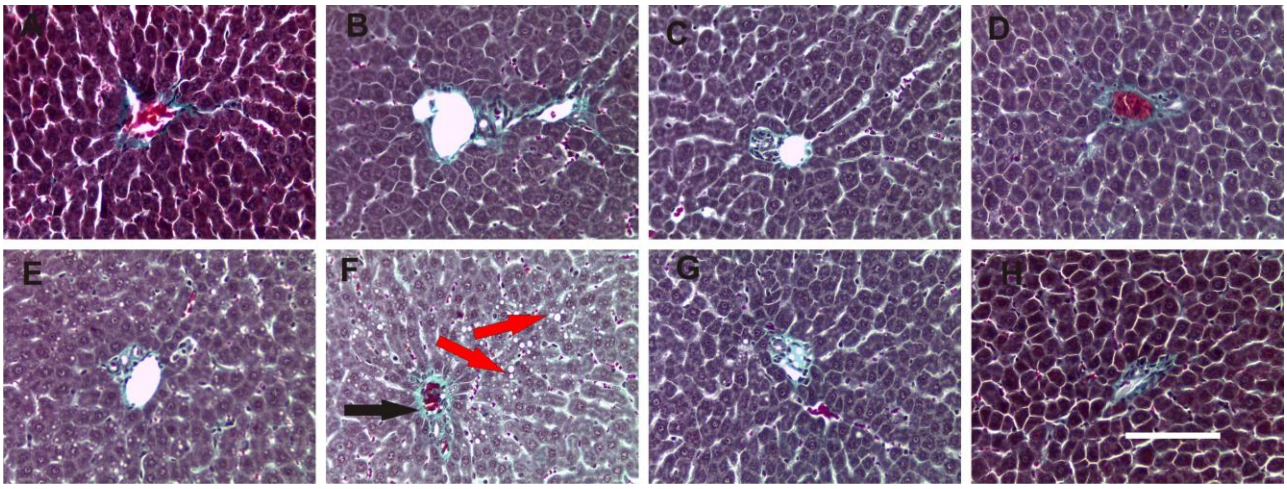


Figure 5.4: Photomicrographs showing histopathological features after Masson's trichrome staining of liver cross-sections from a representative male rat from each experimental treatment group.

A) DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **B) DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). Red arrows indicate steatosis and black arrows indicate fibrosis. Scale bar = 30 µm in the MT stain sections.

In female rats, steatosis was higher in the rats that received a single late hit (DW+F) and a double hit of fructose neonatally and in adulthood (HF+F) compared to all the other experimental groups (Kruskal Wallis; $P<0.05$; Table 5.4). In addition, periportally distributed macrovesicular steatosis was observed in the rats that received a single late hit (DW+F) and those that received a double hit of fructose neonatally and in adulthood (HF+F) (Figure 5.5B and 5.5F). Scores for hepatic inflammation were also higher in the rats that received a single late hit (DW+F) and a double hit of fructose neonatally and in adulthood (HF+F) compared to all the other experimental groups (Table 5.4). Furthermore, fibrosis was 3 times higher in the in rats that received DW+F and HF+F compared to other treatment groups ($P<0.05$; Table 5.4). Fibrosis in these two groups was mainly periportal (Figure 5.6B and F). Neonatal oral administration of OA prevented the development of steatosis and fibrosis induced by a late (DW+F) and a double hit of fructose (HF+F).

Table 5.4: The effect of neonatal oral administration of oleanolic acid or fructose on steatosis, fibrosis and inflammation scores in female rats fed a high fructose diet as neonates and, or in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
Steatosis	0.0±0.0	2.7±0.6	0.3±0.6	0.7±0.6	0.7±0.8	3.0±0.0	0.3±0.6	1.0±0.0
Inflammation	0±0	1.3±0.6	0.3±0.6	0.3±0.6	0.3±0.6	1.7±0.6	0.7±0.6	0.3±0.6
Fibrosis (Area fraction)	1.6±0.4	[a]12.5±0.7	1.6±0.7	4.2±0.3	2.5±0.5	[b]12.5±2.3	1.8±0.8	4.3±0.9

All data presented as mean ± standard deviation. [ab]Significantly higher hepatic fibrotic area fraction in rats that received a single late hit (DW+F) and a double hit of fructose neonatally and in adulthood (HF+F) than other experimental treatment groups ($P<0.05$). **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7).

