THE POTENTIAL OF β-SITOSTEROL TO PROTECT AND TO PROGRAMME FOR PROTECTION AGAINST DIET-INDUCED METABOLIC DERANGEMENTS IN SPRAGUE-DAWLEY RATS

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DECLARATION

I, **Nontobeko Myllet Gumede**, 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. It has not been submitted before for any degree or examination at any other University. Where other sources have been used; they have been appropriately cited and acknowledged.

N. Gumede

Nontobeko Myllet Gumede

Signed on the…18……..day of…November…….2020

DEDICATION

To my mother

Elizabeth Thembi Gumede

Phumula kahle Ndlondlo

1985 to 2003

RESEARCH OUTPUTS

PUBLICATIONS

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ABSTRACT

The consumption of high-fructose diets contributes to the development of metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD), chronic kidney disease (CKD) in children and adults. Neonatal fructose intake programmes for metabolic derangements in later life. β-sitosterol has been shown to have nutritional and health benefits. In two major experiments, I interrogated the potential protective effects of β-sitosterol against the development of high-fructose diet-induced metabolic dysfunction, NAFLD and renal derangements. In the first experiment, I investigated the long-term daily intake of β-sitosterol against high-fructose diet-induced metabolic derangements. Seventy 21-day old Sprague-Dawley rat pups (35 female ; 35 male) were, in an interventional study, randomly allocated to and administered treatments as follows: group I- standard rat chow (SRC) + plain drinking water (PW) + plain gelatine cube (PC); group II- SRC+ 20% w/v fructose solution (FS) as drinking fluid + PC; group III- SRC + FS + 100 mg/kg fenofibrate in gelatine cubes; group IV- SRC + FS + 20 mg/kg β-sitosterol in gelatine cube (Bst) and group V- SRC + PW + Bst. Following 12 weeks of feeding, the rats were fasted overnight, weighed, then euthanised and tissues collected for analyses. Surrogate biomarkers of liver and kidney function, cholesterol, insulin, triglyceride and adiponectin concentrations were determined from plasma. Dietary fructose caused ($p < 0.05$) visceral obesity, hypoadiponectinaemia, micro- and macro-vesicular hepatic steatosis, decreased glomerular density and increased kidney-injury molecule-1 (KIM-1) in growing female and male rats. Additionally it caused ($p < 0.05$) hypertriglyceridaemia in adult female rats only. β-sitosterol prevented the high-fructose diet-induced derangements except the increase in KIM-1.

The second experiment evaluated the potential of neonatal orally administered β-sitosterol to programme for protection against high-fructose diet-induced metabolic derangements in adulthood. Seventy 4-day old Sprague-Dawley rat pups (34 female; 36 male) were acclimatised to handling for two days (PND 4-5) following which they were, in an interventional study, randomly allocated to one of the following treatment regimens: group I - DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v dimethyl sulfoxide (DMSO) at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; group II- FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) in 0.5% v/v DMSO at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning, group III- Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol in 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning and, group IV- Bst + FS = gavaged daily

with a bolus of 20 mg/kg β-sitosterol dissolved in 0.5% v/v DMSO plus 10 mL/kg body mass of 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. The pups were administered their respective treatments daily for 15 days (PND 6-21) and weaned on PND 21. On PND 125 the rats were fasted overnight and then euthanised on PND 126. Fasting blood glucose and triglyceride concentrations were determined and visceral fat pad collected for analyses of visceral obesity.

Long-term high-fructose diet feeding caused NAFLD in adult female and male rats but caused visceral obesity in female rats only. The NAFLD progressed to non-alcoholic steatohepatitis (NASH) only in male rats. Neonatal β-sitosterol (Bst + FS) protected ($p < 0.05$ vs FS + FS) against the high-fructose diet-induced NAFLD in adult female and male rats but failed to prevent ($p > 0.05$ vs FS + FS) the fructose-induced visceral adiposity in female rats.

In conclusion, findings from the first experiment suggest that chronic daily intake of β-sitosterol could potentially be exploited as a preventative intervention against high-fructose diet-induced visceral obesity, hypertriglyceridaemia, hypoadiponectinaemia, macro-vesicular hepatic steatosis and decrease in glomerular density. Findings from the second experiment demonstrated that neonatal orally administered β-sitosterol strategically targeting the developmentally plastic pre-weaning phase may have long-term prophylactic effects against long-term high-fructose diet-induced NAFLD.

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ABBREVIATIONS AND SYMBOLS

♀:	Female
♂:	Male
ALP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
AMPK:	Adenosine monophosphate activated protein kinase
ANOVA:	Analysis of variance
AOAC:	Association of Official Analytical Chemists
AST:	Aspartate aminotransferase
BMI:	Body mass index
BUN:	Blood urea nitrogen
COX-2:	Cyclooxygenase-2
CRP	C-reactive protein
DH:	Distilled water
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DOHaD	Developmental Origins of Health and Disease
ELISA:	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Ex-4	Exendin-4
F-1-P	Fructose-1-phosphate
FS:	20% fructose solution (w/v)
GIT:	Gastro-intestinal tract
GLUT:	Glucose transporter
GPDH:	Glycerol-3-phosphate dehydrogenase
GPx	Glutathione peroxidase
H and E:	Haematoxylin and eosin
HDL-cholesterol	High-density lipoprotein cholesterol
HFCS	High-fructose corn syrup
HMG-CoA	Hydroxy-3-methyl-glutaryl-coenzyme A
IDF:	International Diabetes Federation

IGF	Insulin-like growth factor
IHME	Institute for Health Metrics and Evaluation
IL:	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
IRS-1	Insulin receptor substrate
KIM-1	Kidney-injury molecule-1
LDL-cholesterol	Low-density lipoprotein cholesterol
LI:	Large intestines
MCP-1	Monocyte chemotactic protein-1
MetS:	Metabolic syndrome
NAFLD:	Non-alcoholic fatty liver disease
NAS:	NAFLD Activity Score
NASH:	Non-alcoholic steatohepatitis
OGTT:	Oral glucose tolerance test
PND:	Postnatal day
PPAR:	Peroxisome proliferator-activated receptor
PW:	Plain drinking water
RG:	Reagent grade
SI:	Small intestine
SOD	Superoxide dismutase
SRC	Standard rat chow
STZ:	Streptozotocin
TBIL:	Total bilirubin
T-I-DM:	Type I diabetes mellitus
T-II-DM:	Type II diabetes mellitus
TNBS	Trinitrobenzene sulfonic acid
TNF:	Tumour necrosis factor
TPA	Tetradecanoylphorbol acetate
VLDL	Very-low-density lipoprotein
w/v:	Weight/volume
WC	Waist circumference

WHO:	World Health Organisation
WHR	Waist-to-hip ratio
α:	Alpha
β:	Beta
γ:	Gamma

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

1.1 Introduction/ Justification

Obesity is a major risk factor in the development of metabolic diseases (Pedersen, 2013). In 2016, 1.3 billion people were reported to be obese globally (NCD Risk Factor Collaboration (NCD-RisC), 2017). The prevalence of obesity and its associated complications are increasing at an alarming rate in children and adults (Ogden et al., 2016). Childhood obesity has been shown to translate into a costly disease burden in the provision of healthcare and is worsening the development of metabolic diseases in adulthood (de Onis et al., 2010). The increased prevalence of obesity has been paralleled by an increase in the prevalence of non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MetS) (Wong and Ahmed, 2014). Metabolic syndrome is a combination of risk factors that increase the risk of developing type II diabetes mellitus (T-II-DM), cardiovascular diseases and chronic kidney disease (Chen et al., 2004; Peeters et al., 2014). Non-alcoholic fatty liver disease is characterised by increased hepatic lipid infiltration and accumulation (>5-10 % of liver mass) in the absence of excessive alcohol consumption or the use of steatotic medications (Carr et al., 2016). Non-alcoholic fatty liver disease is the most prevalent chronic liver disease (Younossi et al., 2016) and ranges from simple hepatic steatosis to NASH and ultimately may lead to cirrhosis (Chalasani et al., 2012).

Recent studies have attributed the increase in the prevalence of obesity, NAFLD and MetS to the increase in the adoption of sedentary lifestyles (Bentov, 2014) and the consumption of fructose in processed foods and beverages (Ramos et al., 2017a). Fructose metabolism by-passes the main regulatory step of glycolysis resulting in rapid *de novo* lipogenesis, hepatic lipid accumulation, visceral obesity which contribute to reduced sensitivity to insulin and impaired glycaemic control (Basciano et al., 2010).

The neonatal growth phase is a critical window of developmental phenotypic plasticity (Devaskar and Thamotharan, 2007; Léonhardt et al., 2003). Dietary interventions during the neonatal developmental phase can result in epigenetic changes that subsequently cause disease or good health outcomes in subsequent developmental periods of life (Aalinkeel et al., 1999; Huynh et al., 2008). The consumption of high-fructose diets during the suckling period predisposes to the development of obesity, MetS, NAFLD, diabetes mellitus and cardiovascular diseases later in adulthood by adversely programming metabolic pathways (Ibrahim et al., 2019; Lembede et al., 2018; Nyakudya et al., 2018b). This development of adverse health outcomes can result from a ‘single-hit’, ‘double-hit’ and or ‘multiple-hit’metabolic programming process. The ‘single-hit’

hypothesis states that exposure to suboptimal nutrition during various developmental periods (pre-conception, prenatal, gestation and the early post-natal period) can result in the manifestation of metabolic derangements in an individual either immediately after exposure or after a period of latency (Almond and Currie, 2011). On the contrary, the 'double-hit' hypothesis of metabolic programming states 'single-hit' exposure to suboptimal nutrition is insufficient to cause the manifestation of metabolic disease (Stewart et al., 2013). According to the 'double- hit' hypothesis of metabolic programming, the first single-hit insult merely primes the individual to be more susceptible to developing metabolic disease when exposed to a 'second-hit' insult later in life (Tamashiro and Moran, 2010). The'multiple-hit' hypothesis of metabolic programming states that multiple dietary or environmental insults throughout the various developmental phases of life are necessary to cause the onset of metabolic derangements (Tamashiro and Moran, 2010).

