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THE POTENTIAL PROTECTIVE EFFECTS OF CURCUMIN AGAINST DIET-INDUCED NEONATAL PROGRAMMING OF METABOLIC DYSFUNCTION IN GROWING RATS



Kasimu Ghandi Ibrahim

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

Johannesburg, 2019

DECLARATION

I, **Kasimu Ghandi Ibrahim,** hereby declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg, Republic of South Africa. It has not been submitted before for any degree or examination at any other University.



Kasimu Ghandi Ibrahim

Signed on the 19th day of June 2019.

DEDICATION

To the loving memory of

My father, Late Alhaji Ibrahim Gandi Dogondaji (1935-2001) and

My sister, Late Amina Bello (1962-2001)

May Allah forgive all their shortcomings and grant them eternal rest in Jannatul Firdaus, amin.

PRESENTATIONS ARISING FROM THE STUDY

Oral presentations

1. **K.G. Ibrahim**, E. Chivandi, P. Nkomozepi and K.H. Erlwanger. The protective effects of neonatal administration of curcumin against non-alcoholic steatohepatitis in high fructose-fed female adolescent Sprague Dawley rats. Faculty of Health Sciences, University of the Witwatersrand Research day, 9th September 2018.
2. **K. G. Ibrahim,** E. Chivandi, K. H. Erlwanger and R.L. Brooksbank. The impact of pre-weaning curcumin and fructose administration on the relative leucocyte telomere lengths of adolescent rats fed a high fructose diet post-weaning. Physiological Society of Nigeria Annual Conference, Zaria, Kaduna State Nigeria (Accepted for oral presentation).

Poster presentations

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2. **K.G. Ibrahim**, E. Chivandi and K.H. Erlwanger. The effect of administration of curcumin during suckling on the developmental programming of metabolic function in male and female adolescent Sprague Dawley rats. Physiological Society of Nigeria Annual Conference, Zaria, Kaduna State Nigeria (Accepted for poster presentation).

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1. **K. G. Ibrahim,** E. Chivandi and K. H. Erlwanger. The effect of neonatal oral curcumin administration on the metabolic response and renal histology of adolescent male and female Sprague Dawley rats fed a high fructose diet until adolescence.

ABSTRACT

The current global epidemic of metabolic syndrome in children and adolescents, though driven in part by increased consumption of fructose-rich diets, has been linked to nutritional perturbations in critical developmental periods. Plant-derived polyphenols when administered during the suckling period have been shown to program for protection against diet-induced metabolic dysfunction in childhood or adolescence. Curcumin, a hydrophobic polyphenol extracted from turmeric, has antioxidant, anti-diabetic and anti-obesity properties. It was hypothesised that the oral administration of curcumin during the suckling period would protect against the development of high fructose-induced metabolic dysfunction in adolescence.

One hundred and twenty-eight 6-day old suckling Sprague-Dawley pups (65 males; 63 females) were initially randomly allocated to four treatment groups and administered with either a 0.5% dimethyl sulfoxide (vehicle control), 500mg.kg^{-1} body mass of curcumin, fructose (20%, w/v) or a combination of curcumin and fructose. The treatments were administered once daily via oral gavage at 10ml.kg^{-1} body mass from postnatal day 6 to 21 in order to induce programming. At postnatal day 21, the rats were weaned onto normal rat chow. Each of the initial groups was subdivided into two subgroups: one had only plain tap water while the other had fructose (20%, w/v) as their drinking solution from postnatal day 21 to 63 when the rats were euthanised and tissues collected for analyses.

In both sexes the metabolic responses of the rats (fasting plasma glucose, triglycerides, total cholesterol, insulin and adiponectin concentration) were similar ($p>0.05$, ANOVA) across the treatment groups. Feeding with fructose only in the postweaning period induced mesangial proliferation and mild tubular secretions in the male rats; and moderate glomerular atrophy, increased Bowman capsular space and increased tubular secretions in the female rats. In both sexes, neonatal administration of curcumin prevented the postweaning fructose only-induced changes in kidney tissues. Administration of curcumin during the suckling period with no fructose at all caused glomerular atrophy, increased Bowman capsular space and mild to moderate tubular secretions in both male and female rats. In both sexes, fructose administration induced marked hepatic inflammation ($p= 0.0015$ males, $p= 0.0112$ females, Kruskal Wallis) and hepatic fibrosis ($p<0.0001$, ANOVA), the hallmarks of non-alcoholic steatohepatitis, which were prevented by the neonatal oral administration of curcumin. The hepatic gene expression ratios for 5' Adenosine-monophosphate activated protein kinase alpha (AMPKα) in both sexes were significantly downregulated ($p<0.0001$, ANOVA). The

expression ratios of tumour necrosis factor alpha (TNFα) were significantly upregulated ($p<0.0001$) in rats fed a high fructose diet pre and/or postweaning compared to the other groups. Male rats administered with curcumin and fructose pre-weaning and fed a high fructose diet post weaning had significantly shorter femora ($p= 0.0038$, ANOVA) compared to their counterparts administered a similar treatment pre-weaning and plain tap water post-weaning. Male rats that received curcumin alone or curcumin and fructose during the suckling period and subsequently fed a high fructose diet had significantly lower ($p\leq0.05$, ANOVA) plasma total osteocalcin concentration compared with that from rats in other treatment groups. In female rats, administration of curcumin alone or curcumin and fructose during the suckling period and subsequent feeding with high fructose resulted in significantly lower ($p\leq0.05$, ANOVA) osteocalcin concentration compared to administration of DMSO during suckling and then feeding with a high fructose diet post weaning. Both male and female rats had similar ($p>0.05$, ANOVA) leucocyte telomere lengths across the treatment groups.

Neonatal oral administration of curcumin programmed for protection against non-alcoholic steatohepatitis, kidney injury and increased bone turnover induced by a postweaning high fructose diet. Therefore, the administration of oral curcumin during the suckling period could potentially protect against the high fructose diet-induced liver, kidney and bone diseases in adolescence and can be useful in mitigating the current epidemic.

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LIST OF ABBREVIATIONS

α:	Alpha
β:	Beta
γ:	Gamma
AACE:	American Association of Clinical Endocrinologists
ADCY 3:	Adenylate cyclase three
AHA:	American Heart Association
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
AMPK:	Adenosine monophosphate-activated protein kinase
ARRIVE:	Animal Research: Reporting of *In Vivo* Experiments
AST:	Aspartate aminotransferase
ATP:	Adenosine triphosphate
ATP III:	Adult treatment panel three
AGEs:	Advanced glycation end products
BMI:	Body mass index
BP:	Blood pressure
BUN:	Blood urea nitrogen
CCl_4:	Carbon tetrachloride
ChREBP:	Carbohydrate responsive element binding protein
CRP:	C-reactive proteins
CVD:	Cardiovascular diseases
DM:	Diabetes mellitus
DNA:	Deoxyribonucleic acid
DNMT-1:	Deoxyribonucleic acid methyl transferase one

DOHaD:	Developmental Origins of Health and Disease
EGSIR:	European Group for the Study of Insulin Resistance
ER:	Endoplasmic reticulum
FBG:	Fasting blood glucose
FDA:	Food and Drug Administration
FFAs:	Free fatty acids
GIT:	Gastrointestinal tract
GluT:	Glucose transporter
GRAS:	Generally Regarded As Safe
HDAC:	Histone deacetylase
HDL-C:	High density lipoprotein cholesterol
HGI:	High glycaemic index
HIV:	Human immunodeficiency virus
HOMA-IR:	Homeostasis model of insulin resistance
IAS:	International Atherosclerosis Society
IASO:	International Association for the Study of Obesity
ICO:	Index of central obesity
ICR:	Institute of Cancer Research
IDF:	International Diabetes Federation
IFG:	Impaired fasting glucose
IGT:	Impaired glucose tolerance
IL:	Interleukin
IUGR:	Intrauterine growth restriction
LDL-C:	Low density lipoprotein cholesterol
LTL:	Leucocyte telomere length

MAPK:	Mitogen-activated protein kinase
MC4R:	Melanocortin-4-recetor
MCP-1:	Monocyte chemotactic protein 1
MDA:	Malondialdehyde
MetS:	Metabolic syndrome
MIC:	Mean inhibitory concentration
NAFLD:	Non-alcoholic fatty liver disease
NASH:	Non-alcoholic steatohepatitis
NBPEP:	National Blood Pressure Education Program
NCEP:	National Cholesterol Education Program
NEFA:	Non-esterified fatty acids
NF-kB:	Nuclear factor kappa B
NHANES:	National Health and Nutrition Examination Survey
NHLBI:	National Heart, Lung and Blood Institute
PAI:	Plasminogen activator inhibitor
PAR:	Predictive adaptive response
PES:	Paediatric Endocrine Society
POMC:	Proopiomelanocortin
PPAR:	Peroxisome proliferator-activated protein
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
SCFE:	Slipped capital femoral epiphysis
SOD:	Superoxide dismutase
SREBP:	Sterol regulatory element binding protein
STZ:	Streptozotocin

T2DM:	Type 2 diabetes mellitus
TGs:	Triglycerides
TNF:	Tumour necrosis factor
UCP-1:	Uncoupling protein 1
USA:	United States of America
WBC:	White blood cells
WC:	Waist circumference
WHF:	World Heart Federation
WHO:	World Health Organisation
WHR:	Waist: hip ratio

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PREFACE

This study investigated the potential of neonatal orally administered curcumin to protect against the development of high fructose diet-induced metabolic dysfunction in adolescense. This thesis is presented in seven chapters. Chapter one introduces the study and some key concepts that would be discussed in detail in the subsequent chapters. A justification for the study, aim and objectives and hypotheses are also presented in chapter one. A survey of literature relevant to the study is reported in chapter two. This includes a discussion on metabolic syndrome, childhood obesity and the diagnosis of metabolic syndrome in children and adolescents. A discussion on NAFLD, skeletal system complications of childhood obesity and leucocyte telomere lengths is also included. Developmental programming of metabolic function is also reviewed including the different experimental models used in several studies on programming and reprogramming of metabolism and physiology. The relevant available literature on the polyphenol curcumin including its chemistry, pharmacology and toxicity are also reviewed in chapter two. In the original study, the combination of high cholesterol and high fructose diet was to be used to induce metabolic dysfunction in the rats. However, the rats did not tolerate the dietary cholesterol and for welfare purposes, the study had to be terminated prematurely. Some of the growth performance data from the initial study are included in Appendix D.

Thereafter a high fructose diet without added cholesterol was used in the study. Thus, the core focus of this thesis was on the second study using the high fructose diet intervention. The study itself was divided into four components, each component reflecting a major objective for the study and is written such that each major objective is reported in separate chapters (Chapters 3 to 6). Chapter three reports the first component of the study which is the impact of neonatal oral administration of curcumin on the metabolic functions of adolescent rats that were fed a high fructose diet post-weaning. It comprises of an introduction, materials and methods that were used to answer the research question, results and discussion. Chapter four reports on the protective effects of curcumin administered to suckling pups on the development of the fructose-induced NAFLD. It also comprises of an introduction of the research problem and identification of the knowledge gap, materials and methods (relevant to the objective) used in answering the research question, results and discussion of the findings of the study in the context of available literature. In chapter five, the third component of the study, the impact of neonatal administration of curcumin on the bone health of adolescent rats was reported. The chapter comprises of an introduction, materials and methods, results and discussion. Chapter six reports on the impact of curcumin and fructose administered to

suckling pups on the relative leucocyte telomere length of adolescent rats. It starts with an introduction where the background literature on the telomere biology and its susceptibility to epigenetic modifications were reviewed. The effects of curcumin and fructose on telomeres were also discussed briefly. A description of the materials and methods used in answering the research question was also presented. The results and discussion in the context of the study were also presented. In chapter seven, concluding remarks, limitations and directions for future studies were presented. A list of references that were cited in the study and the appendices including a brief insight into the first prematurely terminated study in which a high fructose and high cholesterol dietary intervention were used were provided at the end of the thesis.

CHAPTER ONE: INTRODUCTION AND JUSTIFICATION

1.0 Introduction

Metabolic syndrome (MetS) is currently an epidemic that affects all age groups worldwide (Alberti et al., 2009; Kaur, 2014). The major outcome of MetS, a complex of cardio-metabolic risk markers, is the development of cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM) (Grundy, 2008; Punthakee et al., 2018). MetS affects about a quarter of the global adult population (Kaur, 2014), with a higher prevalence of the risk factors occurring in women than in men (Attig et al., 2010).

Paediatric obesity is another global epidemic (Isabelle, 2018), which has increased the risk for the development of MetS in growing children and later in adolescence (Lin, 2018). As a result of this, there is an associated surge in the occurrence of the complications of MetS including non-alcoholic fatty liver disease (NAFLD) which were hitherto not common in this age group (Merav et al., 2018). The major drivers for this increase in the prevalence of MetS in both adults and children are the complex interaction of genetic and environmental factors such as physical inactivity and increased consumption of fructose-rich diets (Mamikutty et al., 2014; O'Neill and O'Driscoll, 2015). The role of epigenetic mechanisms in the expression and repression of certain genes involved in carbohydrate and lipid metabolism has also been emphasised as a major cause of the global epidemic (Desai et al., 2015; Smith and Ryckman, 2015).

The consumption of fructose, a sweetener used in many processed foods and drinks has increased globally and is linked to the increased prevalence of obesity (Tappy and Lê, 2010; Kelishadi et al., 2014). The special transporter system (GLUT5) that is required for shuttling fructose across cell membranes is absent in pancreatic β-cells (Bray et al., 2004). Therefore, even when fructose is present in high concentrations in the blood, it fails to stimulate the release of insulin from the pancreas (Mamikutty et al., 2014). The appetite regulatory system in the hypothalamus are modulated by the adipocytokine leptin (Fruhwürth et al., 2018). The concentration of insulin in the circulation is inversely related to leptin secretion by adipocytes (Caron et al., 2018). Fructose does not stimulate insulin release, consequently, the regulatory influence of insulin on leptin secretion is lost, resulting in sustained appetite, overeating and obesity (Qiu et al., 2018).

When fructose binds to biomolecules in a process called glycation (as opposed to glycosylation) reaction, it forms advanced glycation end products (AGEs) which accelerate ageing in cells (Peters, 2012; Nowotny et al., 2018). Leucocyte telomere length (LTL) which

correlates with somatic telomere length is usually measured as an indicator of biological ageing (Aviv and Shay, 2018). It is now suggested that the length of the telomere, whether short or long is associated with particular ageing-related diseases (Aviv and Shay, 2018). Shorter telomeres have been shown to be associated with and increase the risk of chronic diseases such as CVD and diabetes mellitus (Morgan et al., 2018) while longer telomeres may be associated with proliferative conditions such as cancers (Bernal and Tusell, 2018; De Vitis et al., 2018).

Data available from several studies have highlighted the connection between nutritional perturbations in the critical developmental periods and subsequent permanent alteration of the metabolism and physiology of the individual in adolescence and adulthood (Iyare and Nwagha, 2009; Halah et al., 2018; Spiegel et al., 2018). This is the concept called 'neonatal metabolic programming' (Hales and Barker, 1992) which will be discussed in greater detail in chapter two. It is however worthy of mention at this point, that neonatal metabolic programming is an epigenetic phenomenon triggered by stressors in the early periods of relative tissue plasticity that necessitate adaptive responses (Patel and Srinivasan, 2002). These adaptations are mostly sex-specific (Dearden et al., 2018), with a tendency for transgenerational transmission even in the absence of the original initiating stimulus (Aiken and Ozanne, 2014).

Sometimes the effect of programming manifest immediately or they may do so following a latent period (Heindel et al., 2015; Rodríguez-Rodríguez et al., 2018). This period of latency is due to tenuous alterations in gene expression or repression that only become obvious in adolescence or adulthood when the individuals are subjected to a second hit or challenge (Heindel et al., 2015; Rodríguez-Rodríguez et al., 2018).

Despite the availability of orthodox medications, a large portion of the population still prefer natural products due to their perceived safety, availability and affordability (Ayyanar et al., 2008). There is also a challenge with compliance to treatment amongst patients especially with chronic diseases that require lifelong medications (Brown and Bussell, 2011; Tung et al., 2017). Moreover, the World Health Organisation (WHO) is currently encouraging the use of natural bioactive products in the management of diseases (Bahmani et al., 2014; Meng et al., 2018). Natural bioactive compounds are a group of secondary metabolites derived from plants and animal products (Zullaikah et al., 2018). Though a large number of plants and plant products have been fully characterised and their biological activities against many diseases documented (Giglio et al., 2018; Jamshidi-Kia et al., 2018), there is a larger number of such

plant products such as flavonoids and polyphenols used in folklore that have not yet been fully studied.

Curcumin (diferuloylmethane) is a yellowish dietary spice from the dried rhizome of *Curcuma longa* of the ginger family Zingiberaceae (Panahi et al., 2018a). It (curcumin) has received great attention from researchers for over two centuries and is also widely used in Asia especially in Indian Ayurveda and Chinese medicine for the treatment of many diseases including diarrhoea, liver problems and heart ailments (Panahi et al., 2018a). Research has shown curcumin to possess anti-cancer (Epstein et al., 2010; Vallianou et al., 2015), anti-inflammatory (Panahi et al., 2012; Dai et al., 2018a) and cardio-protective properties (Hu et al., 2013). Curcumin has also been shown to be a promising candidate in the treatment of obesity, NAFLD and other metabolic diseases (Shehzad et al., 2011; Son et al., 2013).

1.1 Justification of the study

At birth, most of the body systems of altricial species like rats are poorly developed, corresponding to the level of development in the last trimester of gestation in humans (Vanier et al., 1971; Romijn et al., 1991). The immediate postnatal period is a period of rapid organ development and maturation (Armitage et al., 2005) and therefore is a critical window for epigenetic modifications that in addition to having a lifelong impact in the individual can also be transmitted across generations (Aiken and Ozanne, 2014; Vickers, 2014). The concept of developmental origins of health and disease (DOHaD) aptly describes the linkage between environmental perturbations, including nutritional experiences in the early plastic periods of development and the onset of metabolic diseases in childhood through adolescence and adulthood (Armitage et al., 2005; Bruce and Hanson, 2010). Increased consumption of high fructose diets is believed to be partly responsible for the increased global prevalence of MetS (Biro and Wien, 2010; Mamikutty et al., 2014) and the development of NAFLD (Mamikutty et al., 2015; Alwahsh and Gebhardt, 2017) in childhood and adolescence. Telomere length, (TL) usually measured as relative leucocyte TL, is a marker of cellular ageing that negatively correlates with obesity and other metabolic diseases (Zannolli et al., 2008; García-Calzón et al., 2014).

One of the ways by which neonates can be exposed to high fructose diets is through breastfeeding practices. Exclusive breastfeeding in the first four to six months of life is highly recommended due to its enormous benefits on both the infants and the mothers (Victora et al.,

2016). Breast milk enhances postnatal intestinal function, development of the immune and autocrine systems (Martin et al., 2015) and also inhibits programming of obesity and T2DM in later life (Savino et al., 2013). It is not always possible for mothers to practice exclusive breastfeeding for several reasons which include early resumption to workplace, disease conditions and cultural practices (Desai et al., 2014). Thus, infant feeding formulae are introduced early in the life of the infant, thereby shortening the period of exclusive breastfeeding and hastening the commencement of solid food (Tang et al., 2015). Though the composition of the infant feeding formulae is usually as close to that of breastmilk as possible, their introduction, especially within the first six months of life, is associated with the development of childhood obesity (Gibbs and Forste, 2014). In some communities, mothers try to locally formulate their own infant food (Masters et al., 2017; James et al., 2018) and consequently may use additives that may contain a high fructose concentration. Additionally, maternal consumption of a high fructose diet has been shown to increase the concentration of fructose in breast milk (Goran et al., 2017; Berger et al., 2018). It is pertinent to examine the impact of this early fructose administration on the future metabolic health of the individuals. Thus far, however, there are few studies that involve direct administration of fructose to neonatal rats (Huynh et al., 2008) probably due to experimental constraints of keeping the pups away from their mothers (Srinivasan and Patel, 2008). Several studies have however found the administration of natural polyphenols such as oleanolic acid (Nyakudya et al., 2018), s-allyl-s-cysteine (Lembede et al., 2018) and some hormones such as leptin (Firth et al., 2017) to suckling pups to protect them against fructose-induced metabolic changes including non-alcoholic liver disease (NAFLD) later in life. Peroxisome proliferator-activated receptors (PPAR) serve as important regulators between nutritional programming and MetS (Tain et al., 2015). PPAR modulators like curcumin are speculated to be useful in reprogramming against MetS of developmental origin (Lee et al., 2018). Curcumin is an essential component of the diet in many homes. Due to its ability to interact with a wide range of molecular targets, it (curcumin) has been reported to exert several biological/ therapeutic effects in the body systems. However, whether curcumin is an ideal compound for neonatal programming possibly preventing adverse health outcomes in adolescence when administered during the suckling period is unknown. Hence the current thesis explored this important gap in knowledge.

1.2 Aim and objectives

The aim of this study was to determine whether curcumin administered to suckling pups would confer protection or predispose them later in adolescence to the subsequent development of the adverse health effects of a high-fructose diet.

Four specific objectives of the study were set to:

1. investigate the potential protective effects of oral curcumin administered to suckling pups against the adverse programming effects of a high fructose diet by
    - determining the body mass changes of the rats as a marker of their growth performance.
    - determining adiposity and some markers of obesity: body mass index (BMI), abdominal circumference, visceral and epididymal (in males) fat pad mass.
    - determining the concentration of some key hormones that regulate metabolism: insulin, adiponectin and computation of the HOMA-IR.
    - measuring the concentration of plasma fasting metabolic substrates: glucose, triglycerides and total cholesterol.
2. investigate the potential protective effects of oral curcumin administered to suckling pups against the development of high fructose-induced non-alcoholic fatty liver disease by
    - quantifying the hepatic storage of lipids.
    - determining the liver scores of steatoses by carrying out a histomorphometric analysis of the liver tissue.
    - assaying the liver for key genes involved in the pathogenesis of NAFLD: tumour necrosis factor alpha (TNFα) the enzyme adenosine monophosphate-activated protein kinase (AMPK).
3. investigate the potential effect of neonatal administration of curcumin on bone health in adolescence by
    - determining the lengths, dry masses and computation of the seedor index of the femora and tibiae of the rats
    - carrying out a radiographic examination of the femora and tibiae
    - assaying for the protein hormone, osteocalcin as a marker of bone formation
4. investigate the effect of oral curcumin and/or fructose administered to suckling pups on cellular ageing by

- determining the relative leucocyte telomere length.

Sex differences have been reported in the developmental programming of metabolism (Dearden et al., 2018; Sarkar and Baffy, 2018). This sexual dimorphism results from sex-specific transcriptional regulation that starts with the placenta (Sundrani et al., 2017). To account for this dimorphism, for each of the above objectives male and female rats were used. Thus, unlike most other studies where only one sex is studied, in the current study both male and female rats were used so as to enusre there is no gap in outcomes regarding sex of rats and hence contributing meaningfully to the generation of new knowledge.

It is important to note, as described earlier, that initially high fructose, high cholesterol diet was used. However, due to poor dietary tolerance and for animal welfare reasons, the study was terminated prematurely. Appendix D provides some of the growth data.

1.3 Hypotheses

1.3.1 Hypothesis for objective one

H_0: The administration of curcumin daily to suckling pups has no effect on the adverse programming effects of a high fructose diet in Sprague Dawley rats.

H_1: The administration of curcumin daily to suckling pups affects the adverse programming effects of a high fructose diet in Sprague Dawley rats.

1.3.2 Hypothesis for objective two

H_0: The administration of curcumin to suckling pups does not protect adolescent male and female Sprague Dawley rats against the development of high fructose-induced non-alcoholic fatty liver disease when they are fed a high fructose diet post-weaning.

H_1: The administration of curcumin to suckling pups protects adolescent male and female Sprague Dawley rats against the development of high fructose-induced non-alcoholic fatty liver disease when they are fed a high fructose diet post-weaning.

1.3.3 Hypothesis for objective three

H_0: The administration of curcumin to suckling pups does not affect the bone health of male and female Sprague Dawley rats in adolescence when they are fed a high fructose diet post-weaning.

H_1: The administration of curcumin to suckling pups affects the bone health of male and female Sprague Dawley rats in adolescence when they are fed a high fructose diet post-weaning.

1.3.4 Hypothesis for objective four

H_0: The administration of curcumin and or fructose to suckling pups does not affect the relative leucocyte telomere lengths of male and female Sprague Dawley rats fed a high fructose diet post-weaning.

H_1: The administration of curcumin and or fructose to suckling pups affects the relative leucocyte telomere lengths of male and female Sprague Dawley rats fed a high fructose diet post-weaning.

In the next chapter, a survey of literature pertinent to the study is presented.

CHAPTER TWO: LITERATURE REVIEW

2.1 Metabolic syndrome

The presence of a collection of cardio-metabolic risk factors in an individual that predispose to the development of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) is referred to as metabolic syndrome (MetS) (Samson and Garber, 2014; Punthakee et al., 2018). It has been established that obesity and insulin resistance underlie the development of MetS (Grundy, 2008; Saltiel and Olefsky, 2017).

Though MetS was described as early as 1923 when an aggregation of metabolic abnormalities were observed in some patients (Parikh and Mohan, 2012), it was not until several decades later that Reaven (1988) proposed the term syndrome X. Syndrome X as described by Reaven consisted of hypertension, dyslipidaemia and glucose intolerance occurring together in individuals with insulin resistance as an underlying cause (Grundy, 2004; Parikh and Mohan, 2012). However, the term "metabolic syndrome" was introduced to disperse the assertion that insulin resistance was the sole or primary cause of this aggregation of metabolic abnormalities (N.C.E.P., 2002). This became necessary because of the increasing body of evidence supporting the key position of abdominal obesity in the development of the syndrome (Amihaesei and Chelaru, 2014).

Whereas aggregating the risk factors into a syndrome may not add value to clinical management (Grundy et al., 2005), it changes the focus to identifying the underlying causes and hence more focus on lifestyle changes (Grundy et al., 2005). Since MetS is associated with an array of cancers, and its primary outcomes, CVD and T2DM are major causes of mortality globally, it becomes imperative to agree on a precise definition (O'Neill and O'Driscoll, 2015; Punthakee et al., 2018). Consequently, several definitions and diagnostic criteria have been proposed for MetS. While the World Health Organization (W.H.O) criteria considered insulin resistance as the major cause of the syndrome (Lemieux, 2018), the National Cholesterol Education Program (N.C.E.P) identified increased waist circumference (abdominal obesity) as the major cause (N.C.E.P., 2002). Other definitions include those of the International Diabetes Federation (IDF), European Group for the Study of Insulin Resistance (EGSIR) and the American Association of Clinical Endocrinologists (AACE).

The characteristic definitive diagnostic criteria for the different organisations mentioned above are summarised in table 2.1 below.

Table 2. 1: Summary of diagnostic criteria for metabolic syndrome

WHO 1998	NCEP ATP III 2001	IDF 2005	EGSIR	AACE
Glucose intolerance, IGT or DM and/or insulin resistance together with 2 or more of the following:	Any 3 or more of the following:	Central obesity based on race and gender-specific cut-offs plus any two of the following:	A modification of the WHO using insulin resistance syndrome.	Used insulin resistance syndrome. Diagnosis left to clinical judgement with no specific criteria
Waist/hip ratio (WHR) >0.9 in men and > 0.85 in women and/or Body Mass Index (BMI) > 30kg.m^2	Waist circumference (WC) ≥ 102cm in men and ≥ 88cm in women	Plasma TGs ≥ 150mg.dl^{-1} or history of specific treatment for this lipid abnormality. Low HLD-C <40mg.dl^{-1} in men and < 50mg.dl^{-1} in women or history of specific treatment for this lipid abnormality	Elevated plasma insulin (>75th percentile) plus 2 of the following:	Some degree of glucose intolerance: IFG/IGT
Plasma TGs (≥ 150mg.dl^{-1}) and/or low HDL-C (<35mg.dl^{-1} in males and< 39mg.dl^{-1} in females).	Plasma TGs ≥ 150mg.dl^{-1} Low HDL-C <40mg.dl^{-1} in men and < 50mg.dl^{-1} in women	Raised BP: ≥ 130mmHg systolic or ≥85mmHg diastolic or treatment for previously diagnosed hypertension	WC: ≥ 94cm in men and ≥ 80cm in women	Abnormal uric acid metabolism Plasma uric acid metabolism Renal uric acid clearance

Arterial pressure ≥ 140/90mmHg	High blood pressure: >130/85mmHg	Raised FPG ≥ 100mg.dl^{-1} or previously diagnosed T2DM	Hypertension ≥ 140/90mmHg or on antihypertensive treatment	Dyslipidaemia: TGs, HDL-C, LDL particle diameter, postprandial accumulation of TG-rich lipoproteins,
Microalbuminuria ≥ 20µg.min^{-1} or albumin/creatinine ratio ≥ 30 µg.mg^{-1}	High fasting glucose: > 110mg.dl^{-1}		Elevated TGs (≥ 150mg.dl^{-1}) or reduced HDL-C (≤ 39mg.dl^{-1} for both men and women)	Haemodynamic changes: sympathetic nervous system activity, renal sodium retention, blood pressure (~ 50% of patients with hypertension are insulin resistant
			Elevated plasma glucose: IFG or IGT, but no diabetes	Prothrombotic factors: PAI, fibrinogen, CRP, WBC count
				Endothelial dysfunction

Table modified from (Parikh and Mohan, 2012).

WHO; World Health Organisation, NCEP ATP; National Cholesterol Education Program Adult Treatment Panel, IDF; International Diabetes Federation, EGSIR; European Group for the study of Insulin Resistance, AACE; American Association of Clinical Endocrinologists, IFG; impaired fasting glucose, IGT; impaired glucose tolerance, DM; diabetes mellitus, T2DM, type 2 diabetes mellitus, HDL-C; high-density lipoprotein cholesterol, LDL; low-density lipoprotein cholesterol, CRP; c-reactive proteins, WBC; white blood cells, PAI; plasminogen activator inhibitor.

In 2005, the American Heart Association and the National Heart, Lung and Blood Institute (AHA/NHLBI) also proposed a criterion for the definition of MetS that was essentially similar to that of the NCEP ATP III (Grundy et al., 2005). Thus two major definitions, the AHA/NHLBI and the IDF were proposed (Yamagishi and Iso, 2017). The major difference was that while the former definition gave a central role for abdominal obesity, the latter definition centred on the aggregation of other cardiometabolic risk markers (Yamagishi and Iso, 2017). However, the IDF, AHA/NHLBI, World Heart Federation (WHF), International Atherosclerosis Society (IAS) and the International Association for the Study of Obesity (IASO) harmonised their criteria and eliminated the use of WC as an important component of the definition of MetS (Alberti et al., 2009). This development led to the use of the index of central obesity (ICO) as a replacement for WC in the diagnosis of MetS.

2.1.1 The index of central obesity

The index of central obesity (ICO), which is the relationship between the waist circumference and height, has been advanced as a replacement for waist circumference because it eliminates the need for race and gender-specific considerations (Parikh et al., 2012). An ICO of 0.5 was calculated using the average heights of individuals from different countries and the WC cut off points suggested by the IDF diagnostic criteria for MetS (Parikh et al., 2007; Parikh et al., 2009). This (ICO) is suggested as a possible criterion for the selection of patients for screening for high blood pressure, prediabetes and dyslipidaemia.

2.1.2 Prevalence of obesity and metabolic syndrome

The global prevalence of MetS has reached an epidemic proportion (Wang, 2013). The W.H.O. global status report on non-communicable diseases published in 2014 showed that 39% of adults over the age 18 (38% men and 40% women) were overweight; while 11% of men and 15% of women, representing about 500 million people were obese (W.H.O., 2014). Figure 2.1 below shows the age-standardized prevalence of obesity in adults aged 18 years and over (BMI $\geq$30kg.m^{-2}) by WHO region and World Bank income groups, comparable country estimates, 2014.

In the United States, data from the National Health and Nutrition Examination Survey (NHANES) for metabolic syndrome revealed a prevalence of 34.7% for 2011-2012 with women having a higher prevalence (35.6%) than men (30.3%) (Aguilar et al., 2015). In China, a cross-sectional study using the NCEP criteria reported an overall prevalence of 27.4% with a higher prevalence reported in men (27.9%) compared to females (26.8%) (Song et al., 2015). The prevalence was higher among men with higher educational qualifications and higher family income but lower in women of the same status (Son et al., 2013). Also using the NCEP criteria, a prevalence of 19.8% and 14.1% was reported for men and women in Korea (Woo et al., 2014).

In North Western Nigeria, Sabir et al. (2016) reported an overall prevalence of 35.1% (42.83% in women and 27.36% in men).

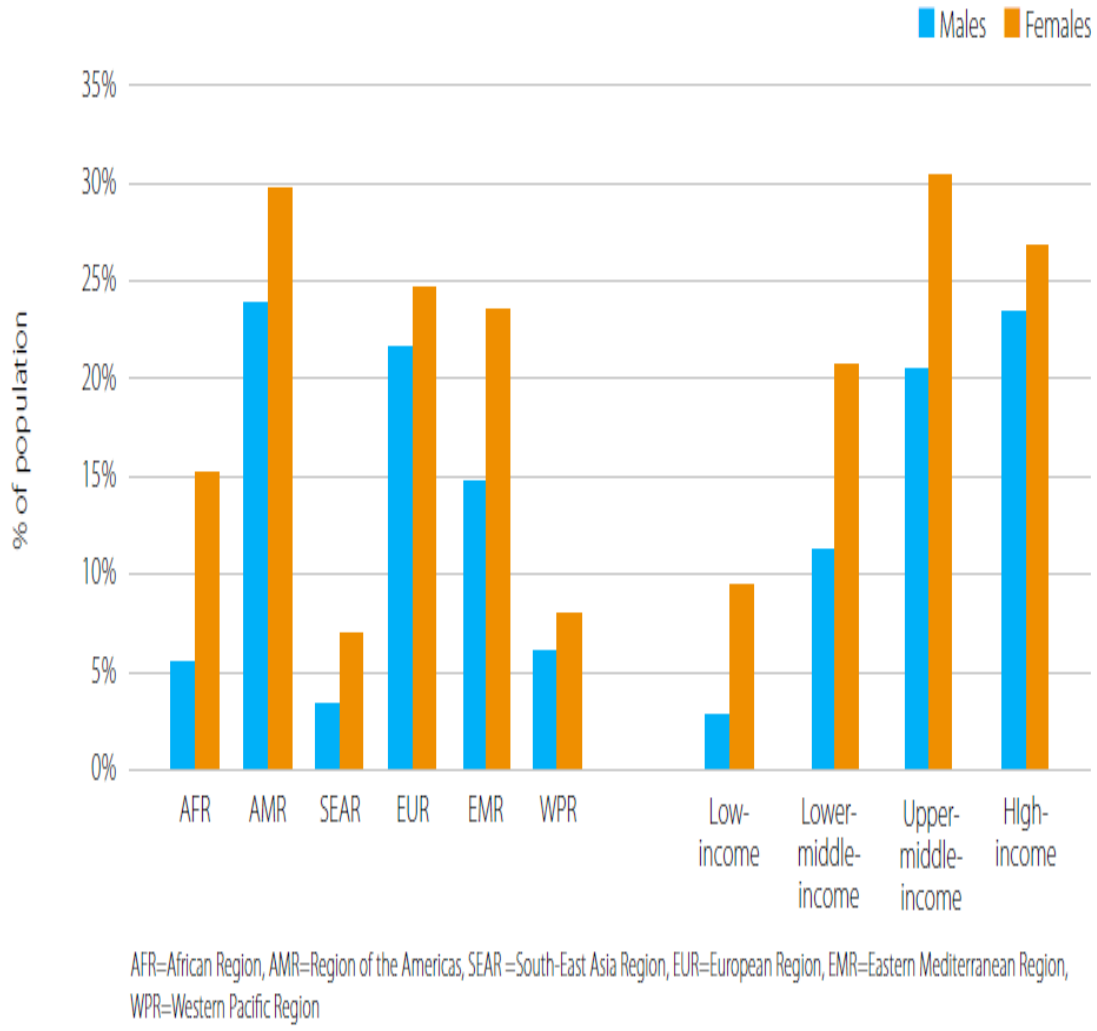


Figure 2. 1: The age-standardized prevalence of obesity in adults aged 18 years and over (BMI ≥30kg.m^{-2}) by WHO region and World Bank income groups, comparable country estimates, 2014.

(Source: WHO, 2014).

A prevalence of 30.7% was reported in South Africa using the 2009 harmonized criteria with women (43.5%) having a higher prevalence than men (16.5%) (Peer et al., 2015).

The picture is not much different in children and adolescents as to be discussed in the next section.

2.1.3 Metabolic syndrome in children and adolescents

The epidemic of childhood obesity is the main driver of metabolic syndrome in the paediatric population group. Childhood obesity is currently one of the most serious public health issues globally (Sartorius et al., 2017). Whereas a definition of MetS in the adult population is possible using one of the available criteria (with ethnicity and sex-specific adjustments), it is quite complicated to do so in children (Zimmet et al., 2007). It is known that anthropometric and other metabolic variables change over the developmental period due to the hormonal changes that occur (Vijayakumar et al., 2011). For instance, the increased activity of growth hormone in this period is known to cause insulin resistance (Bloch et al., 1987; Kelsey and Zeitler, 2016). Because of these peculiarities, it is not possible to use specific cut off points in the diagnosis of MetS in children, rather values above the 90^{th} to 97^{th} percentile are used (Zimmet et al., 2007).

Cook et al. (2003) were the first to propose a defining criterion for MetS in the adolescent population by modifying the NCEP ATP-III criteria. It placed emphasis on an abdominal circumference greater than the 90^{th} percentile, blood pressure, glycaemia and lipids over the cut off limits set by the National Blood Pressure Education Programme (NBPEP) and NCEP (Cook et al., 2003). Another criterion that was proposed by de Ferranti et al. (2004) was similar to that of Cook et al. (2003) but with lower cut off points which use the 75^{th} percentile, thereby leading to the reporting of a higher prevalence of the syndrome. Because abdominal circumference is influenced by ethnicity, a third proposal that is based on the BMI was put forward (Weiss et al., 2004).