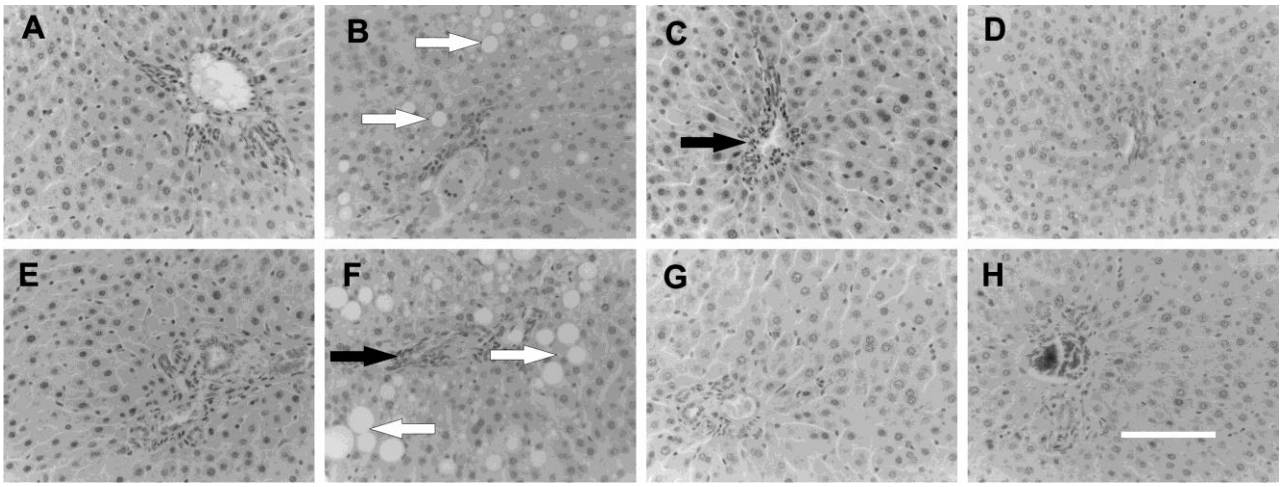


Figure 5.5: Photomicrographs showing histopathological features after haematoxylin and eosin staining of liver cross-sections from a representative female rat of each experimental treatment group.

A) DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **B) DW +** F = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). Solid black arrows indicate clusters of inflammatory cells. The open arrows indicate steatosis. Scale bar = 30 µm in the HE stain sections.

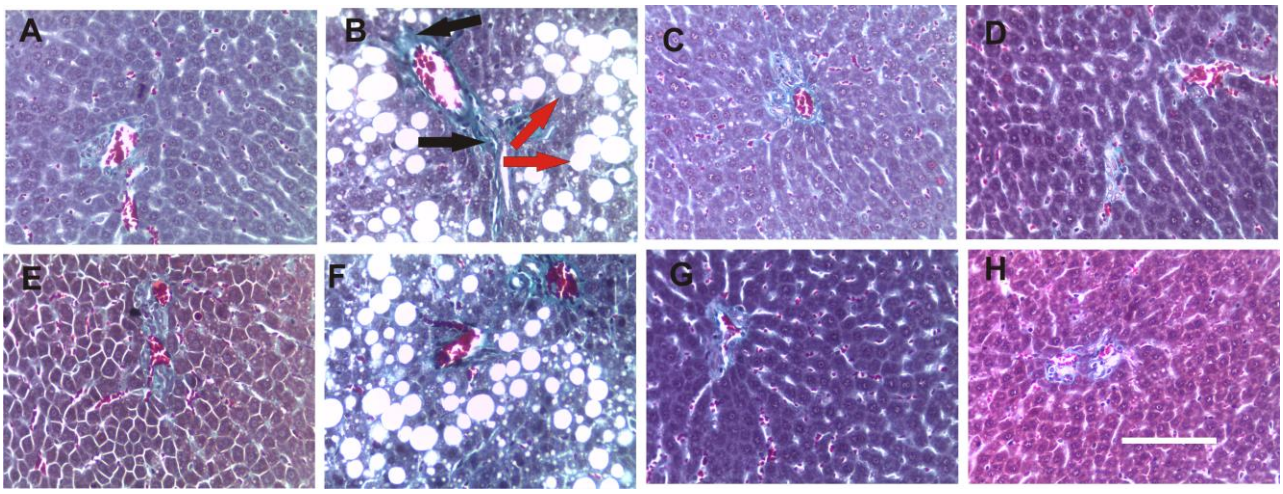


Figure 5.6: Photomicrographs showing histopathological features (fibrosis and steatosis) after Masson's trichrome staining of liver cross-sections from a representative female rat of each experimental treatment group.

A) DW + W = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **B) DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **C) OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **D) OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **E) HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **F) HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **G) OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **H) OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). Red arrows indicate steatosis and black arrows indicate fibrosis. Scale bar =30 µm in the MT-stained sections.

5.3.3 The effect of neonatal oral administration of oleanolic acid on a surrogate marker of liver function (alanine amino transferase) and non-tissue specific (alkaline phosphatase) in fructose-fed adult male and female rats

The serum levels of liver enzymes were similar in all experimental treatment groups for both male and female rats (P>0.05; Table 5.4 and 5.5).

Table 5.4: The effect of neonatal oral administration of oleanolic acid or fructose on a surrogate marker of liver function (alanine amino transferase) and non-tissue specific alkaline phosphatase levels of male rats fed a high fructose diet in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
ALT (U/ℓ)	81.9±10.6	82.7±27.8	93.1±39.7	82.1±34.5	76±10.1	88.0±40.3	66.4±28.9	78.0±15.3
ALP (U/ℓ)	87.3±5.7	108.5±12.5	102.1±17.7	111.4±12.0	96.9±29.8	140.7±21.8	80.6±9.5	86.14±15.3

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 7); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **HF + F** = high fructose solution neonatally +high fructose solution as drinking fluid in adulthood (n = 7); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). ALT = alanine amino transferase; ALP = alkaline phosphatase.

Table 5.5: The effect of neonatal oral administration of oleanolic acid or fructose on a surrogate marker of liver function (alanine amino transferase) and non-tissue specific alkaline phosphatase of female rats fed a high fructose diet in adulthood.