Some of the features of MetS and NAFLD can be treated or managed by the use of synthetic pharmacological agents such as fenofibrate, statins and metformin (Franco et al., 2014). Synthetic pharmacological agents elicit side effects, are expensive and relatively inaccessible to the majority of communities in developing countries (Ahmadiani and Nikfar, 2016). Furthermore, most synthetic pharmacological agents are monotherapeutic, hence cannot mitigate the multiple effects associated with MetS. This has resulted in the increased use of plant-derived ethnomedicines and phytochemicals that possess multiple health beneficial activities (Rajalakshmi et al., 2014) including among others anti-oxidant, anti-inflammatory, hypocholesterolaemic and hypoglycemic effects (Njerua et al., 2013; Rajalakshmi et al., 2014). Plant-derived ethnomedicines are deemed to have minimal side effects, are cost effective and are relatively better accessed. Additionally, they have multiple-bioactivities therefore target most of the metabolic derangements associated with metabolic diseases. Thus, in recent years, the number of studies investigating the medicinal properties of plant-derived ethnomedicines and phytochemicals has increased (Rajalakshmi et al., 2014). The non-toxic, but bioactive β-sitosterol will be the focus of this study. β-sitosterol is a phytosterol with a chemical structure similar to that of cholesterol (Sugano et al., 1977) and is found in high concentrations in plants such as *Amaranthus hydridus* (Marcone et al., 2003) and *Hymenocrather calycinus* (Gohari et al., 2009). The beneficial bioactivities of β-sitosterol include anti-microbial, anti-oxidant, immunomodulatory, anti-inflammatory, anti-hyperlipidaemic, anti-obesogenic and anti-diabetic activities (Baskar et al., 2010; Chai et al., 2011; Field et al., 1997; Gupta et al., 2011). *In vitro* work by Hwang *et al.* (2008) demonstrated that β-sitosterol reduces intracellular level of

triglycerides and cholesterol in L6 myotube cells. β-sitosterol exerts its anti-hyperlipidaemic effects by reducing the expression of the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase gene which catalyses the synthesis of cholesterol (Field et al., 1997). Oral administration of 1.3 mg/kg of β-sitosterol restored basal liver and kidney function in cadmium chloride-treated rats (Olaiya et al., 2014). This shows the potential of β-sitosterol to be used to restore liver and kidney functions following hepatotoxicity and nephrotoxicity, respectively. In a study by Gupta et al. (2011), oral administration of 10, 15 and 20 mg/kg of β-sitosterol to streptozotocin (STZ)-diabetic rats for 21 days, resulted in decreased blood glucose concentration and therefore could potentially be used to improve glycaemic control. Gupta et al. (2011) attributed the anti-hyperglycaemic effects of β-sitosterol to its anti-oxidant activity and its ability to stimulate the regeneration of pancreatic β-cells. Nonetheless, the anti-hyperlipidaemic, anti-diabetic, anti-oxidant, hepatoprotective and renoprotective activities of β-sitosterol (Gupta et al., 2011; Hwang et al., 2008; Olaiya et al., 2014) have been investigated mainly in adult male rat models. A number of studies have targeted the neonatal period for administering interventions that have prophylactic potential against the development of metabolic disease in adulthood. It has been shown from such studies that neonatal intake of anti-obesogenic, anti-oxidant and anti-diabetic phytochemicals including resveratrol (Franco et al., 2014), oleanolic acid (Nyakudya et al., 2018b), s-allyl cysteine (Lembede et al., 2018) and curcumin (Ibrahim et al., 2019) programme protection against diet-induced metabolic disorders in adulthood. At the moment, there are no studies that have investigated the potential of β-sitosterol to protect female and male rats against high-fructose diet-induced metabolic derangements. It is an established fact that in female animals oestrogen has a protective effect against some metabolic diseases (Ballestri et al., 2017; Summart et al., 2017) resulting in sexual dimorphism to insults that elicit metabolic derangements. Recently in my laboratory sexual dimorhism induced by dietary intervention have been reported (Lembede et al., 2018; Mapfumo et al., 2019; Nyakudya et al., 2018b). Therefore in evaluating the prophylactic potential of ethnomedicines and or purified phytochemicals, it is imperative to do studies in both males and females.

1.2 Aims and objectives

The overall aim of the study was to determine whether β-sitosterol protects against high-fructose diet-induced metabolic derangements. This study was carried out in two main experiments with specific aims and defined objectives.

1.2.1 The aim of the first experiment was to determine the potential of β-sitosterol to protect against long-term high-fructose diet-induced metabolic derangements from weaning through to adulthood in growing female and male Sprague-Dawley rats modelling children fed obesogenic diets. Specifically, the study sought to determine the effect of β-sitosterol on

i. metabolic dysfunction by assessing its effects on:

a) body mass
b) ability to tolerate an oral glucose load.
c) plasma triglyceride and cholesterol concentration.
d) plasma concentrations of metabolism-regulating hormones, insulin and adiponectin.
e) whole-body insulin sensitivity by computing homeostatic model assessment index-IR (HOMA-IR).
f) visceral fat and epididymis fat adiposity.

ii. non-alcoholic fatty liver disease (NAFLD) by assessing its (Bst) effects on:

a) liver (absolute and relative to body mass) mass.
b) liver lipid content.
c) liver histo-morphometry
d) hepatic steatosis, lobular inflammation, ballooning and total NAFLD activity score (NAS).
e) surrogate markers of liver function (plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST).

iii. renal health by assessing its effects on:

a) kidney (absolute and relative to body mass) mass
b) kidney histo-morphometry.
c) surrogate markers of kidney function (plasma creatinine and urea).
d) the surrogate marker of renal tubular damage (plasma kidney injury molecule-1).

1.2.2 The aim of the second experiment was to determine if neonatal orally administered β-sitosterol programmes for protection against high-fructose diet-induced metabolic derangements in adulthood by specifically focusing on effects, in adulthood, of neonatal oral administration of β-sitosterol's on:

a) body mass
b) visceral fat adiposity
c) plasma triglyceride and glucose concentration
d) liver (absolute and relative to body mass) mass
e) liver histo-morphometry
d) hepatic steatosis, lobular inflammation, ballooning and total NAFLD activity score (NAS)

1.3 Hypotheses

1.3.1 Experiment 1

H_0: The daily oral administration of β-sitosterol from weaning does not protect against high-fructose diet-induced metabolic derangements in growing Sprague-Dawley rats.

H_1: The daily oral administration of β-sitosterol from weaning protects against high-fructose diet-induced metabolic derangements in growing Sprague-Dawley rats.

1.3.2 Experiment 2

H_0: Neonatal orally administered β-sitosterol does not programme for protection against high-fructose diet-induced metabolic derangements in adult Sprague-Dawley rats.

H_1: Neonatal orally administered β-sitosterol programmes for protection against high-fructose diet-induced metabolic derangements in adult Sprague-Dawley rats.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Metabolic syndrome and obesity have become a global public health problem (Zimmet et al., 2005). Approximately 20–25% of the world's adult population is estimated to have MetS (Asrih and Jornayvaz, 2015; Kaur, 2014). The increase in the prevalence of MetS over the past two decades has been paralleled by the obesity pandemic (Global Burden of Disease, 2016). The World Health Organisation (WHO) guidelines state that adults that have a body mass index (BMI) ≥ 30 kg/m^2 or more are obese (WHO, 2016). Childhood obesity is an important predictor of adult obesity (Engeland et al., 2004). Globally over 41 million children under 5-years old were reported to be overweight in 2016 (WHO, 2016). A quarter were reported to be living in Africa (WHO, 2016). According to the WHO, (2016) children under five years of age with a weight-for-height greater than 3 standard deviation above the WHO child growth standards median are considered obese. Children aged from 5 to 19 years are deemed obese when their weight-for-height is greater than two standard deviation above the WHO growth reference median (WHO, 2016). Obesity is associated with comorbidities including diabetes mellitus (DM), dyslipidaemia, hypertension, NAFLD and MetS (Han and Lean, 2016; Herouvi et al., 2013).

2.2 Metabolic syndrome

Generally, MetS refers to the concurrent manifestation of risk factors, for the development of T-II-DM, NAFLD and cardiovascular disease (Kaur, 2014). The risk factors include insulin resistance, hyperglycaemia, visceral obesity, dyslipidaemia and hypertension (Saklayen, 2018). There are several definitions of MetS and these affect the reportage of statistics for the condition. According to the International Diabetes Federation (IDF), one-quarter of the world's adult population has MetS (Rennie, 1981). Worldwide, the prevalence of MetS is greater in women (56%) than in men (44%) (Cameron et al., 2004). The WHO mandates that MetS be confirmed by the presence of insulin resistance and any other three risk factors (WHO, 1999). However, according to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), MetS is diagnosed when an individual has any three or more risk factors (Grundy et al., 2005). On the other hand, the IDF guidelines state that abdominal obesity is an essential factor in the diagnosis of MetS (Rennie, 1981).

Research has attributed the obesity, NAFLD, T-II-DM and MetS epidemics mainly to increased physical inactivity and the consumption of western-style diets that are rich in obesogenic

nutrients such as fructose (San-Cristobal et al., 2015; Viscogliosi et al., 2013). Fructose, a highly lipogenic monosaccharide, has been demonstrated to contribute significantly to the etiology of obesity, NAFLD and MetS (Pan and Kong, 2018; Rizkalla, 2010). Obesity is characterised by excess accumulation of body fat (Purnell, 2000). Excess body fat, particularly visceral adiposity is significantly associated with increased risk of development of metabolic abnormalities (Tchernof and Després, 2013).

2.2.1 Visceral obesity

Visceral obesity, a key risk factor for MetS (He et al., 2015), refers to the excessive accumulation of lipids in central adipose stores (Mittendorfer, 2011). It is associated with the development of metabolic conditions including dyslipidaemia, insulin resistance, hyperglycaemia, hypertension and NAFLD (Shuster et al., 2012). Different techniques are used to measure visceral adiposity including among others waist-to-hip ratio (WHR), waist circumference (WC) and computed-assisted tomograpy (CT) (Pouliot et al., 1994) but the latter is too expensive for routine use (Albu et al., 1997). However, in addition to being relatively cheaper, WC and WHR are non-invasive techniques (He et al., 2015). It is important to note that the use of WHR is considered a more precise predictor of MetS and cardiovascular disease risk (Shen et al., 2017; Yang et al., 2017). Visceral adipose tissue secretes protein signalling molecules known as adipokines (Mittendorfer, 2011). While leptin, resistin, tumour necrosis factor (TNF)-α, and interleukin (IL)-6 are pro-inflammatory adipokines, adiponectin is an anti-inflammatory adipokine (Shuster et al., 2012). Adiponectin binds to AdipoR1 and AdipoR2 receptors which trigger AMPK-induced fatty acid oxidation and increases insulin sensitivity (Kadowaki and Yamauchi, 2005; Petta et al., 2016). Visceral obesity is negatively correlated with the expression and secretion of adiponectin (De Rosa et al., 2013). Thus, an increase in central adipose stores results in hypoadiponectinaemia, decreased fatty acid oxidation and insulin resistance (Moon et al., 2019).

Leptin is secreted by adipose tissue and it acts on leptin receptors to increase energy expenditure, satiety and insulin sensitivity (Mantzoros et al., 2011). Visceral obesity increases leptin secretion thus causing hyperleptinaemia which consequently leads to leptin receptor desensitisation and increased secretion of TNF-α, and IL-6 by adipose tissue (Brennan and Mantzoros, 2006). An increase in TNF-α and IL-6 upregulates the expression and secretion of resistin and inhibits the phosphorylation of insulin receptor substrate (IRS-1) as well as tissues' expression of GLUT4 transporters (Jager et al., 2007). This will result in insulin resistance, hyperglycaemia and

impaired glycaemic control. Additionally, an increase in TNF-α and IL-6 will result in increased lipolysis in visceral adipose tissue leading to increased release of free fatty acid into circulation and dyslipidaemia (Uysal et al., 1997). Dyslipidaemia results in increased delivery of free fatty acids to the liver causing NAFLD (Ipsen et al., 2018). Additionally, increased plasma FFA concentration results in increased very-low-density lipoprotein (VLDL) triglyceride production which competes with glucose for uptake by peripheral tissues thus resulting in hyperglycemia (Bosello and Zamboni, 2000). It is, therefore, no surprise that visceral obesity is clinically regarded as a key predictor for insulin resistance (Hardy et al., 2012).