However, the IDF proposed definition (Table 2.1) is believed to be more appropriate, and it classifies children and adolescents into age categories; 6 to 10 years, 10-16 years and greater than 16 years (Mancini, 2009). Due to lack of data, no clear diagnostic proposal was established for children under 6 years of age.

Table 2. 2: International Diabetes Federation criteria for the diagnosis of MetS in children and adolescents

Criteria/components	**Age**		
	6 to < 10 years	**10 to 16 years old**	**More than 16 years old**
Adiposity definition	WC ≥ 90th percentile	WC ≥ 90th percentile	WC ≥ 90cm (boys) or ≥ 80cm (girls)
Glucose metabolism	No cut-off definition	FBG ≥ 100mg.dl^{-1}	FBG ≥ 100mg.dl^{-1}
Dyslipidaemia	No cut-off definition	TG ≥ 150mg.dl^{-1} or HDL-C ≥ 40mg.dl^{-1}or taking lipid lowering drugs	TG ≥ 150mg.dl^{-1}or HDL-C ≥ 40mg.dl^{-1} (boys), ≥ 50mg.dl^{-1} (girls) or taking lipid lowering drugs
Arterial hypertension	No cut-off definition	Systolic BP ≥ 130mmHg or diastolic BP ≥ 85mmHg or taking medications for hypertension	Systolic BP ≥ 130mmHg or diastolic BP ≥ 85mmHg or taking medications for hypertension

WC= waist circumference, FBG= fasting blood glucose, TG= triglycerides, HDL-C=+ high-density lipoprotein cholesterol, BP= blood pressure.

Metabolic syndrome in children and adolescents, when projected into adulthood, further increases the risks of developing cardiovascular disease and T2DM (Sun et al., 2008; Kelsey et al., 2014).

A review of the literature on the prevalence of MetS in children by Friend et al. (2013) revealed a whole population prevalence of 3.3%, 11.9% among over-weight and 29.2% among obese children with a higher prevalence in boys (5.6%) than girls (2.9%). In a study of the prevalence of MetS among adolescents aged 12-19 years in the NHANES using the NCEP criteria, an overall prevalence of 8.6% was reported with males having a higher prevalence (10.8%) compared to females (6.1%) (Johnson et al., 2009). From the above statistics and the earlier discussion on childhood obesity, it is obvious that there is a serious problem requiring concerted efforts by all concerned to bring it under control.

However, though urbanisation characterised by poor dietary preferences and sedentary lifestyles have been implicated as the main drivers of the increased prevalence of MetS, there is also evidence that future phenotypes are determined by certain events occurring in the early critical periods of developmental plasticity. This is a phenomenon called neonatal programming which will be discussed later in the chapter.

The increased prevalence of childhood and adolescent metabolic syndrome is closely associated with childhood obesity which is the focus of discussion in the next section.

2.2 Childhood obesity

Childhood obesity with its attendant complications has witnessed an increased prevalence globally (Ebbeling et al., 2002; Crespo et al., 2007; Bass and Eneli, 2015). It is no doubt of public health importance because of the possibility of its projection into adulthood, thus leading to MetS (Sun et al., 2008; Atay and Bereket, 2016). Being overweight or obese in adolescent years contributes to increased adult mortality from various obesity-related complications (Bjørge et al., 2008).

Previously, childhood obesity was thought to be a health issue of the industrialised world. However, most of the industrialised nations have since halted or slowed down the epidemic due to intensive awareness campaigns and other health measures (Atay and Bereket, 2016). For example, in the USA, the increased prevalence in childhood obesity that was seen in the

1970s through the 2000s (6.5- 18% in children and 5-18.4% in adolescents) stabilised and even declined in some population groups (Ogden et al., 2012; Ogden et al., 2014). A review of body mass index (BMI) data from 11.1 million children from 40 states in the USA showed a 0.4-0.9% decrease in the prevalence of obesity among children aged 2-4 years from 2008-2011 (Pan et al., 2016). Also, from 2008-2013, a decrease of 1.6% in the prevalence of childhood obesity was observed among young children from California (Koebnick et al., 2015). In the State of Israel, the prevalence of childhood obesity stabilised from 2000-2012 (11.5% in boys and 9.5% in girls) with no increases recorded (Almagor et al., 2015). Similarly, in Sweden, there was no increase in the prevalence of overweight among teenagers from 2003-2011 (Munter et al., 2016). From the year 1999-2007, the prevalence of childhood overweight reduced by 1.7% among Dutch girls (De Wilde et al., 2014).

Perhaps, a good example of a developed country where childhood obesity has reached epidemic proportions is China. A study conducted in the Shandong province showed an alarming increase in prevalence from 0.45-18.16% in boys, and from 0.11- 6.58% in girls from 1985-2014 (Zhang et al., 2016b). The oil-rich countries have also witnessed an increased prevalence over time: Saudi Arabia, which had a prevalence of childhood obesity of 5.98% in boys and 6.74% in girls in 2002 (El-Hazmi and Warsy, 2002) witnessed an increase to 34.8% in girls and 17.3% in boys aged 6-10 years in 2014 (Al-Mohaimeed et al., 2015).

Unfortunately, in the developing countries, the reverse is the case, as the prevalence is still on the increase (Atay and Bereket, 2016). According to reports, about 40 years ago the prevalence of childhood obesity was similar in both the developed and the developing countries (Poskitt, 2014). However, with increasing technological advancement came the problems of sedentary lifestyles and consumption of ready-made energy-dense foods that are believed to be responsible for the epidemic (Atay and Bereket, 2016).

Data from Benue State, North Central Nigeria in a study involving 3240 male and female children and adolescents showed a higher prevalence of overweight in females compared to males (20.3% vs 16.2%) (Musa et al., 2012). Another study involving school-aged children and adolescents in Southern Nigeria showed an overall prevalence of childhood obesity of 2.8%, with girls having a higher prevalence compared to boys (Ene-Obong et al., 2012).

In the Republic of South Africa, an overall prevalence of 22.9% was reported in a study conducted among 1559 learners aged 7-18 years (Negash et al., 2017). Sekokotla et al. (2017) reported an overall prevalence of obesity of 40.2% in 13-18-year-old participants in Mthatha, South Africa, with the girls having a higher prevalence compared to the boys (47.5% vs

22.5%). Thus, chidlhood obesity in South Africa as in other parts of the world, is a public health issue of great concern that should receive priority attention from researchers and policy makers in order to mitigate the growing epidemic.

Childhood obesity is caused by several factors.

2.2.1 Causes of childhood obesity

The regulation of body weight is achieved via tightly controlled physiological mechanisms that strike a balance between energy intake and expenditure (Stewart, 2015). However, genetic, epigenetic and environmental factors alter these physiological control systems and lead to obesity (Bass and Eneli, 2015). These various factors will be considered in turn.

2.2.1.1 Genetic factors

Genetic factors are responsible for about half of all cases of increased adiposity (Queiroz et al., 2015) with specific ethnic groups (such as blacks, Asians and American Indians) being more predisposed than others (Gurnani et al., 2015). Usually, genetic causes of obesity are not investigated in children unless they also present with other problems such as developmental delays or dysmorphic features (Styne et al., 2017). However, it is now recommended by the paediatric endocrine society (PES) to carry out genetic screening for any child under the age of 5 years presenting with severe obesity or who has a positive family history of severe obesity (Dayton and Miller, 2018). Single gene defects such as mutations in the gene encoding for leptin have been identified in children (Weiss and Lustig, 2014). Leptin, a hormone secreted by adipocytes, plays a role in appetite regulation (Farr et al., 2015). A few other single gene defects like mutations of the adenylate cyclase 3 (ADCY3), an important gene in energy homeostasis have been associated with severe obesity in children (Saeed et al., 2018). The melanocortin-4-receptor gene (MC4R) is important in preventing obesity through its action of reducing food intake and increasing energy expenditure (Siljee et al., 2018). About 3-5% of all cases of severe obesity result from a mutation of the MC4R gene which impairs its activation (Siljee et al., 2018). Both homozygous and heterozygous mutations of the proopiomelanocortin (POMC) gene have been found to lead to adrenal insufficiency early in life, enhanced appetite and obesity (Nordang et al., 2017; Dayton and Miller, 2018). A

homozygous mutation in the proprotein convertase subtilisin/kexin 1 (PCSK1) gene presents with malformation of gastric peptides, proinsulin and development of obesity (Stijnen et al., 2016). However, a larger proportion of obesity appears to be caused by a complex interaction of several obesity-associated genes (Queiroz et al., 2015). For example, in Prader-Willi syndrome were a mutation on paternal chromosome 15q11-q13 leads to early onset of obesity in childhood among other developmental problems (Miller, 2012). Similarly, genetic mutations that lead to the repression of the brain-derived neurotropic factor (BDNF) and/or its receptor tropomyosin-related kinase B (TrkB) alter the balance between energy intake and metabolism and subsequently lead to the development of obesity early in childhood (Styne et al., 2017). However, only about 5% of all cases of childhood obesity can be linked to specific gene defects, the majority of cases are as a result of interactions between the child's genetic, ethnic and environmental factors (Atay and Bereket, 2016).

2.2.1.2 Epigenetic factors/metabolic programming

Both maternal over-nutrition and under-nutrition have been implicated in the development of childhood obesity (Han et al., 2010). This is supported by clinical and epidemiological data (Srinivasan and Patel, 2008). In maternal obesity, excess nutrients that are transferred via the placenta can permanently modify and cause changes in appetite regulation, neuroendocrine functioning and metabolism in the foetus (Lau et al., 2014).

Under-nutrition during important stages of gestation leads to intra-uterine growth restriction (IUGR) which compels the foetus to adopt strategies for survival (Lau et al., 2014). These strategies eventually induce permanent functional changes that lead to obesity (Patel and Srinivasan, 2002). This is the phenomenon referred to as foetal/neonatal metabolic programming which will be discussed in greater detail later in this chapter.

Bottle-fed infants were also found to be more predisposed to obesity in childhood compared to exclusively breastfed infants (Lefebvre and John, 2014; Woo and Martin, 2015; Chiasson et al., 2016). For example, a higher plasma concentration of insulin is found in bottle-fed when compared to breastfed infants, and this is thought to facilitate the deposition of fat and early-onset obesity (Socha et al., 2011). Additionally, bottle-fed infants tend to have a higher protein intake compared to their breastfed counterparts (Oropeza-Ceja et al., 2018). This higher protein intake in bottle-fed infants has been found to strongly correlate with obesity in childhood (Oropeza-Ceja et al., 2018).

2.2.1.3 Physical inactivity

A sedentary lifestyle is one that is characterised by a lack of physical activity or excessive inactivity (Fröberg, 2015). Obese children have been found to be less involved in moderate and vigorous exercises compared to their non-obese counterparts and usually incur imbalanced energy expenditure and thus more weight gain (Malik et al., 2013; Brown et al., 2015). Television viewing is not only associated with reduced physical activity (Guran and Bereket, 2011), but also with increased food intake (energy consumption) and less sleep (Lumeng et al., 2007). Additionally, children are exposed to food advertising which could affect eating habits (Kaur et al., 2003; Stettler et al., 2004) and thus predispose them to the consumption of energy-dense foods and consequently the development of obesity.

The increasing distance between the home and the school, neighbourhood security and increased car ownership have led to a decline in active transport in form of walking and cycling to schools among children and adolescents attending school (Ham et al., 2008; McDonald et al., 2011). In some instances, the school curriculum is structured in such a way that there is little time allocated for physical activities (Hills et al., 2015). There is also an increased tendency for the children to sit and talk during lunch and short breaks instead of engaging in physical activities like organised games (Lewis et al., 2007).

2.2.1.4 Diets

An important cause of the imbalance between energy intake and expenditure that leads to the development of obesity is the type of diet consumed. Fast foods are convenient and inexpensive especially in families where both parents go to work (Niehoff, 2009). The increased consumption of fast foods has been linked to childhood obesity (Zhao et al., 2017). Fast foods are usually energy dense with low nutritional value and supply more calories than would be consumed in a typical home setting (Fulkerson, 2018).

Sugar-sweetened soft drinks are speculated to increase appetite because of their high glycaemic index (Maki et al., 2015). These sugar-sweetened drinks increase the BMI of their consumers gradually by small amounts over several years (Anderson and Butcher, 2006).

Increased consumption of snack foods and increased portion sizes have resulted in increased caloric intake that has been linked to the development of obesity in childhood and adolescence (Fulkerson, 2018; Kral, 2018).

High glycaemic index (HGI) foods are those that produce large increases in postprandial blood glucose concentrations (Cooper et al., 2017). These high glycaemic index foods are capable of regulating appetite and inducing hormonal changes that stimulate hunger and cause over-eating in adolescents (Lennerz and Lennerz, 2018). Consequently, HGI foods have been linked to the development of central adiposity, CVD and T2DM (Cooper et al., 2017; Lennerz and Lennerz, 2018). Milk including yoghurt, on the other hand, has a low glycaemic index and protects over-weight young adults from becoming obese (Wolever, 2017).

Metabolic syndrome follows a common pathway in both children and adults and therefore the pathogenesis of MetS will be discussed in the next section.

2.3 Pathogenesis of metabolic syndrome

The three potential aetiological mechanisms for MetS are obesity, insulin resistance, the gut microbiota and a collection of other independent factors (Grundy et al., 2005). The next sections discuss these aetiological factors.

2.3.1 Obesity in the pathogenesis of metabolic syndrome

The increasing prevalence of obesity is thought to drive the epidemic of MetS (Bosello and Zamboni, 2000; Grundy, 2004). Visceral obesity is central in the pro-inflammatory state underlying MetS (Ritchie and Connell, 2007) and associates with other metabolic risk factors (Grundy, 2004). The excess adipose tissue releases adipocytokines into the circulation which alter the physiology and worsen the other metabolic risk factors (Martin et al., 2015). Some of these adipocytokines include adiponectin, interleukin 2 (IL-2), non-esterified fatty acids (NEFA) and plasminogen activator inhibitor- 1(PAI-1) (Tilg and Moschen, 2006). The chronic inflammatory response found in obesity is also linked with increased acute phase proteins, for example, CRP (Tilg and Moschen, 2006). The accumulation of NEFA leads to

excess accumulation of lipids in the liver and the muscles, promoting NAFLD and insulin resistance (Liu et al., 2010). The increased PAI-1 brings about the prothrombotic state associated with obesity (Eckel et al., 2005; Alessi and Juhan-Vague, 2008). The concentration of adiponectin, an important adipokine that has anti-diabetic, anti-inflammatory, antiatherogenic, and cardioprotective effects, is decreased in obesity (Nigro et al., 2014). These decreased levels of adiponectin are thought to exacerbate other metabolic risk factors such as hyperglycaemia and dyslipidaemia (Grundy, 2004; Nigro et al., 2014). It is in recognition of this powerful connection between obesity and the other risk factors that the NCEP defined MetS as a collection of metabolic complications of obesity (N.C.E.P., 2002).

2.3.2 Insulin Resistance in the pathogenesis of metabolic syndrome

Insulin resistance results when there is a reduced ability of insulin to facilitate cellular glucose uptake and its subsequent utilisation and storage in the peripheral tissues (Gobato et al., 2014). This results in a compensatory increase in the secretion of insulin by the pancreatic β-cells leading to hyperinsulinaemia (Brea and Puzo, 2013; Gobato et al., 2014). It is difficult to assign a role for insulin in the causation of MetS because it is intricately linked to obesity. Though insulin resistance usually rises with increased obesity, there is a wide range of insulin sensitivities for any given level of fat content (Grundy et al., 2005; Chen et al., 2014). Most morbidly obese patients have postprandial hyperinsulinaemia and low insulin sensitivity (Grundy, 2004). In the insulin-resistant state, the fat tissue becomes unresponsive to the antilipolytic effects of insulin resulting in peripheral lipolysis and diversion of free fatty acids to the liver (Browning et al., 2004), as well as increasing the rate/amount of *de novo* lipogenesis (Brea and Puzo, 2013). Peripheral insulin resistance may cause glucose intolerance which can be exacerbated by increased liver gluconeogenesis in the insulin-resistant liver (Roberts et al., 2013).

2.3.3 The gut microbiota in the pathogenesis of metabolic syndrome

The gut microbiota plays an important role in the pathogenesis of MetS (Festi et al., 2014). There is an important symbiotic relationship between gut microbiota and the host in the regulation of metabolism and immunity (D'Aversa et al., 2013). This symbiotic relationship enhances the optimum extraction, utilization and subsequent storage of excess calories in the adipose tissues of the host (DiBaise et al., 2008). Dysbiosis or disruption of the intricate balance between the gut microbiota and the host leads to changes in the functioning of the intestinal barrier and the gut-associated lymphoid tissues (Gomes et al., 2018). These changes result in increased permeability of the intestinal barrier that then allows for the translocation of bacterial fragments into the systemic circulation thereby inducing inflammation and subsequently insulin resistance (Festi et al., 2014; Gomes et al., 2018). The activation of inflammatory pathways in the liver by the presence of bacterial fragments in the portal blood (portal endotoxin influx) leads to the development of NAFLD (Hou et al., 2017; Feng et al., 2018). Both high fat and high fructose diets have been shown to induce metabolic dysfunction by disrupting the gut microbiota and subsequently causing a low-grade inflammation and increased intestinal permeability (Wang et al., 2015; Mastrocola et al., 2017). High fructose diets are thought to exert their effect by reducing the diversity of the gut microbiota and influencing the expression of genes involved in carbohydrate and lipid metabolism (Mastrocola et al., 2017).

2.3.4 Other factors that mediate specific risk markers of metabolic syndrome

Apart from obesity and insulin resistance, each of the cardiometabolic risk factors associated with MetS has its own unique genetic control mechanisms (Grundy, 2004). These control mechanisms account for the variability in expression of each of the metabolic risk factors (O'Neill and O'Driscoll, 2015). A good example is the metabolism of lipoproteins and blood pressure which are both genetically regulated and therefore have varied expressions in response to obesity or insulin resistance in different individuals (Grundy, 2008).

The prevalence of MetS has also been shown to increase with advancing age (Ford, 2005). A pro-inflammatory state and several endocrine factors have been associated with body fat distribution abnormalities and hence, indirectly with MetS (Doyle et al., 2012).

2.4 Complications of childhood obesity

Childhood obesity is a multisystem disorder that has led to an increased occurrence in children of certain diseases previously thought to affect only adults (Han et al., 2010). A summary of some of the complications is hereby provided (Table 2.3). It is thus important that preventative strategies are put in place to forestall the occurrence of these complications.

Table 2. 3: Selected complications of childhood obesity

	Physiological system	Complications	References
1.	Endocrine	Insulin resistance, prediabetes, T2DM, precocious puberty, polycystic ovary syndrome (girls), hypogonadism (boys)	(Ludwig and Ebbeling, 2001; Marcus et al., 2010; Oester et al., 2016)
2.	Cardiovascular	Dyslipidaemia, hypertension, coagulopathy, Chronic inflammation, endothelial dysfunction	(Maggio et al., 2014; Chang et al., 2015)
3.	Gastrointestinal	NAFLD, gall stones	(Koebnick et al., 2012; Nunes et al., 2014; Anderson et al., 2015)
4.	Genetic	Accelerated aging (increased telomere attrition)	(Révész et al., 2014; Bonomini et al., 2015)
5.	Musculoskeletal	Slipped capital femoral epiphysis, Blount's disease, forearm fracture, flat feet	(Maggio et al., 2014)
6.	Renal	Glomerulosclerosis	(Adelman et al., 2001)
7.	Neurological	Pseudotumour cerebri	(Brara et al., 2012)
8.	Psychosocial	Poor self-esteem, depression, eating disorders	(Helseth et al., 2015)

In the present study, some components of complications 1-6 (table 2.3) were considered.

2.4.1 Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the most common liver pathology worldwide affecting about 30% of the general population in the developed countries (Asrih and Jornayvaz, 2015; Fazel Nabavi et al., 2015). NAFLD is found more in men (30-40%) than in women (15-20%) and is much higher in diabetic patients (70%) (Tsuneto et al., 2010). In children and adolescents, the statistics for NAFLD are not much different. About 10% of all children present with NAFLD (Hartmann and Schnabl, 2018). The prevalence of NAFLD increases with age; about 0.7% of 2-4 year old, 3.3% of 5-9 year old, 17.3% of 15-19-year-old and 38% of obese children are affected (Hartmann and Schnabl, 2018).

NAFLD is a clinical condition characterised by the deposition of excess fat (usually triglycerides, > 5% weight or volume) in the liver in individuals without a history of significant alcohol consumption or other known causes of liver disease such as viruses and drugs (Chalasani et al., 2012; Ahmed et al., 2015). The term NAFLD is used to describe a spectrum of liver conditions that range from simple steatosis through non-alcoholic steatohepatitis (NASH) to subsequent fibrosis, cirrhosis and hepatocellular carcinoma (Than and Newsome, 2015). It has however now been established that steatosis may not always precede inflammation but rather NASH may be the primary initial liver injury (Tilg and Moschen, 2010; Buzzetti et al., 2016). Even though NASH is one of the commonest cause of liver disease in children and adolescents, there is no definitive treatment yet for the condition (Mann et al., 2018). Although a lot of authors still refer to NAFLD as the hepatic manifestation of MetS, evidence from several longitudinal studies has found the development of NAFLD to precede that of MetS (Lonardo et al., 2015).

NAFLD is a multisystem disease that was initially thought to develop on the basis of a 2-hit hypothesis where accumulation of triglycerides (due to sedentary lifestyle, obesity, insulin resistance, high fructose/fat diet) in the liver constituted the first hit while the subsequent sensitization and activation of the inflammatory pathways in the liver was considered the second hit (Peverill et al., 2014). However, the complex molecular and metabolic changes

that are involved in the development and progression of NAFLD cannot be properly explained using the 2-hit hypothesis (Tilg and Moschen, 2010; Buzzetti et al., 2016).

The multiple-hit hypothesis is now proposed to account for the complex interactions arising from simultaneously derived insults from insulin resistance, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, gut microbiota, adipose tissues and diets in genetically and epigenetically susceptible individuals (Treeprasertsuk et al., 2011; Buzzetti et al., 2016). Insulin resistance is an important factor in the pathogenesis of steatosis and NASH because it increases *de novo* lipogenesis while inhibiting lipolysis and consequently increasing entry of free fatty acids into the liver (Bugianesi et al., 2010). Insulin resistance also distorts the normal endocrine function of adipose tissues causing abnormal secretion of adipocytokines and other inflammatory cytokines (Blüher, 2016; Samuel and Shulman, 2016). The accumulation of triglycerides, free fatty acids and other lipid metabolites induce hepatic lipotoxicity which leads to structural and functional mitochondrial dysfunction with its associated oxidative stress and production of reactive oxygen species (Krishnan et al., 2018). Mitochondrial dysfunction results in insufficient adenosine triphosphate (ATP) synthesis which then leads to the accumulation of unfolded proteins in the ER thereby causing ER stress (Lebeaupin et al., 2018). Diets such as high fructose induce NAFLD by several mechanisms which include augmenting lipogenesis, mitochondrial dysfunction, activating inflammatory pathways and insulin resistance (Chen et al., 2017).

The increased diagnosis of NAFLD in children at younger ages is thought to be linked to the developmental origins of the disease (Wesolowski et al., 2017). It has been suggested that the predisposing factors for NAFLD come into play early in life (Friedman, 2016). Nutritional perturbations in the early periods of developmental plasticity affect the placenta and induce permanent alterations in the liver and the gut microbiota which then prime the infant liver for NAFLD and increase its susceptibility to NASH postnatally (Wesolowski et al., 2017). These perturbations induce epigenetic changes in the liver that favour the development of liver injury (Treviño and Katz, 2018). Maternal malnutrition which results in intrauterine growth restriction of the foetus is known to negatively impact the development of the pancreas, adipose tissue and the liver and program for hepatic insulin resistance and NAFLD (Spradley et al., 2018).

2.4.2 Accelerated aging in childhood obesity

Ageing is a normal biological process that involves continuous depreciation of body functions including metabolism and leads to morbidity and mortality (Paradies et al., 2010; Rani et al., 2016). Ageing is the single most important risk factor for most chronic diseases (Kennedy et al., 2014). Obesity accelerates biological ageing of cells and tissues and consequently results in the early onset of many age-related diseases in obese individuals (Horvath et al., 2014; Hohensinner et al., 2018). In obesity and MetS, a major consequence is a generation of reactive oxygen species (ROS) from the mitochondria or other sites in the cytoplasm which then cause degradative and toxic processes that lead to accelerated ageing (Bonomini et al., 2015; Furukawa et al., 2017). Indeed the persistent low-grade inflammation and oxidative stress found in obesity and MetS have been associated with accelerated ageing (Abbas, 2017) and telomere attrition (Zgheib et al., 2018).

Telomeres are specialised double-stranded repetitive sequences of six nucleotides that form the cap at the ends of eukaryotic chromosomes and are bound to a protein called shelterin which has six telomere binding proteins (Rodier et al., 2005; Zgheib et al., 2018). Telomere length is an index of chronological ageing (Demissie et al., 2006; Abbas, 2017). Telomeres protect against the fusion of the ends of adjacent chromosomes and preserve the integrity of the coding DNA (Zierer et al., 2016; Aviv and Shay, 2018).

Leucocyte telomere lengths are usually used to determine telomere lengths because of the easy accessibility of blood (Aubert and Lansdorp, 2008; Aubert et al., 2012). The mean telomere length represents the telomere lengths in all the different leucocyte lineages and the somatic cells (Daniali et al., 2013; Sabharwal et al., 2017). The rate of telomere attrition is faster during the early developmental stages of life (Aubert et al., 2012). It has also been previously shown that females have longer telomeres than males due to the transcriptional effects of oestrogen on telomerase, the enzyme responsible for maintaining telomere length (Nawrot et al., 2004). Offspring of older fathers have longer telomeres due to Mendelian inheritance from the long telomeres in sperm of older males (Kimura et al., 2008).

2.4.3 Bone diseases in childhood obesity

Childhood obesity is associated with a number of skeletal complications such as Blount's disease (Maggio et al., 2014), osteoporosis and osteopenia (Muka et al., 2015; Ozturk, 2017). Osteoporosis, a metabolic bone disease characterised by skeletal fragility is a major health problem responsible for huge health care costs (Sugimoto et al., 2016; Zheng et al., 2018b). It results from a decreased bone mass and a progressive distortion of the bone microarchitecture that leads to easy fracture of the bones (Sugimoto et al., 2016).

Blount's disease or tibia vara is a developmental skeletal disease that usually presents as bowed legs and tibial torsion (Sabharwal, 2015). The exact aetiology of Blount's disease is unknown but females are affected more than males and it has a strong association with childhood obesity (Sabharwal, 2015). The degree of obesity correlates with the extent of tibial deformity (Calder, 2014).

Another skeletal comorbidity of childhood obesity is the slipped capital femoral epiphysis (SCFE) (Maggio et al., 2014). SCFE is a skeletal disorder that distorts the hip by displacing the capital femoral epiphysis from the metaphysis through the femoral growth plate (Perry et al., 2018). It affects about 1% of all peripubertal children and is strongly associated with childhood obesity (Aversano et al., 2016).

Though the adolescent period is the period of major bone growth (Lee, 2013), the nutritional and other experiences of the individual from intrauterine life till adolescence or young adulthood determine the strength and quality of the skeleton at any given time (Cooper et al., 2006; Harvey et al., 2014). The risk of future bone fractures is thought to be programmed during the early critical periods of developmental plasticity of an individual (Rapp et al., 2018). Therefore, interventions targeted at these periods of development may provide an opportunity to program for future good bone health.

2.5 Prevention/treatment of childhood obesity and adolescent metabolic syndrome

2.5.1 Lifestyle modifications

The prevention of childhood obesity is usually based on programs directed at developing good eating habits and increased physical activity (Verrotti et al., 2014). Obesity prevention interventions based on lifestyle changes though difficult to implement (Dolinsky et al., 2013), are more practical and easier for children than adults (Llargues et al., 2011). The school setting provides an ideal opportunity to implement obesity control programs because the children spend a lot of time in school, take several meals and engage in sports and physical education (Khambalia et al., 2012). Moreover, school-based programs are more likely to be sustained on a long-term basis, thereby providing maximum benefits (Khambalia et al., 2012). Adult eating habits are usually as a consequence of the eating habits developed in childhood (Cizza and Rother, 2012). Therefore, the inculcation of healthy eating habits early in life is important in the prevention of obesity.

In obese children and those with MetS where lifestyle modifications do not yield the desired outcomes, pharmacotherapy becomes necessary.

2.5.2 Pharmacotherapy

The use of pharmacotherapy in the management of severe obesity in adolescence (BMI ≥ 99 percentiles) is recommended when there are comorbidities and lifestyle changes are not proving beneficial (Spear et al., 2007; August et al., 2008; Dolinsky et al., 2013). Metformin and orlistat are the two drugs currently showing promise in the treatment of childhood obesity (Dolinsky et al., 2013). Orlistat is an inhibitor of pancreatic lipase and thus reduces the absorption of dietary fats via the intestines by about one-third (Iughetti et al., 2010). Metformin is a biguanide drug used as an oral hypoglycaemic agent in the treatment of T2DM and/or insulin resistance (Iughetti et al., 2010). Though metformin acts by inhibiting hepatic gluconeogenesis and lipogenesis, it is also thought to suppress appetite (August et al., 2008), and may, therefore, be used as a weight reduction agent (Dolinsky et al., 2013).

Dyslipidaemia in metabolic syndrome is treated with three classes of drugs viz: the 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG CoA) inhibitors or statins, fibrates and niacin (Wagh and Stone, 2004). The statins, in addition to inhibiting the enzyme HMG CoA, also induce the expression of LDL-receptors which ensure its clearance from the body (Caparrós-Martín et al., 2017). The fibrates act as agonists of the peroxisome proliferator-activated receptor alpha (PPARα) and are used to lower triglycerides and convert small dense LDL to less atherogenic forms (Kamijo, 2018). Niacin or nicotinamide acid is used to control lipids by raising the HDL-c and lower the LDL-c levels (Montserrat-de la Paz et al., 2017).

It is important to control blood pressure in order to prevent serious complications such as stroke, myocardial infarction or chronic kidney disease (Grundy, 2008). There are several classes of drugs used in controlling hypertension but those that regulate the renin-angiotensin system are usually preferred in MetS (Cabandugama et al., 2017). The angiotensin-converting enzyme inhibitors are usually the first line drugs especially when there is also T2DM or chronic kidney disease (Barnett et al., 2004). The inhibition of the renin-angiotensin system is thought to lower the risk of developing T2DM in MetS (Scheen, 2004).

2.5.3 Surgery

Severely obese adolescents may benefit from bariatric surgery which involves surgery aimed at reducing the size of the stomach and also decreasing the absorptive capacity of the stomach (Inge et al., 2007). This procedure has been shown to reduce BMI and comorbidities associated with childhood obesity. A less invasive procedure that does not require a permanent distortion of the gastrointestinal tract, is the placement of a stomach band with an inflatable balloon around the gastroesophageal junction (Inge et al., 2007). By varying the amount of saline injected into the balloon, the size of the stomach can be adjusted (Inge et al., 2007).

Even though the increased prevalence of metabolic syndrome has been linked to increased consumption of diets rich in carbohydrates and physical inactivity, there is strong evidence that most metabolic diseases have developmental origins, a concept called developmental programming which will be discussed in the next section.

2.6 Developmental programming of metabolic function

The periods of gestation and lactation are critical periods of developmental plasticity characterised by rapid growth and organ maturation (Armitage et al., 2005). When stressor events such as nutritional, pharmacological, hormonal or other perturbations occur during these periods, they modify the physiology and metabolism of the individual permanently via epigenetic means (de Moura et al., 2009; Portha et al., 2014; Simeoni et al., 2014; Firth et al., 2017). These perturbations can play important roles in the selection of genes to be expressed or silenced and therefore impact on the metabolic evolution of the individual (Bruce and Hanson, 2010; Park et al., 2017a). Even though the normal growth and development of individuals are a function of the genome (Armitage et al., 2004; Warner and Ozanne, 2010), they can only be achieved if maternal conditions are able to support the associated high rates of proliferation, growth and differentiation (Warner and Ozanne, 2010). Environmental or nutritional perturbations in the parents either during gestation or in the early postnatal period are also known to affect the physiology of the individual (Sales et al., 2017). This phenomenon is called developmental programming.

Hales and Barker (1992) first proposed this hypothesis when they described the thrifty phenotype in the aetiology of T2DM. The authors found an association between early nutritional deprivation and subsequent development of T2DM because of impaired development of the pancreas (Hales and Barker, 1992). Apparently, due to inadequate nutrients, there is a metabolic trade-off (Gluckman and Hanson, 2004) leading to selective distribution in favour of vital organs such as the brain and the heart to the detriment of other organs like the pancreas and muscles (Srinivasan and Patel, 2008). It has been shown that even brief dietary manipulations in early life could lead to major health implications later in life (Armitage et al., 2004).

Disturbances in the environment are responded to either through homeostatic modalities, which confer instant benefits for survival or via a predictive adaptive response (PAR) that may only become beneficial or harmful in future (Gluckman and Hanson, 2004). When homeostatic responses to environmental perturbations overlap with the periods of developmental plasticity, they may also have long-lasting effects on the physiology of the individual (Gluckman and Hanson, 2004). PAR is a form of adaptation that occurs because of experiences during periods of developmental plasticity that predict an appropriate phenotype

suited for the expected environment in later life (Bateson et al., 2014; Nettle et al., 2014). When there is a maladaptation between the predicted and actual environment such that conditions in the actual environment exceed the range of the predictive adaptive responses, adverse health outcomes result (Wells, 2012; Hayward and Lummaa, 2013).

Epigenetic changes which involve DNA methylation, histone modification and micro-RNAs is one of the basic mechanisms by which neonatal developmental programming is achieved (Aiken and Ozanne, 2014). These epigenetic marks alter how genes are expressed without affecting the genome (Park et al., 2017a; Park et al., 2017b). Epigenetic effects in the germline and somatic cells facilitate the transgenerational transmission of developmental programming via both maternal and paternal lines without necessarily having to repeat the initial insult in the subsequent generations (Aiken and Ozanne, 2014; Vickers, 2014). It has also been suggested that generational transmission of programming through the maternal line might be a function of a sub-optimally developed uterine environment or maternal adjustments to pregnancy (Aiken and Ozanne, 2014).

The second mechanism by which developmental programming can be achieved is through altered tissue differentiation. This is demonstrated by the finding of a reduced number of nephrons in adult humans that were born small and subsequently developed hypertension in adulthood (Mackenzie and Brenner, 1995). The reduced number of nephrons is probably the price paid in a trade-off for selective nutrients supply to the brain and other vital organs.

The third mechanism for achieving developmental programming is via altered homeostatic mechanisms. An example that readily comes to mind is the reduction in the reserve of ovarian follicles, the frequency and rate of ovulation and the age at puberty or menarche in females who experienced early life nutritional adversity which disrupted the establishment of the primordial follicle pool (Chan et al., 2015). Another example is the altered ratio of periportal to peri-venous hepatocytes seen in offspring of protein-restricted rats (Peterside et al., 2003) which results in the increased liver production of glucose due to an altered ratio of phosphoenolpyruvate carboxykinase to glucokinase-enriched cells (Desai et al., 1997; Peterside et al., 2003).

In humans, there is epidemiological evidence in support of developmental programming of metabolic diseases. This evidence also shows that the impact of poor nutrition on the programming of future obesity depends on the particular critical time it occurs (Cripps et al., 2005). In the Dutch Hunger Winter of 1944-1945, adolescent male offspring that were

exposed to famine in early gestation showed increased rates of obesity unlike those exposed during late gestation and early infancy who showed reduced rates of obesity (Ravelli et al., 1976). However, obesity only results when there is improved nutrition in later life (de Moura and Passos, 2005). In a study in Russia, adult offspring of mothers who experienced famine during gestation as a result of the siege of Leningrad did not show any signs of programming for obesity, diabetes or coronary artery disease (Stanner et al., 1997). Rapid postnatal catch-up growth has also been shown to have adverse effects on the health of individuals in adulthood (Leon et al., 1996; Forsen et al., 2000).

In rats and similar altricial species, the lactation period (immediate postnatal) is the most critical period for establishment of programming (de Moura et al., 2009), though only a few studies are available on the impact of malnutrition in the lactation period only, perhaps due to experimental difficulties of rearing the pups away from their mothers (Passos et al., 2000; de Moura et al., 2009). At birth, most of the organ systems in altricial species are immature and poorly developed with marked maturation occurring during the suckling period (Armitage et al., 2004; Felderhoff-Mueser et al., 2004; Kiray et al., 2014). However, the critical periods of developmental plasticity in mammals extend from the periconceptual, gestation through the infancy period (Firth et al., 2017).

The risk of developing metabolic diseases in adulthood can be significantly reduced via the modification of the prenatal environment (Srinivasan and Patel, 2008). It has also been shown that dietary interventions and other environmental factors can initiate or reverse epigenetic risk markers thereby programming or reprogramming for metabolic dysfunction (Park et al., 2017a).

The programming effects of neonatal administration of fructose were demonstrated in Sprague Dawley rats when they showed increased body mass, hyperinsulinaemia and increased transportation of fatty acids in their skeletal muscles in adulthood (Huynh et al., 2008).

However, several studies have demonstrated the benefits of dietary interventions during the suckling period in programming and reprogramming of metabolic function later in life. S-allyl-l-cysteine administered in the suckling period protected male and female Wistar rats from fructose-induced liver injury in adulthood (Lembede et al., 2018). Treatment of offspring of malnourished Wistar rat dams with leptin in the neonatal period reversed adverse effects on bone development (Firth et al., 2017), body mass, adiposity and concentrations of

fasting blood glucose, insulin and leptin (Vickers et al., 2005) associated with the phenotype in adulthood.