Parameter	**DW+W**	**DW+F**	**OA+W**	**OA+F**	**HF+W**	**HF+F**	**OAHF+W**	**OAHF+F**
ALT (U/ℓ)	67.8±2.3	81.6±9.7	72.3±8.7	78.1±10.4	77.3±11.0	79.6±7.4	63.5±9.8	67.9±8.8
ALP (U/ℓ)	64.3±3.8	67.1±14.7	68.9±13.9	62.6±12.8	63.5±15.8	69.7±14.3	65.0±24.6	63.7±13.1

All data presented as mean ± standard deviation. **DW + W** = distilled water neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **DW + F** = distilled water neonatally + high fructose solution as drinking fluid in adulthood (n = 6); **OA + W** = oleanolic acid neonatally + plain drinking water post-weaning and throughout adulthood (n = 8); **OA + F** = oleanolic acid neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **HF + W** = high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 7); **HF + F** = high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 8); **OAHF + W** = combination of oleanolic acid and high fructose solution neonatally + plain drinking water post-weaning and throughout adulthood (n = 6); **OAHF + F** = combination of oleanolic acid and high fructose solution neonatally + high fructose solution as drinking fluid in adulthood (n = 7). ALT = alanine amino transaferase; ALP = alkaline phosphatase.

5.4 DISCUSSION

In the current study, the potential protective effects of neonatal oral intake of oleanolic acid (OA) on the development of fructose-induced non-alcoholic fatty liver disease (NAFLD) were investigated in male and female rats. As confirmed by the terminal liver masses, total hepatic lipid content (more than 5% of mass as fat) and histological findings, a double hit of fructose (neonatally with the subsequent administration in adulthood) induced the development of NAFLD in both male and female rats. Moreover, the late fructose hit alone also induced NAFLD in the female, but not in male, rats. Neonatal oral administration of oleanolic acid was effective in attenuating the development of the fructose-induced NAFLD in both male and female rats.

It is notable that the timing of fructose intake had an impact on the liver. When administered early in the neonatal period only, both male and female rats did not develop the fatty liver disease. When administered in adulthood only, only the females developed the fatty livers. When the rats were administered with fructose as neonates and then later as adults both male and females rats had a significant increase in liver lipid accumulation. In addition, the intake of excessive fructose early and late (double hit) resulted in a greater increase in fatty liver compared to the rats that only had fructose late. Thus whilst our results confirm the findings from other studies that have shown that exposure to dietary insults during critical windows of cellular plasticity results in increased risks of developing NAFLD later in life (Stewart et al., 2013, Li et al., 2015), the sex differences in response to the high fructose diets emphasize the need for studies to be designed so as to account for possible differences between sexes and to avoid extrapolating findings from one sex to another without scientific verification. The importance of sex-specific medicine is indeed taking centre stage of late (Taqueti and Bairey Merz, 2017).

5.4.1 The effect of neonatal oral administration of OA on liver masses and hepatic lipid content in fructose-fed adult male and female rats.

The liver masses of male and female rats that received a double hit of fructose, initially as neonates and later in adulthood (HF+F) were up to 19% higher than other experimental groups.

These findings are consistent with those of Bruggeman et al. (2011) who also reported that dietary fructose in female rats increases the relative liver masses. Studies have shown that the increase in liver masses in fructose-fed rats can be attributed to the hepatic lipid storage from *de novo* lipogenesis and glycogen accumulation from hepatic metabolism of fructose (Rippe and Angelopoulos, 2013). I speculate that the increase in terminal liver masses observed in this study could most likely be due to lipid accumulation rather than glycogen since I fasted the rats for several hours prior to termination, as such glycogen would not have been present in significant amounts. Fasting rats overnight (8-12 hours) would have initiated a catabolic state and resulted in the depletion of glycogen stores (Ayala et al., 2010). The fructose-induced increase in liver masses was prevented by neonatal oral administration of OA, suggesting that OA potentially programs against the increase in liver mass possibly through modulating hepatic lipogenic enzymes (Liu et al., 2013). Other studies performed in adult mice have shown that short-term administration of OA protected against the increase in body and liver masses (Wang et al., 2013). An exciting finding in our study is the fact that the OA administered in the neonatal period showed long-lasting effects, about 14 weeks, after the last intake of OA.

Total liver lipid content followed a similar trend as recorded for liver masses, with the double hit of fructose resulting in an increase in hepatic lipid accumulation in both male and female rats. The accumulation and storage of hepatic lipids in male and female rats reported in this study confirm previous findings that demonstrated the role of fructose in the pathogenesis of hepatic steatosis and NAFLD (Lê et al., 2009, Lim et al., 2010). The increase in the liver lipid content observed in the current study could be attributed to the fructose-induced accumulation of lipids through its stimulation of hepatic *de novo* lipogenesis (Softic et al., 2016). The fructose-induced increase in hepatic liver lipid content was attenuated by the neonatal administration of OA. Studies have shown that OA, in addition to protecting against liver and body mass gain, also protects against fructose-induced hepatic triglyceride accumulation and hepatic morphological changes associated with NAFLD (Liu et al., 2013).

NAFLD is a common feature of MetS that is characterised by hepatic steatosis, inflammation, hepatocellular ballooning and fibrosis (Honda et al., 2016, Leitão et al., 2016).