2.2.2 Insulin resistance

Insulin resistance refers to decreased responsiveness of target tissues to circulating insulin (Sesti, 2006). Insulin promotes glucose uptake and storage, lipogenesis and inhibits gluconoegenesis (Edgerton et al., 2006; Petersen and Shulman, 2018). As a consequence, insulin resistance results in decreased uptake of glucose, decreased glycogenesis and lipogenesis (Edgerton et al., 2006; Petersen and Shulman, 2018). This leads to hyperglycaemia and dyslipidaemia which increases oxidative stress and activates the inflammatory response which causes cell and or organ damage (Ormazabal et al., 2018). Insulin resistance causes a decreased nitric oxide production by endothelial cells and activation of the mitogen-activated protein kinase (MAPK) pathways, which manifest with increased vasoconstriction (Muniyappa and Sowers, 2013). The increase in vasoconstriction stimulates the production of pro-inflammation activity and thus hypertension (Soleimani, 2015). Additionally, insulin resistance together with hyperglycaemia, causes hyperinsulinaemia which leads to increased hepatic lipogenesis and consequently worsens the dyslipidaemia (Robins et al., 2011). Therefore, insulin resistance results in numerous clinical disorders, including T-II-DM , obesity, MetS and dyslipidaemia (Sesti, 2006).

2.2.3 Dyslipidaemia

According to the WHO dyslipidaemia, a major risk factor for cardiovascular disease, is estimated to contribute to 2.6 million deaths annually worldwide (WHO, 2011). Dyslipidaemia is a condition that is typified by abnormal blood lipid concentration (Najafipour et al., 2016). It encompasses elevated blood triglycerides, total cholesterol and low-density lipoprotein (LDL)-cholesterol as well as decreased blood high-density lipoprotein (HDL)-cholesterol (Ginsberg and Huang, 2015). Low-density lipoproteins (LDLs) transport lipids from the liver to peripheral tissues where they are stored (Wadhera et al., 2016) hence hyper-LDL-cholesterolaemia is

directly implicated in the development of hypercholesterolaemia, hypertriglyceridaemia, atherosclerosis and obesity (Suma et al., 2008). On the contrary, HDLs clear excess lipids from circulation by transporting them to the liver (Singh et al., 2010). Several studies have shown that an increase in blood HDL-cholesterol concentration attenuates dyslipidaemia, improves anti-inflammatory and anti-oxidant status contributing to the reduction in the incidence of atherosclerosis in MetS patients (Singh et al., 2010).

Hypertriglyceridaemia can occur as a consequence of decreased β-oxidation, increased lipolysis in peripheral adipose stores and enhanced hepatic LDL/VLDL production (Hassing et al., 2012). While 'primary' hypertriglyceridaemia is caused by hereditary factors, 'secondary' hypertriglyceridaemia is a characteristic of obesity, T-II-DM and MetS (Yuan et al., 2007). Hypertriglyceridaemia increases the incidence of cardiovascular disease by 32% in men and 76% in women (Hokanson, 2002) and that of insulin resistance (Ginsberg et al., 2005). The increased hepatic triglyceride accumulation and insulin resistance are associated with abnormal production of reactive oxygen species causing oxidative stress which results in mitochondrial dysfunction (Bantle, 2009; Ipsen et al., 2018) which all contribute to the development of NAFLD.

2.3 Non-alcoholic fatty liver disease

2.3.1 Epidemiology

Non-alcoholic fatty liver disease is the most common cause of liver failure and it is associated with an increased risk of developing MetS and T-II-DM (Ballestri et al., 2015). In the western world the prevalence of NAFLD is reported to range from 20–30% (Bellentan, Federica Scaglioni Mariano, Marino Giorgio Bedogni et al., 2010). The prevalence of NAFLD in the general population of Western countries is higher in obese (80–90%), diabetic (30–50%) and dyslipidaemic (90%) adults (Farrell and Larter, 2006). In both children and adults, sedentary lifestyles and the consumption of obesogenic diets contribute to the increase in the prevalence of NAFLD (Kim and Lee, 2018).

2.3.2 Definition

Non-alcoholic fatty liver disease, a spectrum of liver disorders, is characterised by increased hepatic lipid accumulation (>5-10 % of liver mass) in the absence of excessive alcohol consumption (Abd El-Kader and El-Den Ashmawy, 2015). The disease spans the spectrum of

simple steatosis or non-alcoholic fatty liver (NAFL) which may progress NASH if untreated (Perla et al., 2017). Non-alcoholic steatohepatitis is characterised by hepatic steatosis, hepatic inflammation, and hepatocellular ballooning (Ibrahim et al., 2018). It can progress to cirrhosis and subsequently to hepatocellular carcinoma and liver failure (Carr et al., 2016).

2.3.3 Causes/aetiology

Insulin resistance plays a fundamental role in the pathogenesis of NAFLD (Petta et al., 2016). It results in increased lipolysis of visceral adipose tissue and dyslipidaemia which contributes to increased hepatic lipid infiltration and accretion (Choudhury and Sanyal, 2004). Furthermore, insulin resistance leads to hyperinsulinaemia and consequently increased hepatic lipogenesis and suppressed fatty acid oxidation (Adams and Angulo, 2006). In addition to insulin resistance, metabolic and hormonal disturbances, gut microbiota, inflammation and oxidative stress play a role in development of NAFLD (Choudhury and Sanyal, 2004).

2.3.4 Treatment/management

The first line of intervention against NAFLD involves lifestyle changes: increased physical activity and adherence to healthy low-calorie diets (Cholankeril et al., 2018). Other therapeutic methods include the use of pharmacological synthetic agents. The most commonly used pharmacological synthetic agent against NAFLD is orlistat (Harrison et al., 2004). Orlistat inhibits enteric lipase resulting in compromised fat digestion and absoprtion thus promoting weight loss (Harrison et al., 2004). Anti-hyperlipidaemic pharmacological agents such as statins and fenofibrate are also used to manage aspects of NAFLD (Cholankeril et al., 2018). Fenofibrate treats hypertriglyceridaemia and increases HDL-cholesterol in patients with dyslipidaemia and MetS (Filippatos et al., 2008). The fenofibrate-mediated increase in HDL-cholesterol lowers circulating cholesterol concentration (Valasek et al., 2007). Furthermore, fenofibrate attenuates the progression of NAFLD by improving hepatic insulin sensitivity which limits hepatic lipid accumulation in high-fat diet fed and type II diabetic rats (Seo et al., 2008; Shin et al., 2009). The presence of NAFLD increases the risk of developing of T-II-DM and aggravates NAFLD to more severe forms of steatohepatitis, cirrhosis and hepatocellular carcinoma.

2.4 Diabetes mellitus

2.4.1 Epidemiology

It is reported that the number of people that have diabetes mellitus (DM) rose from 108 million in 1980 to 422 million in 2014 (WHO, 2016). In 2016, an estimated 1.6 million deaths were directly caused by DM (WHO, 2016). T-II-DM is reported to be the most common type of diabetes, accounting for 90% of all diabetes cases (Zheng et al., 2018).

2.4.2 Definition

Diabetes mellitus is a metabolic disease characterised by hyperglycaemia and its associated complications (Linnemann et al., 2014). T-I-DM is an autoimmune disease that results from the destruction of insulin-secreting pancreatic β-cells which manifests in insulin deficiency (Siddiqui et al., 2013). In T-II-DM, the pancreatic β-cells are functional and secrete insulin however tissues do not respond effectively to the effects of insulin (Siddiqui et al., 2013).

2.4.3 Cause(s) / aetiology

While the etiology of T-II-DM is not clearly known, different risk factors such as genetic defects, lowered pancreatic β-cell mass and obesity are thought to be involved (Kahn et al., 2014). Obesity and MetS are key contributors to the development of T-II-DM in children and adults (Al-Goblan et al., 2014). In peripheral tissues such as muscle, fat, and liver it has been demonstrated that obesity decreases sensitivity to insulin through compromised access to insulin receptors resulting in insulin resistance (Kaku, 2010). Under such circumstance the pancreas secretes more insulin in a bid circumvent insulin resistance. This results in hyperinsulinaemia. If untreated, chronic hyperglycaemia result in the development of macro- and microvascular pathological complications.

2.4.4 Microvascular complications of diabetes mellitus

2.4.4.1 Diabetic neuropathy

Diabetic neuropathy, which is characterised by peripheral nerve injury, affects 90% of the diabetic patients (Schreiber, 2015). The diabetes mellitus induced peripheral nerve injury is exacerbated by upregulation of the polyol pathway which causes exaggerated oxidative stress (Yagihashi et al., 2011).

2.4.4.2 Diabetic retinopathy

Diabetic retinopathy is a common microvascular diabetes mellitus induced complication which can lead to blindness (Fong et al., 2004). According to (Fong et al. (2004), this diabetes mellitus induced blindness affects over 10,000 people annually. A sustained hyperglycaemic state induces the upregulation of the polyol pathway resulting in abnormal production of sorbitol which (sorbitol) accumulates in retinal capillary cells. This buildup of sorbitol in the these cells results in osmotic damage (Cai and Boulton, 2002) hence the blindness.

2.4.4.3 Diabetic nephropathy

According to the American diabetes association more than 40% of people with diabetes mellitus develop chronic kidney disease (American Diabetes Association, 2014). The sustained hyperglycemia upregulates the polyol pathway (Gheith et al., 2016) resulting in increased proteinuria, reduced GFR which increases (reduced GRF) glomerular and proximal tubular injury (Toth-Manikowski and Atta, 2015). In addition to upregulation of the polyol pathways, the sustained hyperglycaemic state also upregulates the hexosamine pathway resulting in increased glucosamine-6-phosphate formation (Toth-Manikowski and Atta, 2015) which (glucosamine-6-phosphate) increases transcription of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) (Brownlee, 2001). The latter results in renal cell hypertrophy and increased mesangial matrix components (Schleicher and Weigert, 2000).

The management of diabetic nephropathy involves monitoring and controlling blood pressure using antihypertensive agents (Toth-Manikowski and Atta, 2015). A prospective study demonstrated a decline in GFR from 0.94 to 0.29 mL/min per month during the first three years of effective antihypertensive treatment (Parving et al., 1993). Treatment with angiotensin converting enzyme inhibitor (ACEI) exert a renoprotective effect, in addition to mitigating hypertension (Xue et al., 2017). Treatment with ACE inhibitors block the renin-angiotensin-aldosterone system (RAAS) pathway, which is an important component in the development and progression of diabetic nephropathy (Xue et al., 2017).