Supplementation with specific bioactive food components has also been found to reprogram the effects of maternal high fructose (Ching et al., 2011) and high-fat diet (Albert et al., 2017) on the phenotypes of adult offspring. Fish oil supplementation in high fat fed Sprague Dawley dams during gestation and lactation prevented the development of insulin resistance associated with the phenotype in adult male offspring (Albert et al., 2017). Supplementation with bitter melon (*Momordica charantia*) during gestation and lactation, on the other hand, ameliorated the fructose-induced phenotypic changes (dyslipidaemia and hepatic oxidative stress) in adult offspring of Sprague Dawley rats (Ching et al., 2011).

Polyphenolic compounds have now become an important subject of research due to their reported effects on various chronic diseases including metabolic syndrome and its sequelae (Sohrab et al., 2013). For the purpose of this review of the literature, the focus will be on curcumin (derived from turmeric) which was the phytochemical chosen for the current study.

2.7 Turmeric

Turmeric is a bright yellow powder, derived from the dried roots of the rhizome, *Curcuma longa*, a member of the ginger family Zingiberaceae that is commonly used as a dietary spice, mostly as curry or mustard (Peirce, 1999). *Curcuma longa* L. is a herbaceous plant grown mostly in tropical climates (Prasad et al., 2014). Turmeric has a long history of use in Ayurveda and Chinese medicine as a treatment for diarrhoea, dermatitis, angina pectoris, cough and liver ailments (Gupta et al., 2013; Sahebkar, 2013; Deogade and Ghate, 2015; Kocaadam and Şanlier, 2017).

The major constituents found in *Curcuma longa* extract include an abundance of carbohydrates (60-70%), curcuminoids (1-6%), volatile oils which contain turmerone(3-7%), fibre (2-7%), minerals (3-7%), proteins (6-8%), fats (5-10%), moisture 6-13%) (Nelson et al., 2017). The curcuminoids present in turmeric include curcumin, demethoxycurcumin, bisdemethoxycurcumin and cyclocurcumin (Anand et al., 2008; Jiang et al., 2017).

Curcumin is the most bioactive of the curcuminoids and is perhaps responsible for the popularity of turmeric in medical treatment (Jurenka, 2009; Kocaadam and Şanlier, 2017).

2.8 Curcumin

Curcumin, a diferuloylmethane, was first isolated over 200 years ago by two German Scientists, Vogel and Pelletier and described as a "substance that gives the yellow colour" (Kocaadam and Şanlier, 2017; Kunnumakkara et al., 2017b).

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a lipophilic polyphenol that exerts a wide range of pharmacological activities and has been a subject of research for more than two centuries (Noorafshan and Ashkani-Esfahani, 2013; Sahebkar, 2013).

2.8.1. Structure and pharmacokinetics of curcumin

Several studies on the structural chemistry of curcumin have demonstrated its ability to exist in two tautomeric forms: keto and enol (Kunnumakkara et al., 2017b), which both exert biological activities including antioxidant (Gupta et al., 2012), anti-inflammatory (Aggarwal and Harikumar, 2009) and anti-cancer (Gupta et al., 2010) properties among others.

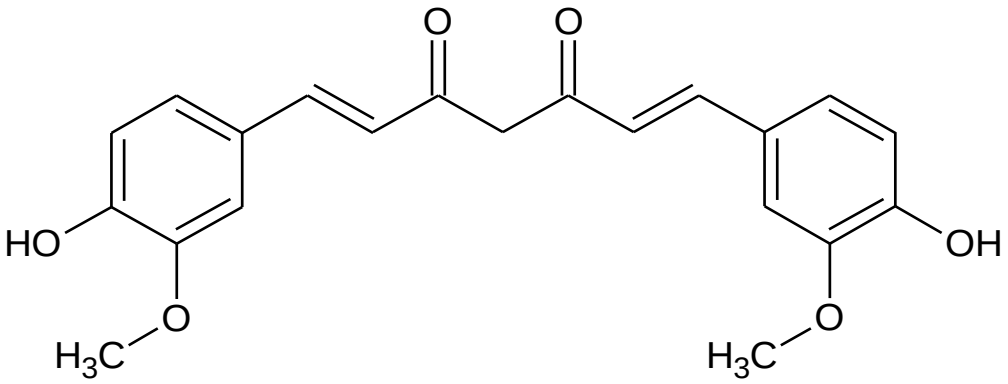


Figure 2. 2: Chemical structure of curcumin

[Structure drawn by the candidate using the Chemsketch software (ACDLABS 12.0)]

Despite the wide array of reported pharmacological activities of curcumin, the major problem with its usage is poor bioavailability (Anand et al., 2007; Sharma et al., 2007; Shen and Ji, 2012; Noorafshan and Ashkani-Esfahani, 2013; Devassy et al., 2015).

Upon administration, curcumin is rapidly metabolised and biotransformed in the liver and subsequently excreted in the faeces (Fadus et al., 2017). Its poor bioavailability is largely due to its very efficient first-pass metabolism, poor absorption from the GIT, intestinal metabolism and rapid excretion (Epstein et al., 2010; Jäger et al., 2014). To further confound the bioavailability problems of curcumin, its metabolites that result from conjugation and glucuronidation have been found to be biologically inactive in some studies (Pan et al., 2000; Ireson et al., 2001; Sandur et al., 2007).

When ingested orally, curcumin is either sulphated or it undergoes glucuronidation into curcumin sulphate or curcumin glucuronide (Aggarwal and Sung, 2009; Shen and Ji, 2012). An intravenous or intraperitoneal administration of curcumin results in its reduction to tetra-, hexa- or octahydrocurcumin (Aggarwal and Sung, 2009; Shen and Ji, 2012).

Several studies investigating the concentration of curcumin in the serum or other tissues following its administration revealed absent or low concentrations of curcumin and its metabolites (Sharma et al., 2007; Dhillon et al., 2008; Shen and Ji, 2012).

Curcumin is unstable at alkaline pH and when irradiated with ultraviolet and visible light (Metzler et al., 2013) and it breaks down to form degradation products. The degradation products of curcumin which include ferulic acid, dihydroferulic acid and vanillin have been demonstrated to have similar beneficial effects as curcumin and may thus contribute to its pharmacological activities (Wang et al., 1997; Masuda et al., 1999; Srinivasan et al., 2007; Shen and Ji, 2009; Makni et al., 2011; Shen and Ji, 2012; Tsuda, 2018). These degradation products have also been shown to preferentially inhibit certain enzymes such as cyclooxygenase-1 and cyclooxygenase-2 when compared to curcumin itself (Shen and Ji, 2009; Shen and Ji, 2012; Tsuda, 2018).

Curcumin also exhibits poor solubility in aqueous solutions but is soluble in organic solvents such as dimethyl sulfoxide, ethanol and acetone (Shen and Ji, 2012). Whereas the hydrophobic nature of curcumin and its poor solubility contribute to its poor bioavailability (Aggarwal and Sung, 2009), the degradation products of curcumin are free of these handicaps and therefore exhibit more bioavailability (Shen and Ji, 2012).

It is, therefore, safe to assert that the poor bioavailability and solubility of curcumin are compensated for by the pharmacological activity of its degradation products. Curcumin's poor bioavailabilty may also be the reason for its administration in relatively high doses (Ejaz et al., 2009; Miao et al., 2015).

2.8.2 Molecular targets of curcumin action

Curcumin exerts its biological activities by interacting with and modulating the expression and functions of a wide range of molecular targets including several signalling pathways (Shehzad et al., 2011; Kocaadam and Şanlier, 2017; Kunnumakkara et al., 2017b). It is thought that curcumin's ability to interact with its molecular targets is made possible via epigenetic regulation (Tuorkey, 2014).

In this regard, curcumin has been found to be a DNA hypomethylating agent by its ability to inhibit deoxyribonucleic acid methyltransferase-1 (DNMT-1) (Liu et al., 2009; Shu et al., 2011), inhibit histone deacetylase (HDAC) (Qureshi and Mehler, 2012; Kalani et al., 2015) and regulate micro RNA (Boyanapalli and Kong, 2015; Remely et al., 2015). By these epigenetic properties, curcumin plays a role in the selection or repression of genes and therefore selectively modulates several signalling pathways that are associated with different lifestyle diseases (Hasima and Aggarwal, 2012; Aggarwal et al., 2014; Kunnumakkara et al., 2017b).

Some of the important molecular targets of curcumin include transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ), sterol regulatory element binding protein-2 (SREBP-2) and nuclear factor-kappa B (NF-kB) (Kang and Chen, 2009; Shao-Ling et al., 2009). Curcumin also targets adipokines, inflammatory mediators, growth factors, kinases, enzymes and several receptors (Goel et al., 2008; Gupta et al., 2011; Yadav and Aggarwal, 2011; Gupta et al., 2012).

2.8.3 Pharmacological properties of curcumin

Curcumin, the active component of turmeric, fondly referred to as "the golden spice" has been used in folk medicine for the prevention and treatment of many disease conditions. Most of the folkloric claims have been verified scientifically and will be presented in this section.

2.8.3.1 Anti-obesity and hypolipidaemic properties of curcumin

The potential therapeutic benefits of curcumin in the treatment of obesity and its associated complications have been extensively researched. A persistent low-grade proinflammatory state has been linked with the pathophysiology of obesity (Weisberg et al., 2008; Aggarwal, 2010; Bradford, 2013; Ganjali et al., 2014). In addition to serving as a valuable energy store, fat tissue also performs important endocrine functions via the production and release of adipocytokines such as adiponectin, leptin, resistin and PAI (Aggarwal, 2010; Bradford, 2013; Ganjali et al., 2014). White adipose tissue also secretes inflammatory cytokines such as tumour necrosis factor (TNF), interleukins (1 and 6) and monocyte chemotactic protein-1 (MCP-1) which are very important in inflammation, insulin resistance and obesity (Aggarwal, 2010; Bradford, 2013).

Curcumin has been found to downregulate the expression of these proinflammatory adipocytokines (Chan, 1995; Singh and Aggarwal, 1995; Aggarwal et al., 2006; Shehzad et al., 2011; Shehzad et al., 2012) but increase the expression of adiponectin messenger ribonucleic acid (mRNA) via its action on PPARγ and suppression of NF-kB pathway (Alappat and Awad, 2010). By suppressing the activity of these proinflammatory cytokines, curcumin prevents the development of obesity by arresting and reversing symptoms linked to obesity such as insulin resistance and hyperglycaemia (Aggarwal, 2010).

When curcumin, 500mg.kg^{-1} of diet was administered for 12 weeks to 4-week-old male C57BL/6 mice that were fed a high-fat diet, it prevented the high fat diet-induced weight gain, significantly reduced serum cholesterol concentration and downregulated the expression of two key transcription factors for adipogenesis and lipogenesis- PPARγ and CCAAT/enhancer binding protein alpha (CCAAT/EBPα) (Ejaz et al., 2009). In the *in vitro* component of their experiment, 3T3-L1 (mouse embryonic fibroblasts) cells were infused with curcumin dissolved in dimethyl sulfoxide (concentrations ranging from 5-20µmol/L) and this resulted in suppressed adipocyte differentiation, adipokine-induced angiogenesis and induced adipocytes apoptosis (Ejaz et al., 2009).

Shao et al. (2012) fed a 4g.kg^{-1} diet of curcumin to high fat fed male C57BL/6J mice for 28 weeks to investigate the lipogenic and anti-inflammatory effects of curcumin in a model of insulin resistance and obesity. Curcumin was found to inhibit the expression of SRBEP-1c and carbohydrate-responsive element-binding protein (ChREBP) in the liver and inhibit the

high fat-induced inflammatory response in the adipocytes without stimulating the Wnt signalling pathway in mature adipocytes (Shao et al., 2012).

When the Wnt signalling pathway is activated, it increases the levels of β-catenin in the nucleus and cytoplasm resulting in the repression of adipogenesis (Krishnan et al., 2006). Similarly, the inhibition of the Wnt signalling pathway is required for the induction of PPARγ and preadipocyte differentiation (Krishnan et al., 2006). Curcumin was found to suppress adipocyte differentiation in 3T3-L1 preadipocytes by suppressing mitogen-activated protein kinase (MAPK) and stimulating the Wnt/β-catenin signalling pathway (Ahn et al., 2010).

Curcumin, 40 or 80mg.kg^{-1} daily administered to 6-week old male C57BL/6 mice via oral gavage for 12 weeks prevented the high fat diet-induced weight gain and hepatic steatosis; and also improved insulin sensitivity and serum lipid levels (Ding et al., 2016). Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate the expression of genes responsible for lipid metabolism in the liver and adipose tissues (Sodi et al., 2018; Xu et al., 2018). Curcumin inhibits SREBPs and consequently inhibits the biosynthesis of lipids including cholesterol and fatty acids (Ding et al., 2016).

In an *in vitro* study, curcumin was found to enhance the expression of genes associated with a brown fat phenotype such as the uncoupling protein 1 (UCP1) and subsequently induced browning of 3T3-L1 cells and primary white adipocytes (Lone et al., 2016).

Curcumin 0.1% (w/w) was supplemented in a 45% high-fat diet and fed to 4-week old male Sprague Dawley rats for 8 weeks. The intervention resulted in reduced serum and hepatic triglycerides, total cholesterol, LDL-c and atherogenic index (Kim and Kim, 2010). The authors speculated that the upregulation of the hepatic CYP7A1 mRNA they observed in the rats could be responsible for the hypocholesterolaemic activity of curcumin (Kim and Kim, 2010). In a randomised crossover trial in obese patients, 1g.day^{-1} of curcuminoids resulted in reduced serum triglycerides but did not have a significant impact on the lipid profile and BMI of the participants (Mohammadi et al., 2013).

2.8.3.2 Antidiabetic property of curcumin

Diabetes mellitus is a chronic endocrine disorder characterised by a high blood glucose concentration (Ghosh et al., 2015a). It has been established that oxidative stress and inflammatory cytokines are central to the pathogenesis of diabetes mellitus (Manna et al., 2013; Ghosh et al., 2015b; Rashid and Sil, 2015). The antioxidant and anti-inflammatory properties, therefore, position it (curcumin) as a promising candidate in the treatment of diabetes and/or its complications (Jin et al., 2018).

Since curcumin exerts its biological activity through several molecular targets, it interacts with receptors in the liver (Kim et al., 2009; Ruderman et al., 2013), skeletal muscle (Na et al., 2011), adipose tissues (Weisberg et al., 2008) and pancreatic β-cells (Best et al., 2007; Rouse et al., 2014) to bring about its hypoglycaemic action.

In a randomised double-blind placebo-controlled trial involving T2DM patients, curcumin 1g daily (co-administered with 10mg of piperine to enhance absorption) for 8 weeks increased the total antioxidant capacity and reduced the malondialdehyde (MDA) levels in the patients (Panahi et al., 2017a).

Curcumin improved the glycaemic status and insulin sensitivity of high-fat fed male C57BL/6J (wild-type and *ob/ob*) mice that were fed a 3% by weight admixture of curcumin (Weisberg et al., 2008). Curcumin was also found to improve glucose tolerance and atherosclerosis induced by a high-fat high cholesterol diet in LDL knock out mice (LDLR -/-) that were administered 100mg.kg^{-1} of curcumin in 0.5% carboxymethyl cellulose for 16 weeks (Ghosh et al., 2014).

Curcumin-enriched yoghurt (500mg.kg^{-1} body mass) administered for 15 days to 6-week old male streptozotocin (STZ)-induced diabetic Wistar rats improved their glucose tolerance and insulin sensitivity (Gutierres et al., 2015). The authors associated these curcumin actions with increased phosphorylation of the serine-threonine kinase (Akt) and translocation of GLUT4 transporters in skeletal muscles (Gutierres et al., 2015).

In another study, Chougala et al. (2012) administered a diet containing 0.5% curcumin to STZ-induced diabetic male Wistar rats. Diabetic rats that received curcumin showed a decreased FBG, decreased urine glucose and urine volume (Chougala et al., 2012). Curcumin solution in doses of 200, 100 and 500mg.kg^{-1} administered to STZ-induced Wistar rats

reduced FBG, FFAs and increased serum superoxide dismutase (SOD) levels (Miao et al., 2015).

Type 1 diabetes mellitus is an autoimmune disease that results in the destruction of the β-cells of the pancreas (Sperling et al., 2018). Curcumin with its antioxidant and anti-inflammatory properties was found promising in preventing the T-cell mediated autoimmunity by preventing leucocyte infiltration of the pancreas and thus, preserving β-cell function (Castro et al., 2014). Curcumin impaired T-cell proliferation and interferon- γ production, reduced NF-kB activation in T-cells and inhibited the T-cell stimulation of dendritic cells leading to reduced antigen presenting cell activity (Castro et al., 2014).

Curcumin has also been found to exert beneficial biological activity against several complications of diabetes (Fazel Nabavi et al., 2015). For example, curcumin has been shown to have renoprotective (Ibrahim et al., 2016), cardioprotective (Guo et al., 2018; Zheng et al., 2018a) and anti-atherosclerotic (Hajihoseini et al., 2017) properties in diabetes. Curcumin also induced angiogenesis and relieved hindlimb ischaemia in a diabetic mouse model (You et al., 2017). Additionally, topical application of curcumin in type 1 diabetic rats promoted skin wound healing (Zhang et al., 2016a; Soleimani et al., 2018). Erectile dysfunction is one of the frequently occurring complications of diabetes mellitus (Maiorino et al., 2017). Curcumin protected Zucker diabetic rats from the erectile dysfunction associated with diabetes mellitus (Draganski et al., 2018). Curcumin was also found to be useful in treating diabetic retinopathy in STZ-induced diabetic rats by inhibiting the activity of calcium-dependent protein kinase and thus attenuating the vascular leakage associated with the condition (Li et al., 2016). Cerebral infarction is a major complication of diabetes mellitus (Al-Rubeaan et al., 2016). Curcumin was found to improve diabetes-associated cerebral infarction by enhancing the expression of GLUT1 and GLUT3 in patients, following middle cerebral artery surgery (Xia et al., 2018).

2.8.3.3 Anti-inflammatory property of curcumin

Inflammation has long been established as the underlying cause of most chronic diseases including diabetes mellitus, obesity, non-alcoholic steatohepatitis (NASH), CVD and cancers (Ptaschinski and Lukacs, 2018). Inflammation is mediated by a group of proteins called

cytokines secreted by specific cells of the immune system (Carson and Kunkel, 2018; Dinarello, 2018).

TNF-α, an important inflammatory mediator in most chronic diseases (Aggarwal et al., 2006) acts via the activation of signalling pathways such as NF-kB and signal transducer and activator of transcription 3 (STAT-3) (Reuter et al., 2010; Kunnumakkara et al., 2018). However, NF-kB is also activated by several other cytokines, pathogenic microbes, high blood glucose, free fatty acids, cigarette smoke and other disease-causing agents (Hop et al., 2017; Panahi et al., 2018a). Most of the inflammatory mediators including some enzymes, kinases and cell adhesion molecules are also regulated by NF-kB (Aggarwal and Harikumar, 2009). Curcumin exerts its anti-inflammatory property via the downregulation of NF-kB and its associated regulated gene products (Sadeghi et al., 2018).

Curcumin, through its activity on its molecular targets, inhibits the inflammatory process by regulating proliferation, metastasis and angiogenesis in the inflamed area (Shehzad et al., 2013; Elham et al., 2018). Therefore, most of the activities of curcumin against chronic diseases are because of its anti-inflammatory property (Shehzad et al., 2013; Prasad et al., 2014; Deogade and Ghate, 2015).

2.8.3.4 Antioxidant property of curcumin

It has been reported that curcumin's strong antioxidant property is due to its free radical scavenging activity (Takahashi et al., 2014; Deogade and Ghate, 2015; Maithilikarpagaselvi et al., 2016) and upregulating the expression of transcription factors for several antioxidant genes (Xie et al., 2018). Curcumin, 200mg.kg^{-1} daily for 60 days was found to lower lipid peroxidation and increase total antioxidant capacity in an experimental model of oxidative stress using male Wistar rats (Al-Rubaei et al., 2014). In an *in vitro* model of colistin-induced neurotoxicity, curcumin decreased the concentration of intracellular reactive oxygen species (ROS) and increased the activity of superoxide dismutase, catalase and glutathione in neuroblastoma-2a (N2a) cells (Dai et al., 2018a). In another study where 2-month-old Wistar rats were restrained to induce stress-induced oxidative stress in the brain, liver and kidney, intraperitoneal administration of curcumin resulted in attenuation of oxidative stress and lipid peroxidation (Samarghandian et al., 2017).

Ali et al. (2018) reported increased activity of the antioxidant enzymes glutathione reductase and glutathione when curcumin (ranging from 37.5 to 150mg.kg^{-1}) was orally gavaged to male Sprague Dawley rats in an experimental model of chronic disease (Ali et al., 2018). Apparently, curcumin enhanced the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), the transcription factor that regulates the activities of the two enzymes.

In a rat model of STZ-induced type 1 diabetes mellitus, supplementation with 0.1% curcumin in the diet (weight ratio) for 21 days reduced the plasma levels of MDA, glutathione peroxidase and catalase but increased the plasma levels of SOD (Xie et al., 2018).

2.8.3.5 Hepatoprotective property of curcumin

The liver is a key organ in the body which performs numerous functions including first-pass metabolism, detoxification of drugs and toxins, production and storage of proteins and hepato-biliary excretion (Keiding et al., 2018). The liver is frequently affected in MetS, resulting in NAFLD which has been described as its (MetS) hepatic manifestation (Vanni et al., 2010; Than and Newsome, 2015). Curcumin has been shown to possess anti-steatotic (Varatharajalu et al., 2016; Ganesan et al., 2018) and anti-fibrotic effects in the liver (Varatharajalu et al., 2016; Amel Zabihi et al., 2017; Kabirifar et al., 2018) and therefore protects the liver from the consequences of MetS.

In a rat model of dimethylnitrosamine (DMN)- induced liver cirrhosis using 8-week old Sprague Dawley rats, curcumin, 100mg.kg^{-1} attenuated liver fibrosis (Kyung et al., 2018) and could thus be considered as a potential agent to stop the progression of liver damage in cirrhosis. Curcumin, 200mg.kg^{-1} body mass also relieved carbon tetrachloride (CCl4)-induced liver injury in adult male C57BL/6 mice (Peng et al., 2018).

A hydroalcoholic extract of curcumin at dose ranges from 30-120mg.kg^{-1} body mass for 21 days reversed the cadmium-induced elevation of liver enzymes (AST, ALT and ALP) in adult male Wistar rats (Darabi and Jahromi, 2018).

2.8.3.6 Reno-protective property of curcumin

MetS is frequently complicated by renal failure due to the presence of hypertension, dyslipidaemia and insulin resistance (Cohen and Cohen, 2015). Curcumin was effective in ameliorating cyclosporin A (CsA)-induced elevated serum levels of creatinine, blood urea nitrogen (BUN) and decreased creatinine clearance in a rat model (Huang et al., 2018a). It (curcumin) also reversed the histological finding of vacuolar degeneration of glomerular cells associated with the CsA-induced nephrotoxicity (Huang et al., 2018a). In another study, curcumin was also found to protect rats from doxorubicin (DXR)-induced nephrotoxicity by decreasing the serum levels of inflammatory and apoptotic markers associated with DXR-induced nephrotoxicity (Benzer et al., 2018). In chronic kidney disease, curcumin's antioxidant and anti-inflammatory activities inhibit the inflammatory and oxidant pathways in the kidney and thus protect the kidney from further damage (de Almeida Alvarenga et al., 2018).

2.8.3.7 Anti-hypertensive/cardiovascular-protective properties of curcumin

Globally, cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality (Mozaffarian et al., 2015). The complex cardio-metabolic risk correlates of MetS predispose the individual to the development of CVD (Mongraw-Chaffin et al., 2018). Curcumin has been found in several studies to exhibit anti-hypertensive and cardiovascular-protective activities and thus has the potential for use in the treatment of cardiovascular diseases.

For example, in two separate Institute of Cancer Research (ICR) mouse models of cadmium-induced hypertension and vascular dysfunction, 50 and 100mg.kg^{-1} curcumin (Kukongviriyapan et al., 2014) and 50 and 100mg.kg^{-1} of tetrahydrocurcumin (Sangartit et al., 2014) administered every other day and concurrently with cadmium were found to normalise blood pressure and improve vascular function, remodelling of the hypertrophic aortic wall and oxidative status of the mice (Kukongviriyapan et al., 2014; Sangartit et al., 2014). In another study using spontaneously hypertensive rats, curcumin was shown to lower blood pressure and improve the histopathological changes in the coronary arteries (Hu et al., 2017).

The anti-hypertensive and cardiovascular protective effects of curcumin are thought to be exerted via its antioxidant activity and by increasing the availability of nitric oxide (Boonla et al., 2014).

2.8.3.8 Anti-cancer property of curcumin

Curcumin exerts its anti-cancer activity mainly because of its ability to target and modulate multiple signalling pathways in the cells (Kunnumakkara et al., 2017a). Its ability to act on multiple pathways makes curcumin an excellent chemosensitizer for many cytotoxic drugs (Bordoloi and Kunnumakkara, 2018). Due to the inhibition of the inflammatory pathways involving NF-kB and STAT-3, curcumin plays an important role in arresting cancer cells growth and progression (Vallianou et al., 2015). NF-kB has been shown to promote the differentiation and proliferation of tumour cells (Gall Troselj and Novak Kujundzic, 2014). Unlike other cytotoxic drugs that enhance the activity of NF-kB, curcumin promotes cellular apoptosis by inhibiting it (Gall Troselj and Novak Kujundzic, 2014; Vallianou et al., 2015).

Additionally, curcumin has been shown to target cancer stem cells thereby acting on multiple cancer cell lines simultaneously (Zang et al., 2014). Curcumin induces apoptosis (Terlikowska et al., 2014; Agarwal et al., 2018; Banuppriya et al., 2018), cell cycle arrest (Agarwal et al., 2018), tumour angiogenesis and metastases (Terlikowska et al., 2014).

2.8.3.9 Antibacterial, antifungal and antiviral properties of curcumin

Due to its widely reported usage in folklore medicine in the treatment of infections, curcumin has been extensively investigated for its antimicrobial activities.

Curcumin demonstrated antibacterial activity against *Helicobacter pylori* in both *in vitro* and *in vivo* studies with Mean Inhibitory Concentration (MIC) between 5 and 50µg.ml^{-1} (De et al., 2009). Tyagi et al. (2015) demonstrated the anti-bacterial activity of curcumin against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* in an *in vitro* study.

Curcumin has shown promise as an antifungal agent with broad-spectrum activity and minimal side effects. In an *in vitro* study using broth microdilution assay, the antifungal activity of curcumin was investigated on 23 fungal strains. Curcumin was up to 32-fold more potent than fluconazole against several of the fungal strains (Martins et al., 2009).

Several studies have also shown curcumin to exhibit antiviral activity. For example, in an *in vitro* study, curcumin inhibited the human immunodeficiency virus (HIV) integrase and degraded Tat-protein in HIV (Ali and Banerjea, 2016). Using both in vitro (human lung cancer cell line A549) and an *in vivo* mouse model, Shuguang et al. (2018) found curcumin effective in relieving pneumonia secondary to the influenza virus by arresting pulmonary injury and downregulating cytokine production.

2.8.3.10 Neuroprotective property of curcumin

The "golden nutraceutical" curcumin was also found to exhibit neuroprotective activity making it useful in the prevention of neurotoxicity resulting from injury, oxidative stress or drugs (Dai et al., 2018b; Dong et al., 2018; Huang et al., 2018b). Curcumin restored brain mitochondrial function by ameliorating oxidative stress induced by oxaliplatin in adult male Wistar rats (Waseem and Parvez, 2016). Using a rat model of Alzheimer's disease, curcumin was found to protect against endothelin-1- induced neuronal death by inhibiting its pro-apoptotic signalling in the hippocampal neurons (Stankowska et al., 2017).

2.8.3.11 Gastroprotective property of curcumin

Among the many biological activities of the pleiotropic compound curcumin, is its gastroprotective activity as shown in several studies. Curcumin (1.5 to 100mg.kg^{-1}, intragastric) administered to male and female Wistar rats reduced ethanol-induced gastric ulcers, increased gastric blood flow with an increase in the plasma gastrin levels (Czekaj et al., 2018). When adult female Sprague Dawley rats were administered with 200mg.kg^{-1} of curcumin orally and then fasted for 16 hours, the inner linings of the gastrointestinal tract did not show any lesions (Haider et al., 2013). This suggests a gastroprotective role for curcumin.

Because of its potent anti-inflammatory activity, curcumin has also been touted as a possible treatment for inflammatory bowel diseases (Rafaela et al., 2018).

2.8.3.12 Osteoprotective properties of curcumin

In a recent controlled clinical trial, 110mg.kg^{-1} of curcumin orally daily for six months inhibited osteoporosis by modulating markers of bone resorption and increasing bone density in spinal cord injury patients (Hatefi et al., 2018). Another study also found curcumin supplementation (1g daily for six months) in patients with low bone density to increase the bone density, thereby pointing to its benefits in the management of osteopenia (Riva et al., 2017).

2.8.4 Toxicity of curcumin

Though natural products have the advantage of being less toxic than orthodox medicines, some plants and plant products have shown toxic effects in humans and animals (Ogbole et al., 2017). Curcumin, however, has a very high safety index and has been classified as a "Generally Regarded As Safe" (GRAS) compound by the United States Food and Drug Administration (FDA) (Chainani-Wu, 2003; Epstein et al., 2010; Kocaadam and Şanlier, 2017). In humans, Lao et al. (2006) conducted a dose escalation study to determine the safety of a single dose of curcumin and found a maximum tolerated dose to be between 8-12g per day. Though it is difficult to achieve single doses higher than 8g due to the large quantity of the powder involved (Hsieh, 2001). In rats, chronic administration of doses as high as 1.8g.kg^{-1} daily for 90 days has been reported without any adverse consequences (Epstein et al., 2010). However, hepatotoxicity was reported in female Swiss mice (Kandarkar et al., 1998) but not Wistar rats (Deshpande et al., 1998) that were fed with whole turmeric for 14 days (0.2-5%). However prolonged feeding with 5% whole turmeric for 90 days resulted in a reduction in body and liver masses, and hepatotoxicity in the rats (Deshpande et al., 1998).

The next chapter will describe the first component of the study, which is the effect of administration of oral curcumin to suckling pups on the neonatal programming of fructose-induced metabolic dysfunction.

CHAPTER THREE: The effect of neonatal oral curcumin administration on the metabolic response and renal histology of male and female Sprague Dawley rats fed a high fructose diet until adolescence

3.1 Introduction

The global epidemic of childhood obesity (Ludwig, 2018) is a public health crisis affecting communities in developed and developing countries (Powell, 2018). Due to this increase in the prevalence of childhood obesity, there is a consequent increase in the occurrence of metabolic syndrome (MetS) in childhood and adolescence (Falkner and Cossrow, 2014). The danger that childhood MetS carries is that it has the potential to be projected into adulthood (Magnussen et al., 2018), thereby further increasing the adult prevalence of MetS (Kaur, 2014).

Increased consumption of fructose-rich diets and sedentary lifestyles have been identified as major drivers of this global epidemic (Mamikutty et al., 2015; Hannou et al., 2018) linked to a background of interactions between genetic and environmental factors (Kelishadi et al., 2014). Ding et al. (2018) reported that the cardiometabolic risk factors that cluster together in MetS are also independent risk factors for chronic kidney disease (CKD). The consumption of a high fructose diet has been shown to cause structural and functional changes in the kidneys that increase the risk of developing CKD in both human and experimental animal models (Yu et al., 2018). The assessment of kidney function using surrogate markers of kidney function such as plasma levels of urea and creatinine has limited ability to detect early disease as their levels only become elevated when more than 50-60% of renal function is lost (Scarfe et al., 2015). The histopathological analysis of renal tissues reveals early changes that may point to developing kidney disease (Scarfe et al., 2015). Thus, although invasive, kidney biopsies and histological assessment have long been considered the gold standard for investigating renal function.

Early life stressor events, especially those occurring during gestation and the suckling phases can cause epigenetic changes that could then result in the permanent alteration of the energy homeostasis, physiology and metabolism of the individual, a phenomenon called neonatal metabolic programming (Patel and Srinivasan, 2002; Collden et al., 2015). Such stressor events include nutritional, hormonal or pharmacological interventions (Patel and Srinivasan, 2002). Perturbations in the environment and the type of diet introduced early in life can thus either initiate or reverse some epigenetic risk markers of MetS (Park et al., 2017a).

Deriving from the concept of developmental programming, dietary interventions have been used to either program or reverse the effects of programming on future metabolic health. For example, when a high fructose diet was administered to suckling Sprague Dawley rats, it

produced permanent changes in body mass and induced insulin resistance in adulthood (Huynh et al., 2008). Further, in a maternal model of undernutrition, the female offspring administered with subcutaneous leptin showed a reversal of the adverse metabolic programming effects of undernutrition in the offspring (Vickers et al., 2005).

Due to the difficulties in instituting exclusive breastfeeding practices especially in working-class women, infant feeding formulae are usually introduced early in life. Formula feeding has been implicated in the development of obesity early in childhood (Gibbs and Forste, 2014). Moreover, some parents introduce other solid foods or beverages with high sugar content early in life (Anzman-Frasca et al., 2018). Therefore, there is a risk of programming for metabolic diseases in childhood due to these early life feeding practices.

Previous studies have shown the benefits of interventions with natural products and phytochemicals in the suckling period in programming the metabolic health of adolescent and adult rats (Ibrahim et al., 2017; Lembede et al., 2018; Nyakudya et al., 2018). The use of natural products to improve metabolic health is attracting international interest due to their (natural products) perceived safety, availability and affordability (Bahmani et al., 2014). It is, therefore, necessary to identify other compounds that can be used to either program or reprogram against the development of adverse metabolic dysfunction.

Curcumin, a dietary polyphenol, is one of the biologically active components of turmeric, from the rhizome of *Curcuma longa* from the ginger family, Zingiberaceae (Sahebkar, 2013). Historically, it has been used for several centuries especially in Asia, as a remedy for common ailments including diarrhoea, cough and jaundice (Sahebkar, 2013). It (curcumin) has been shown to have anti-obesity (Shao et al., 2012), anti-diabetic (Panahi et al., 2017a), antioxidant (Ali et al., 2018), anti-inflammatory (Elham et al., 2018) and reno-protective (Huang et al., 2018a) properties among others.

Due to the wide array of therapeutic properties reported for curcumin against metabolic conditions including the renal complications of MetS, and the need to identify and validate natural compounds for use in programming or reprogramming against MetS, we hypothesised that orally administering curcumin to suckling pups will protect them later in adolescence against adverse metabolic programming effects of a high fructose diet pre- and postweaning.

3.2 Materials and methods

3.2.1 Ethical approval, compliance to ARRIVE guidelines and study location

The protocols and procedures used in the study were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg (AESC 2016/04/18/B) and complied with international ethical guidelines and standards for the care and use of laboratory animals. The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines have been used in writing up this manuscript. The study was conducted in the Central Animal Service (CAS) animal research facility of the University of the Witwatersrand, Johannesburg, Republic of South Africa.

3.2.2 Chemicals and reagents used

For this study, dimethyl sulfoxide (DMSO; Sigma-Aldrich, Missouri, USA), used as vehicle solvent, was made up to a final concentration of 0.5% in distilled water. Curcumin (Sigma-Aldrich, Missouri, USA) 500mg was dissolved in 10ml of a 0.5% DMSO solution. However, the curcumin did not dissolve completely and was therefore administered as a suspension. Fructose (Nature's choice, Randvaal, South Africa) solution was made to 20% w/v using 0.5% DMSO in distilled water in the pre-weaning period and tap water post-weaning. According to the manufacturer's product information label, the nutritional composition of the fructose was 1680 kJ energy, 0 g protein, 0.2 mg/100 g sodium, 99.8 g/100 g carbohydrates, 0 g fat and 0 g fibre. The commercial rat chow (Epol®, Centurion, South Africa) had the following nutritional composition: 170 g/kg protein, 25 g/kg fat, 70 g/kg fibre, 25 g/kg calcium, 6 g/kg phosphorus and 6.5 g/kg lysine. A drop of food colouring (no nutritional value) (Robertsons, Retailer Brands (Pty) Ltd, South Africa) was added to 5 l of the drinking fluids and used to distinguish the fluids from one another.

3.2.3 Study animals, housing and general care

Six-day-old male and female Sprague Dawley pups from fourteen dams that were sourced from the Central Animal Services Unit of the University of the Witwatersrand, Johannesburg

were used in the study. Each dam had a litter size of between 8-12 pups. The suckling pups nursed freely with their dams while housed in perspex cages which were lined with wood shavings and shredded paper for environmental enhancement. The cages had steel mesh covers. The dams had *ad libitum* access to pelletized rat chow (Epol®, Johannesburg, South Africa) and plain tap water. The cages and bedding were changed twice weekly to promote hygiene. The ambient temperature in the room was maintained at 26 ± 2ºC with adequate ventilation and a 12- hour light/dark cycle (lights on at 0700hrs).

At postnatal day 21, the rats were weaned from their dams and placed in individual cages. The weaned rats were allowed *ad libitum* access to normal rat chow and either tap water or fructose solution (20%, w/v) as their drinking fluid. The dams were returned to stock.

3.2.4 Study design

The study was carried out in two interventional phases (see figure 3.1). The date of delivery of the pups was taken as day zero. The pups were allowed to acclimatise to the study environmental conditions till postnatal day (PN) six. The first phase of the study was conducted in the pre-weaning period from PN six to PN 21 and it was aimed at inducing programming with curcumin and fructose. The second phase of the interventions extended from PN 21 and continued for six weeks until PN63 (late adolescence). The aim of the second phase of the experiment was to determine whether the initial interventions would have any effect on the response of the rats to a high fructose diet post-weaning.