Histomorphological examination of the liver confirmed the presence of sex-specific steatosis in rats from different treatment groups. The present study reports the presence of periportal microvesicular steatosis and periportal macrovesicular steatosis in male and female rats that received fructose neonatally and in adulthood. However steatosis was only observed in females that received a late hit of fructose in adulthood (DW+F), but not in male rats that received the same treatment. Female rats that received fructose had a more severe form of steatosis (macrosteatosis) and appeared to be more vulnerable to the development of NAFLD compared to males given the same fructose treatments. The sex-differences in liver masses and hepatic steatosis that I observed could possibly be explained by alterations in hepatic gene expression in the neonatal rats due to fructose administration which results in sex-dependent changes in lipid metabolism of the adult rats as previously reported (Clayton et al., 2015). It is also possible that the sex differences observed in liver masses and hepatic steatosis could be due to the differential sex-specific neonatal programming of metabolic dysfunction as previously reported (Vickers, 2011, Clayton et al., 2015, Rodríguez et al., 2016).

My findings on the vulnerability of female rats to develop NAFLD are in contradiction with several human epidemiological studies which showed that male subjects had a higher prevalence of NAFLD than females (Weston et al., 2005, Williams et al., 2011, Caballería et al., 2010). These observed sex differences were considered to be a reflection the role of sex steroid hormones and genetic vulnerability in the pathogenesis of NAFLD (Torres et al., 2012). In addition to the human studies, differences in lifestyle of males and females were also suggested as playing a role wherein it was observed that the weekly consumption of non-diet soda by males was significantly greater than by females(Williams et al., 2011). This would translate to an increased consumption of fructose by the males, the implications of which were discussed earlier. In my study the male and female rats had *ad libitum* access to the fructose solutions and similar housing conditions resulting in the females being more susceptible to developing NAFLD. There is thus need to investigate in human populations where males and females have the same lifestyles whether the susceptibility to develop NAFLD shows a similar trend to that of rats.

Future studies are recommended to establish the mechanisms responsible for our observed findings.

I nevertheless propose that neonatal OA administration may have prevented fructose-induced hepatic metabolic dysfunction through neonatal programming of hepatocellular protective mechanisms. The possible mechanism through which OA could have prevented hepatic lipid storage may be the neonatal programming of mechanisms regulating lipogenic pathways such as antagonizing the action of fructokinase activity which in turn alters hepatic conversion of fructose to fructose-1-phosphate.

5.4.2 The effect of neonatal oral administration of OA on hepatic histomorphometry of fructose-fed adult male and female rats.

Histomorphological analyses of the livers showed the presence of inflammatory cells in female rats that received a double hit of fructose neonatally and in adulthood, but not in males receiving the same treatment. Excess fructose in the liver causes fructosylation of proteins and superoxide formation which contributes to hepatic inflammation (Cichoż-Lach and Michalak, 2014). Accumulation of visceral white adipose tissue in fructose-consuming obese individuals is an important source of pro-inflammatory cytokines in the development of NAFLD (Mulder et al., 2016, Mulder, 2017). Hepatic inflammation is promoted by infiltration of macrophages (Seki and Schwabe, 2015) and the chemokine monocyte chemoattractant protein (MCP)-1 and its receptor C-C chemokine receptor-2 (CCR2) which play an important role in the recruitment of macrophages to the sites of hepatic inflammation (Mulder et al., 2016). Other studies have shown that malondialdehyde (MDA) generated during hepatic metabolism of fructose causes inflammation by activating NF-kB, a transcription factor regulating the expression of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 8 (Jaeschke et al., 1996). Excessive fructose intake has also been reported to cause hepatic inflammation by enhancing production of pro-inflammatory cytokines such as TNF-α and the activation of the c-Jun amino-terminal kinase (JNK) (Shimatmoto and Nobuyuki, 2006).

After 8 weeks of fructose feeding in adulthood following neonatal administration of fructose, male and female rats presented with fibrotic septa and there was evidence of accumulation of collagen fibres, as shown by a tripling of the fibrosis area fraction in rats receiving a late hit and a double fructose hit. Excessive fructose consumption causes hepatic steatosis and accumulation of hepatic lipids which eventually causes lipid peroxidation. Aldehyde products of lipid peroxidation such as 4-hydroxynonenal and MDA activate hepatic stellate cells (Lettéron et al., 1996, Day and James, 1998), the main collagen-producing cells in the liver, resulting in fibrosis (Reeves and Friedman, 2002). The dual effect of increased inflammation and oxidative cellular damage often result in accumulation of connective tissue and possibly contributes to the progression of NAFLD to NASH (Miller and Adeli, 2008).

5.4.3 The effect of neonatal oral administration of OA on surrogate markers of liver function in fructose-fed adult male and female rats.