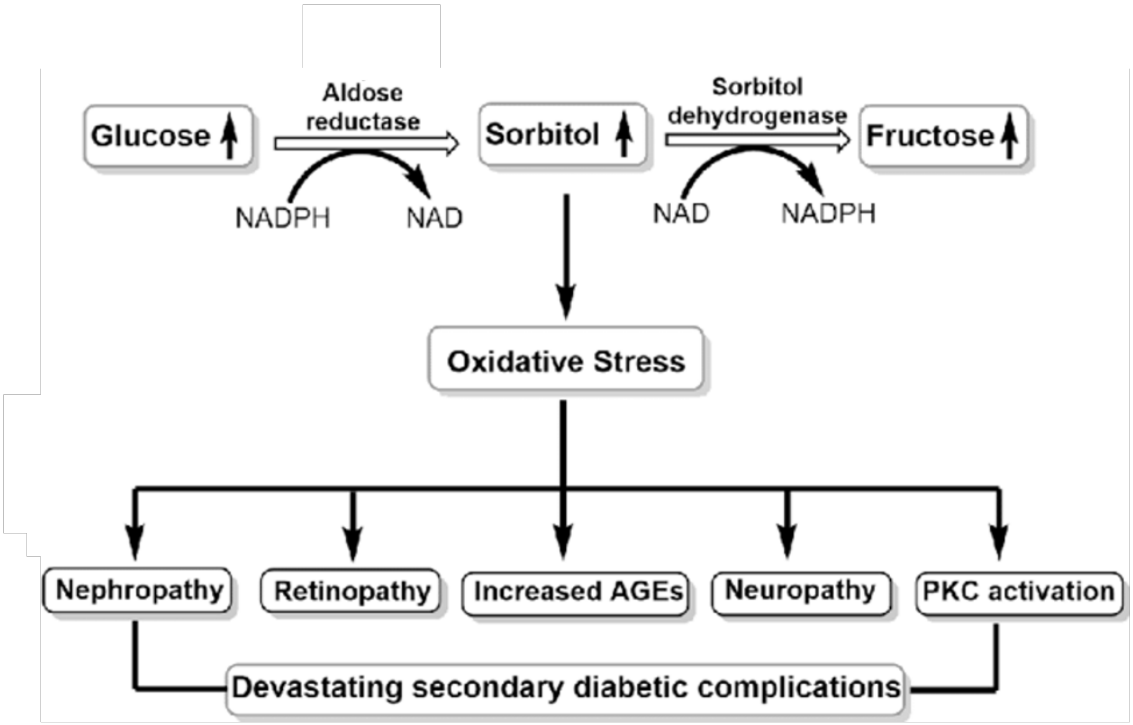

Figure 2.1: Mechanism of the polyol pathway. Adapted and modified from Shehzad (2018).

2.5 Experimental models of metabolic disorders

According to the IDF, an estimated 20-25 % of the world's adult population have MetS (IDF, 2006). The strain placed on the health system by metabolic disorders has prompted researchers to design various animal models for investigating metabolic disorders (Wayhart and Lawson, 2017). Dietary manipulation, genetic modifications and chemical intoxication are some of the approaches that are commonly used in research to induce metabolic disorders in animal models (Wayhart and Lawson, 2017).

2.5.1 Genetically-induced models

Genetically-induced models of MetS include leptin receptor-deficient (*db/db)* mice, leptin-deficient (*ob/ob)* mice and Zucker diabetic fatty rats (Augstein and Salzsieder, 2009; Park et al., 2006; Van Den Bergh et al., 2008). These genetically-induced models of MetS are beneficial in evaluating metabolic disorders that have monogenetic aetiology (Brown and Panchal, 2011; Mazen et al., 2009). Nonetheless, in humans metabolic disorders are typically multifactorial thus

making genetically-induced models inappropriate for investigating the pathophysiology of diet-induced metabolic disorders which are observed in humans.

2.5.2 Chemically-induced models

Intraperitoneal injection of streptozotocin (40-70 mg/kg) and or alloxan (75 mg/kg) are commonly used to induce pancreatic β-cell necrosis (Gajdošík et al., 1999; Masukawa and Nakanishi, 1994). The streptozotocin and alloxan induced models mimic T-I-DM (Lenzen, 2008). A key advantage of chemically-induced models is that they are time and cost effective compared to diet-induced models (Wayhart and Lawson, 2017). Chemically-induced models are suitable for studies interrogating T-I-DM (Zhang et al., 2008). They, however, do not mimic the pathophysiology of diet-induced metabolic disorders which are prevalent in humans (Dheer and Bhatnagar, 2010; Lenzen, 2008).

2.5.3 Diet-induced models

Diet-induced models have become the core approach for investigating metabolic disorders (Wong et al., 2016). Various dietary-approaches inclusive of high-fructose, high-carbohydrate and high-fat diets are used to induce metabolic disorders (Wayhart and Lawson, 2017). Studies report that high-carbohydrate and or high-fat diet models develop hypertension, impaired glucose tolerance, and dyslipideamia (Hao et al., 2015; Senaphan et al., 2015). The increase in the intake of sweetened beverages has resulted in excessive dietary fructose consumption worldwide (Wayhart and Lawson, 2017). Research indicates that dietary fructose contributes to the growing prevalence of metabolic disorders in children and adults (Bantle, 2009; Bray, 2013). There is thus a growing interest in the use of high-fructose diet models (Lozano et al., 2019; Rizkalla, 2010). In animal and human studies, excessive amounts of dietary fructose has been reported to cause obesity, NAFLD, MetS and renal disturbances (Oron-Herman et al., 2008; Sánchez-Lozada et al., 2007; Shahraki et al., 2011). Additionally, studies have also shown that the consumption of fructose during the early developmental periods of life can result in adverse metabolic programming outcomes which predispose to metabolic disease (Zheng et al., 2016).

2.6. Metabolic programming

Organisms have the ability to respond and adapt to environmental stimuli through biochemical, cellular, molecular and physiological mechanisms (Patel and Srinivasan, 2002). Suboptimal

events and nutritional manipulations during the windows of developmental plasticity can result in epigenetic alternations inclusive of DNA methylation, histone modification and transcription of non-coding ribonucleic acid (Choi and Friso, 2010; Vickers, 2011). These epigenetic changes can temporarily or permanently impact gene expression and consequently affect metabolic development and function of an organism, a phenomenon known as metabolic programming (Low et al., 2017; Seki et al., 2017).

The perinatal period is a critical window of developmental plasticity where the phenotypic outcomes of an individual can be influenced by events and interventions during this period (Tarry-Adkins and Ozanne, 2011). Therefore, the consumption of suboptimal diets during this period can result in adverse metabolic programming outcomes that predispose to metabolic disease (Marciniak et al., 2017). Maternal consumption of suboptimal diets during gestation and lactation alters nutrients availability, foetal growth as well as maternal metabolic hormone expression and production (Li et al., 2011). This consequently predisposes the offspring to developing metabolic disorders (Alzamendi et al., 2010; Bruce et al., 2009; Gregorio et al., 2010). Several studies have shown that maternal consumption of high-fat and or high-fructose diets during gestation and lactation increases the expression of lipogenic and pro-inflammatory genes and enzymes in the resulting offspring (Saad et al., 2016; Williams et al., 2014). Consequently, this increases the propensity to develop obesity, NAFLD and MetS in childhood and or in adulthood (Bruce et al., 2009; Gregorio et al., 2010; Li et al., 2011).

Rodents are altricial species that rely solely on their dam's milk for nourishment prior to weaning (Scheiber et al., 2017). In rats postnatal day (PND) 7-10 is equivalent to the last trimester of human gestational development which is typified by rapid brain development (Sengupta, 2013) while postnatal day 11-21 equate to the neonatal period in humans which is characterised by the proliferation and development of the endocrine systems (Sengupta, 2013). Researchers have therefore developed the perinatal rat models to understand the mechanisms involved in perinatal metabolic programming (Vickers, 2016).

A study by Lembede et al. (2018) reported that neonatal high-fructose diet (20% fructose solution) predisposed rats to develop fatty liver disease in adulthood. Lembede et al. (2018) also found that neonatal (PND 6-20) and adulthood (PND 21 -116) high-fructose diet consumption increased liver lipid accretion and caused microvesicular steatosis in adult rats. Similarly, Nyakudya et al. (2018b) reported that neonatal (PND 7-21) fructose (20%) intake resulted in

visceral obesity, hypertriglyceridaemia and insulin resistance in adult rats. Work by Ibrahim et al. (2019) revealed that long-term (from PND 6 - 63) high-fructose diet intake caused NASH in adolescent rats. Ibrahim et al. (2019) attributed their findings to the upregulation of TNF-α and downregulation of AMP-activated protein kinase (AMPK)-α in the liver by fructose. Other studies have reported that neonatal fructose consumption predisposes to metabolic disease through upregulating lipogenic and adipogenic genes (Baker and Friedman, 2018; Burgueño et al., 2013).

2.7 The role of fructose in the development of metabolic disorders

Fructose is an inexpensive monosaccharide found naturally in fruits and vegetables (Vos et al., 2008). It is mainly consumed in sweetened carbonated non-alcoholic drinks where high-fructose corn syrup (HFCS 55) is a key ingredient (Vos et al., 2008). Dietary fructose contributes to the documented persistent increase in the prevalence of obesity and metabolic disorders (Ang and Yu, 2018).

In the gastrointestinal tract, dietary fructose is transported across the small intestinal luminal membrane through glucose transporter-5 (GLUT5) facilitated diffusion (Drozdowski and Thomson, 2006). The fructose then exits the absorptive enterocytes on their basolateral membranes via GLUT2 transporters into the blood where it eventually is channeled into the hepatic portal vein which transports it to the liver (Mirtschink et al., 2018).

In the liver, fructose is metabolised by fructokinase to fructose-1-phosphate (F-1-P) which is then converted into the triose phosphates: dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G-3-P) which then enter the glycolytic pathway (Tappy, 2018). Unlike glucose whose metabolism is regulated by the rate-limiting step catalysed by phosphofructokinase, the metabolism of fructose by-passes this key regulatory step (Seung et al., 2008).

This by-pass results in increased *de novo* triglyceride and VLDL synthesis These substrates mediate the development of NAFLS, obesity and MetS (Pan and Kong, 2018). The triglycerides are exported from the liver by VLDL particles to peripheral adipose stores where they cause dyslipidaemia and visceral obesity (Klop et al., 2013; Rizkalla, 2010). Additionally, increased *de novo* lipogenesis results in endoplasmic reticulum stress, mitochondrial dysfunction and increased oxidative stress (Ren et al., 2012). In the liver, oxidative stress triggers hepatocyte

damage and activates the pro-inflammatory response which contributes to the progression of NAFLD to NASH (Sumida et al., 2013). In addition to mediating obesity, MetS and NAFLD excess dietary fructose intake has been shown to induce kidney damage (Gaby, 2005; Kizhner and Werman, 2002). Its consumption results in increased uric acid which induces pro-inflammatory mechanisms by stimulating monocyte chemotactic protein-1(MCP-1) production in proximal renal tubular cells (Cirillo et al., 2009). This eventually leads to progressive renal injury (Cirillo et al., 2009; Vasdev et al., 2004). It is therefore vital that interventions with therapeutic and or prophylactic efficacy but with minimal side effects be sought in order to be deployed to mitigate the complications associated with diet-induced metabolic disorders including MetS.