The litters from fourteen Sprague Dawley dams (n=128, males = 65, females = 63) were used in the study. During the first phase of the study, the pups were allocated in a split-litter pattern to four treatment groups replicated by sex. The pups were uniquely identified using colour-coded permanent non-toxic marker ink on their tails. The treatment groups were as follows:

Group 1 (negative control)**:** administered 10ml.kg^{-1} of 0.5% DMSO.

Group 2: administered curcumin, 500mg.kg^{-1} body mass. Our choice of dosage was based on previous studies that have used a similar dose to achieve anti-obesity (Ejaz et al., 2009) and

anti-diabetic (Miao et al., 2015) effects. Higher doses have been used previously but because the gastrointestinal tract of the suckling pups is underdeveloped, subjecting it to a thick suspension was avoided.

Group 3: administered fructose (20% w/v)

Group 4: administered curcumin 500mg.kg^{-1} + fructose (20%, w/v)

All the treatments were administered once daily at 10ml.kg^{-1} daily (between 08h30 and 10h00) via oral gavage using a size 20G plastic cannula mounted on a 1ml syringe.

In the second phase of the study, the rats from each of the initial four groups were further sub-divided into two subgroups. While the two subgroups were weaned onto normal rat chow, one of the subgroups had tap water and the other had fructose solution (20%, w/v/) as their drinking fluids. The treatments in phase two were administered for six weeks until PN 63.

Thus, at post weaning there were eight groups of rats which were replicated by sex as follows:

1. DMSO + TW= received 10ml.kg^{-1} of 0.5% DMSO during suckling and plain tap water postweaning
2. DMSO + FW= received 10ml.kg^{-1} of 0.5% DMSO during suckling and fructose (20%, w/v) postweaning
3. CC + TW= received 500mg.kg^{-1} curcumin during suckling and plain tap water postweaning
4. CC + FW= received 500mg.kg^{-1} curcumin during suckling and fructose (20%, w/v) postweaning
5. FW + TW= received fructose (20%, w/v) during suckling and plain tap water postweaning
6. FW + FW= received fructose (20%, w/v) during suckling and fructose (20%, w/v) postweaning
7. CCFW + TW= received a combination of 500mg.kg^{-1} curcumin and fructose (20%, w/v) during suckling and plain tap water postweaning
8. CCFW + FW= received a combination of 500mg.kg^{-1} curcumin and fructose (20%, w/v) during the suckling period and fructose (20%, w/v) postweaning

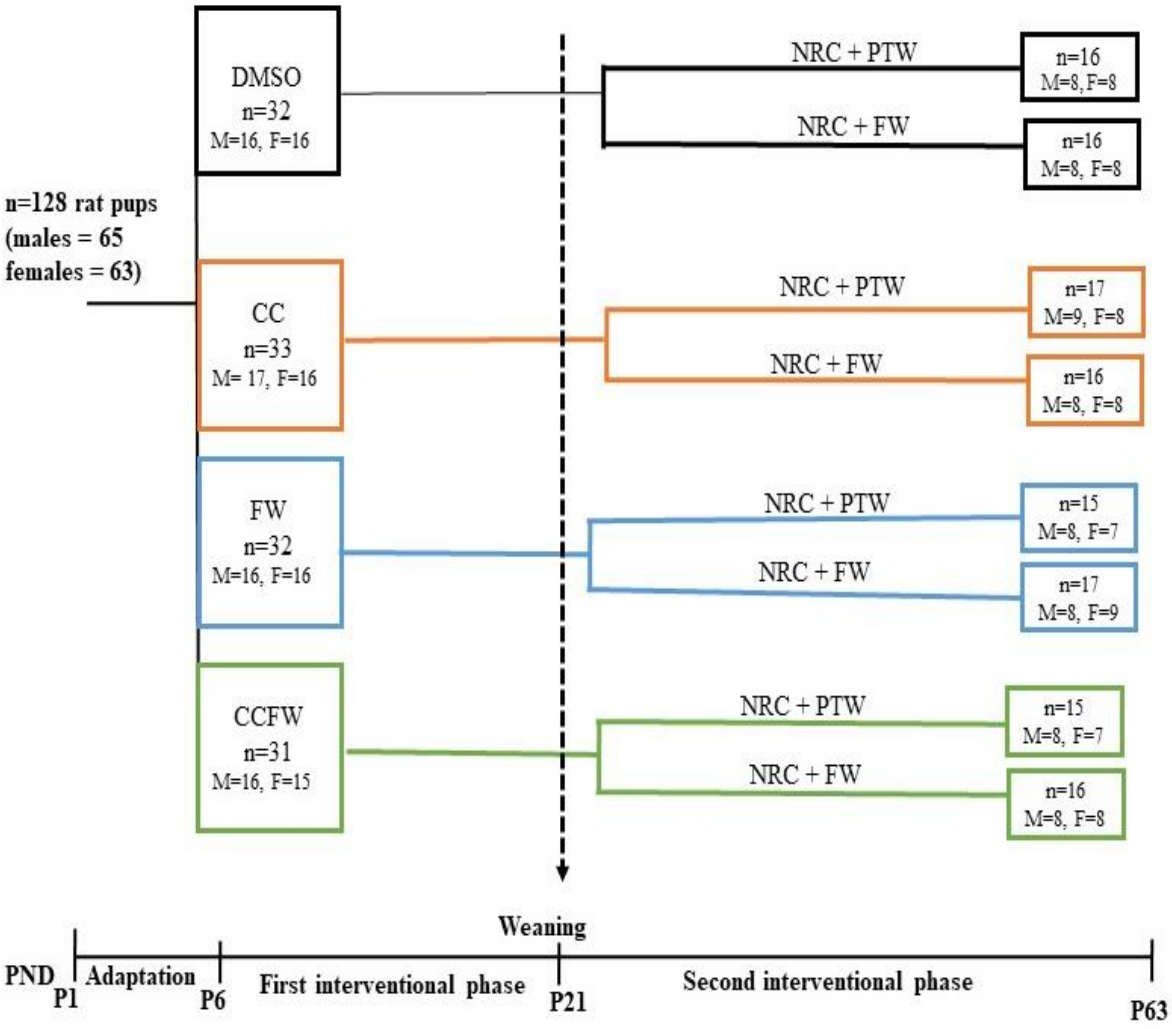


Figure 3. 1: schematic diagram of the study design

DMSO, 10ml.kg^{-1} of 0.5% dimethyl sulfoxide; CC, 500mg.kg^{-1} curcumin; FW, fructose (20%, w/v); NRC, normal rat chow; PTW, plain tap water; M, males; F, females.

3.2.5 Body mass measurements

The pups were weighed daily using a digital weighing scale (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa) during the first phase of the study to adjust their treatments to ensure constant dosing. In the second phase, the rats were weighed twice weekly to monitor their growth and general health.

3.2.6 Terminal procedures

On postnatal day 62, the rats were subjected to a 12-hour overnight fast in preparation for termination. In the morning of termination, the body masses of the rats were determined using a weighing scale (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa). A drop of blood collected via a pin prick to the tail vein was used to determine the fasting blood glucose (FBG) concentration of the rats using a calibrated glucometer (Contour Plus ™, Bayer Corporation, Mishawaka, USA). The length of the rats, from the nasal opening to the anus (Poudyal et al., 2010) and abdominal circumferences of the rats were measured using a tape measure (Novelli et al., 2007) and recorded. The body mass index of the rats was computed using the formula, BMI = body mass (g)/ [length (cm)]^2. The rats were subsequently euthanised by anaesthetic overdose using intraperitoneal sodium pentobarbitone (150mg.kg^{-1} body mass, Euthapent; Kyron laboratories, South Africa). The thorax was opened and blood was taken via cardiac puncture and transferred into heparinised blood collecting tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The blood was centrifuged (Hermle Z 230A, B Hermle AG, Germany) at 4000 x g at 4ºC for 15 minutes and the plasma was collected and stored in microtubes (Eppendorf, Hamburg, Germany) at -20ºC for biochemical and hormonal assays. The kidneys, pancreas, visceral and epididymal (in male rats) fat pad mass were carefully removed and weighed with an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). The kidneys were preserved in 10% phosphate-buffered formalin for histology.

3.2.7 Determination of the plasma concentration of insulin and computation of HOMA-IR

Plasma concentration of insulin was determined using a sandwich-enzyme-linked immunosorbent assay (ELISA) kit [Elabscience ® INS (Insulin) ELISA kit, Houston, TX, USA] according to the manufacturer's instructions (sensitivity, 0.19mg.ml^{-1}; detection range, 0.31-20ng.ml$^{-1,}$ standard curve range, 0-20ng.ml^{-1}). The homeostatic model of insulin resistance was computed using the following formula:

HOMA-IR = [fasting insulin concentration (ng.dL^{-1}) x fasting glucose concentration (mg.dl^{-1})]/405 (Matthews et al., 1985).

3.2.8 Determination of the plasma concentration of adiponectin

Plasma concentration of adiponectin was determined by a sandwich- ELISA method using the rat adiponectin ELISA kit (Elabscience ® Rat ADP/Acrp30 ELISA kit, Houston, TX, USA) according to the manufacturer's instructions (sensitivity, 28.13pg.ml.ml^{-1}; detection range, 46.88-3000pg.ml^{-1}, standard curve range, 0-3000pg.ml^{-1}). Briefly, standards and samples were pipetted into the Rat ADP/Acrp30 antibody coated microplates. A biotinylated detection antibody specific for Rat ADP/Acrp30 and the Avidin-Horseradish Peroxidase conjugate were added to the wells. The reaction was terminated with a stop solution and the optical density was determined with a microplate reader (Multiskan Ascent, Lab system, model nº 354, Helsinki, Finland) at 450nm. A standard curve was plotted for the standards using their optical densities and concentration. Thereafter the concentration of adiponectin was extrapolated from the standard curve.

3.2.9 Determination of the plasma concentration of triglycerides and total cholesterol

Plasma concentration of triglycerides (TGs) and total cholesterol were determined using a colourimetric analyser IDEXX Catalyst Dx™ connected to an IDEXX Vet Lab station (IDEXX Laboratories Inc., Westbrook, Maine, USA) according to the manufacturer's instructions.

3.2.10 Histomorphology of the kidney tissues

The kidney tissues were processed using an automatic tissue processor (Micron STP 120, Thermo Fischer Scientific, USA) and then embedded in paraffin wax. The samples were sectioned onto glass slides at 3µm thickness using a rotary microtome (Leica Biosystems, USA) and stained with Masson's trichrome (Bancroft and Gamble, 2008). The MT-stained kidney sections were assessed for histopathological changes. Representative photomicrographs of the MT-stained sections were acquired using An Olympus BH2 RFCA microscope (Olympus Corporation, Japan).

3.2.11 Statistical analysis

Data were analysed using the GraphPad Prism version 7.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) and expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA), followed by a comparison of the means using the Bonferroni *post hoc* test was used. A statistical significance was assumed when $p \leq 0.05$.

3.3 Results

3.3.1 Morbidity and mortality

There were no incidental or iatrogenic morbidities or mortalities recorded throughout the study duration.

3.3.2 Effect of curcumin and fructose on body mass changes

The growth pattern of male and female rats is shown in figures 3.2 and 3.3. Both male and female rats gained significant body mass ($p<0.0001$, ANOVA), from induction through weaning and termination. However, there were no significant differences across the treatment groups at different time points in male and female rats.

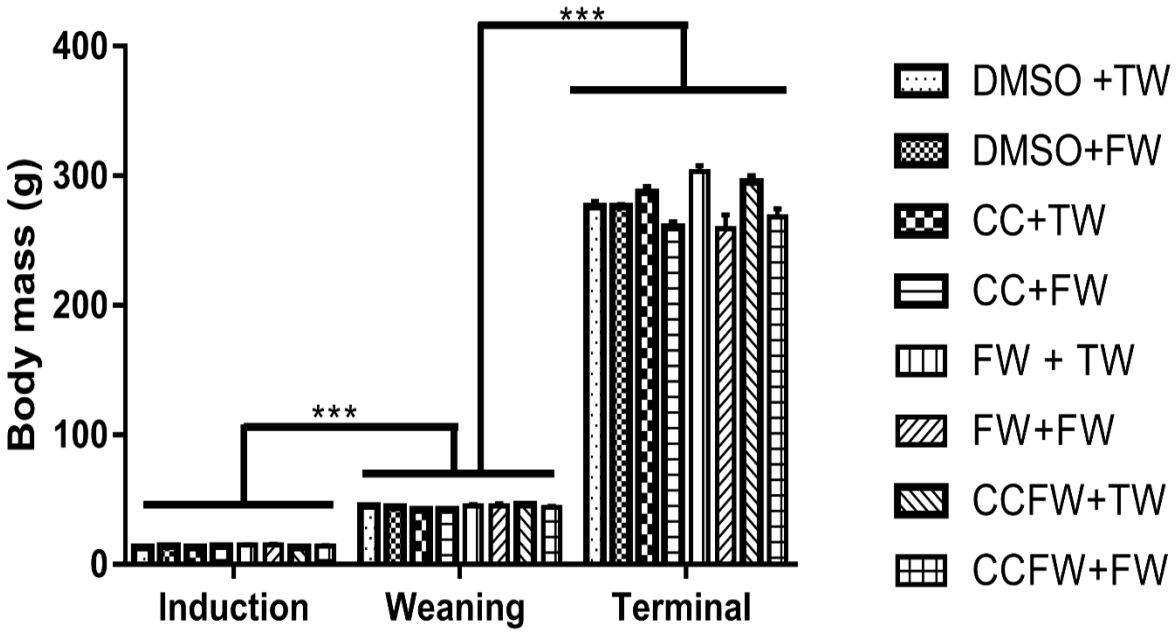


Figure 3. 2: Effect of a high fructose diet on the growth pattern of male Sprague Dawley rats that were administered with oral curcumin during suckling

***= significantly different at $p<0.0001$. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 7-9 per group.

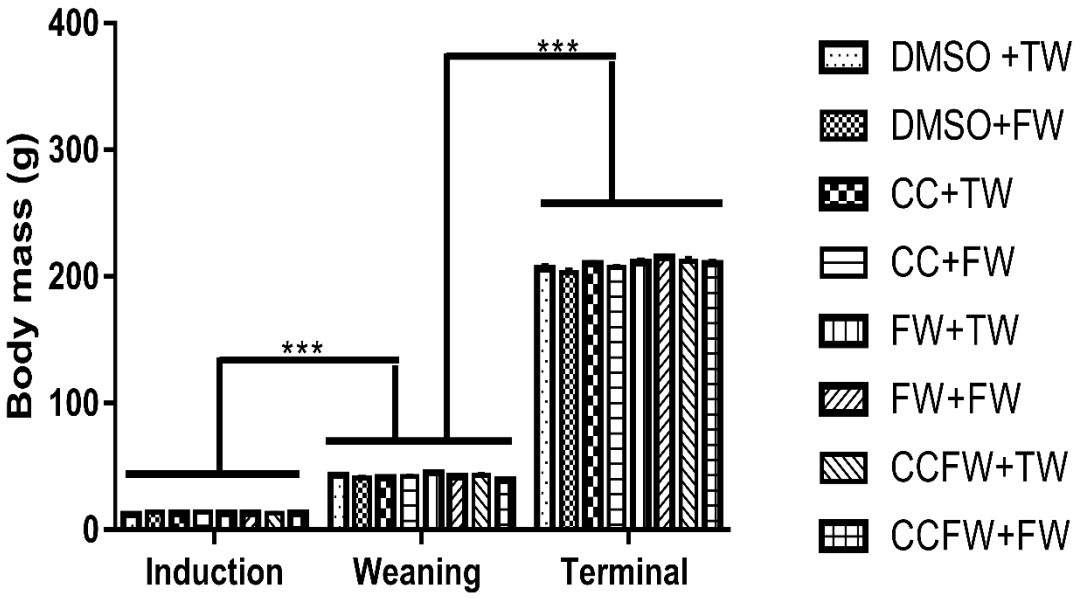


Figure 3. 3: Effect of a high fructose diet on the growth pattern of female Sprague Dawley rats that were administered with oral curcumin during suckling

***= significantly different at $p<0.0001$. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 7-9 per group.

3.3.3 Effect of curcumin and fructose on markers of adiposity

Tables 3.1 and 3.2 show the effect of neonatal administration of curcumin and fructose on markers of adiposity in male and female Sprague Dawley rats that were fed a high fructose diet post-weaning. In both sexes, there were no differences observed ($p>0.05$, ANOVA) across the treatment groups in all the measured parameters.

Table 3. 1: Effect of fructose administration on the abdominal circumference, body mass index, absolute (g) and relative to body mass (% BM) visceral fat pad mass, absolute (g) and relative (% BM) epididymal fat pad mass of growing male Sprague Dawley rats

Treatment groups	Abdominal circumference	Body Mass Index	Visceral fat pad mass	Visceral fat pad	Epididymal fat mass	Epididymal fat mass
	(cm)	($g.cm^2$)	(g)	(%BM)	(g)	(%BM)
DMSO + TW	15.00 ± 0.67	0.60 ± 0.01	3.00 ± 0.22	1.10 ± 0.11	1.70 ± 0.08	0.62 ± 0.03
DMSO + FW	16.00 ± 0.19	0.61 ± 0.01	2.90 ± 0.55	1.00 ± 0.07	1.90 ± 0.09	0.67 ± 0.03
CC + TW	16.00 ± 0.59	0.63 ± 0.02	3.40 ± 0.42	1.20 ± 0.15	1.80 ± 0.13	0.62 ± 0.03
CC + FW	16.00 ± 0.49	0.62 ± 0.02	2.50 ± 0.40	0.94 ± 0.13	1.60 ± 0.19	0.62 ± 0.05
FW + TW	16.00 ± 0.45	0.64 ± 0.02	3.20 ± 0.55	1.10 ± 0.22	2.10 ± 0.17	0.63 ± 0.02
FW + FW	15.00 ± 0.42	0.59 ± 0.02	2.60 ± 0.34	1.00 ± 0.10	1.60 ± 0.09	0.63 ± 0.02
CCFW + TW	16.00 ± 0.55	0.62 ± 0.02	2.90 ± 0.39	0.98 ± 0.13	1.80 ± 0.15	0.62 ± 0.03
CCFW + FW	16.00 ± 0.46	0.60 ± 0.02	2.80 ± 0.49	0.98 ± 0.14	1.60 ± 0.23	0.58 ± 0.06

There were no differences ($p>0.05$) observed in the abdominal circumference, BMI, absolute (g) and relative (BM) visceral fat pad mass, absolute (g) and relative (BM) epididymal fat pad mass of the rats. BM=body mass. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 7-9 per group.

Table 3. 2: Effect of fructose administration on the abdominal circumference, body mass index, absolute (g) and relative (BM) visceral fat pad mass of growing female Sprague Dawley rats

Treatment groups	Abdominal circumference	Body Mass Index	Visceral fat pad mass	Visceral fat pad Mass
	(cm)	($g.cm^2$)	(g)	(%BM)
DMSO + TW	14.00 ± 0.58	0.54 ± 0.02	5.00 ± 0.47	2.40 ± 0.22
DMSO + FW	14.00 ± 0.31	0.56 ± 0.02	4.40 ± 0.58	2.10 ± 0.24
CC + TW	14.00 ± 0.43	0.56 ± 0.01	3.70 ± 0.37	1.80 ± 0.17
CC + FW	15.00 ± 0.26	0.56 ± 0.01	4.20 ± 0.29	2.00 ± 0.13
FW + TW	14.00 ± 0.36	0.58 ± 0.02	4.20 ± 0.54	2.00 ± 0.31
FW + FW	15.00 ± 0.31	0.56 ± 0.02	5.10 ± 0.23	2.40 ± 0.10
CCFW + TW	13.00 ± 0.57	0.57 ± 0.01	4.30 ± 0.49	2.00 ± 0.19
CCFW + FW	15.00 ± 0.28	0.56 ± 0.01	5.00 ± 0.50	2.40 ± 0.19

There were no differences ($p>0.05$) observed in the abdominal circumference, BMI, absolute (g) and relative (BM) visceral fat pad mass of the rats. BM= body mass. DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 7-9 per group.

3.3.4 Effect of curcumin and fructose on plasma concentration of adiponectin

The effect of neonatal administration of curcumin and/or fructose on the plasma concentration of adiponectin in adolescent male and female Sprague Dawley rats that were fed a high fructose diet post weaning are shown in figures 3.4 and 3.5 respectively. There were no statistically significant differences ($p>0.05$, ANOVA) in the plasma concentration of adiponectin across the treatment groups in both sexes.

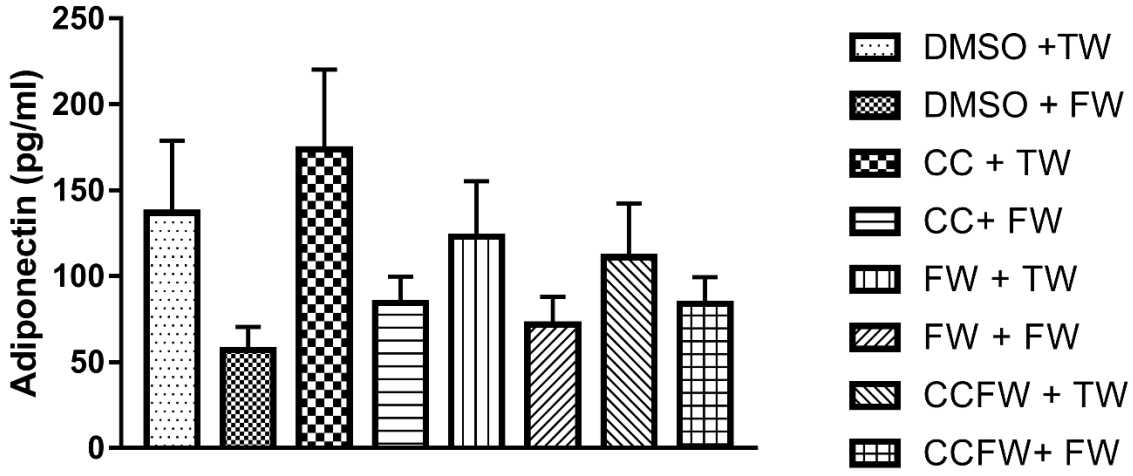


Figure 3. 4: Effect of a high fructose diet on the plasma adiponectin concentration of male Sprague Dawley rats that were administered with oral curcumin during suckling

There were no differences ($p>0.05$) in the plasma adiponectin concentration of the rats across the treatment groups. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post weaning. Data expressed as mean ± SEM, n= 7-9 per group.

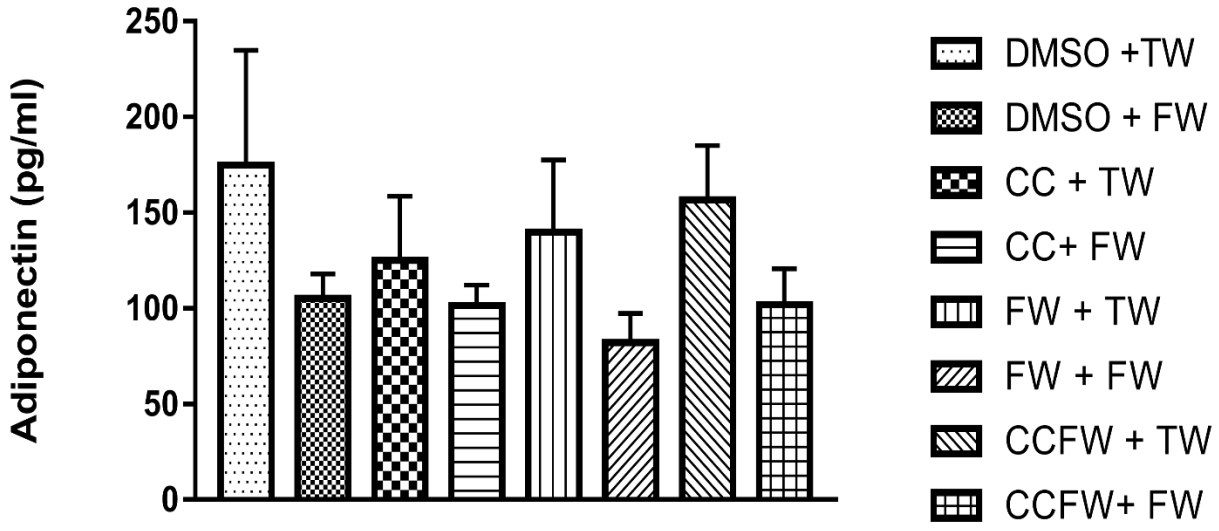


Figure 3. 5: Effect of a high fructose diet on the plasma adiponectin concentration of female Sprague Dawley rats that were administered with oral curcumin during suckling

There were no differences ($p>0.05$) in the plasma adiponectin concentration of the rats across the treatment groups. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post weaning. Data expressed as mean ± SEM, n= 7-9 per group.

3.3.5 Effect of curcumin and fructose on fasting blood glucose, insulin concentration and homeostatic model of insulin resistance

Tables 3.3 and 3.4 show the effect of neonatal administration of curcumin and fructose on the concentrations of plasma fasting blood glucose and insulin, and the HOMA-IR in adolescent male and female Sprague Dawley rats that were fed a high fructose diet post-weaning. In both sexes, plasma fasting blood glucose, insulin and HOMA-IR were similar ($p>0.05$) across the treatment groups.

Table 3. 3: Effect of fructose administration on fasting blood glucose, insulin and HOMA-IR of adolescent male Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Fasting blood glucose ($mg.dl^{-1}$)	Insulin ($ng.ml^{-1}$)	HOMA-IR
DMSO + TW	74.00 ± 3.40	4.80 ± 1.20	0.91 ± 0.25
DMSO + FW	81.00 ± 4.00	4.30 ± 1.10	0.81 ± 0.20
CC + TW	71.00 ± 0.85	5.70 ± 1.10	0.99 ± 0.20
CC + FW	73.00 ± 2.20	4.70 ± 1.20	0.85 ± 0.21
FW + TW	81.00 ± 4.50	3.50 ± 0.83	0.68 ± 0.16
FW + FW	73.00 ± 2.00	6.40 ± 1.00	1.20 ± 0.18
CCFW + TW	79.00 ± 5.50	5.20 ± 0.94	0.99 ± 0.16
CCFW + FW	80.00 ± 4.10	4.30 ± 0.99	0.81 ± 0.15

There were no differences ($p>0.05$) in the fasting blood glucose, plasma insulin concentration and HOMA-IR of the rats across the treatment groups. FBG= fasting blood glucose, HOMA-IR= homeostatic model of insulin resistance. DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post weaning. Data expressed as mean ± SEM, n= 7-9 per group.

Table 3. 4: Effect of a high fructose diet on fasting blood glucose, insulin and HOMA-IR of female Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Fasting blood glucose ($mg.dl^{-1}$)	Insulin ($ng.ml^{-1}$)	HOMA-IR
DMSO + TW	78.00 ± 3.50	4.40 ± 0.10	0.88 ± 0.24
DMSO + FW	78.00 ± 2.90	5.60 ± 0.95	1.10 ± 0.20
CC + TW	77.00 ± 1.80	5.60 ± 0.11	0.94 ± 0.23
CC + FW	71.00 ± 2.90	4.60 ± 0.93	0.83 ± 0.17
FW + TW	73.00 ± 5.20	3.40 ± 0.56	0.62 ± 0.12
FW + FW	72.00 ± 1.80	4.40 ± 0.11	0.81 ± 0.23
CCFW + TW	76.00 ± 2.20	5.40 ± 0.10	0.88 ± 0.22
CCFW + FW	72.00 ± 2.30	6.30 ± 0.86	1.10 ± 0.16

There were no differences ($p>0.05$) in the fasting blood glucose, plasma insulin concentration and HOMA-IR of the rats across the treatment groups. FBG= fasting blood glucose, HOMA-IR= homeostatic model of insulin resistance. DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post weaning. Data expressed as mean ± SEM, n= 7-9 per group.

3.3.6 Effect of curcumin and fructose on fasting plasma triglycerides and total cholesterol

The effect of curcumin and fructose on fasting plasma triglycerides and total cholesterol of adolescent male and female Sprague Dawley rats that were fed a high fructose diet post-weaning are shown in tables 3.5 and 3.6 respectively.

There was no significant difference ($p>0.05$, ANOVA) in the fasting plasma triglycerides and total cholesterol across the treatment groups in both sexes.

Table 3. 5: Effect of a high fructose diet on fasting plasma concentrations of triglycerides and total cholesterol in male Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Triglycerides ($mg.dl^{-1}$)	Total cholesterol ($mg.dl^{-1}$)
DMSO + TW	16 ± 1.50	35 ± 2.10
DMSO + FW	12 ± 1.10	34 ± 2.00
CC + TW	15 ± 1.60	31 ± 1.70
CC + FW	15 ± 2.10	31 ± 2.80
FW + TW	10 ± 1.60	24 ± 2.20
FW + FW	14 ± 1.60	33 ± 2.20
CCFW + TW	15 ± 1.90	32 ± 2.70
CCFW + FW	16 ± 1.40	32 ± 2.30

There were no differences ($p>0.05$) observed in the plasma concentration of triglycerides and cholesterol across the treatment groups.TG= triglyceride, HDL-C= high density lipoprotein cholesterol, LDL-C= low density lipoprotein cholesterol. DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Data expressed as mean ± SEM, n= 7-9 per group.

Table 3. 6: Effect of a high fructose diet on fasting plasma concentrations of triglycerides and total cholesterol in female Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Triglycerides ($mg.dl^{-1}$)	Total cholesterol ($mg.dl^{-1}$)
DMSO + TW	12.0 ± 3.50	37.0 ± 1.40
DMSO + FW	15.0 ± 1.90	45.0 ± 3.60
CC + TW	11.0 ± 2.60	39.0 ± 2.30
CC + FW	12.0 ± 1.60	39.0 ± 1.90
FW + TW	9.6 ± 1.60	34.0 ± 0.62
FW + FW	13.0 ± 1.70	40.0 ± 2.40
CCFW + TW	8.9 ± 2.40	36.0 ± 2.00
CCFW + FW	15.0 ± 1.60	40.0 ± 1.60

There were no differences ($p>0.05$) observed in the plasma concentration of triglycerides and cholesterol across the treatment groups.TG= triglyceride, HDL-C= high density lipoprotein cholesterol, LDL-C= low density lipoprotein cholesterol. DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Data expressed as mean ± SEM, n= 7-9 per group.

3.3.7 Effect of curcumin and fructose on the masses of some viscera

The absolute and relative (%BM) masses of the kidneys and pancreas of adolescent male and female Sprague Dawley rats are presented in tables 3.7 and 3.8.

There were no differences ($p>0.05$, ANOVA) observed across the treatment groups in both sexes in the measured viscera.

Table 3. 7: Effect of fructose administration on the absolute and relative masses of kidneys and pancreas of male Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Kidneys (g)	Kidneys (%BM)	Pancreas (g)	Pancreas (%BM)
DMSO + TW	2.00 ± 0.08	0.72 ± 0.01	0.96 ± 0.08	0.35 ± 0.04
DMSO + FW	1.90 ± 0.06	0.71 ± 0.02	1.00 ± 0.06	0.36 ± 0.02
CC + TW	2.00 ± 0.08	0.71 ± 0.02	1.00 ± 0.05	0.36 ± 0.02
CC + FW	1.90 ± 0.08	0.73 ± 0.01	1.00 ± 0.05	0.40 ± 0.03
FW + TW	2.20 ± 0.07	0.72 ± 0.02	1.20 ± 0.13	0.40 ± 0.04
FW + FW	2.20 ± 0.14	0.86 ± 0.11	0.92 ± 0.08	0.35 ± 0.02
CCFW + TW	2.20 ± 0.10	0.74 ± 0.01	1.20 ± 0.08	0.42 ± 0.02
CCFW + FW	2.00 ± 0.10	0.76 ± 0.02	1.10 ± 0.12	0.41 ± 0.03

There were no differences ($p>0.05$) observed in the masses of the viscera across the treatment groups. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Data expressed as mean ± SEM, n= 7-9 per group.

Table 3. 8: Effect of a high fructose diet on the absolute and relative masses of the kidneys and pancreas of female Sprague Dawley rats that were administered with curcumin during suckling

Treatment groups	Kidneys (g)	Kidneys (%BM)	Pancreas (g)	Pancreas (%BM)
DMSO + TW	1.50 ± 0.05	0.71 ± 0.01	0.77 ± 0.03	0.38 ± 0.02
DMSO + FW	1.50 ± 0.03	0.74 ± 0.02	0.89 ± 0.05	0.44 ± 0.03
CC + TW	1.50 ± 0.03	0.71 ± 0.02	0.84 ± 0.04	0.40 ± 0.02
CC + FW	1.60 ± 0.03	0.78 ± 0.01	0.88 ± 0.04	0.43 ± 0.02
FW + TW	1.50 ± 0.03	0.72 ± 0.01	0.94 ± 0.09	0.44 ± 0.03
FW + FW	1.60 ± 0.03	0.74 ± 0.02	0.88 ± 0.04	0.41 ± 0.02
CCFW + TW	1.50 ± 0.05	0.72 ± 0.01	0.85 ± 0.03	0.40 ± 0.01
CCFW + FW	1.60 ± 0.05	0.75 ± 0.01	0.94 ± 0.08	0.45 ± 0.03

There were no differences ($p>0.05$) observed in the masses of the viscera across the treatment groups. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Data expressed as mean ± SEM, n= 7-9per group.

3.3.8 Effect of curcumin and fructose on kidney histomorphology

Figures 3.6 and 3.7 show representative photomicrographs of MT-stained kidney tissues of male and female Sprague Dawley rats. In both male and female rats, the negative control groups (DMSO + TW) showed normal glomerular, tubular and interstitial architecture. While the group that received fructose postweaning only (DMSO + FW) in the males showed some mesangial proliferation, mild tubular secretions and normal Bowman capsular spaces, the same group in the females showed moderate glomerular atrophy, increased Bowman capsular space and increased tubular secretions. In both male and female rats, neonatal administration of curcumin prevented the postweaning fructose only-induced changes in kidney tissues. Additionally, the kidney tissues of male and female rats that were administered with curcumin during suckling and no fructose at all, showed glomerular atrophy, increased Bowman capsular space and mild to moderate tubular secretions. In the male rats, an early single hit fructose and double hit fructose did not affect the architecture of the kidney tissues. In the female rats, a preweaning only administration of fructose-induced moderate glomerular atrophy with increased Bowman capsular space while a double hit fructose resulted in mesangial proliferation and mild tubular secretions. In both male and female rats, combined administration of curcumin and fructose during suckling and subsequent feeding with plain tap water induced mild mesangial proliferation in the kidney tissues. However, a combined administration of curcumin and fructose during suckling and fructose feeding postweaning induced mesangial proliferation in the male and mild glomerular atrophy in the female Sprague Dawley rats.

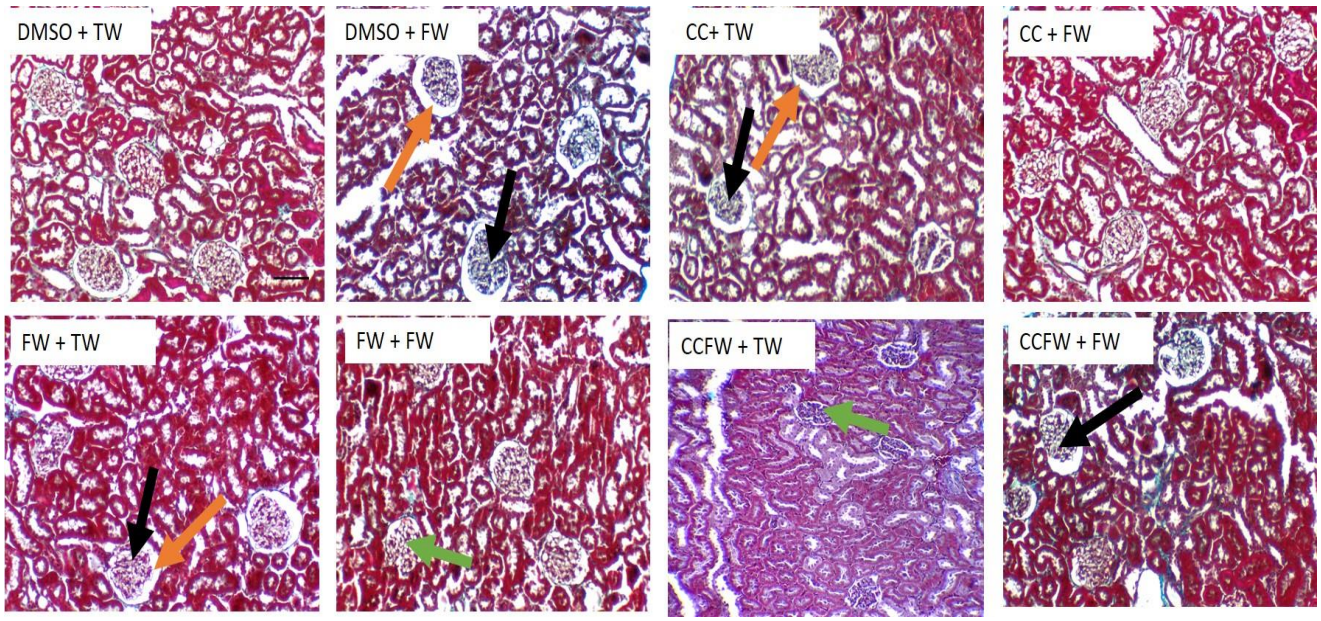


Figure 3. 6: Photomicrographs showing the effect of a high fructose diet on kidney histology (MT staining) of representative adolescent male Sprague Dawley rats that were orally administered with curcumin during suckling.

The black arrows point to glomerular atrophy, the brown arrow points to increased Bowman capsular space while the green arrows point to mesangial proliferation. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Scale bar in DMSO + TW = 100µm, applies to all the figures.