My study did not reveal any significant differences in the blood levels of the enzymes, ALT and non-tissue specific ALP. Similarly, Zarghani et al. (2016) reported no significant changes in liver enzymes following diet-induced NAFLD. Human studies have shown that histologically confirmed NAFLD and NASH may exist without elevation of liver function enzymes (Calvaruso and Craxì, 2009). In this study, I have confirmed the presence of NAFLD in fructose-fed rats through histological analyses, liver masses and hepatic lipid storage, although the enzymes serving as surrogate markers of liver function were normal. The lack of significant increase in liver enzymes in rats that were administered with OA means that OA did not cause any hepatotoxicity and is safe for use as a phytochemical that potentially programs against the development of NAFLD. Although liver enzymes are useful indicators of hepatocellular damage, they may not be used as conclusive diagnostic tools of liver damage as such there is need to make use of other confirmatory diagnostic test panels such as non-invasive molecular biomarkers such as hyaluronic acid, tissue inhibitor of metalloproteinase 1 and amino-terminal pro-peptide of type III collagen to confirm liver damage (Pearce et al., 2013). Histology is considered a gold standard method for the diagnosis of NAFLD but is invasive and requires biopsies, as such the use of newer techniques is advisable.

5.5 CONCLUSION

In this study, I have demonstrated that a high fructose diet can induce fatty liver disease, however, the timing of the fructose intake in the life stage of rats has an impact on the phenotype. Sex-specific differences were also noted in response to the high fructose diets. It is thus important to note that studies should not just focus on a single sex but should be comparative between the sexes. I have also demonstrated, for the first time, that neonatal administration of oleanolic acid attenuates the development of fructose-induced NAFLD by reducing hepatic lipid storage, terminal liver masses and hepatic histomorphological changes associated with NAFLD. I conclude that dietary supplementation with OA in the neonatal phase of development potentially programs for hepatoprotection against the development of NAFLD in adult life. This opens the potential for further exploration of the use of prophylactic interventions during periods of developmental plasticity for long-term health benefits.

CHAPTER 6:

CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

In this study which was divided into two main experiments, I investigated, in the first experiment, the potential protective effect of neonatal oral administration of OA against fructose-induced oxidative damage, the development of adverse health outcomes and maturational or developmental changes of the gastrointestinal tract (GIT) in suckling male and female pups. My hypothesis was that neonatal oral administration of OA would protect against dietary fructose-induced oxidative damage, would not cause the development of poor health outcomes or precocious development of the GIT in suckling rats. Following the experimental treatments over a 7-day period, growth performance, clinical health profiles, biological molecular analyses were performed to assess the protective effect of neonatal oral administration of OA (60 mg/kg) following the administration of 20% (w/v) fructose solution. I have now shown that fructose administered acutely in the neonatal period caused the development of oxidative damage through the downregulation of the gene expression of anti-oxidant enzymes (GPx1 and SOD2). Neonatal administration of OA however prevented the fructose-induced downregulation of the genes for anti-oxidant enzymes. Neither OA nor fructose orally administered in the neonatal period had adverse effects on growth performance, biomarkers of metabolic function, surrogate markers of renal, hepatic function, hepatic lipid accumulation and GIT development when administered in suckling male and female rats.

The prophylactic use of OA in the fight against metabolic dysfunction, administered in the critical developmental period, prevents induced oxidative damage, and would not cause health risk or negative effects on gastrointestinal development. This confirms the safety of OA for use during the neonatal period. In the first experimental study, the gene expression in skeletal muscles and plasma levels of leptin, insulin and growth hormones, which play an important role in the regulation of growth and metabolism were not measured. The plasma samples collected were not enough to perform these assays. I therefore recommend that future studies should assess the gene expression of metabolic hormones in order to elucidate the exact mechanism(s) through which dietary treatments in the neonatal period influences metabolism. I also recommend the performance of proteomic analyses to quantify anti-oxidant enzymes in order to understand and relate the effect dietary treatments on gene expression and levels of anti-oxidant enzymes.

In the second experiment, I investigated the long-term potential protective effect of neonatal oral administration of OA against the development of metabolic dysfunction and non-alcoholic fatty liver disease (NAFLD) in fructose-fed adult male and female rats. To my knowledge, this is the first study in which a neonatal programming model has been used to investigate the potential of targeting the neonatal period for the prophylactic treatments with OA. After administering treatments to the rats as outlined in section 4.2.4, growth performance, morphometry of the gastrointestinal tract, glycaemic control, visceral adiposity, circulating metabolites, hepatic function and hepatic histomorphometry were assessed to determine the effect of administering OA in the neonatal period on the development of metabolic dysfunction. I have shown that OA administered in the neonatal period protects against the development of fructose-induced metabolic phenotype and NAFLD.

Findings from the long-term experimental study show that neonatal treatment with OA offers an exciting potential for the new advances in our understanding of the importance of the critical window of developmental plasticity as a target for dietary or pharmacological manipulations to confer health benefits later in adulthood. The findings from the current study also show that, in our fight against metabolic syndrome, it is feasible to intervene during the critical period of development in order to ensure lifelong positive health outcomes later in adulthood. It is notable that the rodents are altricial species, and thus the interventions in the neonatal period were easily administered by oral gavage ensuring delivery of the OA and fructose directly to the animals. In humans the equivalent developmental stage would be the third trimester and hence there is a need to explore the bioavailability of the OA across the placenta to ensure adequate delivery to the foetus.

Although I have demonstrated the potential protective effect of neonatal oral administration of OA against the development of MetS-associated negative outcomes and NAFLD, there is still a need to interrogate the specific programming mechanisms through which OA confers these health benefits. I therefore recommend the performance molecular analyses for genes involved in the metabolism of carbohydrates and lipid for future studies.