2.8 Interventions for metabolic disorders

2.8.1 Lifestyle modification

Lifestyle modifications largely encompassing increasing physical activity and the consumption of healthy diets (Khalfa et al., 2017) are key management approaches in mitigating metabolic disorders. In fact, performing physical activity such as walking, gardening, and housework for 30 minutes a day is reported to reduce the risk of developing MetS and T-II-DM by 50% (Hamasaki, 2016; Ingle et al., 2017). The consumption of a Mediterranean diet rich in whole grains, fruits, vegetables, nuts and olive oil has been shown to reduce the risk of developing MetS by 51% (Esposito et al., 2004; Viscogliosi et al., 2013). Research suggests that this is likely due to the Mediterrenian diet-mediating an increase in HDL-cholesterol concentration which transports cholesterol from the blood to the liver, and by reducing elevated blood glucose, triglycerides and LDL-cholesterol (Babio et al., 2009; Paletas et al., 2010). Although lifestyle modifications are considered the core intervention for metabolic disorders, poor compliance is a major drawback (Alefishat et al., 2017) hence the use of pharmacological synthetic agents against metabolic disorders.

2.8.2 Conventional pharmacological interventions

Pharmacological agents, such as, fenofibrate, metformin, thiazolidinediones and sulfonylurea and statins have been and continue to be used to treat some of the complications of MetS (Chapman et al., 2010; Florez et al., 2014). In my study I chose to use fenofibrate as a positive control.

Fenofibrate, a peroxisome proliferator-activated–receptor (PPAR)-α agonist (Harano et al., 2006) is an antihyperlidaemic agent that decreases plasma triglyceride concentration by inhibiting hepatic fatty acid synthesis (Staels et al., 1998). It also decreases plasma cholesterol concentration through suppressing the expression and activity of HMG-CoA synthase and HMG-CoA reductase enzymes (Guo et al., 2001) and increasing the elimination of cholesterol in bile (Guo et al., 2001). Research has also shown that fenofibrate increases plasma HDL-cholesterol concentration (Winegar et al., 2001) and decreases plasma LDL-cholesterol, C-reactive protein (CRP), IL-6 and TNF-α concentrations (Filippatos and Elisaf, 2015; Krysiak et al., 2013; Ye et al., 2011). This effectively improves plasma lipid profile and exerts anti-inflammatory effects. While synthetic pharmaceutical agents such as fenofibrate can be used to manage specific features of metabolic dysfunction, they are unaffordable and inaccessible to resource-limited communities (Hamine et al., 2015; Nathan et al., 2009). Moreover, these synthetic pharmacological agents are associated with adverse outcomes including lactic acidosis, liver toxicity and hypoglycaemia (Soleimani, 2015), and are typically used as curative medicines (Adams and Angulo, 2006). There is a need to find alternative and or complementary interventions administered during the neonatal period that can be used as long-term prophylactic agents against diet-induced metabolic disease.

2.9 Plant-derived medicines

Worldwide, there is an increase in the use of plant-derived medicines. It is estimated that 80% of the world population relies on traditional ethnomedicines for primary health care (Shakya, 2016). Ethnomedicinal plants are comparatively less costly compared to synthetic pharamacological agents, and easily accessible to resource-limited communities (Sofowora et al., 2013). The plant-derived ethnomedicines contain phytochemicals that exhibit health beneficial properties such as anti-obesity, anti-inflammatory, anti-oxidant and anti-diabetic activities (Dembinska-Kiec et al., 2008). Although some phytochemicals have been shown to have health benefits, others have been shown to negatively impact certain physiological systems (Baumann et al., 2000; Yasuoka et al., 2003). In recent years, the interest in phytochemicals such β-sitosterol which possesses health-beneficial pharmacological activities has significantly grown (Baskar et al., 2012, 2010; Gupta et al., 2011).

2.10 β-sitosterol

2.10.1 Source and structure

β-sitosterol is a naturally occurring plant sterol whose principle structure (figure 2.1) is similar to that of cholesterol (Gallová et al., 2011). It is reported to exist in different plant parts including rhizomes, fruits and leaves with high occurrence in seeds, nuts and seed oil (Bin Sayeed et al., 2016; Khan et al., 2008; Sultana and Khanam, 2016).

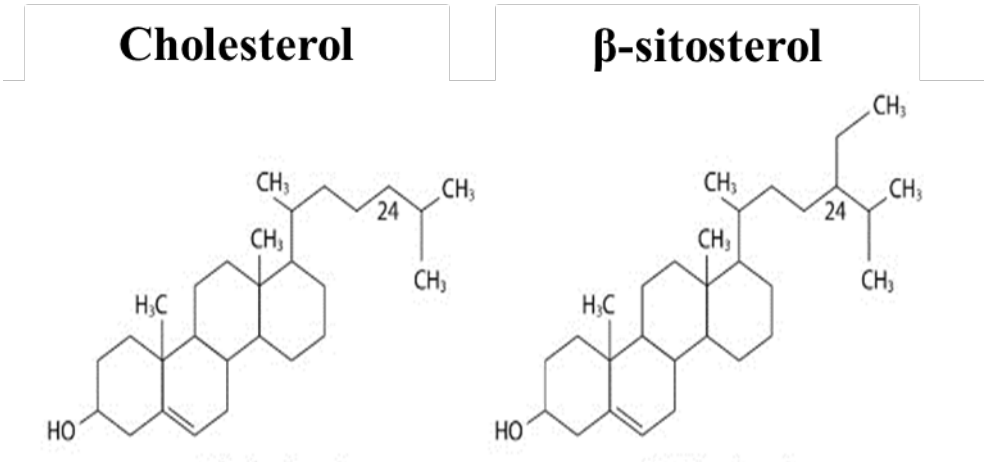

Figure 2.2: Chemical structure of β-sitosterol and cholesterol (Larry R. Engelking, in Textbook of Veterinary Physiological Chemistry Third Edition).

2.10.2 Bioavailability

β-sitosterol has low fat solubility and is hydrophobic and is poorly emulsified by bile thus is poorly absorbed (Yuan et al., 2019). Emara et al. (1999) reported that the absorption of β-sitosterol in humans is slower than cholesterol as evidenced by a delayed time of maximum absorption (t_{max}), however its absorption is higher in females compared to males (Sanders et al., 2000).

2.10.3 Metabolism

In the gastrointestinal tract β-sitosterol is converted to autoxidation products such as 24-ethyl-coprostanol, coprostanol and 24-ethyl-coprostanone. The metabolites are excreted via faeces and the skin (Song et al., 2000). Once absorbed β-sitosterol is transported to the liver and metabolised to the bile acids cholic and chenodeoxycholic acid (Salen et al., 1970) which are used in the synthesis of the bile salts sodium glycocholate and potassium taurocholate, respectively. About

80% of the absorbed β-sitosterol is excreted in bile as free sterol and eliminated from the body mainly in faeces (Sanders et al., 2000).

2.10.4 Toxicity

While literature is thin with regards to the toxicicty of β-sitosterol, it has been reported that administration of β-sitosterol 250µg/ 100 bwt to albino rats for 60 days did not compromise liver or kidney function (Malini and Vanithakumari, 1990). Additionally, Best et al. (1954) indicated that subjects to whom β-sitosterol was administered did not present with any side effects. Thus on the basis of the reviewed literature it can be inferred that β-sitosterol might not be toxic.

2.10.5 Biological activity

It is regarded as a safe nutritional supplement with a multitude of health-beneficial properties including anti-microbial, anti-diabetic, anti-oxidant, anti-obesogenic, anti-inflammatory, anti-cancer, hepatoprotective and renoprotective effects (Bin Sayeed et al., 2016).

2.10.5.1 Anti-diabetic effects of β-sitosterol

The oral administration of β-sitosterol (10 mg/kg bwt) to streptozotocin (STZ)-nicotinamide-induced diabetic mice decreased blood glucose concentration (Kumar et al., 2013). Similarly, Ramu et al. (2016) reported decreased blood glucose concentration in alloxan monohydrate-induced diabetic adult Wistar rats following oral administration of 100 mg/kg β-sitosterol. The anti-hyperglycaemic effects of β-sitosterol were attributed to its ability to reduce intestinal glucose absorption and suppress glycogenolysis and gluconeogenesis pathways (Ramu et al., 2015). Intraperitoneal administration of β-sitosterol (10, 15, 20 mg/kg) for 21 days decreased glycated haemoglobin as well as blood glucose and nitric oxide concentrations in STZ-diabetic adult rats (Gupta et al., 2011). It exerted its anti-diabetic effects through its anti-oxidant activity and protective effects on pancreatic β-cells (Gupta et al., 2011). It has been shown to improve tolerance to an oral glucose load and stimulate insulin secretion in *in vitro* and *in vivo* studies (Ivorra et al., 1990, 1988). The anti-diabetic properties of β-sitosterol suggest that it could potentially be used as an intervention in the prevention and management of diet-induced MetS and T-II-DM.

2.10.5.2 Anti-dyslipidaemic effect of β-sitosterol

β-sitosterol has been demonstrated to be an effective agent in the management of hypercholesterolaemia (Lei et al., 2017). It (β-sitosterol) reduces serum cholesterol concentration by inhibiting cholesterol absorption in the small intestines (Gylling and Miettinen, 1996; Ostlund, 2002). Additionally it decreases cholesterol synthesis through its ability to reduce the expression of the HMG-CoA reductase; a key enzyme in the synthesis of cholesterol (Field et al., 1997). *In vitro*, β-sitosterol has been shown to reduce triglyceride content in L6 myotube cells through AMPK activation (Chai et al., 2011). AMPK has been reported to inhibit glycerol-3-phosphate acyltransferase (GPAT) which is involved in triglyceride synthesis (Henriksen et al., 2013). It (β-sitosterol) has been shown to stimulate lipolysis in adipocytes (Chai et al., 2011). Taken together, these findings on the anti-obesogenic activities of β-sitosterol suggest that it could be used in the prevention and management of obesity-related metabolic disorders.

2.10.5.3 Anti-oxidant effect of β-sitosterol

An *in vitro* study showed that β-sitosterol enhances superoxide dismutase (SOD) and glutathione peroxidase (+GPx) activities in murine RAW 264.7 macrophages through the activation of the oestrogen receptor/PI3 kinase-dependent pathway (Vivancos and Moreno, 2005). Baskar et al. (2012) showed that intraperitoneal administration of β-sitosterol (5, 10, and 20 mg/kg) for 16 weeks protected against 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis by elevating enzymatic and non-enzymatic anti-oxidant activity in the colon. Metabolic disorders are associated with oxidative stress and inflammation thus β-sitosterol could be an effective intervention against oxidative stress.