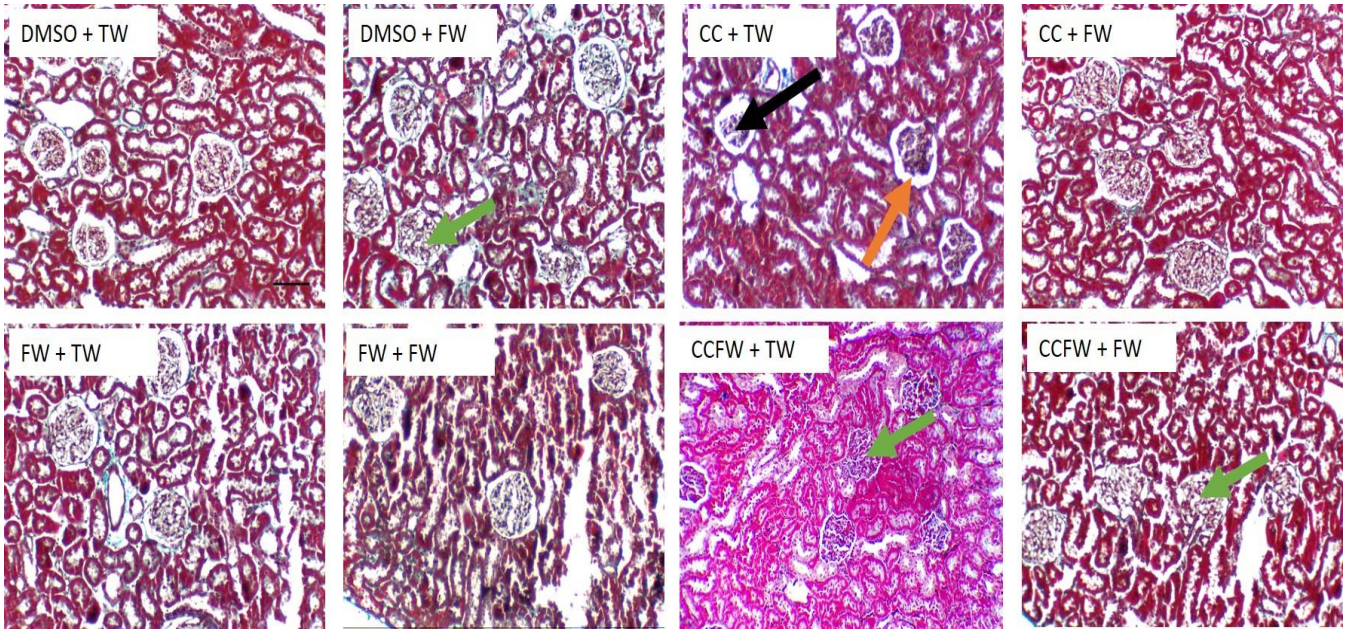


Figure 3. 7: Photomicrographs showing the effect of a high fructose diet on kidney histology (MT staining) of representative adolescent female Sprague Dawley rats that were orally administered with curcumin during suckling.

The black arrows point to glomerular atrophy, the brown arrow points to increased Bowman capsular space while the green arrow point to mesangial proliferation. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the growing period, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, FW + TW= fructose (20%, w/v) as neonates and plain tap water in the growing period, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid in the growing period, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water in the growing period, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) in the growing period. Scale bar in DMSO + TW= 100µm, applies to all the figures.

3.4 Discussion

In the present study, neither the pre-weaning nor post-weaning administration of fructose to the male and female Sprague Dawley rats induced features of MetS usually associated with the consumption of a high fructose diet. In this regard, the terminal body masses, BMI, visceral fat pad (absolute and relative to body mass) and epididymal fat mass (in males) were all similar across the treatment groups in both sexes. Additionally, the concentrations of insulin and adiponectin and metabolic substrates (FBG, TG and total cholesterol) were similar across the treatment groups in both male and female rats. However, the administration of curcumin during suckling generally conferred protection on both male and female adolescent rats against the histopathological changes in the kidney tissues induced by a high fructose diet.

In several studies using adult rodents, a high fructose diet was shown to induce features of metabolic syndrome. For instance, when fructose (10%, w/v) was fed to adult male Sprague Dawley rats for eight weeks, it increased the concentrations of fasting blood glucose, insulin, triglycerides, total and low-density lipoprotein cholesterol (Fadlalla and Khojah, 2017). Mamikutty et al. (2014) also found features of obesity such as hypertrophy of adipocytes, elevated levels of serum triglycerides and hyperglycaemia when they administered a 20 % fructose w/v solution for eight weeks to adult male Wistar rats. Recently, Aguilera-Mendez et al. (2018) administered a 30% fructose w/v solution in six week old male Wistar rats for 14 weeks and found significantly raised hepatic and plasma total cholesterol and triglycerides, increased low-density lipoprotein cholesterol and reduced high-density lipoprotein cholesterol. However, in the current study, both the pre-weaning and post-weaning administration of fructose did not induce overt features of MetS. The absence of features of MetS in the fructose groups makes it difficult to determine whether curcumin had any effects on programming of metabolic function in this study.

There are possible explanations for the absence of the effects of a high fructose diet in the neonatal and growing rats. Firstly, it is important to state that, the target for this study was the adolescent age group, and thus the use of suckling pups. In children and adolescents, the epidemic of overweight and obesity (Orlando et al., 2018) is strongly linked to increased consumption of high sugar foods (Fidler Mis et al., 2017). The suckling pups and the growing rats have a higher body surface area to volume ratio compared to the adult rats which results

in a higher metabolic rate and subsequent oxidation of the excess fructose without manifesting its deleterious effects (Tillman et al., 2014). Young rats, though also susceptible to fructose-induced metabolic derangements especially NAFLD may not manifest changes in body mass and adiposity (Crescenzo et al., 2018). This resistance of young rats to fructose-induced metabolic derangements such as dyslipidaemia and hyperglycaemia is a protective mechanism acquired early in life (Ghezzi et al., 2012), which may be due to the efficiency of the GLUT4 transporters (Ghezzi et al., 2012). The GLUT5 transporters in the small intestines responsible for shuttling fructose across membranes are few and immature from birth till weaning (Patel et al., 2015; Xu and Ghishan, 2018), although they may precociously mature before weaning when fructose is administered early (Castello et al., 1995; Ferraris, 2001). Unfortunately, in the present study, the GLUT5 transporters were not assayed at weaning to determine their level of expression.

The protective mechanism against fructose-induced derangements was previously demonstrated in weanling male C57bl/6J mice that were fed a 60% fructose w/w diet for 14 weeks without recording changes in body mass, triglycerides, glucose and non-esterified fatty acids (Tillman et al., 2014). Fructose (20% w/v) feeding in 21 day old male Wistar rats for 6 weeks did not produce any change in body mass but increased the total cholesterol to triglyceride ratio (Dupas et al., 2016). In another study, alteration in neonatal nutrition of suckling rats in the form of high carbohydrate milk formula resulted in differences in body mass only at about postnatal day 100 (Patel and Srinivasan, 2010). The age of the rats in the current study at termination was 63 days (adolescent/early adulthood) which might explain the absence of the features of MetS. The study was terminated at postnatal day 63 because my interest was on childhood obesity and that age (postnatal day 63) in rats developmentally is equivalent to late childhood (Sengupta, 2013).

However, we did find some interesting results in the kidney histology of the adolescent rats. Whereas the postweaning administration of fructose-induced histopathological changes in the kidney tissues of the rats, oral administration of curcumin during suckling prevented the development of those changes in both sexes. Curcumin has previously been shown to reverse the vacuolar degeneration of glomerular cells associated with cyclosporin A (Huang et al., 2018a) and cadmium tetrachloride-induced nephrotoxicity (Hismiogullari et al., 2015). We thus speculate that the neonatal administration of curcumin to the rats may have

epigenetically programmed the rats' kidneys to resist the adverse effects of a high fructose diet postweaning. However, this requires further investigation. On the other hand, we found that oral administration of curcumin during suckling alone without a postweaning high fructose diet-induced some degree of glomerular atrophy and increased Bowman capsular space in the kidneys of the adolescent male and female rats. Considering that we did not find any literature ascribing a nephrotoxic effect on curcumin in both *in vitro*, animal and human studies, we propose that curcumin should be used with caution in the neonatal age group.

In rats, the kidneys though functionally immature at birth, undergo rapid development and attain optimal functioning at or around weaning (Seely, 2017). The neonatal kidney is characterised by reduced number and functionality of the tubules and consequently reduced kidney functions such as glomerular filtration rate, tubular secretion and absorption compared to adults (Seely, 2017). Our neonatal interventions with curcumin and fructose may have overwhelmed the scanty tubules and affected their proper functioning on a long-term basis and thus resulting in the increased tubular secretions observed in the kidney sections.

The masses of the kidneys and pancreas were also similar across treatment groups in both sexes. Since organ weights are used in toxicological studies as an indication of early deleterious effects of compounds (Sellers et al., 2007; Shafaei et al., 2015), it could be inferred that both curcumin and fructose did not exert toxic effects on the weighed organs.

3.6 Conclusion

Oral administration of curcumin and fructose during suckling, followed by a high fructose diet post weaning did not negatively affect the general metabolic health of adolescent male and female rats. However, although the administration of curcumin during suckling prevented the fructose-induced histological changes in the kidneys of the rats, it may also predispose to glomerular atrophy in later life. These findings suggest that although curcumin has previously been reported to possess a lot of beneficial health effects, it should be used with caution in the neonatal age group.

The next chapter reports on the second component of the study which is on the potential protective effects of neonatal oral administration of curcumin in rats against the development of non-alcoholic steatohepatitis in rats that were fed a high fructose diet until weaning.

CHAPTER 4: The long-term protective effects of neonatal administration of curcumin against non-alcoholic steatohepatitis in high fructose-fed adolescent rats

4.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is the commonest liver pathology in the developed world, affecting as much as one-third of the population (Fazel et al., 2016). The prevalence of NAFLD is higher in males than females (Tsuneto et al., 2010) and increases with the presence of diabetes mellitus (Blachier et al., 2013). About 10% of all children are affected by NAFLD (Hartmann and Schnabl, 2018). The prevalence increases with age and in obese children it can reach up to 38% (Hartmann and Schnabl, 2018). NAFLD is a spectrum of liver diseases characterised by excessive accumulation of lipids, especially triglycerides (Gautam, 2018). The spectrum of diseases associated with NAFLD includes simple hepatic steatosis and non-alcoholic steatohepatitis (NASH) that are found in the absence of other known causes of liver disease such as excessive alcohol consumption, hepatitis C virus (HCV) infection or chemical agents (van der Poorten et al., 2013). Simple hepatic steatosis is the non-progressive form of the disease that rarely develops into cirrhosis, while NASH usually progresses to cirrhosis and hepatocellular carcinoma that results in mortality (Than and Newsome, 2015). In children, NASH is the commonest presentation of liver disease (Mann et al., 2018).

NAFLD was previously considered as the hepatic manifestation of metabolic syndrome (MetS) (Vanni et al., 2010; Than and Newsome, 2015). However, recent evidence has disputed that assertion by showing NAFLD to precede the development of MetS (Lonardo et al., 2015). Importantly, about 30 per cent of patients with NAFLD do not present with any systemic metabolic abnormalities (Kim et al., 2018). However, most patients with NAFLD concurrently present with insulin resistance which is the hallmark (Asrih and Jornayvaz, 2015) in the development of type 2 diabetes mellitus (T2DM) and MetS (Adams et al., 2009). It is estimated that about one-third of individuals with NAFLD have type 2 diabetes mellitus and/or MetS (Adams et al., 2005).

MetS is a collection of cardio-metabolic risk factors that are driven by obesity and insulin resistance, consequently increasing the chances of developing cardiovascular disease and T2DM in those affected (Samson and Garber, 2014). It is a global epidemic affecting all age groups (Vickers, 2011; Wang, 2013). Sedentary lifestyles and increased consumption of diets rich in carbohydrates and fats have been linked to the significant increase in the prevalence of MetS and NAFLD (Mamikutty et al., 2015). The increased consumption of fructose, a simple

sugar used as a sweetener in many foods and drinks contributes significantly to the development of NAFLD (Vos and Lavine, 2013).

The metabolism of fructose occurs mainly in the liver (Alwahsh and Gebhardt, 2017). In contrast to glucose, the metabolism of fructose is neither regulated by insulin nor inhibited by adenosine triphosphate (ATP) at the level of fructokinase and aldolase B enzymes (Tappy and Lê, 2012). Consequently, there is about 70% fructose uptake and conversion to triose phosphate in the liver; exceeding its oxidative capacity and thus leading to the production of lactate, gluconeogenesis, glycogen synthesis and *de novo* lipid synthesis (Tappy and Lê, 2012). Therefore, high fructose diets can cause a hepatic cellular stress response (Basaranoglu et al., 2013) leading to enhanced lipogenesis, mitochondrial dysfunction, inflammation, fibrosis and hepatic insulin resistance (Chen et al., 2017). The resultant dysregulation of fatty acid synthesis and triglyceride production in the liver leads to steatosis (Alwahsh and Gebhardt, 2017). High fructose diets may promote hepatic inflammation by increasing the production and secretion of tumour necrosis factor alpha (TNFα) from Kupffer cells (Jegatheesan and De Bandt, 2017) and by inducing dysbiosis of the gut microbiota (Jegatheesan et al., 2016). The enzyme 5' Adenosine monophosphate-activated protein kinase alpha (AMPKα) has a key role in the regulation of cellular energy homeostasis (Li et al., 2011). Its activation in the liver favours energy producing and not energy consuming processes such as fatty acid synthesis (Carneiro et al., 2017). This downregulation of energy consuming processes is achieved through the inhibition of the sterol regulatory element binding protein 1 (SREBP1) and consequently repressing key transcription factors such as peroxisome proliferator activated-receptors (PPAR) that are involved in lipid metabolism (Carneiro et al., 2017). It is important however to note that while the upregulation of AMPK protects against hepatic steatosis, its downregulation does not indicate susceptibility to hepatic steatosis (Boudaba et al., 2018).

It has been shown that sedentary lifestyles and metabolic disorders such as obesity and insulin resistance, the main drivers of MetS, can lead to the development of NAFLD through epigenetic means (Lee et al., 2014). Available epidemiological and clinical evidence suggests that stressor events during the early periods of life, especially gestation and lactation can modify the epigenome and alter the physiology and metabolism of the individual on a long-term basis, a phenomenon called developmental metabolic programming (Hales and Barker, 2001; Collden et al., 2015). It has also been shown that it is possible to reverse the effects of

this early developmental programming using nutritional, pharmacological, or hormonal interventions in the period of developmental plasticity (Vickers, 2011). Consequently, the suckling period in rats has been targetted for prophylactic interventions against the future development of MetS by virtue of its being a critical window for epigenetic modifications (Armitage et al., 2005). For example, the adverse effects of a high maternal dietary fructose intake on anti-oxidant status and lipid metabolism in adult offspring were attenuated by dietary supplementation with bitter melon (*Momordica charantia*) from preconception till lactation day 21 in a rat experimental model (Ching et al., 2011). Also, potential fructose-induced fatty liver changes in adulthood were prevented by a prior neonatal administration of S-allyl-s-cysteine to the Wistar rats (Lembede et al., 2018). Further, we have shown that the administration of *Hibiscus sabdariffa* aqueous calyx extracts to neonatal Sprague Dawley rats protected the growing female rats from fructose-induced hypertriglyceridaemia and the male rats from hypercholesterolaemia, when they were fed a high fructose diet (Ibrahim et al., 2017). Presently, there is no approved treatment for NASH in children (Ahmed et al., 2015; Mann et al., 2018) and therefore the search for natural products that could prevent its development is essential.

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], a polyphenol isolated from the roots of turmeric (*Curcuma longa*), has been shown to exhibit hepatoprotective activity mainly via its anti-oxidant and anti-inflammatory properties (Inzaugarat et al., 2017; Panahi et al., 2018b). Curcumin was shown to have anti-steatotic and anti-fibrotic properties in several adult animal models of NAFLD (Amel Zabihi et al., 2017). Despite its hepatoprotective activity, curcumin has not been investigated for its ability to program against fructose-induced NAFLD in growing rats when administered during the suckling period.

Given the high prevalence of NAFLD in adolescents and children, there is a need to develop strategic prophylactic interventions which target periods of early developmental plasticity for long term health benefits. Interventions in early life can impact health immediately or following a latent period (with or without a secondary insult). The objective of this study was to determine whether oral administration of curcumin to neonatal rats would developmentally program the livers of the rats for long term protection against fructose-induced non-alcoholic fatty liver disease. The fructose was provided either as an early neonatal hit or a continual high fructose diet from neonatal stage until termination in adolescence.

4.2 Materials and methods

4.2.1 Ethical approval

As previously stated in chapter three (3.2.1).

4.2.2 Chemicals and reagents

Curcumin, DMSO and fructose were sourced as described previously in chapter three (3.2.2). The SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Austin, TX, USA), Forward and reverse primers (TNF, AMPK and GADPH) and nuclease-free water were purchased from Inqaba Biotech (Pretoria, South Africa). SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA) and trizol (Life Technologies, Carlsbad, CA, USA) were also used in the study.

4.2.3 Animals, housing and general care

This section is as previously described in chapter three (3.2.3).

4.2.4 Treatment protocol

As described in chapter three (3.2.4).

4.2.5 Terminal procedures

In addition to the terminal procedures described in chapter three (3.2.6), the liver was excised and weighed (Presica 310M, Presica Instruments AG, Switzerland). A sample of the liver was taken from the medial lobe and preserved in 10% phosphate-buffered formalin for histology. A second liver sample was taken and preserved in RNAlater (Sigma-Aldrich, St. Louis,

Missouri, USA) and stored at -80°C for the molecular/gene expression study. The rest of the liver was stored in a freezer (Bosch, Stuttgart, Germany) at -20°C for liver lipid determination.

4.2.6 Quantification of hepatic lipid content

The hepatic lipid content was determined using the solvent extraction method as described by Bligh and Dyer (1959). Briefly, the liver samples were retrieved from the freezer and allowed to thaw at room temperature. Known masses of the liver were placed in 150ml of 2:1 chloroform: methanol solution and allowed to extract overnight in a fridge (DEFY, Durban, South Africa) at 4ºC. The solution was then filtered through Whatman™ filter paper (Albert Filtration and Separation Technology filter paper with pore size 7 – 11 µm, size 150 mm) into 250ml separation funnels. Thereafter, 30ml of 0.9% saline was dispensed into the separation funnels, vigorously mixed, covered with lids and allowed to stand overnight at 4ºC. The bottom phase was collected in round bottom flasks and evaporated to dryness using a rotor evaporator (Labex®, Krugersdorp, South Africa). The dried lipid extract in the round bottom flasks was dissolved in 20ml chloroform. An aliquot of 2ml chloroform (from each sample) was then placed in pre-weighed 10ml scintillating bottles and re-dried in an oven (Salvis® vacucenter, Oakton, USA) at 50°C for 30 minutes. The dried bottles were then allowed to cool in a desiccator, re-weighed, and the percentage of liver lipid content was determined using the formula:

$$\text{Liver lipid (\%)} = \frac{M_2 - M_1}{M_0} \times 100$$

Where M_0= Dry mass of the liver sample (g)

M_1= Mass of empty scintillation bottle (g)

M_2= Mass of scintillation bottle and lipid

The residual liver samples following extraction were air dried to constant weight in the laboratory at room temperature and the masses used in the final computation of the hepatic lipid content on a dry mass basis.

4.2.7 Liver histomorphometry

Following fixation, the liver tissues were processed using an automatic tissue processor (Micron STP 120, Thermo Fischer Scientific, USA). The samples were embedded in paraffin wax and sectioned at 3µm thickness using a rotary microtome (Leica Biosystems, USA). The tissue sections were then stained with haematoxylin and eosin (HE) to visualise hepatocellular changes and Masson's trichrome (MT) stain to visualise 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 (Leica Biosystems, USA). Composite images were prepared with CorelDraw X8 Software (Corel Corporation, Ottawa, Canada). No pixelation adjustments of the captured photomicrographs were undertaken except for adjustment of contrast and brightness.
The haematoxylin and eosin-stained (HE) sections of the liver were semi-quantitatively scored for steatosis and inflammation according to criteria described by Liang et al. (2014). Macro-vesicular steatosis, micro-vesicular steatosis and hepatocellular hypertrophy were semi-quantitatively analysed by determining the percentage area affected in each camera field at 40X magnification. Macro-steatosis, micro-steatosis and hepatocellular hypertrophy were graded as follows: Grade 0 = <5%; Grade 1 = 5–33%; Grade 2 = 33–66%; Grade 3 = >66% area affected per camera field of the parenchyma.

Inflammation was scored by counting the number of inflammatory cell aggregates in the liver parenchyma and graded as follows: Grade 0 = none or no foci of inflammation per camera field; Grade 1 = 0.5 to 1.0 foci per camera field; Grade 2 = 1-2 foci per camera field; Grade 3 = >2 foci per camera field at 100X magnification.

Fibrosis was quantitatively assessed from photomicrographs of the MT-stained liver sections at 20X magnification using Image (Schneider et al., 2012). Briefly, the area [$A = ap \times \sum p$] and area fraction ($A_{fraction}$) of each liver section (20X) occupied by connective tissue were measured using the point counting method, where ap is the area per point ($0.002mm^2$; $\sum p$ is the sum of the points falling on the connective tissue within a camera field ($0.149\ mm^2$) of each liver section and $A_{fraction} = [A \div 0.149\ mm^2 \times 100]$. A total of 20 camera fields were used for each section.

To avoid sampling errors, liver samples were all obtained from the medial lobe and all the histological features were semi-quantitatively and quantitatively assessed by a histologist (PN) who was blinded to the animal treatments.

4.2.8 Gene expression studies

4.2.8.1 RNA extraction

Total ribonucleic acid (RNA) was isolated from samples of the liver selected randomly per treatment group using trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA concentration and quality were measured using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, MA, USA) while the integrity of the RNA was analyzed using 1% agarose gel electrophoresis and viewed under a doc machine (Bio-Rad Laboratories, Hercules, CA, USA).

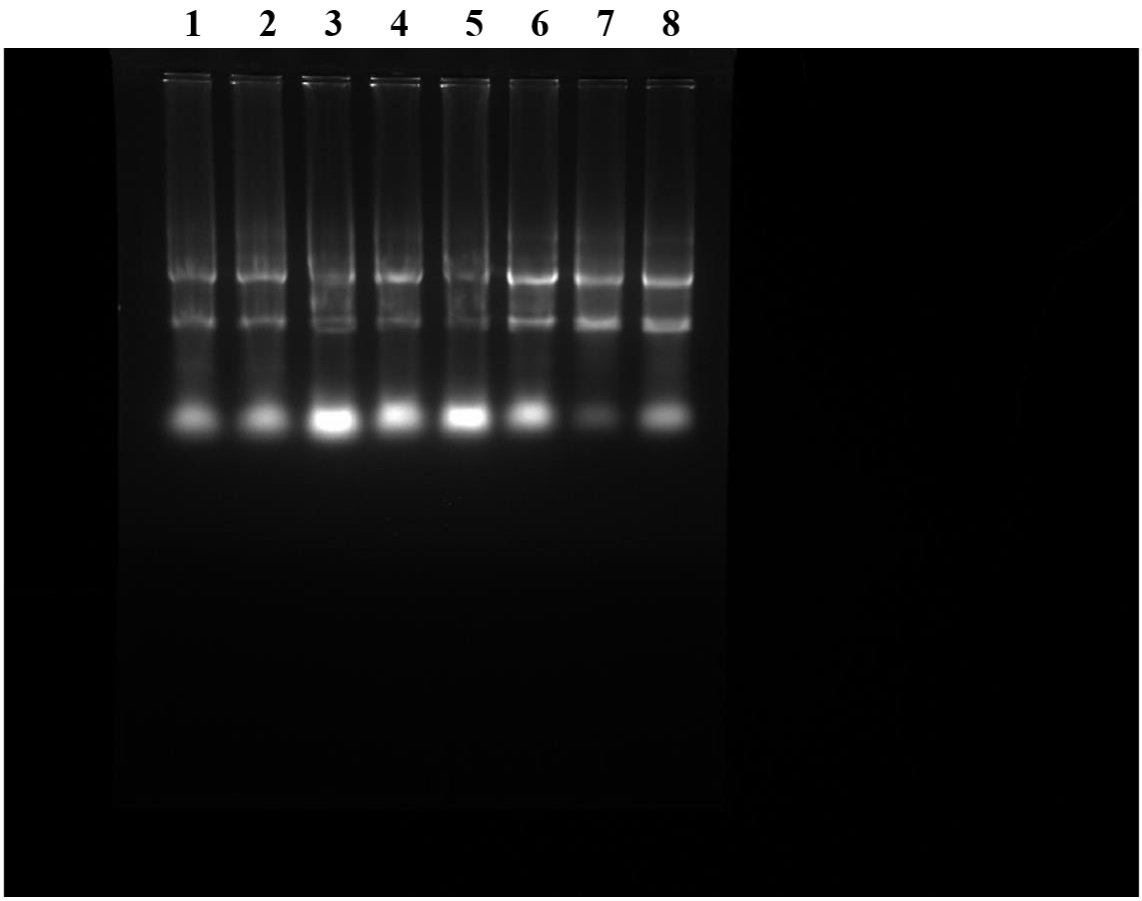


Figure 4. 1: Example of an agarose gel showing the integrity of total RNA extracted from the livers representative of the eight treatment groups in adolescent male rats

1= DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, 2= DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 3= CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, 4= CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 5= FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, 6= FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 7= CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, 8= CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning.

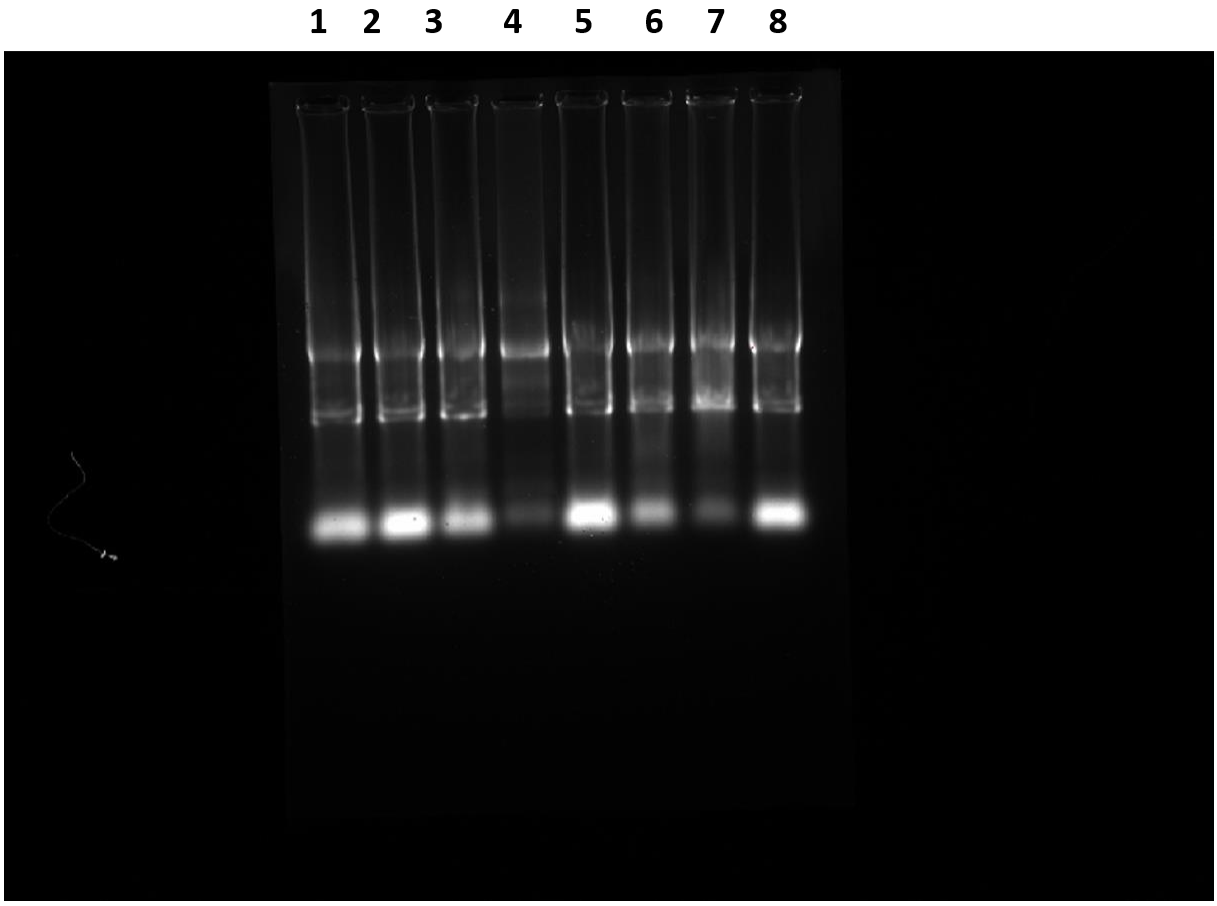


Figure 4. 2: Example of an agarose gel showing the integrity of total RNA extracted from the eight treatment groups in female adolescent rats

1= DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, 2= DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 3= CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, 4= CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 5= FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, 6= FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, 7= CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, 8= CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning.

4.2.8.2 Complementary deoxyribonucleic acid (cDNA) synthesis

The cDNA was synthesized using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. All incubations were done in the T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The cDNA was stored at -20℃ until needed for use.

4.2.8.3 Real-time polymerase chain reaction

Gene expression for AMPKα and TNFα were determined using the StepOnePlus™ Real-time PCR system (Applied Biosystems, Singapore). SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Austin, TX, USA) was used according to the manufacturer's protocol. The forward and reverse primers (Table 4.1) for each of the gene expressions and the SYBR™ Green master mix were mixed together appropriately according to the volumes given by the manufacturer. The cDNA template was mixed with RNase-free water. Solutions were pipetted into appropriate wells in triplicates in the 96 well plate and the template was added last. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. The reaction plate was placed in the instrument and the thermal cycling conditions were set and the run started with appropriate calibrations.

Table 4. 1: List of primers used in the study

Genes	**Forward primer (F) and Reverse prime (R)**
AMPK-α	F: 5′-GGCAAAGTGAAGATTGGAGAACA-3′ R: 5′-AACTGCCACTTTATGGCCTGT C-3′
TNF-α	F: 5′-CCCAGACCCTCACACTCAGAT-3′ R: 5′-TTGTCCCTTGAAGAGAACCTG-3′
GAPDH	F: 5′-GAACATCATCCCTGCATCC-3′ R: 5′-CCTGCTTCACCACCTTCTT-3′

4.2.9 Statistical analysis

Data were analysed using GraphPad Prism Version 7.0 (GraphPad Software Inc., San Diego, CA) and expressed as mean ± standard error of the mean. Hepatic lipid content, hepatic fibrosis and gene expression ratios were analysed using a one-way analysis of variance (ANOVA), followed by a Bonferroni *post hoc* test to compare the means. The Kruskal-Wallis test (non-parametric one-way ANOVA) was used to analyse non-alcoholic fatty liver disease score (NAS) multiple group data followed by Dunn's *post hoc* test to compare the medians.

4.3 Results

4.3.1 Masses of the liver

The absolute and relative to body masses (%BM) masses of the livers of male and female growing Sprague Dawley rats are shown in tables 4.2 and 4.3. There were no statistically significant differences ($p>0.05$, ANOVA) observed in the absolute (g) and relative (%BM) liver masses across the treatment groups in both male and female rats.

Table 4. 2: Masses of the liver of growing male Sprague Dawley rats

Treatment groups	Liver (g)	Liver (%BM)
DMSO + TW	9.6 ± 0.44	3.5 ± 0.08
DMSO + FW	9.6 ± 0.28	3.5 ± 0.07
CC + TW	9.7 ± 0.42	3.4 ± 0.09
CC + FW	9.0 ± 0.46	3.5 ± 0.03
FW + TW	11 ± 0.50	3.6 ± 0.18
FW + FW	9.1 ± 0.42	3.5 ± 0.05
CCFW + TW	11 ± 0.68	3.7 ± 0.19
CCFW + FW	10 ± 0.80	3.7 ± 0.11

No significant differences (P>0.05) were observed in terminal body masses, absolute and relative masses of the liver across the treatment groups. BM= body mass. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

Table 4. 3: Masses of the liver of growing female Sprague Dawley rats

Treatment groups	Liver (g)	Liver (%BM)
DMSO + TW	7.4 ± 0.53	3.6 ± 0.18
DMSO + FW	7.4 ± 0.26	3.7 ± 0.08
CC + TW	7.9 ± 0.30	3.7 ± 0.14
CC + FW	7.3 ± 0.19	3.5 ± 0.08
FW + TW	7.6 ± 0.62	3.6 ± 0.20
FW + FW	7.8 ± 0.19	3.6 ± 0.06
CCFW + TW	7.4 ± 0.27	3.5 ± 0.04
CCFW + FW	7.4 ± 0.27	3.5 ± 0.10

No significant differences ($p>0.05$) were observed in terminal body masses, absolute and relative masses of the liver across the treatment groups. BM= body mass. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

4.3.2 Hepatic lipid content

Figures 4.3 and 4.4 show the effect of neonatal administration of curcumin on hepatic lipid content of growing male and female Sprague Dawley rats that were fed with fructose. There were no differences ($p>0.05$, ANOVA) in the hepatic lipid content of male rats across the treatment groups. In the females, the rats that had fructose as neonates and tap water post weaning had significantly higher hepatic lipids compared to those that had DMSO in the neonatal phase and tap water post weaning ($p= 0.0357$, ANOVA) and those that had curcumin and fructose as neonates and post-weaning ($p= 0.0038$, ANOVA). Female rats that had curcumin and fructose as neonates and fructose solution post weaning had significantly lower hepatic lipids compared to those that had DMSO as neonates and fructose solution post weaning ($p= 0.0189$, ANOVA) and those that had curcumin and fructose solution as neonates and tap water in the growing period ($p= 0.0172$, ANOVA). The hepatic lipid content in the female rats, despite the observed differences, ranged from 2.3-4.7%.

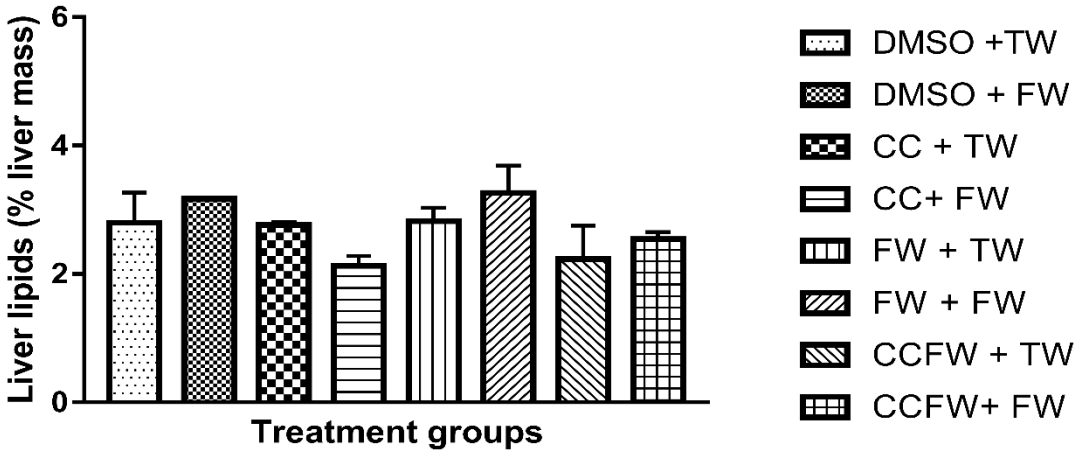


Figure 4. 3: Effect of a high fructose diet on the hepatic liver content of adolescent male Sprague Dawley rats that were administered with curcumin during suckling

No significant differences ($p>0.05$) were observed in percentage lipid content across the treatment groups. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

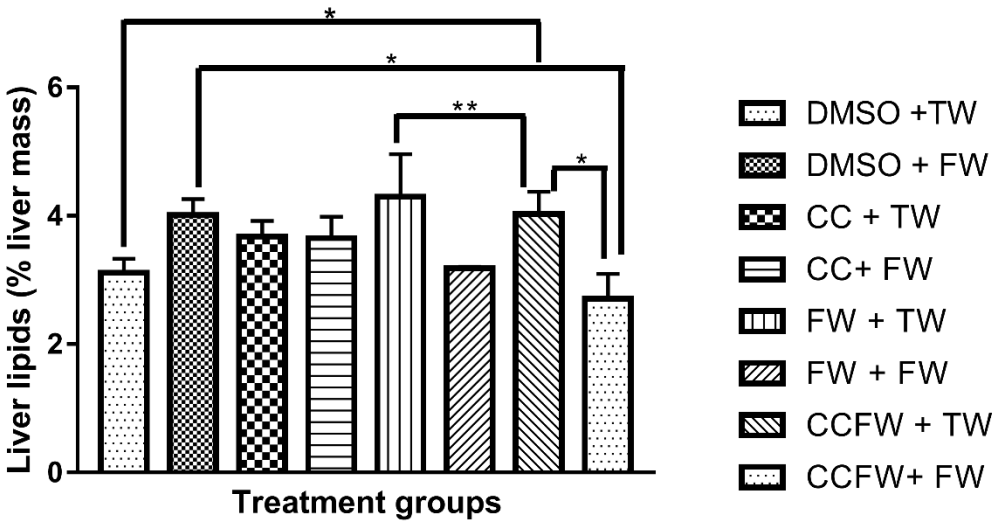


Figure 4. 4: Effect of a high fructose diet on the hepatic liver content of adolescent female Sprague Dawley rats that were administered with curcumin during suckling

*= significantly different at $p<0.05$, **= significantly different at $p<0.01$.