To my knowledge, this is the first study to investigate the effects of neonatal administration of OA on the susceptibility of rats to diet-induced metabolic dysfunction later in life. The effects of fructose were shown to be dependent on the time of intervention. An early fructose hit (fructose administered in the neonatal phase) did not seem to have any discernible effects. A late fructose hit (fructose consumption in adulthood) resulted in differences in body mass, triglyceride concentration and food and fluid intake. Administration of fructose neonatally with the subsequent consumption of fructose in adulthood (double fructose hit) increased hepatic lipid accumulation in both male and female rats. Fructose consumption neonatally and/or in adulthood did not affect linear growth, adiposity, glucose handling, organ morphometry and general health markers. The neonatal oral administration of oleanolic acid was shown to be hepatoprotective as it prevented hepatic lipid accumulation. Although I quantified the lipids that accumulated in the liver, I did not profile the hepatic lipids. In future, hepatic lipid profiles will be worth evaluating as this would provide an insight into the type of lipids that accumulated in the liver as a result of the dietary treatments. Determination of hepatic lipid profiles will also be important since various lipid classes have been implicated in the mechanisms involved in the pathogenesis of hepatic IR and NAFLD and its downstream sequelae.

As part of a strategy to curb the metabolic syndrome scourge, I recommend the further exploration of the use of OA-containing/enriched foods or ethnomedicinal plants in an effort to reduce the effect of high-energy diets and sedentary lifestyles on health outcomes associated with metabolic dysfunction. If bioavailability of OA in milk is high, the consumption of OA-rich foods, especially by breast feeding mothers may be beneficial for the neonates as they may receive the OA through breast milk and be neonatally programmed for the development of positive health outcomes later in adult life. Due to the apparent safety of OA when administered neonatally, another approach for targeting the neonatal period for prophylactic treatments would be to incorporate OA as a supplement to infant formula food. This will enable the oral administration of OA in neonates to protect against MetS-outcomes later in life. Given that rat neonates are equivalent to the third trimester of human development, future murine model

experiments should consider supplementing OA in the diet of nursing dams as well to simulate developmental programming.

In addition to encouraging people to eat foods that have high levels of OA, another approach would be to potentially investigate OA-derived synthetic compounds whose structures are based on the chemical structure of OA that may have a greater potency than OA. Examples of highly potent synthetic OA derivatives are the 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) derivatives, which are strong anti-oxidant compounds (Camer et al., 2014). In particular, the OA CDDO derivative, CDDO-Me (Bardoxolone Methyl) highlights the promising potential of these compounds as it has successfully completed phases I and II of human clinical trials in China.

Overally, I have shown that administration of a high fructose diet had adverse effects on several negative health outcomes associated with metabolic dysfunction and induced the development of NAFLD. However, the timing of the fructose exposure in the life stage of rats has an impact on the development of metabolic dysfunction and NAFLD phenotype. I also observed sex-specific differences in the metabolic response to dietary fructose. It is thus important to note that future metabolic studies should not just focus on a single sex but should be comparative between the sexes. I have also demonstrated, for the first time, that neonatal administration of oleanolic acid protects against the subsequent development of fructose-induced health outcomes associated with metabolic dysfunction and NAFLD by reducing hepatic lipid storage, terminal liver masses and hepatic histomorphological changes associated with NAFLD. I conclude that neonatal interventional treatment with oleanolic acid during the critical window of developmental plasticity protected against the development of fructose diet-induced health outcomes associated with metabolic dysfunction and NAFLD in male and female Sprague Dawley rats.

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APPENDICES

Appendix 1: Plagiarism Declaration

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I TREVOR TAPIWA NYAKUDYA (Student number: 0718789E) am a student registered for the degree of PhD in the academic year 2018.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: _____ Date: 04 JUNE 2018

Appendix 2: Animal Ethics Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/47/D

APPLICANT: Mr TT Nyakudya

SCHOOL: Physiology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *The effect of oleanolic acid on health outcomes associated with diet-induced metabolic dysfunction in rats (Rattus norvegicus)*

Number and Species

40 Sprague-Dawley Rats (adults); 384 pups

Approval was given for to the use of animals for the project described above at an AESC meeting held on 26 August 2014. This approval remains valid until 25 August 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 12 SEPT 2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 11th SEPTEMBER 2014

cc: Supervisor: Prof K Erlwanger & Dr E Mukwevho
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix 3: Modification of the Ethics Clearance

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: **Trevor Nyakudya**

b. Department: **Physiology**

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	**2014**	**47**	**D**
Other M&Es :			1

d. Project Title: **The effect of oleanolic acid on health outcomes associated with diet-induced metabolic dysfunction in rats (*Rattus norvegicus*).**

		No.	Species
e.	Number and species of animals originally approved:	40 dams & 384 pups	Sprague Dawley rats
f.	Number of additional animals previously allocated on M&Es:	0	Sprague Dawley rats
g.	Total number of animals allocated to the experiment to date:	13 dams & 134 pups	Sprague Dawley rats
h.	Number of animals used to date:	23 dams & 185 pups	Sprague Dawley rats

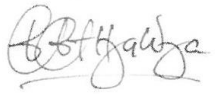
i. Specific modification / extension requested:

a) Addition of the following co-workers:
Dr Nasiru Muhammad
Ms Yvonne Mhosva

j. Motivation for modification / extension:

The co-workers (postgraduates in the School of Physiology) will assist with animal handling and sample collection during the course of the study and terminally.