2.10.5.4 Anti-inflammatory effects of β-sitosterol

The anti-inflammatory properties of β-sitosterol have been reported both *in vivo* and *in vitro* (Desai et al., 2009; Gómez et al., 1999). The administration of β-sitosterol (0.5 mg/ear), twice daily, for five days reduced the 12-O-tetradecanoylphorbol acetate (TPA) inflammation-induced oedema in ears of mice (Gómez et al., 1999). The reported anti-inflammatory activity of β-sitosterol occurs through the inhibition of neutrophil migration and the reduction in neutrophil infiltration into inflamed tissues (Gómez et al., 1999). *In vitro* β-sitosterol up-regulated the expression of IL-10 in bone marrow-derived macrophages. Valerio and Awad (2011) attributed the up-regulation of IL-10 to oppposing the activation of signal transducer and activator of transcription (STAT) thereby decreasing the inflammatory response (Valerio and Awad, 2011).

β-sitosterol also decreased TNF-α, IL-12 and IL-5 and monocyte chemoattractant protein (MCP-1) (Valerio and Awad, 2011). Lee et al. (2012) reported that orally administered β-sitosterol reduced the expression of pro-inflammatry cytokines TNF-α, IL-1β, and IL-6 in the gastrointestinal tract of mice induced by the administration of 2,4,6-trinitrobenzene sulfonic acid. Thus the β-sitosterol mitigated the colitis. A pro-inflammatory status increases the susceptibility to the development of obesity, NAFLD, MetS and T-II-DM. Therefore the anti-inflammatory activities of β-sitosterol make it a feasible intervention against metabolic disorder-related inflammation.

2.10.5.5 Hepatoprotective and renoprotective effects of β-sitosterol

Research by Tamura et al. (1998) reported that feeding a diet containing β-sitosterol (1% w/w) to male mice improved liver ultrastructure by increasing the number of peroxisomes in liver cells. Orally administered β-sitosterol (2.3 mg/kg bwt) restored liver and kidney function in cadmium-induced hypertensive rats (Olaiya et al., 2014). Additionally pre-treatment with 35 µg/kg β-sitosterol for 14 days protected adult female rats against CCl_4-induced hepatotoxicity (Wong et al., 2014). The hepatoprotective effects of β-sitosterol are attributed to its ability to improve mitochondrial function by upregulating mitochondrial glutathione redox cycling (Wong et al., 2014). Metabolic syndrome is associated with liver and kidney damage (Prasad, 2014; Rosselli et al., 2014). Thus the hepatoprotective and renoprotective properties of β-sitosterol makes it potentially a useful intervention against MetS-related liver and kidney damage (LaGuardia et al., 2012; Lim et al., 2010).

2.10.5.6 Antimicrobial activity of β-sitosterol

It has been shown that β-sitosterol has anti-microbial activity against a wide range of bacteria including *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* (Sen et al., 2012). The antibacterial activity of β-sitosterol is attributed to its ability to inhibit bacterial cell surface proteins such as 'sortase' thus preventing transpeptidation (Kanokmedhakul et al., 2005). However a limitation of these studies on the antimicrobial activity of β-sitosterol is the inadequacy in explaining the mechanism of actions (Kanokmedhakul et al., 2005). Recent studies have demonstrated that intestinal microbiota play a role in the onset and progression of metabolic disorders (He and Shi, 2017; Welty et al., 2016). Thus, the anti-microbial activities of β-sitosterol could be utilised strategically as an intervention for obesity, NAFLD and MetS by targeting gut microbiota.

2.10.5.7 Anticancer activity of β-sitosterol

According to the WHO, cancer is one of the leading causes of death worldwide with an estimated 9.6 million deaths in 2018 (WHO, 2018). β-sitosterol is reported to promote apoptosis and inhibit cell proliferation in human colon cancer cell lines (Jayaprakasha et al., 2007). Similarly, in colon and breast cancer studies, the anticancerous activity of β-sitosterol has been attributed to its ability to increase apoptosis and inhibit the growth in tumour cells (Awad et al., 1998; Lee et al., 2007). Imanaka et al. (2008) reported that daily oral intake of β-sitosterol for 7 days prevented tumour metastasis in adult rats through enhancing the gut immune system. Additionally, the anticancer activities of β-sitosterol have also been attributed to its ability to activate caspase-3 and down-regulate Bcl-2 which are key in the regulation of apoptosis (Choi et al., 2003; Park et al., 2007; Von Holtz et al., 1998). Metabolic disorders are associated with breast, endometrial, colorectal, pancreatic and hepatic cancers (Dibaba et al., 2018; Uzunlulu et al., 2016; Watanabe et al., 2019). Thus the anticancer effects of β-sitosterol make it a feasible intervention against metabolic disorder related cancers.

The majority of studies on the heatlh beneficial activities of β-sitosterol were evaluated largely in adult male rat models with the major focus on its therapeutic potential. It is therefore pertinent that the potential prophylatic effects of β-sitosterol against high-fructose diet-induced metabolic derangements be evaluated in growing male and female rats. The next chapter (chapter 3) gives a narrative of the potential prophylactic effects of β-sitosterol against metabolic dysfunction in high-fructose diet-fed growing male and female rats.

CHAPTER 3: β-SITOSTEROL AND THE DEVELOPMENT OF HIGH-FRUCTOSE DIET-INDUCED METABOLIC DYSFUNCTION IN SPRAGUE-DAWLEY RATS

3.0 Introduction

The World Health Organisation (WHO) reported that the global prevalence of obesity in infants and young children, aged 0 to 5 years, had increased from 32 million in 1990 to 41 million in 2016 (WHO, 2014). If this trend continues globally, it is projected that the number of overweight or obese infants and young children will increase to 70 million by 2025 (World Health Organisation, 2014). The prevalence of obesity is reported to be higher in girls compared to boys (Maruf et al., 2013). Obesity is a major risk factor for the development of MetS (Alberti KG et al., 2005; Grundy et al., 2005; Nolan et al., 2017). Metabolic syndrome is defined as the concurrent manifestation of risk factors that increase the risk of developing type II diabetes mellitus (T-II-DM) and cardiovascular disease (Peeters et al., 2014). These risk factors include insulin resistance, visceral adiposity, atherogenic dyslipidaemia and elevated blood pressure (Fiszman et al., 2007). Plasma triglyceride concentration and visceral obesity have been identified as accurate indicators for screening and predicting the risk for MetS and T-II-DM (He et al., 2013; Li et al., 2012; Ren et al., 2017). Sedentary lifestyles and the consumption of obesogenic diets have been identified as the major causes of obesity and MetS (Bentov, 2014).

Some features of MetS can be treated or managed through the use of synthetic pharmaceutical agents such as fenofibrate an anti-hyperlipidaemic drug (Valasek et al., 2007). However, the challenges associated with the use of conventional synthetic pharmacological agents coupled with consumer demand for natural products has resulted in a worldwide increase in the use of natural plant-derived ethnomedicines (Valasek et al., 2007). Plant-derived ethnomedicines have been and continue to be used in disease management and their role in primary health care is expanding (Valasek et al., 2007). Ethnomedicinal plants contain phytochemicals such as phytosterols that are known to have multiple health beneficial biological properties (Florez et al., 2014; Fyhrquist et al., 2002; Valasek et al., 2007). β-sitosterol is one of the most abundant and widely distributed phytosterols (Kim et al., 2014). It possesses anti-obesogenic, anti-inflammatory, and anti-diabetic properties (Gupta et al., 2011; Vivancos and Moreno, 2005).

Despite the evidence that obesity, MetS and T-II-DM are increasing in children, the beneficial effects of β-sitosterol on metabolic health have been investigated mainly in adult animal models and adult human studies (Gerson et al., 1965; Gupta et al., 2011; Ramu et al., 2015). Moreover a large proportion of these studies have investigated the curative properties of β-sitosterol and not its prophylactic potential. Globally, it is well recognised that prevention of diseases reduces the burden of health care to a greater extent than the cure of diseases. It is important to note that key organs such as the pancreas, liver and the heart are damaged as a consequence of the development of obesity (Uranga and Keller, 2019). As such it is important to start prophylactic treatment in early life as it provides better chances for reducing long-term complications associated with obesity (Pandita et al., 2016). This study, therefore, investigated the potential of orally administered β-sitosterol to prevent high-fructose diet-induced metabolic dysfunction in growing female and male rats thereby mimicking children fed obesogenic diet and continuing on the diets through to adulthood.

3.1 Material and methods

3.1.1 Ethical approval

Ethical clearance for the study was granted by the Animal Ethics Screening Committee (AESC) of the University of Witwatersrand (AESC clearance number 2017/08/55/B; **Appendix I**). This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011). Also in accordance with the SANS guidelines.

3.1.2 Chemicals and reagents

All the chemicals and reagents used in the study were of analytical grade. β-sitosterol, fenofibrate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fructose was obtained from Nature's Choice (Randvaal, South Africa).

3.1.3 Animals, housing and general care

Seventy 21-day old female and male Sprague-Dawley rat pups were used in the experiment. Each rat was individually housed in a Perspex cage with stainless steel mesh lid in the Central Animal Services (CAS) unit of the University of the Witwatersrand. The cages were lined with wood shavings for bedding. The bedding was changed once a week. A twelve-hour light and dark cycle was followed with lights on from 7 am to 7 pm. Room temperature was maintained at 26 ± 2˚C. The rats had *ad libitum* access to a standard rat chow (Nutritionhub (PTY) Ltd, Stellenbosch, South Africa) and drinking fluid (water or 20% fructose solution as described below) throughout the experiment. The rat chow had the following nutrition content (stated by the manufacturer): calcium 14 g/kg, phosphorus 8 g/kg, vitamin A $_{(min)}$ 16 000 IU/kg, vitamin D 2000 IU/kg and vitamin E 100 mg/kg, crude protein 240 g/kg, crude oils and fats 53 g/kg, linoleic acid 14 g/kg, crude fibre 45 g/kg and crude ash 75 g/kg. The rat pups were acclimatised to handling for two days (postnatal days 21 and 22) prior to the commencement of the experiment on postnatal day 23.

3.1.4 Experimental design

On post-natal (PND) day 23 the seventy pups were, in an interventional study, randomly distributed to and fed the following treatment regimens for 12 weeks:

group I, the negative control (7 male; 7 female) - standard rat chow (SRC) + plain drinking water (PW) + plain gelatine cube (PC),

group II in which metabolic dysfunction was induced (7 male; 7 female) – SRC + 20% w/v fructose solution (FS) to drink + PC,

group III in which fenofibrate was used as a positive control for the prevention of metabolic dysfunction (7 male; 7 female) - SRC + FS + 100 mg/kg body mass per day of fenofibrate in a gelatine cube (FF),

group IV in which the potential prophylactic effects of Bst against high-fructose diet-induced metabolic dysfunction were explored (7 male; 7 female) – SRC + FS + 20 mg/kg body mass per day of β-sitosterol in a gelatine cube (Bst) and

group V in which the impact of Bst alone on the metabolic health of the rats was explored (7 male; 7 female) - SRC + PW + Bst.