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

4.3.3 Liver histomorphometry

The photos showing liver histomorphometry (sections stained with H and E, X 20 magnification) of representative male and female rats are presented as figures 4.5 and 4.6. The livers of male and female rats that had fructose both pre and post weaning and those that had only fructose post weaning (DMSO+ FW) had significant inflammatory cell aggregation ($p= 0.0015$ males, $p= 0.0112$ females, Kruskal Wallis) which was absent in the rest of the groups.

The representative liver histology (sections stained with Masson Trichrome for fibrosis, X20 magnification) photos for male and female rats are presented as figures 4.6 and 4.7. The high fructose diet induced significant fibrosis ($p<0.0001$, ANOVA) in the livers of male and female rats that had fructose only post weaning and those that had fructose pre and post-weaning. The fructose-induced fibrosis was inhibited by curcumin administration during suckling.

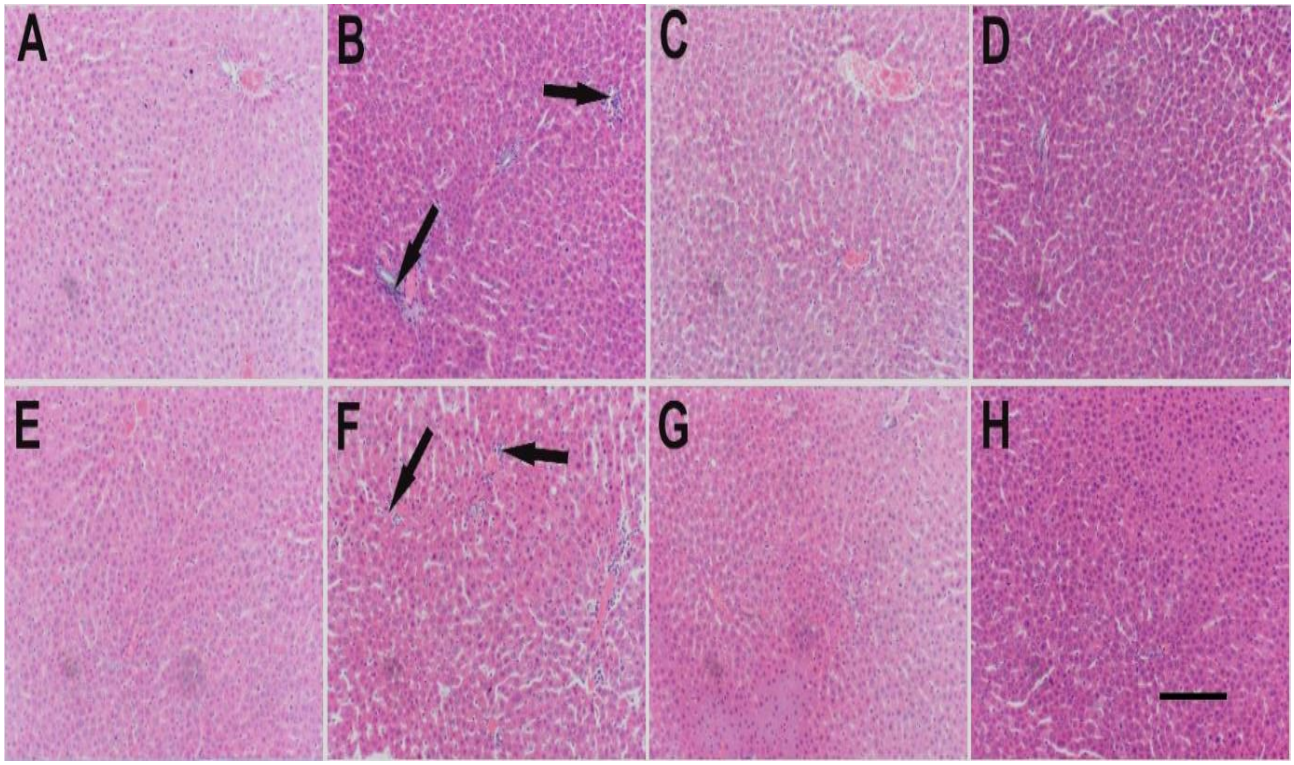


Figure 4. 5: Effects of a high fructose diet on liver histology (H and E staining) of representative adolescent male Sprague Dawley rats that were orally administered with curcumin during suckling.

The arrows point to highlight inflammatory cell aggregates. A=DMSO + TW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning; B= DMSO + FW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning; C= CC + TW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning; D= CC + FW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning; E= FW + TW, fructose (20%, w/v) as neonates and plain tap water post weaning; F= FW + FW, fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning; G= CCFW + TW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning; H= CCFW + FW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group. Scale bar in H= 30µm, applies to all the figures.

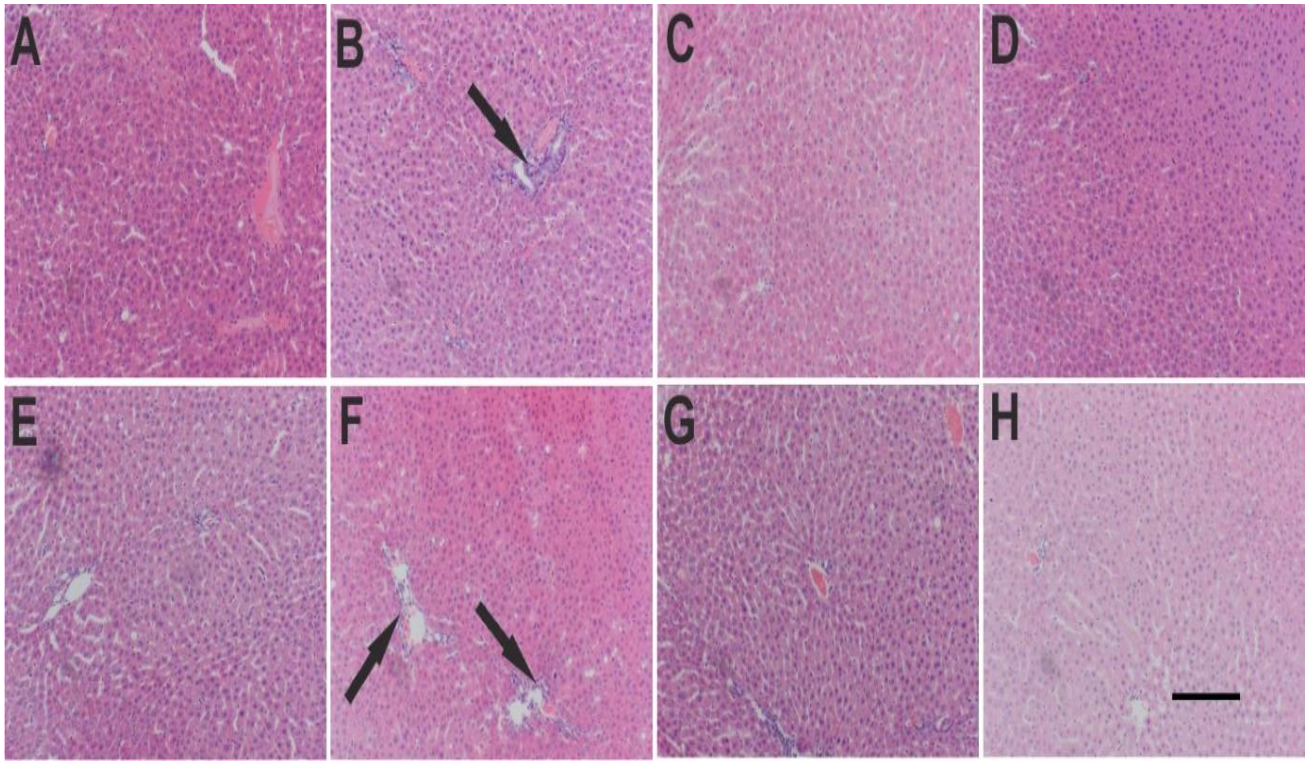


Figure 4. 6: Effects of a high fructose diet on liver histology (H and E staining) of representative adolescent female Sprague Dawley rats that were orally administered with curcumin during suckling.

The arrows point to highlight inflammatory cell aggregates. A=DMSO + TW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning; B= DMSO + FW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning; C= CC + TW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning; D= CC + FW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning; E= FW + TW, fructose (20%, w/v) as neonates and plain tap water post weaning; F= FW + FW, fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning; G= CCFW + TW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning; H= CCFW + FW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group. Scale bar in H= 30µm, applies to all the figures.

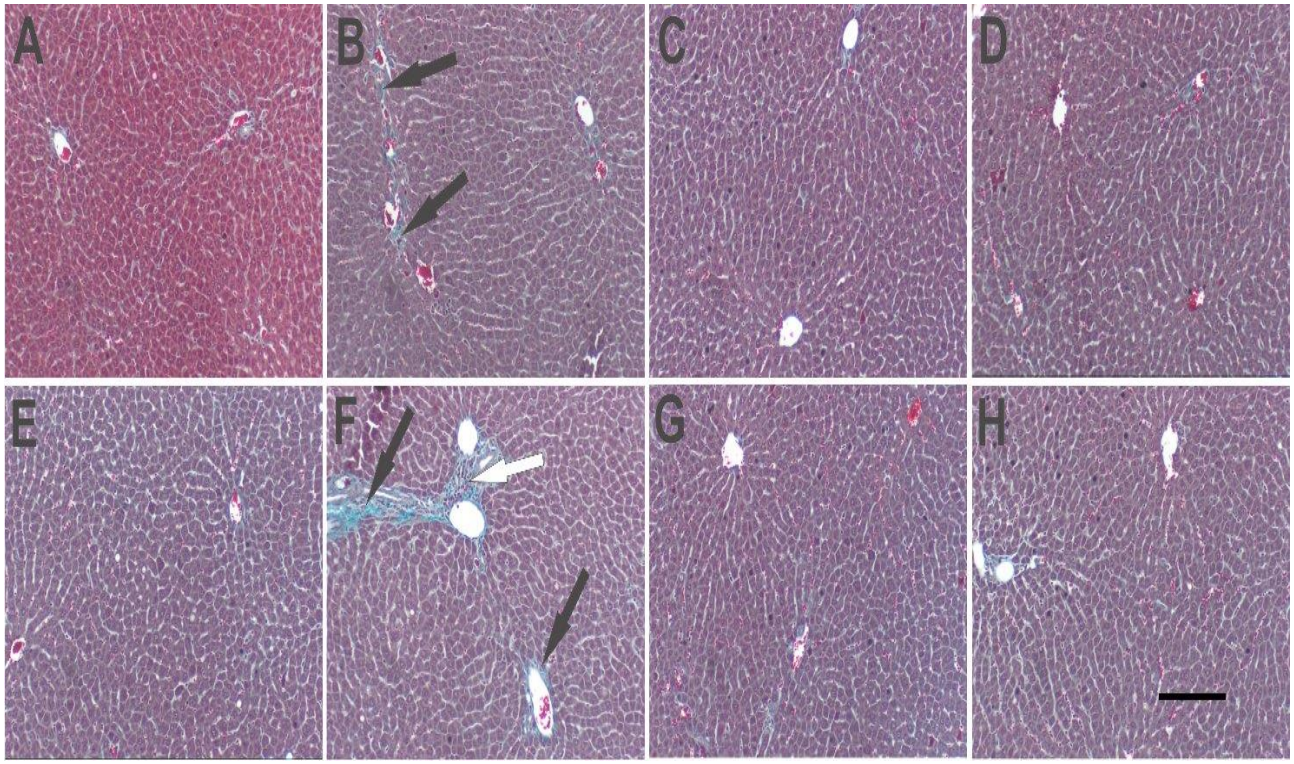


Figure 4. 7: Effects of a high fructose diet on liver histology (MT staining) of representative adolescent male Sprague Dawley rats that were orally administered with curcumin during suckling.

The black arrows point to fibrosis while the white arrow points to inflammatory cell aggregates. A=DMSO + TW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning; B= DMSO + FW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning; C= CC + TW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning; D= CC + FW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning; E= FW + TW, fructose (20%, w/v) as neonates and plain tap water post weaning; F= FW + FW, fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning; G= CCFW + TW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning; H= CCFW + FW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group. Scale bar in H= 30µm, applies to all the figures.

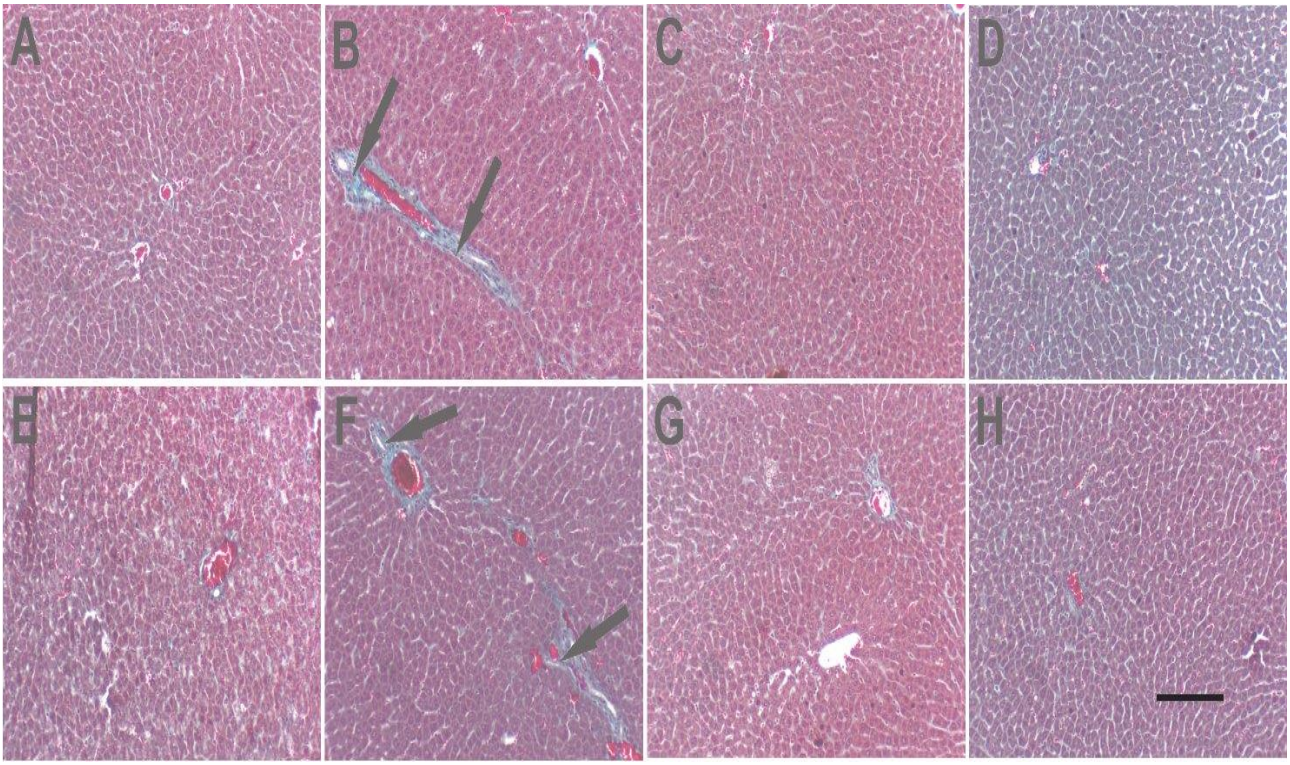


Figure 4. 8: Effects of a high fructose diet on liver histology (MT staining) of representative adolescent female Sprague Dawley rats that were orally administered with curcumin during suckling.

The black arrows point to indicate inflammatory cell aggregates. A=DMSO + TW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning; B= DMSO + FW, 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning; C= CC + TW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning; D= CC + FW, curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning; E= FW + TW, fructose (20%, w/v) as neonates and plain tap water post weaning; F= FW + FW, fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning; G= CCFW + TW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning; H= CCFW + FW, curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group. Scale bar in H= 30µm, applies to all the figures.

Tables 4.4 and 4.5 show the NAS score for macro-steatosis, micro-steatosis and inflammation and percentage fibrosis for livers of male and female rats respectively, following the interventions. Though the NAS score of both male and female rats did not show any significant differences in both macro- and micro-steatosis ($p<0.05$, ANOVA), it showed the presence of marked inflammation in the fructose-fed groups. In both male and female rats, there was a significantly higher ($p<0.0001$, ANOVA) percentage fibrosis in the groups that received fructose post weaning (DMSO + FW) and those that received fructose pre and post weaning (FW + FW) when compared to all the other treatment groups.

Table 4. 4: Effect of a high fructose diet on the non-alcoholic fatty liver disease activity score in the livers of adolescent male Sprague Dawley rats that were orally administered with curcumin during suckling

Treatment groups	Inflammation	Micro-steatosis	Macro-steatosis	Hypertrophy	Fibrosis (%)
DMSO + TW	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	1.00[a]
DMSO + FW	2.2 (2,3) [b]	0.4 (0,2) [a]	0.2 (0,1) [a]	0.4 (0,2) [a]	7.00[b]
CC + TW	0.33 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	0.67[a]
CC + FW	0.75 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	2.00[a]
FW + TW	1 (0,2) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	1.50[a]
FW + FW	3 (3,3) [b]	0.5 (0,1) [a]	0 (0,0) [a]	0.25 (0,1) [a]	7.50[b]
CCFW + TW	0.25 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	0.75[a]
CCFW + FW	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	1.30[a]

[a, b] = means with different superscripts in the same column are statistically significant ($p<0.0001$). DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

Table 4. 5: Effect of a high fructose diet on the non-alcoholic fatty liver disease activity score in the livers of adolescent female Sprague Dawley rats that were orally administered with curcumin during suckling

Treatment groups	Inflammation	Micro-steatosis	Macro-steatosis	Hypertrophy	Fibrosis (%)
DMSO + TW	0.25 (0,1) [a]	0.25 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0.50[a]
DMSO + FW	2.7 (2,3) [b]	0 (0,0) [a]	0 (0,0) [a]	0.67 (0,2) [a]	8.70[b]
CC + TW	0.33 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	1.70[a]
CC + FW	0.5 (0,1) [a]	0.25 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0.75[b]
FW + TW	1 (1,1) [a]	1.3 (1,2) [a]	0.67 (0,1) [a]	0.67 (0,1) [a]	2.30[a]
FW + FW	3 (3,3) [b]	2 (0,3) [a]	0 (0,0) [a]	1.3 (0,2) [a]	8.30[b]
CCFW + TW	0.33 (0,1) [a]	0 (0,0) [a]	0 (0,0) [a]	0 (0,0) [a]	0.67[a]
CCFW + FW	0.25 (0,1) [a]	0.25 (0,1) [a]	0.25 (0,1) [a]	0.25 (0,1) [a]	0.75[a]

[a, b] = means with different superscripts in the same column are statistically significant ($p<0.0001$). DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

4.3.4 Gene expression studies

The effect of administration of curcumin during suckling on the relative expression of AMPKα in male and female rats later when in adolescence after being fed a high fructose post-weaning are shown in figures 4.9 and 4.10. In both sexes, the expression of AMPKα was significantly downregulated ($p<0.0001$, ANOVA) in the groups that were administered with fructose post weaning only, and those that received fructose pre and post weaning when compared to the control group.

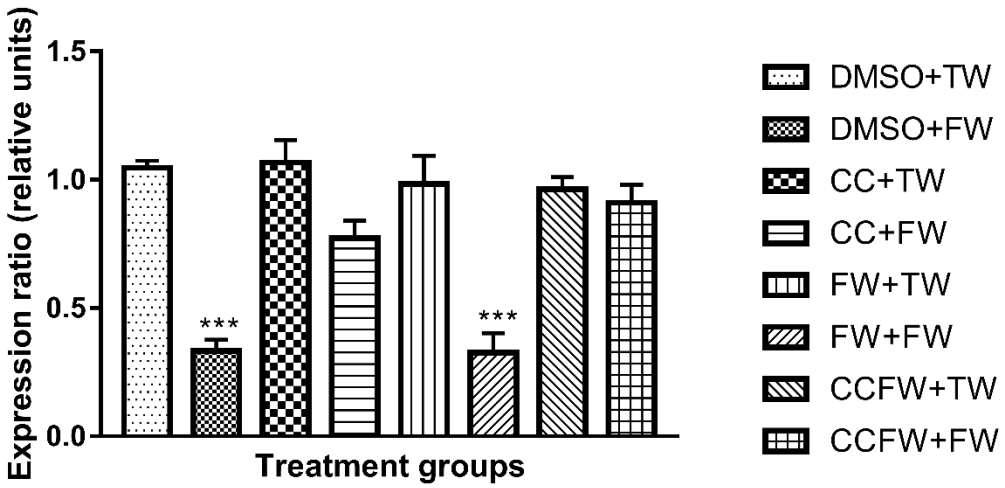


Figure 4. 9: Effects of administration of curcumin during suckling on the gene expression ratio of AMPK α in male adolescent Sprague Dawley rats that were fed a high fructose diet post weaning.

***= significantly different at $p<0.0001$.

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

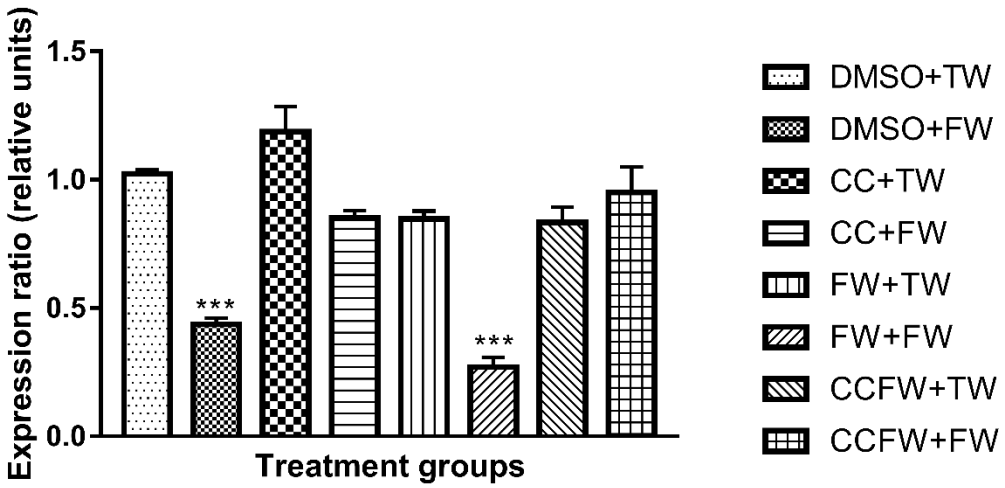


Figure 4. 10: Effects of administration of curcumin during suckling on the gene expression ratio of AMPK α in female adolescent Sprague Dawley rats that were fed a high fructose diet post weaning

***= significantly different at $p<0.0001$.

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

Figures 4.11 and 4.12 show the effect of administration of curcumin during suckling on the expression of TNFα later in adolescence after a high fructose diet post weaning. The expression of TNFα was upregulated significantly ($p<0.0001$, ANOVA) in the groups that were administered with fructose post weaning only, and those that received fructose pre and post weaning when compared to the control group.



Figure 4. 11: Effects of administration of curcumin during suckling on the gene expression ratio of TNFα in male adolescent Sprague Dawley rats that were fed a high fructose diet post weaning.

***= significantly different at p<0.0001, **= significantly different at p= 0.0020

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

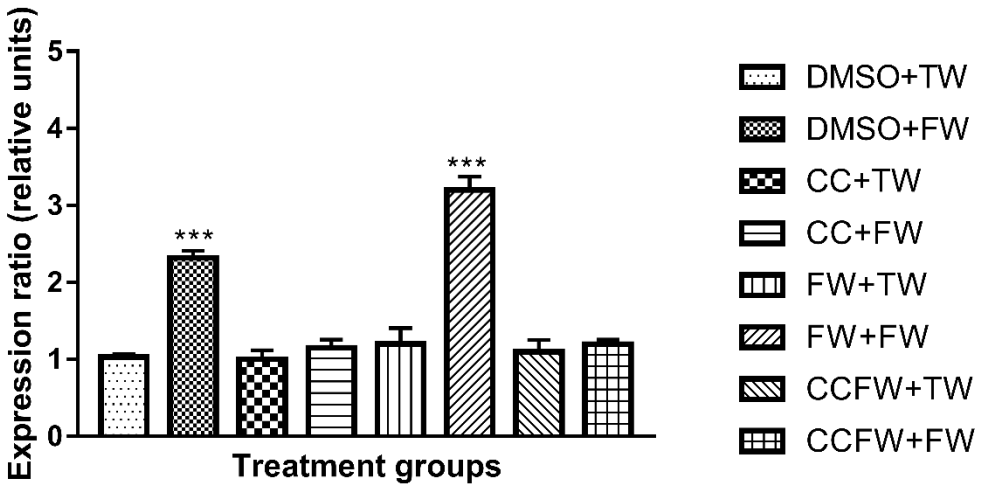


Figure 4. 12: Effects of administration of curcumin during suckling on the gene expression ratio of TNFα in female adolescent Sprague Dawley rats that were fed a high fructose diet post weaning.

***= significantly different at $p<0.0001$.

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

4.4 Discussion

The major findings in this study are those of marked inflammation and fibrosis in the livers of both male and female rats that were orally administered with fructose (during suckling and when administered during suckling and post weaning) in the absence of any significant steatosis and hypertrophy. These inflammatory and fibrotic changes were prevented by neonatal administration of curcumin. Additionally, while the expression of AMPKα was downregulated, that of TNFα was upregulated in the same fructose administered groups described above. As far as we can establish, we are the first to demonstrate that a neonatal intervention with oral curcumin could prevent the future occurrence of high fructose diet-induced liver injury.

We did not observe any differences in the total hepatic lipid content of male rats and though there were some differences between some treatment groups in the female rats, the lipid content was below 5% of the total liver mass in all the groups. A finding of 5% or greater (by weight or volume) steatosis in the livers of individuals, in the absence of alcohol ingestion or other known causes of liver pathology is required for the diagnosis of NAFLD (Chalasani et al., 2012; Ahmed et al., 2015). Although fructose consumption has been implicated in the development of hepatic steatosis (Mamikutty et al., 2015; Alwahsh and Gebhardt, 2017), it was also shown that chronic supplementation with fructose (10%, w/v) for seven months did not induce hepatic steatosis and inflammation in female Sprague Dawley rats (Sangüesa et al., 2018).

Our histological findings of significant inflammation and fibrosis in the absence of significant steatosis are not unusual. It has been suggested that simple hepatic steatosis and NASH may represent different disease entities, thus it is now accepted that in the pathogenesis of NASH, it is not necessarily a case of simple steatosis progressing to NASH (Tilg and Moschen, 2010). The multiple hit theory for the pathogenesis of NAFLD suggests that multiple factors such as high fructose/ fat diets, gut microbiota and insulin resistance acting simultaneously in genetically and epigenetically predisposed individuals result in the development of simple steatosis or NASH depending on the timing and combination of the factors (Buzzetti et al., 2016).

Based on the multiple hit theory of the pathogenesis of NAFLD, we believe there are two possible mechanisms for the hepatic inflammation and fibrosis in our rats. Firstly, it has been established that the unregulated metabolism of fructose in the liver leads to hepatic inflammation and fibrosis (Basaranoglu et al., 2013) mediated through epigenetic means. This inflammation of hepatocytes leads to a stress response in the liver that results in lipid accumulation, indicating that inflammation may precede steatosis in NASH (Tilg and Moschen, 2010).

Secondly, the role of the gut microbiota in the development of NAFLD has been established. For instance, it is known that dysbiosis occurring together with poor diet influences the metabolism of some foods consequently leading to increased production of short chain fatty acids (SCFA), increased endogenous production of ethanol and depleted levels of choline (Abu-Shanab and Quigley, 2010; Leung et al., 2016). The gram-negative bacterial cell wall component, lipopolysaccharide (LPS) induces inflammation which causes liver injury (Abu-Shanab and Quigley, 2010; Vos and Lavine, 2013). Gut microbiota disruption may also cause dysmotility and increased gut permeability to certain bacterial toxic products and hence expose the liver to harmful substances that will lead to inflammation and fibrosis of the liver (Leung et al., 2016). Our first intervention was during the immediate postnatal period when the gut microbiota was most likely still being established and therefore it (intervention) might have interfered with the gut process leading to the inflammation we observed in the livers of the rats. There is need therefore for further studies on the long-term effects of curcumin intake on the gut microbiota.

Curcumin is well known for its anti-inflammatory and antioxidant properties (Aggarwal, 2010; Kunnumakkara et al., 2017b) and acts via interaction with multiple molecular targets, altering gene expression through epigenetic means and signalling pathways (Inzaugarat et al., 2017).

One of the possible mechanisms that could explain the absence of inflammation in the livers of rats that were administered with curcumin as neonates is the gut microbiota modifying effects of curcumin. Curcumin was previously reported to change the composition and abundance of some bacterial species in the gut and reverse endotoxaemia in high-fat diet fed rats by improving the integrity of the intestinal wall (Feng et al., 2017). In high fat fed male Sprague Dawley rats that were orally gavaged with a single daily dose of 200mg.kg^{-1} of

curcumin for eight weeks, it reduced the damage to the intestinal mucosal barrier (Hou et al., 2017). Curcumin apparently increased the expression of occludin and decreased the concentration of tumour necrosis factor alpha (TNFα) and LPS in the intestinal mucosa (Hou et al., 2017).

Secondly, curcumin is thought to protect the liver from steatosis through a mechanism that involves the activation of adenosine monophosphate-activated protein kinase (AMPK). AMPK senses stress in the cells (Rousset et al., 2015) and is also activated by some drugs or xenobiotics (Hawley et al., 2010). In addition, butyrate and acetate generated from the fermentation activities of the gut microbiota have also been shown to enhance AMPK activity (Gao et al., 2009).

AMPK is an important mechanistic pathway for the observed effects of polyphenols in metabolic disorders (Li et al., 2011). Activation of AMPK in the liver confers protection against the accumulation of triglycerides and inhibits *de novo* lipogenesis without influencing fatty acid oxidation (Woods et al., 2017). Curcumin activates AMPK and thus protects the liver from developing steatosis (Jiménez-Flores et al., 2014). Previous studies as will be briefly discussed below have corroborated the ability of curcumin to activate AMPK. Curcumin reportedly inhibited hepatic steatosis and lipogenesis secondary to high-fat diet by activating AMPK and downregulating the activity of sterol regulatory binding element protein 1 (SRBEP1) (Li et al., 2011; Kang et al., 2013). When mice fed a high-fat diet were supplemented with low doses of curcumin for six weeks, it activated hepatic total AMPK and inhibited fatty acid synthase (Lee et al., 2013). Curcumin was also shown to regulate lipid metabolism and inhibit steatosis in the livers of high fat high cholesterol-induced obese mice through the activation of AMPK (Um et al., 2013). Western blot on the livers of 15-week old mice treated with 0.75% curcumin mixed in their diet for 8 weeks showed an increased expression of AMPK and peroxisome proliferator-activated receptor gamma (PPARα) and reduced the expression of the nuclear factor kappa B (NF_KB) protein (Jiménez-Flores et al., 2014). Although AMPK is regulated principally by kinases and ubiquitination (Zungu et al., 2011), the determination of the gene expression ratio is an important indicator of the possible epigenetic influences of curcumin on the gene expression/repression of AMPK (Sabari et al., 2017).

In this study, fructose consumption upregulated the expression of TNFα in the livers of both male and female rats. However, neonatal administration of curcumin prevented the upregulation of TNFα when the rats were subjected to a high fructose diet post weaning. TNFα is a potent proinflammatory cytokine (Lambertucci et al., 2018) that plays an important role in the development of NAFLD, in particular, NASH (Kakino et al., 2018). Fructose consumption is known to increase the activity of TNFα (El-Haleim et al., 2016). The metabolism of fructose in the liver initiates hepatocyte cellular stress and induce inflammation (Zámbó et al., 2013). Fructose metabolism in the liver promotes the production of saturated fatty acids that then activates the toll-like receptors 4 (TLR 4) in the liver (Baffy, 2009). Activation of the TLR4 leads to the production and release of TNFα by Kupffer cells which further worsens the inflammatory process in the liver (Jegatheesan and De Bandt, 2017). We speculate that the anti-inflammatory effects of curcumin epigenetically programmed the livers of the rats to prevent the upregulation of TNFα despite the high fructose feeding. There is need though in future studies to investigate possible epigenetic mechanisms such as modification of histone deacetylases and DNA methylation.

Interestingly, the high fructose diet groups that showed upregulation of TNFα in the liver also showed a significant downregulation of AMPKα. It has been shown previously that long term administration of fructose reduces the activity of AMPKα in several tissues, including the liver (Kosuru et al., 2018; Ojeda et al., 2018). However, we found that the administration of curcumin during suckling prevented the downregulation of the AMPKα genes in the liver following a high fructose diet post weaning. We have mentioned earlier that it has been shown that curcumin activates AMPK in the liver and consequently protects against the development of high fructose-induced NAFLD (Kang et al., 2013; Jiménez-Flores et al., 2014). We, therefore, speculate that curcumin administered to rats during suckling epigenetically programmed the rats to resist the down-regulation of AMPK that is associated with a high fructose diet.

Curcumin has in several human and animal studies been shown to exhibit hepatoprotective properties which may be responsible for the observed protection against inflammatory and fibrotic changes in the livers of our experimental rats. When patients with NAFLD in a randomised double-blind placebo-controlled trial were administered with $70mg.kg^{-1}$ per day of an amorphous formulation of curcumin for eight weeks, it reduced the hepatic lipid content

by up to 79% in the curcumin-administered group (Rahmani et al., 2016). In another randomised controlled trial, NAFLD patients were given curcumin 500mg.kg^{-1} in two doses daily for eight weeks resulting in a reduction in hepatic fat content (Panahi et al., 2017b). Curcumin was found to attenuate high fat diet-induced hepatic steatosis in C57BL/6J mice (Inzaugarat et al., 2017) possibly by regulating liver lipid metabolism through the activation of AMP-activated protein kinase (AMPK) (Um et al., 2013). Curcumin diminished liver ectopic lipid accumulation in high fat fed rats (Feng et al., 2017) and reduced the induction and progression of fibrosis in a mouse model of steatohepatitis (Vizzutti et al., 2010). These studies have demonstrated the therapeutic potential of curcumin. Of novelty is the fact that our study has shown its long-term prophylactic potential against diet-induced noalcoholic steatohepatitis.

4.5 Conclusion

In the present study, we showed that administration of curcumin to neonatal rats protected the rats against the subsequent development of fructose-induced NASH in adolescence. The possible mechanism for this protection is via the prevention of the upregulation of TNFα and the downregulation of AMPKα induced by long term fructose administration. This finding suggests a potential prophylactic role for the use of curcumin in the prevention of NASH and hence mitigating the current epidemic in children and adolescents.

The next chapter is on the potential of neonatal orally administered curcumin to program for good bone health and the plasma total osteocalcin levels in adolescent male and female rats that were fed a high fructose diet until adolescence.

CHAPTER FIVE: The potential developmental programming effect of oral curcumin administered during the suckling period on the bone health and plasma total osteocalcin in male and female Sprague Dawley rats fed a high fructose diet post weaning

5.1 Introduction

The prevalence of metabolic syndrome and its resultant deleterious effects are increasing globally (Falkner and Cossrow, 2014). In children and adolescents, the metabolic syndrome epidemic is driven by a rising prevalence of childhood obesity (Ludwig, 2018; Powell, 2018). The increase in childhood obesity has been linked to an increase in the consumption of high fructose-containing foods and physical inactivity (Fulkerson, 2018; Kral, 2018). Childhood obesity is a multisystem disorder that results in severe complications in most systems of the body, including the skeletal system (Han et al., 2010).

It is estimated that bone diseases such as osteoporosis affect about 200 million people globally, with the prevalence being higher in women and the elderly (Vestergaard, 2014; Sözen et al., 2017). In addition to non-medical costs such as reduction in the quality of life, osteoporosis management costs about 17 to 20 billion dollars per annum in the United States (Fischer et al., 2017).

The skeletal system provides the structural framework for the body and also plays an essential role in the regulation of glucose metabolism in the body by virtue of being an essential insulin target tissue (Tangseefa et al., 2018). Bone metabolism or remodelling continually occurs throughout an individual's life and involves tightly regulated processes of formation of new bone tissue and resorption of old tissues (Sánchez-Duffhues et al., 2015). Osteoblasts originate from the bone marrow mesenchyme and undergo continuous differentiation over childhood/adolescence to become bone matrix tissues as well as having an adipogenic function (Ganguly et al., 2017).

Osteocalcin is a bone-derived hormone that is exclusively secreted by osteoblasts and then released into the general circulation (Kanazawa, 2015). The hormone (osteocalcin) undergoes carboxylation of three of its glutamyl residues which then enhances its binding to hydroxyapatite in the bone matrix (Yeap et al., 2015). There is a second form of osteocalcin which is undercarboxylated. This undercarboxylated form of osteocalcin plays an active role in glucose metabolism by serving as a link between bone metabolism and glucose metabolism (Kanazawa, 2015). Undercarboxylated osteocalcin facilitates insulin secretion directly by inducing the proliferation of the pancreatic β-cells and indirectly through its stimulation of the secretion of glucagon-like peptide 1 from the intestines (Tangseefa et al.,

2018). Osteocalcin also stimulates the secretion of adiponectin from adipocytes, further enhancing insulin sensitivity in the tissues (Kanazawa, 2015). Through a feedforward mechanism, both insulin and adiponectin stimulate the expression of osteocalcin in the osteoblasts (Kanazawa, 2015). The osteoblasts have insulin receptors which control osteoblast development and subsequent secretion of osteocalcin (Fulzele et al., 2010). Therefore a reduction in the blood osteocalcin concentration might disrupt the insulin signalling mechanism possibly leading to the development of insulin resistance (Saleem et al., 2010).

There is a strong association between metabolic syndrome, its individual components and bone health (Muka et al., 2015). Inflammation, a prominent feature of metabolic syndrome is also a common finding in bone disease (Wong et al., 2018). The elevated levels of inflammatory cytokines in metabolic syndrome are thought to activate the receptor activator of nuclear factor kappa B(NF- kB) (RANK)/ receptor of NF- kB ligand (RANKL)/osteoprotegerin pathway and thus promote osteoclast formation and bone resorption (Weitzmann, 2013).