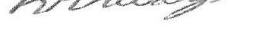
Date: 10/11/2015 Signature:



RECOMMENDATIONS: Approved. Inclusion of Dr Muhammad and Ms Mhosva as co-workers.
Conditions: Both co-workers should attend a 'first time animal users' orientation session facilitated by the CAS before working with the rats.

Date: 12th November 2015

Signature:



Chairman, AESC

Appendix 4: Modification of the Ethics Clearance

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: **Trevor Nyakudya**

b. Department: **Physiology**

c. Experiment to be modified / extended

	AESC NO		
Original AESC number	**2014**	**47**	**D**
Other M&Es :			2

d. Project Title: **The effect of oleanolic acid on health outcomes associated with diet-induced metabolic dysfunction in rats (*Rattus norvegicus*).**

		No.	Species
e.	Number and species of animals originally approved:	40 dams & 384 pups	Sprague Dawley rats
f.	Number of additional animals previously allocated on M&Es:	0	Sprague Dawley rats
g.	Total number of animals allocated to the experiment to date:	23 dams & 185 pups	Sprague Dawley rats
h.	Number of animals used to date:	23 dams & 185 pups	Sprague Dawley rats

i. Specific modification / extension requested:

a) Addition of the following co-workers:
Mmahiine Christinah Molepo (Student no: 27390047)
Simon Isaiah (Student number, 26580748)
b) Extension of the study by an extra two years.

j. Motivation for modification / extension:

a) These co-workers are registered postgraduate students at North West University. They are being added to offer technical assistance with molecular work samples which will be run at the University of the North West where my co-supervisor (Prof Mukwevho) now works.
b) The request for extension of the ethics clearance by two years is based on the fact that there were delays with the supply of animals at some stage during course of the study. The extension of time will also enable me to continue with the remaining phases of my study.

Date: 22/02/2016 Signature: 

RECOMMENDATIONS: Approved.
i. Inclusion of Mmahiine C Molepo and Simon Isaiah as co-workers.
ii. Extension of time to 28 February 2018.

Date: 24 February 2016

Signature: 

Chairman, AESC

Appendix 5: University of Johannesburg Animal Ethics Clearance

UNIVERSITY OF JOHANNESBURG

FACULTY OF HEALTH SCIENCES

RESEARCH ETHICS COMMITTEE

NHREC Registration no: REC-241112-035

REC-01-02-2016

01 March 2016

TO WHOM IT MAY CONCERN:

STUDENT: NYAKUDYA, T
STUDENT NUMBER: -

TITLE OF RESEARCH PROJECT: The Effect of Oleanolic Acid on Health Outcomes Associated with Diet-Induced metabolic Dysfunction in Rats

DEPARTMENT OR PROGRAMME: NON DEGREE PURPOSE

SUPERVISOR: - **CO-SUPERVISOR:** -

The Faculty Academic Ethics Committee has scrutinised your research proposal and confirm that it complies with the approved ethical standards of the Faculty of Health Sciences; University of Johannesburg.

The REC would like to extend their best wishes to you with your postgraduate studies.

Yours sincerely,

Maria Poggenpoel

Prof M Poggenpoel
Chair : Faculty of Health Sciences REC
Tel: 011 559 6689
Email: mariep@uj.ac.za

Appendix 6: Soxhlet Procedure for Lipid Determination

1) Switch on the MAINS/ POWER on the Tecator Soxtec System
2) Take an extraction thimble from the thimble holder and put it in the thimble support. Put the thimble support with the thimble on the balance. Accurately weigh about 0.2 – 2.5 g (dependent on the amount of the sample available and the fat content thereof) of the milled sample with a spatula into the extraction thimble, noting the weight.
3) Place a fat-free cotton wool plug in the top of the thimble. Remove the thimble containing the sample from the thimble support and place it back in the thimble holder.
4) The clean extraction cups with two glass beads in each cup are dried at 90±5ºC for at least 2 hours. Remove cups with tongs from the oven. Place in a desiccator and allow to cool for 15 min and weigh.
5) Check that the temperature setting of the unit is such that the oil temperature is between 85ºC and 95ºC.
6) Turn on the cooling water supply to the condensers.
7) Hold the thimbles in position in the extraction unit and draw them up into the extraction tubes by moving the unit levers from BOILING to RINSING position. EVAPORATION lever should be in the OFF position.
8) Place the extraction cups in the holder gloves.
9) To each extraction, cup add about 60 mℓ of petroleum ether.
10) Place the cups containing the ether on the heating pads and fix in position by pressing down the fastening mechanism lever till it locks.
11) Check that the recovery levers are in the vertical position.
12) Move the unit levers to the boiling position and set the timer for 30 min or 45-60 min for samples with a higher fat content.
13) At the end of the rinsing period close the extraction system by turning the recovery lever to the horizontal position.
14) Allow the ether to collect in the tubes for ±10 min.