The fenofibrate and β-sitosterol doses used were similar to those previously described by Baskar et al. (2010); Ji et al. (2005), respectively. Due to the poor aqueous solubility of Bst, dimethyl sulphoxide [(DMSO); 0.5% v/v] was used as a vehicle to dissolve β-sitosterol and the fenofibrate (Baskar et al., 2010; McCormack et al., 2015). The plain gelatine cubes in the control groups were also made up using 0.5% DMSO. The gelatine cubes were prepared as previously described by Kamerman et al. (2004) with modifications. In place of 16 g of brown sugar, 8 g (Huletts, Tongaat, South Africa Ltd) were used as it was discovered that use of higher concentration of sugar reduced intake. Throughout the experiment, the rats were weighed twice weekly in order to ensure the maintenance of a constant dose of the chemical interventions as well as to monitor growth performance.

3.1.5 Terminal procedures and sample collection

Following 12 weeks of the administration of the treatments the rats were fasted overnight but allowed access to plain drinking water. The rats were then weighed and fasting blood glucose and triglyceride concentration were determined from a drop of blood obtained from the tail vein using a pinprick (Ndong et al., 2007). Blood glucose and triglyceride concentrations were measured using a calibrated glucose meter (Contour Plus Meter, Bayer, Isando-Johannesburg, South Africa) and a calibrated Accutrend triglyceride meter (Roche, Mannheim, Germany), respectively. Immediately thereafter the rats were terminally euthanised with an intraperitoneal injection of sodium pentobarbitone at 200 mg/kg body mass and blood samples were collected via cardiac puncture using 18G needles and 10ml syringes into heparinised blood tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The blood samples were centrifuged at 20°C for 15 min at 5000 x g in a Sorvall RT® 6000B centrifuge (Pegasus Scientific Inc., Rockville USA). The harvested plasma was stored in microtubes at -20°C pending determination of plasma cholesterol, insulin and adiponectin concentrations.

The abdomen of each rat was opened via a midline incision and the visceral fat pad (retroperitoneal, perirenal mesenteric) was dissected out and weighed. The visceral fat mass relative to body mass (%BM) was computed using the formula:

Relative visceral fat mass = [visceral fat mass (g) /fasted terminal body mass (g)] x 100.

3.1.6 Determination of insulin and adiponectin concentration and HOMA-IR

Fasting insulin (ng/mL) and adiponectin (pg/mL) plasma concentrations were determined using rat insulin and adiponectin enzyme-linked immunosorbent assay (ELISA) kits with a detection range of 0.3-20 ng/mL and 46.88-3000 pg/mL, respectively (Elabscience Biotechnology Co, Wuhan, Hubei Province, China). A detailed description of the protocol used is attached in appendices III and IV, respectively. A standard curve, for each hormone, was constructed using calibrator concentrations. The concentrations of insulin and adiponectin in the samples were then determined from the constructed standard curves. Insulin resistance was estimated by computing the homeostatic model assessment index, HOMA-IR, using the fasting blood glucose and insulin concentration using the equation HOMA-IR= [insulin (ng/mL) x blood glucose (mmol/L)]/22.5 (Divi et al., 2012).

3.1.7 Determination of plasma total cholesterol

The plasma cholesterol concentrations of the rats were determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions**.**

3.2 Statistical analysis

GraphPad Prism 6.0 (Graph-pad Software Inc. San Diego, USA) software was used to analyse data. Data are expressed as mean ± SD. Data on weekly body mass was analysed using a repeated-measures analysis of variance (ANOVA). Multiple-group parametric data were analysed using one-way ANOVA, followed by Tukey's *post-hoc* test. Significance was accepted when $p < 0.05$.

3.3 Results

3.3.1 Body mass

Figures 3.1 and 3.2 present the initial and terminal body masses of female and male rats, respectively. The induction body masses of the female rats were similar ($p > 0.05$). Similarly, the induction body mass of the male rats were similar. Following the 12-week intervention, the female and male rats across treatments significantly grew ($p < 0.0001$; induction body masses compared to terminal body masses; Figures 3.1 and 3.2). The terminal body masses of female rats were similar ($p > 0.05$) across treatments (Figure 3.1). In male rats, the administration of β-sitosterol (Bst + FS and Bst + PW) or fenofibrate (FF + FS) significantly reduced ($p < 0.05$) terminal body mass compared to the control (Figure 3.2). A comparative analysis showed that the induction masses of the female and male rats were similar ($p > 0.05$) however, the male rats had significantly higher ($P < 0.0001$) terminal body masses than female rats across all treatments.

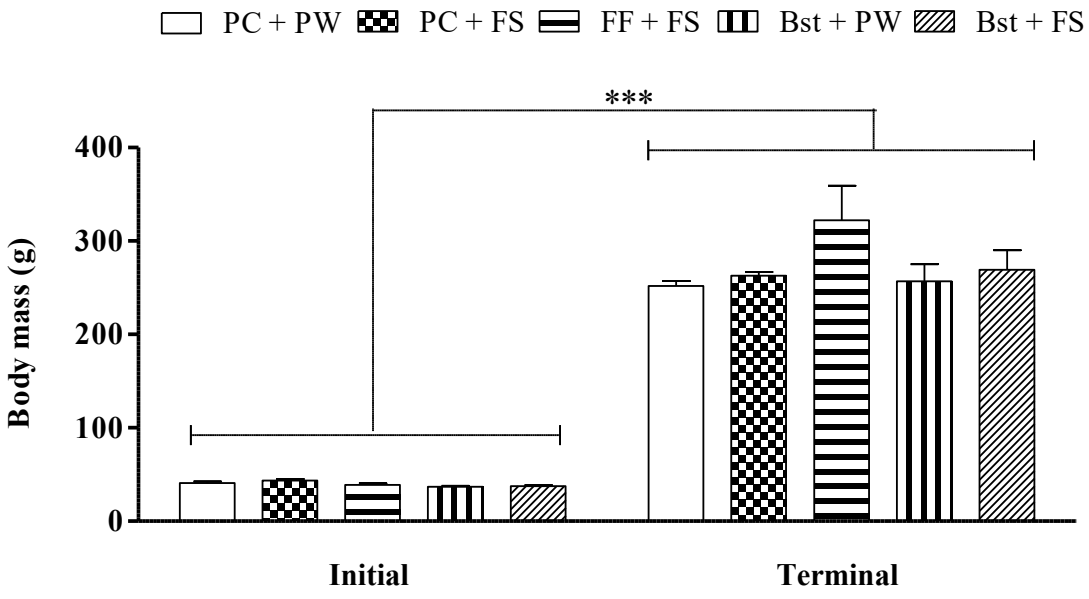

Figure 3.1: Initial and terminal body masses of the female rats.

*** $p < 0.0001$ initial vs terminal. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 per treatment.

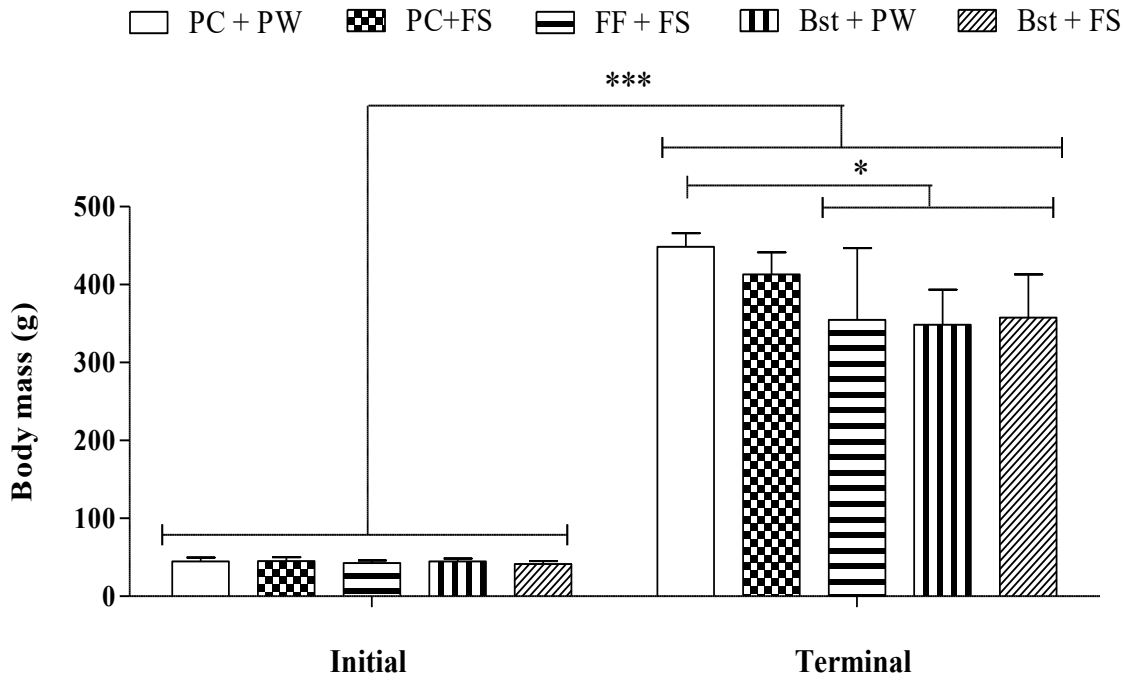

Figure 3.2: Initial and terminal body masses of the male rats.

*** $p < 0.0001$ initial vs terminal; * $p < 0.05$ when terminal masses are compared to the control (PC + PW). PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-

sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 per treatment.

3.3.2 Visceral obesity

Figures 3.3 and 3.4 show the visceral fat pad masses of female and male rats, respectively. The high-fructose diet (PC + FS) fed female and male rats had significantly increased ($p < 0.001$) visceral fat mass (absolute and relative to body mass) compared to their control (PC + PW) counterparts (Figures 3.3 and 3.4). β-sitosterol (Bst + FS) prevented ($p < 0.001$; compared to PC + FS) the high-fructose diet-induced increase in visceral fat mass in female and male rats (Figures 3.3 and 3.4). Fenofibrate (FF + FS) prevented ($p < 0.05$; compared to PC + FS) the high-fructose diet-induced absolute visceral fat mass in female and male rats (Figures 3.3 and 3.4). In male rats, fenofibrate (FF + FS) prevented ($p < 0.05$; compared to PC + FS) the high-fructose diet-induced visceral fat mass (relative to body mass) (Figure 3.4).