Though bone remodelling occurs throughout life (Sánchez-Duffhues et al., 2015), most of the bone mass increases in humans usually occur during adolescence and the peak bone mass is achieved during this developmental stage (Boot et al., 2010). Events that interfere with these bone mass increases result in suboptimal peak bone masses at adolescence and then subsequently increase the risks of developing bone diseases, including fractures in old age (Rizzoli et al., 2010; Lee, 2013). There is evidence that programming for long-term bone health occurs during the early critical periods of developmental plasticity, a phenomenon referred to as developmental programming (Devlin et al., 2013; Vickers, 2014). This phenomenon whereby stressor events like nutritional perturbations in the critical periods of development alter gene expression and modify the individual's physiology, can influence the attainment of peak bone mass and therefore predispose to bone diseases in later life (Firth et al., 2017). The period of developmental plasticity extends from pre-conception to infancy (Firth et al., 2017) and during this critical window, epigenetic changes such as DNA methylation, histone modification and micro RNAs production are usually induced or repressed by dietary and environmental factors (Park et al., 2017a). Interventions in the suckling period in rats have been shown to either program or reprogram for metabolic health

in later life. For example, neonatal administration of leptin in Wistar rats prevented the bone suppressive effects of maternal undernutrition in adult offspring (Firth et al., 2017).

The administration of a high fructose diet to rats is an established model for inducing metabolic syndrome (Tappy and Mittendorfer, 2012; Mamikutty et al., 2014). Because of the important role the skeletal system plays in metabolism, the metabolic changes induced by fructose may affect bone health (Bass et al., 2013). Interestingly, the effects of fructose on bone health have been rather conflicting with some studies reporting beneficial effects while others reported negative effects. For instance, feeding a high fructose diet for 12 weeks was reported to lead to the formation of stronger bones with a better microarchitecture when compared to glucose in a rat model (Bass et al., 2013). Jatkar et al. (2017) also reported no negative effects on the bones of male BALB/cByJ (BALB) mice following 15 weeks of consuming a high fructose diet. However, fructose has been shown to lead to the generation of reactive oxygen species in the body leading to oxidative stress that then causes an imbalance in the bone remodelling process by increasing bone resorption through stimulation of osteoclast activity (Yarrow et al., 2016).

Management of poor metabolic and bone health usually requires chronic or lifelong medications which in addition to poor patient compliance, are also associated with side effects (Inamdar and Kulkarni, 2017). Natural products such as plant polyphenols which are perceived to be safe and readily available are currently being extensively researched for their benefits in treating chronic conditions (Tsuda, 2012; Bahmani et al., 2014). Some of these natural products have been used during suckling in rats as mentioned earlier, to program for long term protection against some metabolic diseases in later life (Lembede et al., 2018; Nyakudya et al., 2018).

Curcumin (diferuloylmethane) is a biologically active component of turmeric. It is a dietary polyphenol from the dried rhizome of *Curcuma longa* of the ginger plant family, Zingiberaceae (Chainani-Wu, 2003; Sahebkar, 2013). Curcumin has demonstrated a wide array of biological activities in both *in vitro* and *in vivo* studies. Some of the documented biological activities of curcumin include anti-obesity (Shao et al., 2012), anti-diabetic (Panahi et al., 2017a), anti-oxidant (Ali et al., 2018) and anti-inflammatory (Elham et al., 2018). Additionally, curcumin has demonstrated beneficial effects in promoting good bone health by increasing bone strength and microarchitecture in an ovariectomised postmenopausal rat model (Putnam et al., 2007; French et al., 2008).

Considering the previously established beneficial activity of curcumin against the components of metabolic syndrome and its ability to promote good bone health, we hypothesised that its (curcumin) administration during the suckling period in rats would program for improved bone health later in adolescence in rats fed a high fructose diet post weaning.

5.2 Materials and methods

5.2.1 Ethical clearance and study location

As previously reported in chapter three (3.2.1).

5.2.2 Study animals: housing and care

As previously reported in chapter three (3.2.3).

5.2.3 Chemicals and fructose used in the study

As previously reported in chapter three (3.2.2).

5.2.4 Study design

As previously reported in chapter three (3.2.4).

5.2.5 Body mass measurements

As previously reported in chapter three (3.2.5).

5.2.6 Terminal procedures

In addition to the terminal procedures described in chapter three (3.2.6), blood was taken via cardiac puncture and transferred into heparinised blood collecting tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The blood was subsequently processed in a centrifuge (Hermle Z 230A, B Hermle AG, Germany) at 4000 x g at 4ºC for 15 minutes and the plasma was collected and stored in microtubes (Eppendorf, Hamburg, Germany) at -80ºC (ESCO, Lexicon® ULT Freezer, Hatboro, PA, USA) for osteocalcin assay. The carcasses of the rats were stored in a freezer until dissection of the long bones.

5.2.7 Determination of bone morphometric parameters

The carcasses were removed from the freezer and allowed to thaw overnight at 4°C. The left hind limbs were carefully detached from the acetabulum and the femora and tibiae were defleshed with a scalpel and scissors. The two bones were disarticulated from each other and dried in an oven (Salvis) at 50°C for 5 days till constant masses were achieved. The masses of the bones were determined using an electronic weighing scale (Precisa 310M, Instrulab, Johannesburg, South Africa). The lengths of the bones were then determined using a digital Vernier calliper (Major Tech (Pty) Ltd., KTV 150 digital calliper, Elandsfontein, South Africa).

The relative densities of the bones were computed using the Seedor index (Seedor et al., 1991) as follows:

Seedor index = mass/length ($mg.mm^{-1}$).

The higher the Seedor index, the denser the bone (Monteagudo et al., 1997).

5.2.8 Radiographic assessment of the relative bone densities

Radiographs of representative samples of tibiae and femora from each treatment group were taken using a Shimadzu Mux 200 X-ray machine (Shimadzu Corporation, Kyoto, Japan)**.** The bones were placed on a Fujifilm FCR 24×30 cm IP Cassette type CC at a 100 cm distance from the radiation light source. Settings of 2.8 MAS and 58 kV were used for exposure. The radiographs were digitised using a Fujifilm FCR PRIMA and viewed using MeDiViewer (version 1.3.2, JDK 1.3.1_08, Schaf Systemtechnik GmbH, Falkenstrabe 22, 91580 Petersaurach).

5.2.9 Determination of plasma osteocalcin concentration

Total plasma osteocalcin concentration was determined using a commercial rat-specific sandwich enzyme-linked immunosorbent assay kit (Elabscience®, Rat OC/BGP (Osteocalcin) ELISA kit, Houston, TX, USA). The ELISA protocol was followed according to the manufacturer's instructions (sensitivity, 0.47ng.ml^{-1}; detection range, 0.78-50ng.ml^{-1}; standard curve range, 0-50ng.ml^{-1}). The plasma osteocalcin concentrations (ng. mL^{-1}) were extrapolated from the generated standard curve.

5.2.10 Statistical analysis

Data were analysed using GraphPad Prism version 7.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) and expressed as mean ± standard error of the mean (SEM). Data were analysed using a one-way analysis of variance (ANOVA), followed by a comparison of the means using the Bonferroni *post hoc* test. Statistical significance was assumed when $p \leq 0.05$.

5.3 Results

5.3.1 Lengths, masses and Seedor indices of femora and tibiae

Tables 5.1 and 5.2 show the effect of the interventions on the lengths, masses and Seedor indices of femora and tibiae of male and female rats respectively. There were no differences ($p>0.05$, ANOVA) across the treatment groups in the lengths, masses and Seedor indices of the femora and tibiae of the male and female rats.

Table 5. 1: Effect of a high fructose diet on the lengths, masses and Seedor index of the bones of male Sprague Dawley rats that were fed curcumin during suckling

	Femur			Tibia		
Treatment groups	**Length (mm)**	**Mass (mg)**	**Seedor index ($mg.mm^{-1}$)**	**Length (mm)**	**Mass (mg)**	**Seedor index ($mg.mm^{-1}$)**
DMSO + TW	32 ± 0.46	479 ± 16	15 ± 0.32	36 ± 0.53	400 ± 13	11 ± 0.23
DMSO + FW	32 ± 0.44	484 ± 11	15 ± 0.17	36 ± 0.29	378 ± 14	10 ± 0.33
CC + TW	33 ± 0.61	488 ± 21	15 ± 0.38	37 ± 0.53	414 ± 14	11 ± 0.26
CC + FW	32 ± 1.20	442 ± 32	14 ± 0.69	36 ± 0.51	364 ± 29	10 ± 0.73
FW + TW	33 ± 1.10	526 ± 29	16 ± 0.37	37 ± 0.57	441 ± 15	12 ± 0.33
FW + FW	31 ± 0.43	468 ± 14	15 ± 0.28	36 ± 0.41	386 ± 12	11 ± 0.29
CCFW + TW	33 ± 0.93	508 ± 21	15 ± 0.45	37 ± 0.56	424 ± 14	11 ± 0.23
CCFW + FW	31 ± 0.70	468 ± 30	16 ± 1.10	36 ± 0.87	394 ± 19	11 ± 0.39

DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 6-8 per group.

Table 5. 2: Effect of a high fructose diet on the lengths, masses and Seedor index of the bones of female Sprague Dawley rats that were fed curcumin during suckling

	Femur			Tibia		
Treatment groups	**Length (mm)**	**Mass (mg)**	**Seedor index ($mg.mm^{-1}$)**	**Length (mm)**	**Mass (mg)**	**Seedor index ($mg.mm^{-1}$)**
DMSO + TW	30 ± 0.42	432 ± 16	14 ± 0.35	35 ± 0.33	354 ± 11	10 ± 0.23
DMSO + FW	30 ± 0.30	414 ± 17	14 ± 0.43	35 ± 0.27	335 ± 14	9.7 ± 0.34
CC + TW	32 ± 0.93	438 ± 11	14 ± 0.39	35 ± 0.27	358 ± 9.6	10 ± 0.20
CC + FW	30 ± 0.16	431 ± 9.5	14 ± 0.28	35 ± 0.21	354 ± 4.2	10 ± 0.08
FW + TW	30 ± 0.15	457 ± 6.2	15 ± 0.15	36 ± 0.91	368 ± 6	10 ± 0.31
FW + FW	31 ± 0.53	449 ± 14	15 ± 0.46	35 ± 0.17	370 ± 5.5	11 ± 0.12
CCFW + TW	31 ± 0.32	463 ± 15	15 ± 0.34	37 ± 1.3	369 ± 8.1	10 ± 0.23
CCFW + FW	30 ± 0.56	433 ± 28	14 ± 0.66	35 ± 0.51	343 ± 15	9.9 ± 0.34

DMSO + TW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= $10ml.kg^{-1}$ of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin ($500mg.kg^{-1}$ in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin ($500mg.kg^{-1}$) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 6-8 per group.

5.3.2 Radiographs of representative samples of femora and tibiae

The representative radiographic images of the femora and tibiae of male and female Sprague Dawley rats that were administered with curcumin during the suckling period and subsequently fed a high fructose diet post weaning are presented as figures 5.1 and 5.2. There were no discernible differences in the density of both the femora and tibiae of male and female rats across the treatment groups.

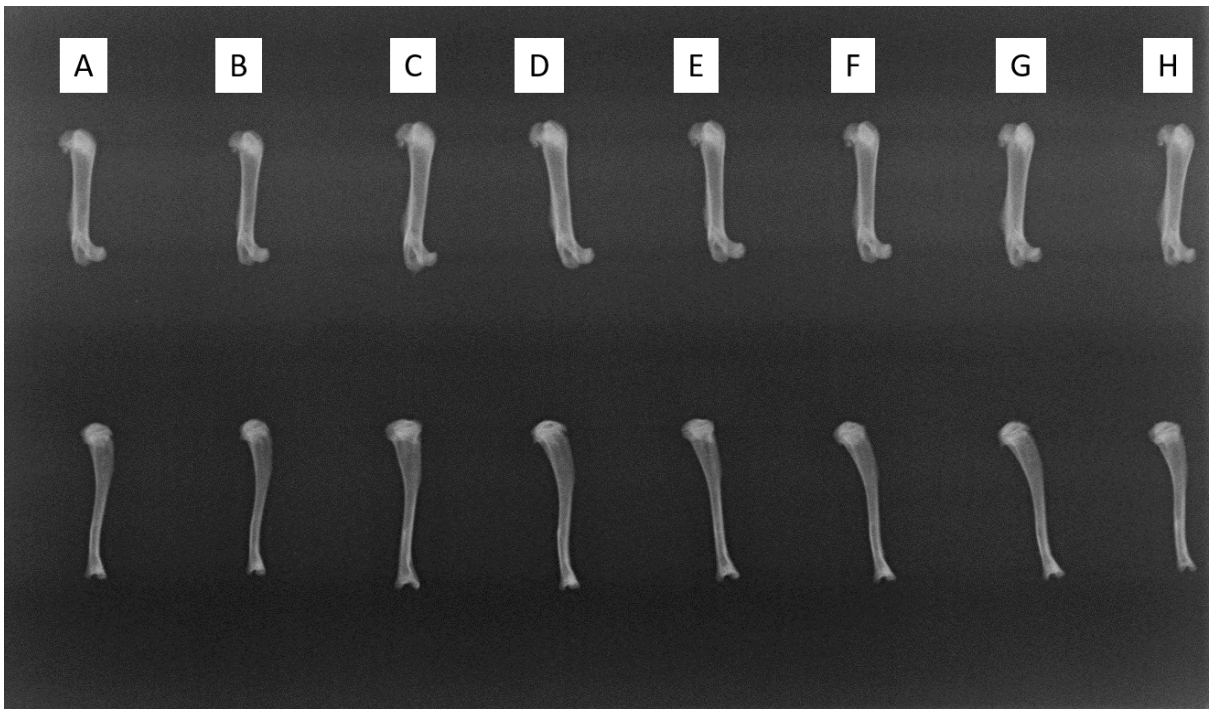


Figure 5. 1: Representative radiographs showing the effect of a high fructose diet on the femora (top) and tibiae (bottom) of adolescent male Sprague Dawley rats that were administered with curcumin during suckling.

A= DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, B= DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, C= CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, D= CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, E= FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, F= FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, G= CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, H= CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Representative images of n= 6-8 per group are shown.

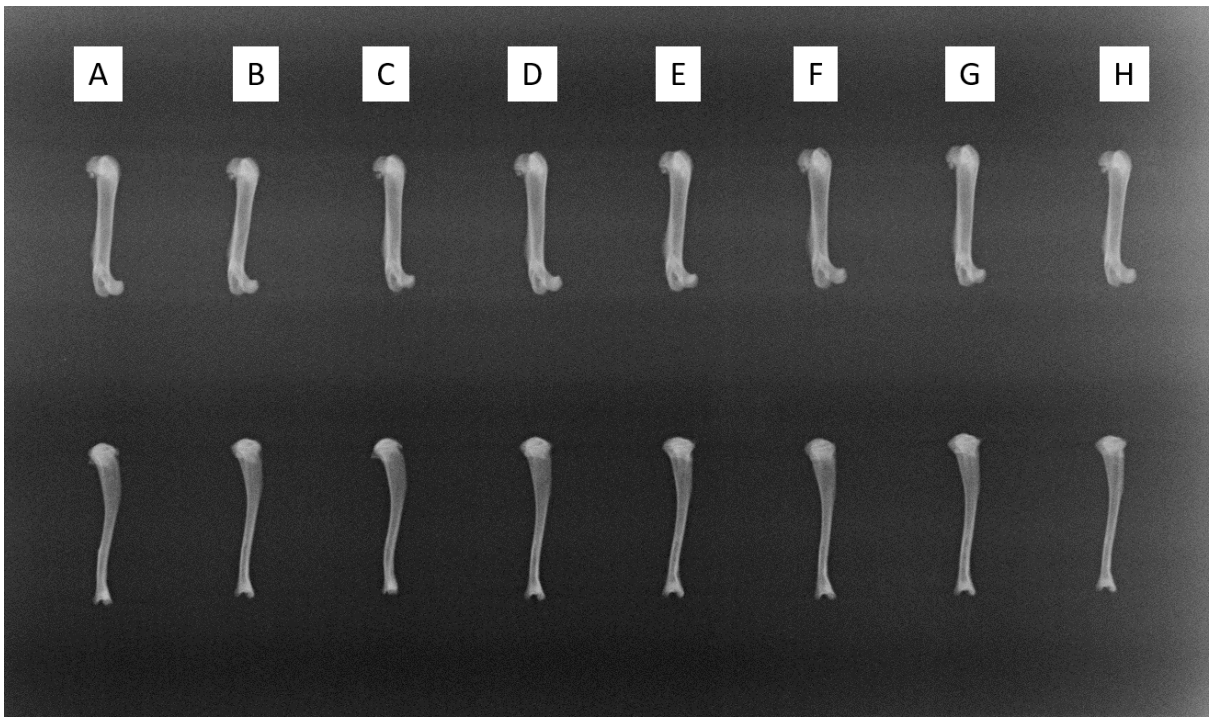


Figure 5. 2: Representative radiographs showing the effect of a high fructose diet on the femora (top) and tibiae (bottom) of adolescent female Sprague Dawley rats that were administered with curcumin during suckling.

A= DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, B= DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, C= CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, D= CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, E= FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, F= FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, G= CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, H= CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Representative images of n= 6-8 per group are shown.

5.3.3 Plasma osteocalcin concentration

The effect of a high fructose diet on the plasma concentration of osteocalcin in male and female Sprague Dawley rats that were orally administered with curcumin during the suckling period are presented in figures 5.3 and 5.4. Male rats that were administered with curcumin alone and those administered with a combination of curcumin and fructose during the suckling period and subsequently fed a high fructose diet had significantly lower ($p \leq 0.05$, ANOVA) total plasma osteocalcin concentration compared with that of rats from other treatment groups. In the female rats, administration of curcumin alone or combined curcumin and fructose during the suckling period and then subsequent feeding with a high fructose post weaning resulted in significantly lower ($p \leq 0.05$, ANOVA) total plasma osteocalcin concentration compared with administration of DMSO during the suckling period and then feeding with a high fructose diet post weaning.

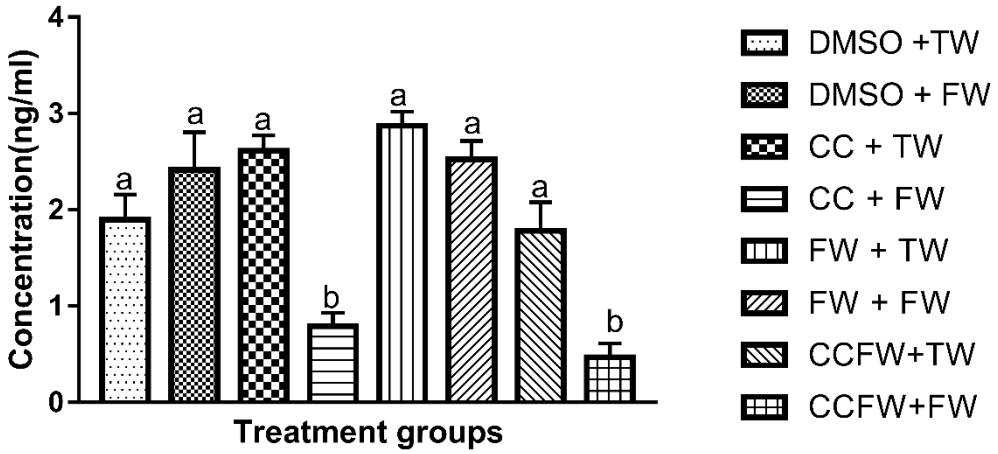


Figure 5. 3: Effect of a high fructose diet on the plasma concentration of total osteocalcin of male Sprague Dawley rats that were administered curcumin during the suckling period.

a, b, c, d= bars with different letters are significantly different at $p \leq 0.05$. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 6-8 per group.

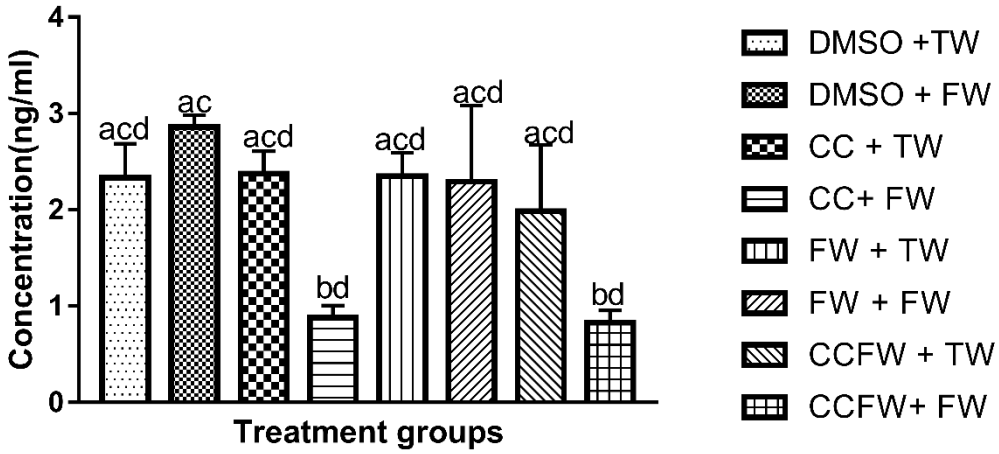


Figure 5. 4: Effect of a high fructose diet on the plasma concentration of total osteocalcin of female Sprague Dawley rats that were administered curcumin during the suckling period.

a, b, c, d= bars with different letters are significantly different at $p \leq 0.05$. DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post-weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water post-weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post-weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post-weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post-weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) post-weaning. Data expressed as mean ± SEM, n= 6-8 per group.

5.4 Discussion

The major finding in this study was that administration of curcumin alone or in combination with fructose during the suckling period resulted in a reduced total plasma concentration of osteocalcin following a postweaning high fructose diet in both male and female rats. The total plasma osteocalcin comprises of the carboxylated and undercarboxylated forms of osteocalcin, thus, the findings could be interpreted in two different ways. Whereas, a low concentration of carboxylated osteocalcin implies a low bone turnover, reduced bone loss and good bone health; a low concentration of undercarboxylated osteocalcin could imply an impairment of glucose metabolism probably because of the postweaning high fructose diet.

A high fructose diet is known to induce features of metabolic syndrome including hyperglycaemia and insulin resistance (Stanhope and Havel, 2010; Tappy and Mittendorfer, 2012). The low levels of osteocalcin that were noted only in the high fructose groups that had previously been administered with curcumin during the suckling period could imply that curcumin predisposed the rats to the adverse effects of the post-weaning high fructose diet. Osteocalcin is an important non-collagenous protein biochemical marker of bone turnover that is found in abundance in the bone matrix (Nikel et al., 2018). Most of the osteocalcin exists in the carboxylated form and is involved in bone remodelling (Namai et al., 2018). Undercarboxylated osteocalcin is involved in glucose metabolism regulation and its deficiency leads to the development of insulin resistance (Saleem et al., 2010; Namai et al., 2018). Previous studies on the effects of neonatal administration of fructose in rats focussed on its programming effects on metabolism (Huynh et al., 2008; Ghezzi et al., 2012) and not on long term bone health. However, it has been previously shown that a 25% fructose (w/v) administered to 12-week old male Wistar rats did not induce any significant changes in the plasma osteocalcin concentration (Dai et al., 2017). It is therefore not surprising that we did not observe significant effects in the groups that had only fructose without curcumin when compared to the control in both sexes.

Contrary to our findings, curcumin was found to induce osteoblast differentiation by increasing the expression of genes that induce osteoblast differentiation, such as runt-related transcription factor 2 (Runx2) and osteocalcin in an *in vitro* study, (Son et al., 2018). The beneficial effects of curcumin on bone health were further shown in another study involving spinal cord injury subjects with low bone density where it prevented further bone loss and improved the bone density (Hatefi et al., 2018). It is possible that curcumin only lowers osteocalcin levels when a high fructose diet disrupts the positive feedback loop that exists

between osteocalcin and insulin concentrations (Kanazawa, 2015). Therefore, the pre-weaning administration of curcumin may have epigenetically programmed the rats for lower osteocalcin concentration later in life.

We did not find any differences in the bone lengths, masses and Seedor indices in both male and female rats. This further suggests that it is probably the endocrine functions of the bones that were affected by our interventions and not the bone density. Though postweaning feeding with fructose has been reported in several studies not to exert any effect on the morphometry of the femora and tibiae of rats (Ibrahim et al., 2017; Lembede et al., 2018); Douard et al. (2013) had previously reported shorter femora in male Sprague Dawley rats that were fed a 63% fructose diet for 4 weeks when compared to control rats due to decreased calcium absorption and vitamin D deficiency caused by the high fructose diet. Fructose metabolism in the body has been associated with the generation of reactive oxygen species leading to oxidative stress (Yarrow et al., 2016). This oxidative stress induces osteoblast apoptosis which inhibits bone growth (Dai et al., 2017). In our present study, we did not observe any obvious inhibition of bone growth as a result of either the administration of curcumin or the high fructose diet.

Though the use of Seedor index to estimate bone density is acceptable (Monteagudo et al., 1997), measuring bone mineral content and bone mineral density of bones with dual-energy x-ray absorptiometry (DXA) (VanGompel et al., 2017) would have been preferable. Bone breaking strength tests would have given more information on the density of the bones. We measured total osteocalcin concentration and not the different forms which may provide different results and suggest an alternative mechanism.

5.6 Conclusion

Oral administration of curcumin alone or with fructose during the suckling period reduced total plasma osteocalcin concentration following a postweaning high fructose diet. These findings suggest that while the administration of curcumin during periods of developmental plasticity may worsen the disruption of glucose homeostasis induced by a high fructose diet, it probably reduces bone turnover. Thus, despite the potential role for curcumin in the prevention of fructose-induced bone diseases, it should be used with caution in the neonatal age groups.

The next chapter (which has been accepted for publication in the Journal of Biological Sciences) reports on the impact of curcumin when administered orally during the suckling period on the leucocyte telomere lengths of adolescent male and female Sprague Dawley rats that were fed a high fructose diet postweaning.

CHAPTER SIX: Neonatal oral curcumin: effect on telomere lengths of adolescent rats fed a high fructose diet postweaning

6.1 Introduction

Telomeres are specialised noncoding repetitive nucleotide sequences (TTAGGG) found at the end of eukaryotic deoxyribonucleic acid (DNA) (Zierer et al., 2016). Their main function is to ensure chromosome stability during mitosis (Lee et al., 2011) and protect the DNA from damage by the cellular repair processes, fusion of adjacent genes and erosion of genetic material (Zierer et al., 2016). Leucocyte telomere length indicates chronological ageing in cells (Demissie et al., 2006) and is negatively associated with chronic diseases including obesity (Iwama et al., 1998).

In addition to genetics, telomere lengths are determined by environmental factors (Müezzinler et al., 2014) that generate oxidative stress and inflammation (Von Zglinicki, 2000). Telomere length has been shown to be susceptible to programmed development during the early ontogenic periods (Tellechea et al., 2015). Although telomere lengths exhibit high heritability (Mangino et al., 2012), embryonic telomere lengths are subject to the influences of *in utero* and post-natal life conditions (Entringer et al., 2012). These conditions exert stable and long-term effects on the telomeres through epigenetic mechanisms such as inhibition of the deoxyribonucleic acid methyltransferase-1 (DNMT-1) (Shalev et al., 2013). For example, a high maternal folate concentration in early pregnancy was found to correlate with longer foetal telomere lengths compared with those with low folate concentration (Entringer et al., 2015). This finding indicates that foetal telomeres show developmental plasticity that is affected by maternal nutrition and which may accelerate ageing (Qasem et al., 2012).

The period extending from gestation to the end of lactation is a window of developmental plasticity in rats when epigenetic changes can modify the expression or repression of genes (Canani et al., 2011). Nutritional interventions in the neonatal period interfere with the establishment of the normal gut microbiome (Yamazaki et al., 2016) which in addition to its essential role in nutrient absorption and energy homeostasis also serves as a source of short chain fatty acids (SCFA) like butyrate, that acts epigenetically as a histone deacetylase inhibitor (Yamazaki et al., 2016).

Fructose showed a hypomethylating effect in mitochondrial DNA of Sprague Dawley rats when administered as a 20% w/v solution for 14 weeks (Álvarez-Errico et al., 2015). DNA methylation is involved in myeloid cell differentiation from their stem cell progenitors (Kim and Costello, 2017) and exhibits remarkable stability that allows it to be transmitted through subsequent cell divisions (Aggarwal, 2010). Thus, the effects of interventions administered

during the periods of developmental plasticity can have a long-lasting impact on the subsequent generations of circulating blood cells.

Curcumin (diferuloylmethane), a polyphenol found in the dried roots of turmeric (*Curcuma longa*) has antioxidant, anti-inflammatory (Bradford, 2013), anti-obesity (Weisberg et al., 2008) and anti-diabetic (Kalani et al., 2013) effects among other metabolic effects. These effects were shown in both *in vitro* and in *vivo* adult rodent models and human studies. Additionally, it has been shown that curcumin not only controls gene expression through DNA methylation, histone modification or production of non-coding micro RNAs (Qureshi and Mehler, 2010; Khaw et al., 2013) but also inhibits telomerase (Khaw et al., 2013).

We thus investigated whether neonatal oral administration of curcumin and/or fructose would induce epigenetic changes that would affect the relative leucocyte telomere lengths of rats later in adolescence after being fed a high fructose diet.

6.2 Materials and methods

6.2.1 Study location and ethical approval

As previously reported in chapter three (3.2.1).

6.2.2 Chemicals and reagents

The details of curcumin, DMSO and fructose are as previously reported in chapter three (3.2.2). SYBR™ Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Austin, TX, USA), Forward and reverse primers (telomeres and pyruvate kinase) and nuclease-free water (Inqaba Biotech, Pretoria, South Africa) were also used in the study.

6.2.3 Study design

As described in chapter three (3.2.4).

6.2.4 Terminal procedures

The terminal procedures were as previously decribed in chapter three (3.2.6). Additionally, whole blood taken by cardiac puncture was transferred into heparinised blood collecting tubes (Becton Dickinson VACUTAINER Systems Europe, Meylan Cedex, France). Whole blood was used for deoxyribonucleic acid (DNA) extraction either immediately or when not possible, the blood was stored in a fridge (DEFY, Durban, South Africa) at 4ºC.

6.2.5 Deoxyribonucleic acid (DNA) extraction

Genomic DNA extraction from whole blood was done using the NucleoSpin Blood QuickPure kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

6.2.5 Real-time quantitative PCR

Leucocyte telomere lengths were determined using the real-time quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was done with the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Singapore). This method has been previously described (Cawthon, 2002; Raymond et al., 2013). In the present study, we determined the factor by which telomere repeat copy number (T) differed from the copy number of pyruvate kinase (S) (Raymond et al., 2013).

A master mix was prepared for each of the runs (telomeres and pyruvate kinase (PK)). The master mix consisted of PowerUp™ SYBR™ Green Master Mix, Forward and Reverse Primers (telomeres and PK) and nuclease-free water. The PCR runs were conducted in two separate 96-well plates, one each for telomeres and PK. The genomic DNA samples, which were run in duplicates were matched in terms of position in the wells to give the lowest variability (Cawthon, 2002). We pipetted 2µl of sample and 18µl of the master mix in each of the wells. A control DNA sample and a blank (nuclease-free water) were included in the plates and served as a reference sample and endogenous control respectively. Each of the amplification was then run in the RT-qPCR machine using appropriate calibrations. The

relative telomere length was expressed as the ratio of the telomere repeat copy number and the single gene copy number (T/S). The primers used in the study are listed in table 6.1 below.

Table 6. 1: List of primers used in the study

Gene	Forward primer (F) and Reverse prime (R)
Telomere	F:5′CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT- 3′ R:5′-GCCTTGCCTTACCCTTACCCTTACCCTTACCCT TACCCT- 3′
Pyruvate kinase	F: 5'-TGTGGGTGATCTGGTGATTGTGGT-3' R: 5'-AGGCATTTCAGGATACGCTCAGCA-3'

6.2.6 Statistical analysis

Data were analysed using the statistical software GraphPad Prism version 7.0 (GraphPad Software Inc., San Diego, CA) and expressed as means ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to analyse the data, followed by a Bonferroni *post hoc* test to compare the means. The level of significance was set at $p \leq 0.05$.

6.3 Results

Figure 6.1 shows the relative leucocyte telomere lengths of the male adolescent experimental rats that were administered with oral curcumin during the suckling period and a high fructose diet postweaning. The relative telomere lengths were similar ($p > 0.05$, ANOVA) across the treatment groups in the rats. The CC + FW group in the male rats had relatively shorter telomere lengths than the FW + FW group, though not statistically significant ($p = 0.0517$, ANOVA). The relative leucocyte telomere lengths of adolescent female Sprague Dawley rats that were administered with oral curcumin during the suckling period and a high fructose diet postweaning are shown in figure 6.2. The relative leucocyte telomere lengths of the female rats were not statistically different ($p > 0.05$, ANOVA) across the treatment groups.

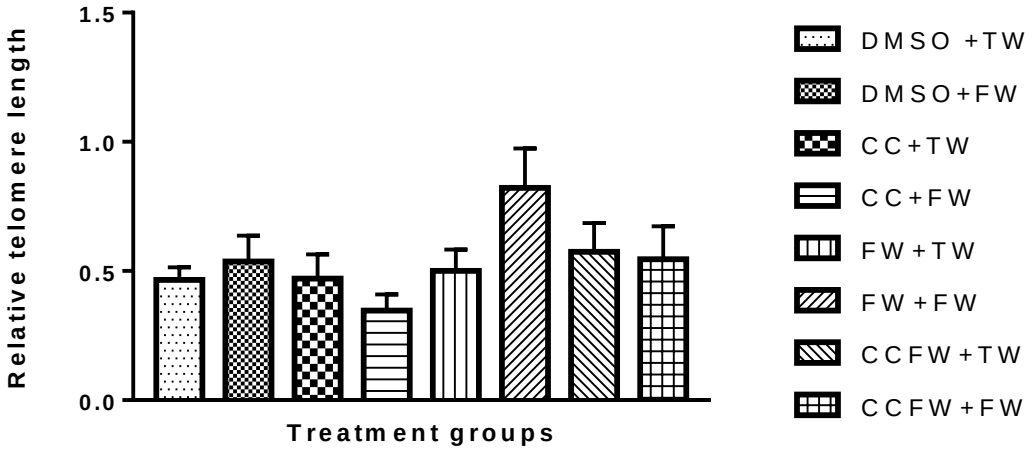


Figure 6. 1: The effect of an oral administration of curcumin and/ fructose during the suckling period on the relative leucocyte telomere lengths of growing male Sprague Dawley rats that were fed a high fructose diet post-weaning

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

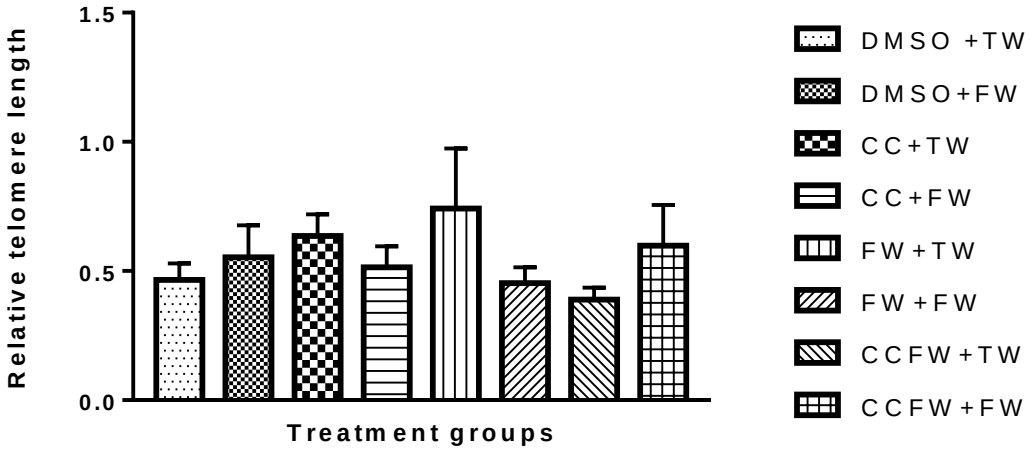


Figure 6. 2: The effect of an oral administration of curcumin and/ fructose during the suckling period on the relative leucocyte telomere lengths of growing female Sprague Dawley rats that were fed a high fructose diet post-weaning

DMSO + TW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and plain tap water post weaning, DMSO + FW= 10ml.kg^{-1} of a 0.5% dimethyl sulfoxide solution as neonates and fructose (20%, w/v) as drinking fluid post weaning, CC + TW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and plain tap water in the post weaning, CC + FW= Curcumin (500mg.kg^{-1} in 0.5% DMSO) as neonates and fructose (20%, w/v) as drinking fluid post weaning, FW + TW= fructose (20%, w/v) as neonates and plain tap water post weaning, FW + FW= fructose (20%, w/v) as neonates and fructose (20%, w/v) as drinking fluid post weaning, CCFW + TW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and plain tap water post weaning, CCFW + FW= curcumin (500mg.kg^{-1}) and fructose (20%, w/v) in 0.5% DMSO as neonates and fructose (20%, w/v) as drinking fluid post weaning. Data expressed as mean ± SEM, n= 7-8 per group.

6.4 Discussion

In this study, there were no observed differences in the relative leucocyte telomere lengths of growing rats of both sexes across the different groups. This means that neither the neonatal oral administration of curcumin and/or fructose nor the post-weaning 6-weeks consumption of a 20% fructose solution (w/v) had any impact on the leucocyte telomere lengths of the rats. Previous studies, as would be presented below, however, have shown the telomere system to be affected by epigenetic modifications while curcumin and fructose are known to induce epigenetic changes.