15) Unlock the fastening mechanism and pull it up, remove the cup holder from the unit.
16) Carefully remove the extraction cups out of the cup holder with gloves and place in an oven set at 90±5ºC until all the ether has evaporated i.e. ±30 min.
17) After drying in the oven the extraction cups are take out and place in a desiccator for 15 min and weighed.
18) The thimbles are removed from the extraction unit. A small plastic beaker is used to collect the ether when the recovery levers are turned to the vertical position.
19) Some of this ether is used for cleaning the extraction cups. The cups are then washed with warm soapy water. Rinse with clean water.
20) Switch off power and turn off the cooling water.
21) CALCULATIONS

The percentage fat content was calculated using the formula:

$$\% \text{ fat} = \frac{MCEE - MC}{MS} \times 100$$

Where: MC = mass of cup
MCEE = mass of cup plus fat
MS = mass of sample

22) Triplicate values to differ by not more than 10%. The criteria depend on the type of sample – usually 4%, but for fatty samples that are not homogenous, the percentage difference can be up to 10%.
23) A control sample will be analysed at least once a day. The results of the control sample will be documented on a control chart. If the result falls outside the limits, the analyses will be repeated.

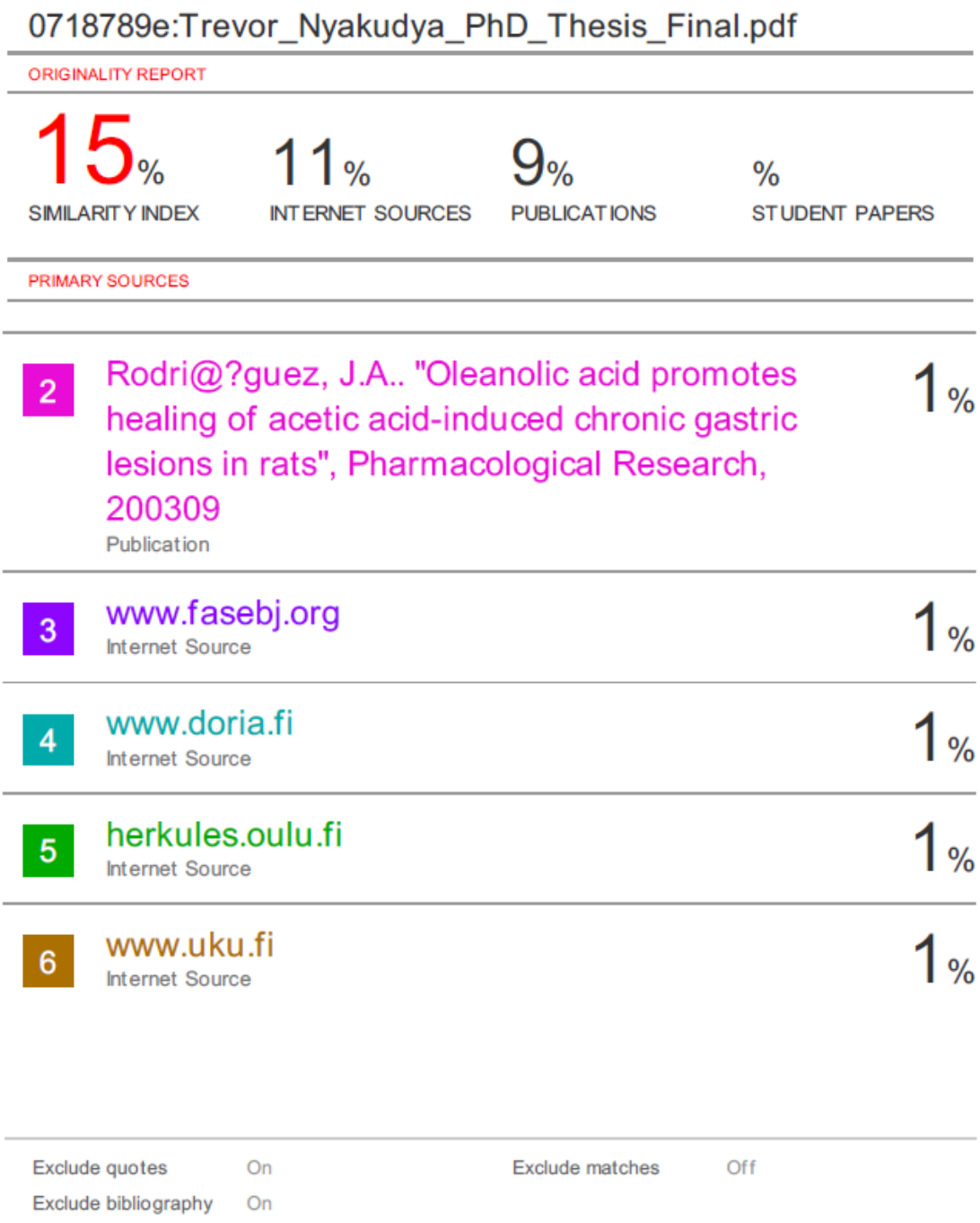
Appendix 7: Manufacturer Instructions for ELISA Kit

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.** It's recommended that all samples and standards be assayed in duplicate.

1. **Add Sample:** Add 100μL of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.
2. **Biotinylated Detection Ab:** Remove the liquid of each well, don't wash. Immediately add 100μL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.
3. **Wash:** Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper.
4. **HRP Conjugate:** Add 100μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C.
5. **Wash:** Repeat the wash process for five times as conducted in step 3.
6. **Substrate:** Add 90μL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.
7. **Stop**: Add 50μLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.
8. **OD Measurement**: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.
9. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Appendix 8: Turn-It-In Score



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