The human telomerase reverse transcriptase (hTERT) gene promoter controls the appropriate expression of the hTERT gene (Zinn et al., 2007). When DNA methylation or histone modification of this promoter occurs, it represses the activity of telomerase, thus contributing to telomere attrition (Zinn et al., 2007). In addition to telomeres being prone to histone modifications (Gonzalo et al., 2005), the sub-telomeric DNA is also susceptible to methylation (Bellon et al., 2010). Several studies have pointed to a functional connection between the epigenetic mark and telomere lengths (Gonzalo et al., 2005; Gonzalo et al., 2006) which indicates a key relationship between epigenetic changes and the regulation of telomere lengths (Blasco, 2007).

Curcumin has been shown to be a potential epigenetic modulator of telomere lengths *in vitro* through its ability to inhibit deoxyribonucleic acid methyltransferase-1 (DNMT-1) and histone deacetylase (HDAC) (Liu et al., 2009; Qureshi and Mehler, 2010) and to regulate micro-RNAs and their multiple target genes (Teiten et al., 2013). However, despite these observed epigenetic activities, its (curcumin) administration to suckling pups in our study did not have any effect on the telomere lengths of the rats later in adolescence.

Additionally, curcumin has direct actions on telomere lengths as reported in several studies. Through its inhibitory action on telomerase, curcumin was found to promote telomere attrition in an *in vitro* study using brain tumour cells (Khaw et al., 2013) and MCF-7 breast cancer cells (Pirmoradi et al., 2018). Telomerase is a reverse transcriptase enzyme that maintains telomere lengths (Criscuolo et al., 2018). It is highly expressed in tumour cells but is almost undetectable in somatic cells, thus making it a potential tumour marker (Khaw et al., 2013). However, in a mouse model of Alzheimer's disease, diets enriched with curcumin were found to enhance DNA stability and favour a trend for longer telomere lengths (Thomas et al., 2009). In the present study, curcumin had no effect on the telomere lengths of the rats

across the treatment groups, suggesting that it did not have an impact on the activity of telomerase.

A National Health and Nutrition Examination Survey revealed a strong association between the consumption of sugar-sweetened sodas and shorter telomere lengths (Leung et al., 2014). Perhaps another link between fructose consumption and telomere lengths might be through the formation of advanced glycation end products which randomly bind to proteins or lipids and impair their functioning (Nedić et al., 2013). Glycation is usually associated with increased oxidative damage which has been linked to shorter telomere lengths (Nedić et al., 2013).

As earlier mentioned in the introduction, fructose, demonstrated a hypomethylating effect in mitochondrial DNA of Sprague Dawley rats when administered as a 20% w/v solution for 14 weeks (Yamazaki et al., 2016), thus we postulated that the impact of a 20% fructose solution during the period of developmental plasticity and rapid growth might have a programming effect on telomere lengths. However, we have demonstrated that the administration of a high fructose diet during suckling and extending through to adolescence did not affect the leucocyte telomere length of the rats. The phenotypic manifestations of programming may sometimes not appear until later life in especially if the epigenetic modifications affect genes that regulate responses to later environmental challeges such as diets (Gicquel et al., 2008). We therefore speculate that programming of the telomere lengths may have occurred but the overt effects would only appear in later life. It is also possible that the duration of the neonatal interventions was not long enough to induce long term changes in telomere lengths.

The interventions did not have any adverse effects on the growth of the rats as shown by the similarity in the body masses of the rats taken at termination. Previous studies have shown that with obesogenic dietary interventions from the neonatal stage, significant differences in body mass were only noted after 100 days (Patel and Srinivasan, 2010). The rats were 63 days old at the termination of the study which was before the onset of the adolescent surge in sex hormones which also have a sex-specific effect on body mass gain.

6.5 Conclusion

Despite the previously established epigenetic modifying effects of curcumin and fructose, and the susceptibility of the telomere system to epigenetic changes, we did not find any difference in the telomere lengths of the rats in this study. This suggests that the telomere attrition associated with curcumin and fructose intake may not manifest in adolescence but later in life during adulthood.

CHAPTER SEVEN: CONCLUSION, LIMITATIONS AND DIRECTIONS FOR FUTURE STUDIES

The present study investigated the potential of the polyphenol curcumin when administered during the suckling period in rats, to protect against the development of postweaning high fructose diet-induced metabolic derangements in adolescence.

In the first specific objective, I hypothesised that an oral administration of curcumin during the suckling period in rats would protect the rats against the adverse effects of a postweaning high fructose diet on body mass, fasting circulating metabolic substrates, insulin, adiponectin, visceral fat, some visceral organs and kidney histology of adolescent rats. The suckling period is a critical window of developmental plasticity especially in altricial species where most of the body systems are still immature and subject to epigenetic modifications. Thus, dietary interventions during this period may modify the structure and function of either specific tissues or the whole-body system. Generally, the postweaning 6-week high fructose diet feeding did not show any overt effect on the body mass, fasting blood glucose, triglycerides, insulin and adiponectin concentration as well as visceral fat of the rats. Unfortunately, I did not record the feed intake of the rats, though I did observe a reduction in feed intake in the high fructose diet-fed groups. It is safe to say that the rats were compensating for the excess calories from the high fructose by decreasing their feed intake. However, since programming is an epigenetic phenomenon, I suspect that if I had assayed the genes responsible for carbohydrate and lipid metabolism such as the peroxisome proliferator receptor activators (PPAR) or the sterol regulatory element binding proteins (SREBP), I may have seen changes in the expression ratios across treatment groups. I, therefore, recommend that future studies on metabolic programming should interrogate the expression of these genes with a view to shedding more light on the surreptitious early changes that may occur. It would also be insightful to do some epigenetic studies such as the determination of DNA methylation and histone modification status in order to establish whether programming occurred or not. I also believe that my postweaning fructose intervention did not induce derangements of the assayed parameters in the adolescent rats probably due to the ambient temperature of the central animal house where the rats were housed.

Thermoneutral zone is the ambient temperature range at which an animal incurs the lowest energy expenditure to either generate or dispose of heat from the body (Ganeshan and Chawla, 2017). In small rodents like rats, the thermoneutral zone is different depending on whether they are housed individually or in groups (Lutz, 2018). Whereas a temperature range of 28-30°C is considered appropriate for individually housed rats (Abegg et al., 2016), lower temperatures may suffice for group-housed rats due to the possibility of the rats coming together to keep warm (Lutz, 2018). At temperatures below the thermoneutral zone, the rats

would use a large proportion of their total energy expenditure to generate heat in order to achieve and maintain their ideal stable body temperature (Lutz, 2018). It is therefore possible that the growing rats in this study were expending the excess fructose-derived energy to achieve thermoregulation and thus the adverse effects of the high fructose diet did not manifest in this study. Thus, future studies must be designed such that the ambient temperature of the animal house is kept within the thermoneutral zone of the rats. However, I do recognise that there are currently guidelines which need to be adhered to until the consensus on housing conditions for rats is amended.

However, it was notable that the postweaning high fructose feeding induced histopathological changes (increased tubular secretions, glomerular atrophy in the male rats and mesangial proliferation in the female rats) which were prevented by a prior administration of curcumin during the suckling period. However, I did also find that when curcumin was administered during the suckling period with no postweaning exposure to a high fructose diet, it resulted in glomerular atrophy in adolescence. The exact mechanism by which the neonatal administration of curcumin protected the rats' kidneys against the adverse effects of the high fructose diet is not clear and should be investigated further. Additionally, although Mason's Trichrome stain was able to show histopathological changes in the kidney tissues, other stains such as the Periodic Acid-Schiff (PAS) would have helped in identifying the specific type of tubular secretion and thus shed light on the possible mechanisms involved in the pathology. In this study, light microscopy which has limitations in magnification and resolution due to the limited wavelength of the viewing light was used. Thus, micro changes in the kidney tissues that may point to early kidney disease could have been missed. In future, studies should utilise the more precise electron microscopy and immunohistochemical studies to demonstrate early changes in kidney architecture.

The second specific objective of the study was preceded by the hypothesis that the administration of curcumin to suckling pups would protect adolescent male and female Sprague Dawley rats against the development of high fructose-induced non-alcoholic fatty liver disease when they are fed a high fructose diet post-weaning. I have shown in this study, that postweaning feeding with a high fructose diet-induced hepatic inflammation and fibrosis in the liver tissues of adolescent male and female rats which were prevented by a prior neonatal administration of curcumin. The exact mechanism by which curcumin prevented these changes (inflammation and fibrosis) still needs further elaboration. However, I hypothesise that the antioxidant activity of curcumin may have programmed the livers of the rats for reduced gene expression of TNFα and enhanced expression of the AMPK gene which

then mitigated the development of inflammation and fibrosis in adolescence. In defining NAFLD, steatosis is referred to as the accumulation of triglycerides in the hepatocytes (Chalasani et al., 2012; Kim et al., 2018). For this study, I quantitatively determined the total lipid content in the livers which includes both triglycerides and other lipids. Therefore, I recommend that future studies could qualitatively and specifically determine the triglyceride content in the liver tissue to assess the steatosis. Future studies should also consider immunohistochemical staining to detect the presence of activated macrophages and their markers in liver tissues. This would help to further evaluate the extent of hepatic inflammation.

My third hypothesis was that the administration of curcumin to suckling pups would affect the bone health of male and female Sprague Dawley rats in adolescence when they are fed a high fructose diet post-weaning. In this study, I have shown that oral administration of curcumin alone or with fructose during the suckling period reduced total plasma osteocalcin concentration in rats that were fed a high fructose diet postweaning until adolescence. Osteocalcin exists in two forms; carboxylated and undercarboxylated. The carboxylated type of osteocalcin is involved in bone health and its low concentration indicates decreased bone turnover and good bone health. Undercarboxylated osteocalcin plays an important role in glucose homeostasis. However, in this study, I assayed for the plasma total osteocalcin and not the different forms, and therefore it might be difficult to draw conclusions on the mechanisms involved. Future studies should, therefore, determine the different specific forms of osteocalcin in order to determine the precise effect of curcumin on the bone. In this study, I used the mass length ratios of the tibiae and femora to estimate the density of the bones. Though the use of the mass length ratio is an acceptable method of determining bone density (Monteagudo et al., 1997), dual-energy x-ray absorptiometry (DXA) is the gold standard for the measurement (VanGompel et al., 2017). Hence, I recommend that future studies should consider using the DXA to determine bone density. Other biomechanical tests such as the bone breaking strength tests could also provide further information on the density of the bones. Additionally, in future studies, the bones could be crushed, and the calcium and phosphate content determined to give more insight into the impact of neonatal curcumin on the bone mineralisation.

My fourth hypothesis was that the administration of curcumin and or fructose to suckling pups would affect the relative leucocyte telomere lengths of male and female Sprague Dawley rats fed a high fructose diet post-weaning and thus cause accelerated ageing. However, despite

existing evidence that curcumin and fructose impact on telomere lengths, I did not find any differences. To my knowledge, this is the first study to investigate the impact of neonatal oral administration of phytochemicals on the telomere lengths in adolescence. I have shown that the telomere system was not affected by adolescence. There is need however to investigate in future whether if susceptible to developmental programming, the resultantly increased attrition may only occur later in adulthood.

In conclusion, data from my study has shown that the administration of a high fructose diet to male and female rats induced non-alcoholic steatohepatitis (NASH), lowered the plasma total osteocalcin levels and induced histopathological changes in kidney tissues of the rats. I also showed for the first time that the oral administration of curcumin to suckling rats mitigated some of the adverse effects of a high fructose diet such as hepatic inflammation and fibrosis, kidney injury and possibly good bone health. I, therefore, conclude that the oral administration of curcumin during the suckling period most likely programmed male and female adolescent Sprague Dawley rats for protection against the adverse health outcomes associated with a high fructose feeding. However, there is a need for its (curcumin) cautious use as hitherto unreported histopathology may occur.

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APPENDICES

Appendix A: Certificate of ethical approval

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2016/04/18/B

APPLICANT: Mr K Ibrahim

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The potential protective effects of curcumin against diet-induced neonatal programming of metabolic dysfunction in growing rats

Number and Species

40 Female nursing dams Sporague Dawley rats and 400 male and female 6 day old pups Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2016/04/19. This approval remains valid until 2018/04/20.

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: 29 APRIL 2016

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: 29/04/16

cc: Supervisor: Professor K Erlwanger
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix B: Modification and extension of the ethics certificate 1

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: IBRAHIM KASIMU GHANDI

b. School and email address: Physiology; ghandi.kasimu@udusok.edu.ng

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2016	04/18	B
Other M&Es :			2

d. Project Title: The potential protective effects of curcumin against diet-induced metabolic dysfunction in growing rats

		No.	Species
e.	Number and species of animals originally approved:	40 dams; 400 pups	SD rats
f.	Number of additional animals previously allocated on M&Es:	3 dams; 32 pups	"
g.	Total number of animals allocated to the experiment to date:	43 dams; 432 pups	"
h.	Number of animals used to date:	25 dams; 228 pups	"

i. Specific modification / extension requested:

1. Additional co-workers
2. Extension of the duration of the ethical clearance by one year

S/NO	NAME	Email address	Phone number
1.	Miss Hayley Wright	1134909@students.wits.ac.za	071 519 5989
2.	Mashudu Matumba	mgmatumba@gmail.com	078 703 0004
3.	Professor Emmanuel Mukwevho	Emmanuel.mukwevho@nwu.ac.za	018 389 2854
4.	Mr Pilani Nkomozepi	pilanin@uj.ac.za	073 404 5331

k. Motivation for modification / extension:

Miss Hayley is a BHSc honours student in the school of physiology. She will be involved in the dissection of the long bones from the carcasses of the rats and assaying for the markers of bone growth from the plasma samples collected as part of her research project.

Mr Mashudu (North West University) and Prof Mukwevho (North West University) will render technical assistance and participate in the molecular work (assaying for genes involved in carbohydrate and lipid metabolism as stated in the original application).

AESC 2012 M&E

The molecular work will be carried out at North West University, Mmabatho in Prof Mukwevho's laboratory and under his direction. His Laboratory is well equipped for the molecular work which it runs routinely.
Mr Nkomozepi will render assistance with the histological analysis of the tissues already collected from the in vivo work (liver, kidneys and visceral fat).

The current ethical clearance certificate is valid till 2018/04/20. Considering that I still have a couple of Laboratory analysis to carry out, I would like the approval to be extended by one year.

All other procedures will remain unchanged from the originally approved protocol.

Date: 2018/03/06

Signature:

RECOMMENDATIONS

Date: 17/3/2018

Signature:

Chairman, AESC

Appendix C: Modification and extension of the ethics certificate 2

AESC 2017 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: IBRAHIM KASIMU GHANDI

b. School and email address: Physiology; ghandi.kasimu@udusok.edu.ng

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2016	04/18	B
Other M&Es :			1

d. Project Title: The potential protective effects of curcumin against diet-induced neonatal programming of metabolic dysfunction in growing rats

		No.	Species
e.	Number and species of animals originally approved:	40 dams; 400 pups	SD rats
f.	Number of additional animals previously allocated on M&Es:	4 dams; 32 pups	SD rats
g.	Total number of animals allocated to the experiment to date:	44 dams; 432 pups	SD rats
h.	Number of animals used to date:	17 dams; 175 pups	SD rats

i. Specific modification / extension requested:

1. Removal of the high cholesterol diet (2%, w/w)
2. Addition of a co-worker: Malehope Churchill Molopo
3. To return eleven rats to stock

k. Motivation for modification / extension:

In the original approved ethics, during the first experiment the pups will upon weaning be further subdivided into two groups; half of the rats would be on normal rat chow and plain tap water while the other half would be on a high cholesterol diet (2%, w/w) and high fructose solution (20%, w/v).

We observed that after the first 2 weeks on the treatment diets, the rats on the high cholesterol diet were not eating as much as their counterparts on the normal rat chow. We had previously used high fructose (20%, w/v) without adverse effects on the growth of the rats (AESC/2013/46/05). The poor food intake is most likely due to the additional dietary cholesterol.

I would therefore like to remove the cholesterol and continue with normal rat chow and the fructose solution (20%, w/v) alone both in the first and second experiments.

I would also like to include Mr Malehope Churchill Molopo as a co-worker to assist with weighing the rats, preparation and administration of the fructose solution.

I would also like to return eleven rats (which were on cholesterol) to stock. They will not be wasted as they will be used for dissections.

All other procedures will remain unchanged from the originally approved protocol.

Date: 3rd July, 2017

Signature:

RECOMMENDATIONS: Approved. i. Removal of additional cholesterol from diet. ii. Return of 12 rats to stock. iii. Inclusion of Malehope Churchill Molopo as a co-worker.

Date: 7/7/2017

Signature:

Chairman, AESC

Appendix D: summary of findings of the initial pilot study

Validating an appropriate method for induction of metabolic syndrome in weanling Sprague Dawley rats that were administered with curcumin and fructose during suckling

Introduction

The administration of a high fructose diet alone has been shown to be sufficient in inducing metabolic syndrome in rodent experimental models of metabolic disorders (Angelova and Boyadjiev, 2013; Mamikutty et al., 2014). It has even been suggested that a duration as short as a 2-week long feeding with a high fructose diet will induce insulin resistance and hypertriglyceridaemia in adult Sprague Dawley rats (Pagliassoti et al., 1996). However, it has also been shown that the age of the rats impacts their response to a simple sugar diet such as high fructose diets (Pagliassotti et al., 2000). Weanling rats that were fed a high sucrose (68% Kcal) diet for five weeks did not develop insulin resistance and hypertriglyceridaemia as opposed to older rats (Pagliassotti et al., 2000). In contrast to the high sucrose diet, a 5-week high fat (45% Kcal) feeding duration resulted in insulin resistance in weanling rats (Pagliassotti et al., 2000).

Interestingly, when a high cholesterol diet was fed together with high fructose in the drinking water of male Wistar rats for four weeks, it induced obesity, hyperglycaemia, hypertension and dyslipidaemia (Barrios-Ramos et al., 2014; Wong et al., 2016). High cholesterol diets are also known to be risk markers for development of non-alcoholic steatohepatitis (NASH) in high fat fed models of non-alcoholic liver disease (Wouters et al., 2008). For example, when C57BL/6J mice were fed a high cholesterol (1.25%) diet for 3 weeks, it induced hepatic inflammation and steatosis (Ibrahim et al., 2016). In another study using young/adolescent (5-week-old) Ossabaw swine, feeding with a western diet that consisted of 43.0% high fat, 17.8% high fructose and 2% high cholesterol for 16 weeks induced obesity, systemic insulin resistance, dyslipidaemia and dysbiosis of the gut microbiota (Panasevich et al., 2017). Additionally (and probably due to the dysbiosis of the gut microbiota), a severe NASH developed (Panasevich et al., 2017).

Considering previous experiences from our laboratory where a high fructose solution (20%, w/v for 6 weeks) alone provided as drinking fluid, in growing rats did not result in a full blown metabolic syndrome (Ibrahim et al., 2017) but caused some metabolic dysfunction, it was hypothesised that we could achieve greater manifestation of the component

characteristics of metabolic syndrome by using a dietary combination of fructose solution to drink and a high cholesterol diet (Barrios-Ramos et al., 2014; Wong et al., 2016).

The objectives of the study therefore were to determine whether a postweaning feeding with a combination of a high cholesterol diet (2%, w/w) and a high fructose (20%, w/v) drink:

1. will induce metabolic dysfunction showing characteristics of metabolic syndrome in adolescent male and female rats
2. will be prevented by neonatal intake of curcumin from developing characteristics which are associated with metabolic syndrome in adolescent male and female rats.

Materials and methods

Ethical clearance

The study protocols were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand, Johannesburg (AESC 2016/04/18/B). The study complied with international ethical guidelines and standards for the use of laboratory animals.

Study location

The study was conducted in the Central Animal Services (CAS) unit of the University of the Witwatersrand, Johannesburg, Republic of South Africa.

Study animals: housing and care

Sixty-four 6-day old male and female Sprague Dawley pups from eight dams sourced from the Central Animal Services Unit of the University of the Witwatersrand, Johannesburg were used in the pilot study. The rats were kept under similar conditions as described previously in the thesis (Chapters 3,4,5 and 6).

Chemicals and fructose used in the study

For this study, dimethyl sulfoxide (DMSO; Sigma-Aldrich, Missouri, USA), curcumin (Sigma-Aldrich, Missouri, USA), cholesterol powder (Sigma-Aldrich, Missouri, USA) and

fructose (Nature's choice, Randvaal, South Africa) were used. The DMSO was used as the vehicle in which the other treatments were dissolved.

Preparation of the feeds

A pelleted commercially sourced normal rat chow (Epol®, Johannesburg, South Africa) was used to reconstitute the feeds. The pellets were ground into fine powder using a manually operated grinder (Corona®, Bogota, Colombia). The high cholesterol diet was formulated by mixing each 98g of the rat chow powder with 2g of cholesterol and mixing thoroughly. Adequate tap water was added to the resultant powder mixture to convert it into a paste which was dried overnight in an oven (Salvis®, Salvis Lab, Switzerland). The normal rat chow feed was also reconstituted by making it into a paste and drying in the oven as with the high cholesterol feed.

Study design

The study design was also as previously described in the thesis (chapters 3,4,5 and 6, materials and methods section). Briefly, the study was designed in two phases. The first phase (neonatal phase) extended from postnatal day 6 to 21. The aim of this phase of the study was to induce programming of metabolic function. The aim of the second phase which was to extend from postnatal day 21 to 63 (late adolescence/early adulthood) was to determine whether the initial interventions would impact the response of the rats to a high fructose high cholesterol diet.

The pups (n=64: males = 32, females = 32) were allocated in a split-litter pattern to four treatment groups replicated by sex in the first phase of the study. The treatment groups were as follows:

Group 1 (control): DMSO (0.5%).

Group 2: administered curcumin, 500mg.kg^{-1} body mass

Group 3: administered fructose (20% w/v)

Group 4: administered curcumin 500mg.kg^{-1} + fructose (20%, w/v)

All the treatments were administered at 10ml.kg^{-1} body mass daily (between 0830hrs and 1000hrs) using an orogastric tube.

In the second phase of the study which was supposed to run from postnatal day 21 to 63, but had to prematurely end at postnatal day 43, the rats from each of the initial four groups were further sub-divided into two subgroups. One subgroup was weaned onto normal rat chow and plain tap water and the other had fructose (20%, w/v/) as their drinking fluid and a high cholesterol feed (2%, w/w). thus, in the second phase of the study there were 8 treatment groups. While the diets were being administered, the rats were routinely monitored for general health and wellbeing.

Body mass measurements

The pups were weighed daily using a digital weighing scale (Snowrex Electronic Scale, Clover Scales, Johannesburg, South Africa) during the first phase of the study to adjust their treatment concentrations so as to ensure constant dose administration.

In the second phase, the rats were weighed twice weekly to monitor their growth and general health.

Statistical analysis

Data were analysed using GraphPad Prism version 7.0 statistical software (GraphPad Software Inc., San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). Data were further analysed using a one-way analysis of variance (ANOVA), followed by a comparison of the means using the Bonferroni *post hoc* test. Statistical significance was assumed when $p \leq 0.05$.

Results

Figures 1a and 1b show the induction and weaning body masses of male and female rats. There were no differences ($p<0.05$, ANOVA) in body masses across the treatment groups in both male and female rats at induction and weaning. However, both the male and female rats gained significant ($p<0.0001$, ANOVA) body masses from induction through weaning.

Figures 2a and 2b show the body masses of male and female rats that were fed a normal rat chow and those that were fed with a high cholesterol diet at 2 weeks into the feeding treatment. Male and female rats that were fed a normal rat chow were significantly heavier

(males= p=0.0004; females= p<0.0001, ANOVA) than those that were fed a high cholesterol diet. There were no significant differences (P>0.05, ANOVA) in the body masses of the rats within different iterations of the high of the high cholesterol fed rats. There were also no significant differences (P>0.05, ANOVA) in the body masses of the rats within different groups that did not receive the high cholesterol diets. Figures 3a and 3b show the body masses of male and female rats that were fed a normal rat chow and those that were fed with a high cholesterol diet at 4 weeks into the feeding treatment. Male and female rats that were fed a normal rat chow were significantly heavier (males= p<0.0001, 38.69%; females= p<0.0001, ANOVA, 37.36%) than those that were fed a high cholesterol diet. There were no significant differences (P>0.05, ANOVA) in the body masses of the rats within different iterations of the high of the high cholesterol fed rats. There were also no significant differences (P>0.05, ANOVA) in the body masses of the rats within different groups that did not receive the high cholesterol diets.

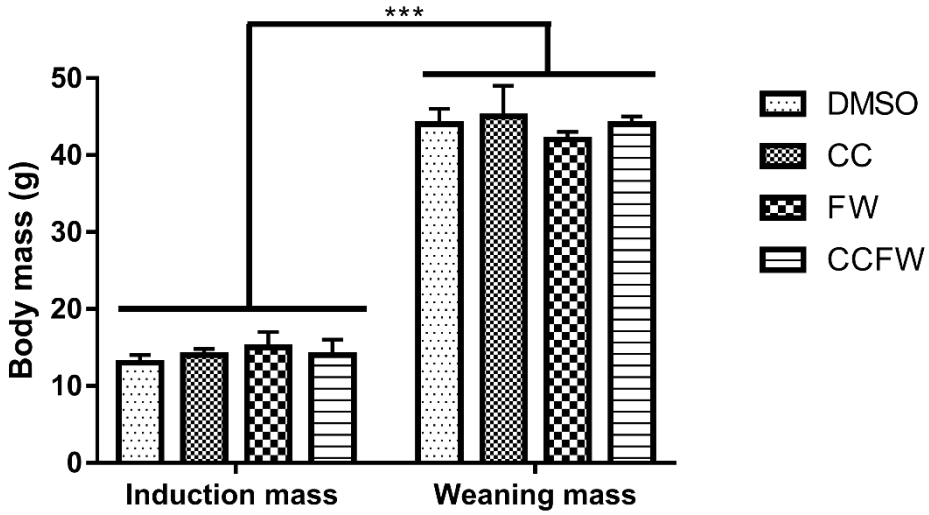


Figure 1a: Body masses of male rats at induction of the study and at weaning

***= significantly different at p<0.0001. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 4 per group.

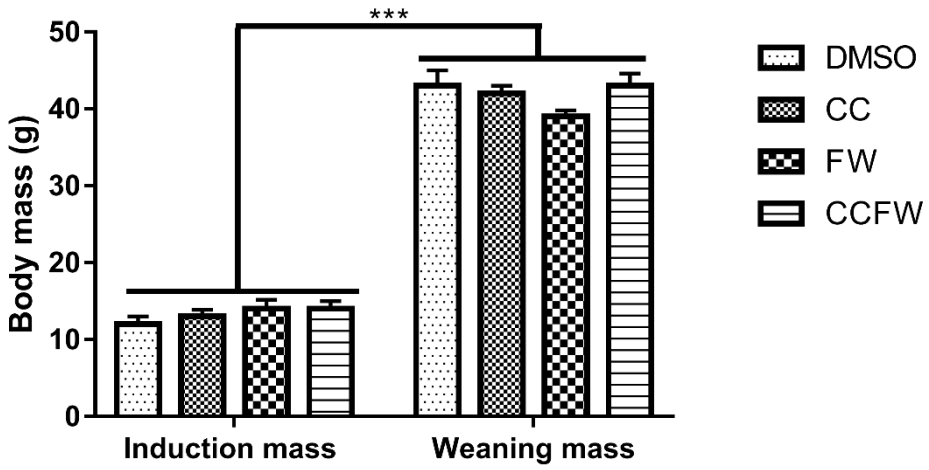


Figure 1b: Body masses of female rats at induction of the study and at weaning

***= significantly different at p< 0.0001. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 4 per group.

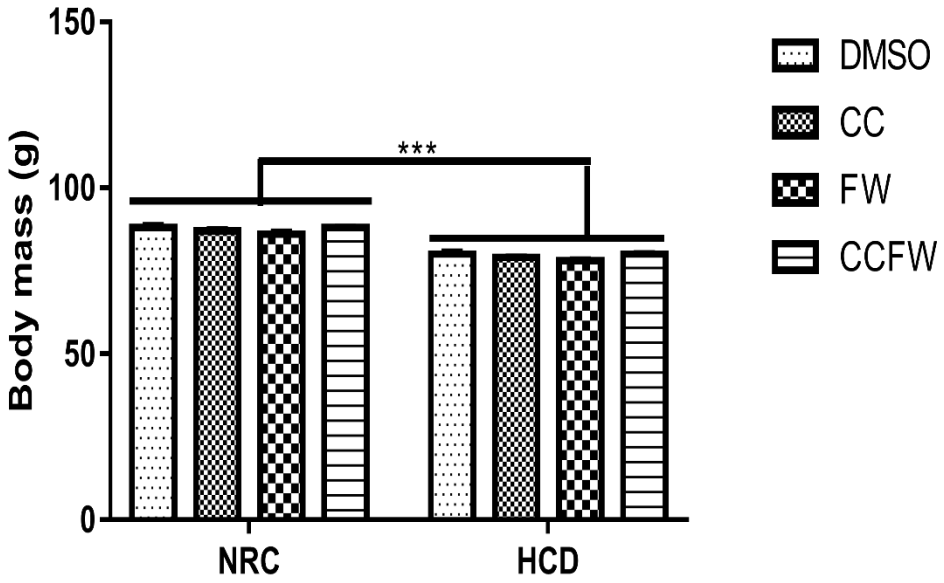


Figure 2a: Body masses of male rats at 2 weeks into the high cholesterol and high fructose diet

***= significantly different at p< 0.0001. NRC= normal rats chow, HCD= high cholesterol (2%, w/w) diet. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 2 per group.

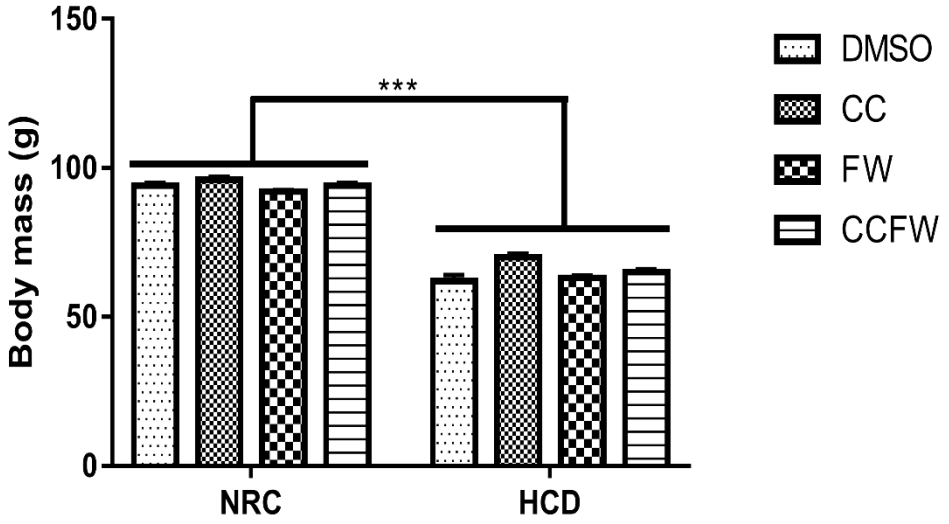


Figure 2b: Body masses of female rats at 2 weeks into the high cholesterol and high fructose diet

***= significantly different at p< 0.0001. NRC= normal rats chow, HCD= high cholesterol (2%, w/w) diet. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 2 per group.

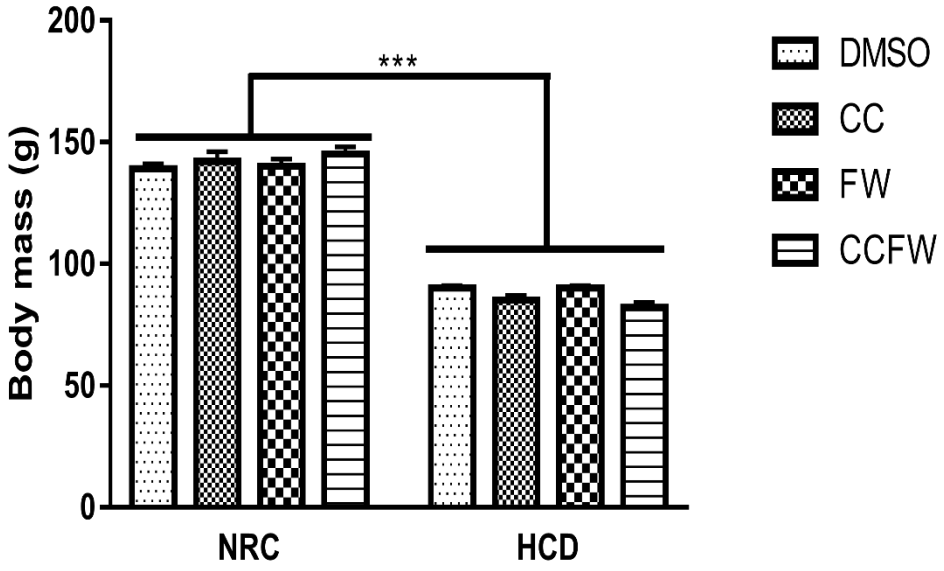


Figure 3a: Body masses of male rats at 4 weeks into the high cholesterol and high fructose diet

***= significantly different at p< 0.0001. NRC= normal rats chow, HCD= high cholesterol (2%, w/w) diet. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 2 per group.

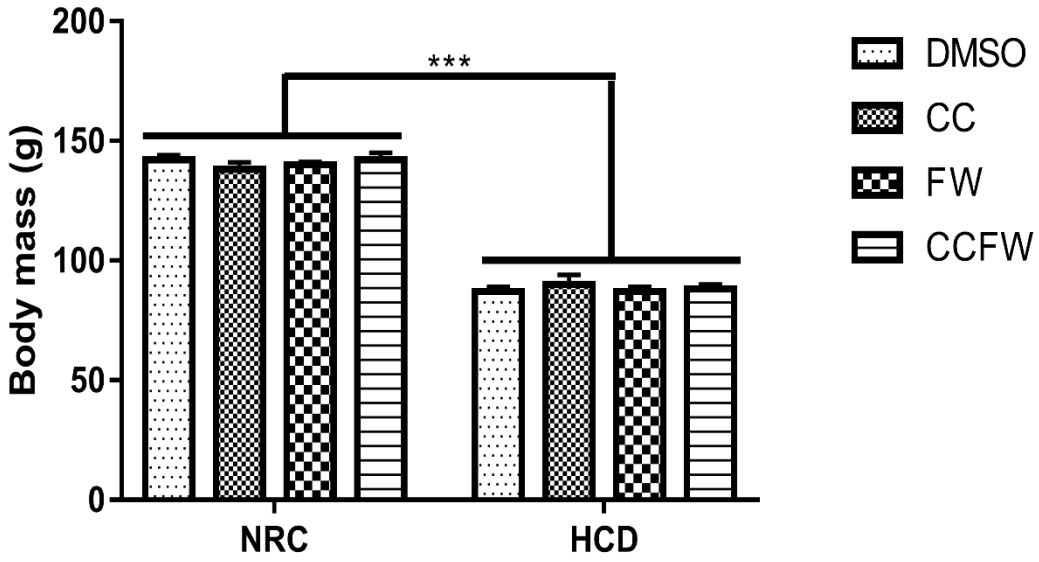


Figure 3b: Body masses of female rats at 4 weeks into the high cholesterol and high fructose diet

***= significantly different at p< 0.0001. NRC= normal rats chow, HCD= high cholesterol (2%, w/w) diet. DMSO= 10ml.kg-1 of 0.5% dimethyl sulfoxide, CC= curcumin (500mg.kg^{-1} in 0.5% DMSO), FW= fructose (20%, w/v in 0.5% DMSO), CCFW= curcumin (500mg.kg^{-1} in 0.5% DMSO) and fructose (20%, w/v in 0.5% DMSO). Data expressed as mean ± SEM, n= 2 per group.

Discussion

By two weeks into the dietary treatments, the rats that were receiving normal rat chow and plain tap water were already significantly heavier than their counterparts that were receiving a high cholesterol diet and fructose drinking water.

Previous studies have shown that animals on a high fat diet tend to show poor weight gain which is followed by catch up growth (Dulloo et al., 2006; Claycombe et al., 2013). As a consequence, the rats were monitored for a further two weeks. At this stage it was noted that the high cholesterol fed rats had body weights which were lagging severely compared to those on the normal feed. In addition, on observation, the rats on the HCD showed poor hair growth and relative lethargy compared to those on the NRC.

The measurement of body mass changes is a convenient method that is employed in monitoring the health and general welfare of laboratory animals (Ahloy-Dallaire et al., 2018). Moreover, our animal research ethics guideline documents identify weight loss or poor weight gain of greater than 15% relative to control animals as a terminal end point for studies. Based on this, the study had to be terminated prematurely by euthanasia of the rats. Due to the relatively poor condition of the rats, and for ethical reasons, no further experiments were conducted on these rats. It would be inappropriate to present metabolic data from the animals showing ill thrift.

Though I did not accurately measure feed intake, I observed that the rats on the high cholesterol diet were not eating as much as their counterparts on the normal rat chow. Previous studies have reported a reduction in feed intake and body mass gain following feeding with high cholesterol diets in female Sprague Dawley rats (Ali et al., 2000) and in male BALB/c mice (Zidani et al., 2017). I had previously used a high fructose diet in growing Sprague Dawley rats without observing any adverse effects on the growth of the rats (Ibrahim et al., 2017). In addition, within the groups of rats fed the high cholesterol diets there were no differences in body mass which could be attributable to the test phytochemical of interest (curcumin). I therefore suspected that the poor feeding in this instance was due to the addition of cholesterol in the diet.

Conclusion

In view of my concerns about the welfare of the rats, I terminated the study prematurely and applied for the modification of my ethical clearance to allow me to start another study using a high fructose solution (20%, w/v) as drinking fluid to induce metabolic dysfunction.

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