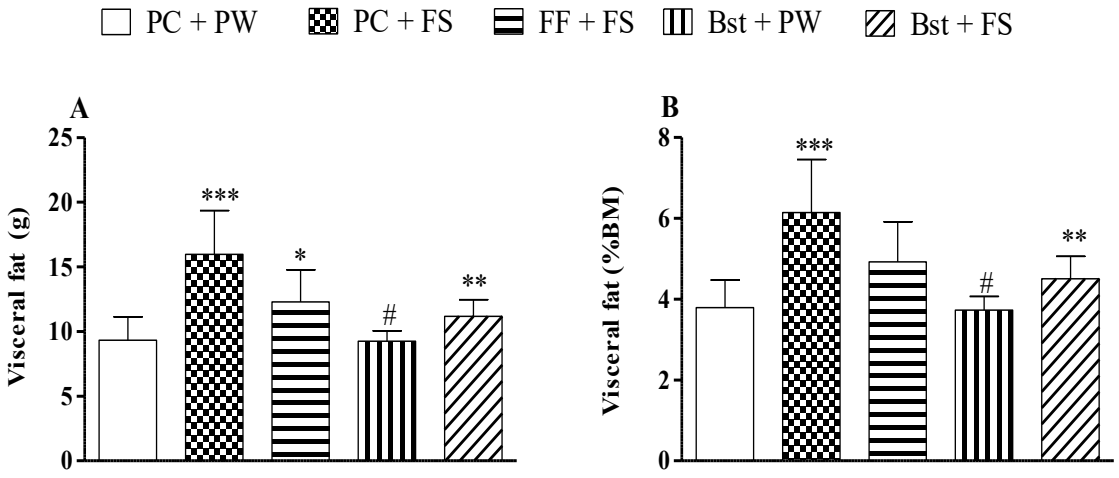

Figure 3.3: Effect of β-sitosterol on absolute (A) and relative (B) visceral fat pad mass of female rats fed a high-fructose diet.

*** $p < 0.0001$ vs control (PC + PW); * $p < 0.05$ vs PC + FS; ** $p < 0.001$ vs PC + FS; # $p < 0.0001$ vs PC + FS. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS =

plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink; BM = body mass. Data presented as mean ± SD; n = 7 per treatment.

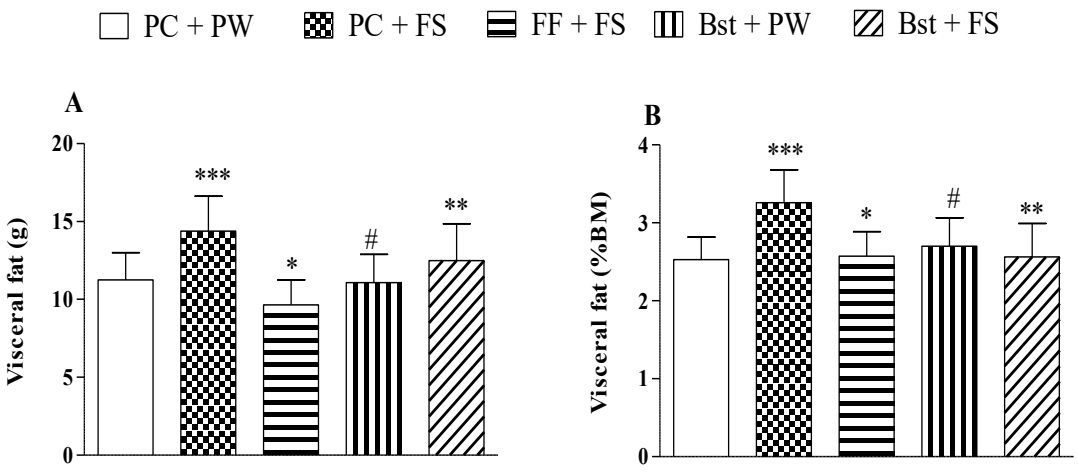

Figure 3.4: Effect of β-sitosterol on absolute (A) and relative (B) visceral fat pad mass of male rats fed a high-fructose diet.

*** $p < 0.0001$ vs control (PC + PW); * $p < 0.05$ vs PC + FS; ** $p < 0.001$ vs PC + FS; # $p < 0.0001$ vs PC + FS. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink; BM = body mass. Data presented as mean ± SD; n = 7 per treatment.

3.3.3 Triglyceride and cholesterol concentration

Figures 3.5 and 3.6 show the plasma triglyceride (A) and cholesterol (B) concentrations in the female and male rats, respectively. The high-fructose diet (PC + FS) fed female rats significantly increased ($p < 0.0001$) plasma triglyceride concentration compared to the control (PC + PW) (Figure 3.5). The high-fructose diet (PC + FS) had no effect ($p > 0.05$) on plasma triglyceride concentration in male rats (Figure 3.6). β-sitosterol (Bst + FS) prevented ($p < 0.0001$; compared to PC + FS) the high-fructose diet-induced hypertriglyceridaemia in female rats (Figure 3.5). Fenofibrate (FF + FS) failed to prevent the high-fructose diet-induced hypertriglyceridaemia seen in female rats (Figure 3.5). Although the high-fructose diet did not significantly increase ($p > 0.05$) the plasma total cholesterol concentration of the female and male rats (Figures 3.5 and 3.6, respectively), it increased the plasma total cholesterol by 26% ($p = 0.08$) in female rats and by 12% in male rats (Figures 3.5 and 3.6). β-sitosterol alone (Bst + PW) had no effect ($p > 0.05$) on plasma total concentration in female and male rats, however, the high-fructose diet-fed female and male rats administered β-sitosterol (Bst + FS) had significantly higher ($p < 0.05$) plasma total cholesterol concentration when compared to PC + PW (Figure 3.5 and 3.6). Female rats fed a high-fructose diet coupled with the administration of fenofibrate (FF + FS) had significantly higher ($p < 0.05$) plasma cholesterol concentration compared to their control (PC + PW) counterparts (Figure 3.5).

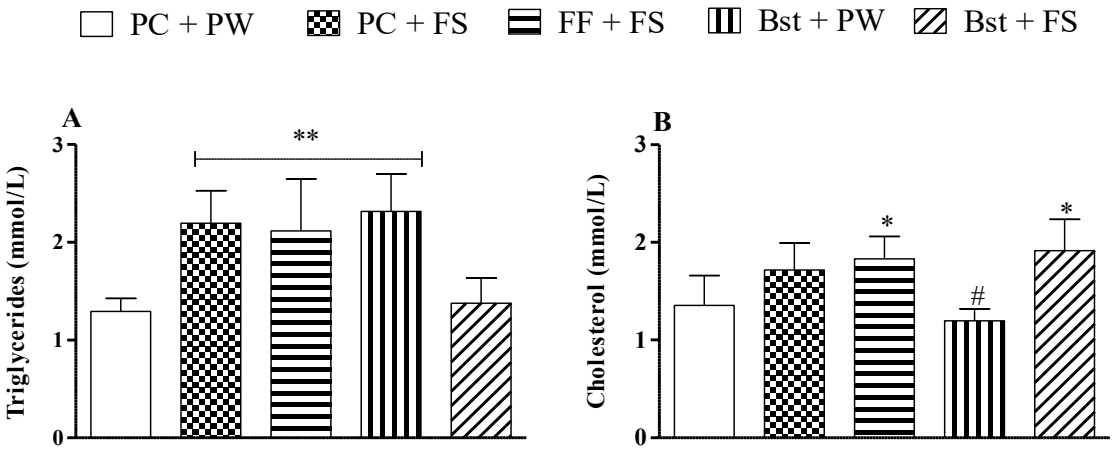

Figure 3.5: Effect of β-sitosterol on plasma triglyceride (A) and cholesterol (B) concentration of female rats fed a high-fructose diet.

# $p < 0.05$ vs PC + FS, FF + FS and Bst + FS; * $p < 0.05$ vs control (PC + PW); ** $p < 0.001$ vs PC + PW and Bst + FS. PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 per treatment.

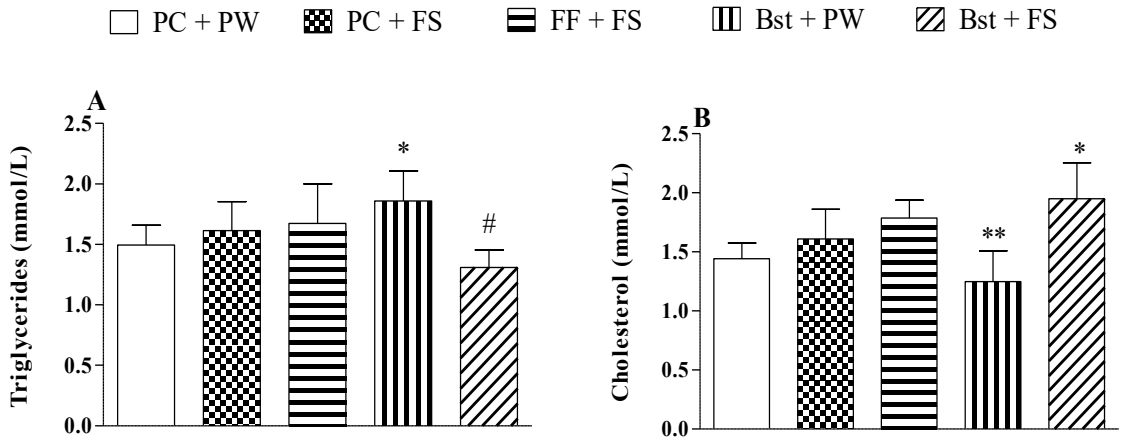

Figure 3.6: Effect of β-sitosterol on plasma triglyceride (A) and cholesterol (B) concentration of male rats fed a high-fructose diet.

# $p < 0.05$ vs FF + FS and Bst + PW; * $p < 0.05$ vs control (PC + PW); ** $p < 0.001$ vs PC + FS, FF + FS and Bst + FS. PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-

sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 per treatment.

3.3.4 Glucose, insulin, adiponectin concentration and HOMA-IR

Figures 3.7 and 3.8 show the glucose (A), insulin (B) and adiponectin (C) concentrations as well as HOMA-IR (D) of the female and male rats, respectively. The high-fructose diet had no effect on glucose and insulin concentrations and HOMA-IR of the female and male rats (Figures 3.7 and 3.8). Rats fed the high-fructose diet (PC + FS) had an undetectable adiponectin concentration and thus were assigned the lowest detectable ELISA adiponectin concentration of 46.88 pg/mL (Figures 3.7 and 3.8). The female rats fed the high-fructose diet in combination with β-sitosterol (Bst + FS) had significantly higher ($p < 0.0001$) plasma adiponectin concentration compared to their counterparts fed PC + PW and PC + FS, respectively (Figure 3.7). Female and male rats fed a high-fructose diet in combination with fenofibrate (FF + FS) had significantly higher ($p < 0.0001$) plasma adiponectin concentration compared to their counterparts fed PC + PW and PC + FS, respectively (Figures 3.7 and 3.8). β-sitosterol alone (Bst + PW) significantly increased ($p < 0.001$) plasma adiponectin concentration compared to PC + PW and PC + FS, respectively but lowered ($p < 0.001$) plasma insulin concentration and HOMA-IR compared to all other treatments (Figure 3.7 and 3.8).

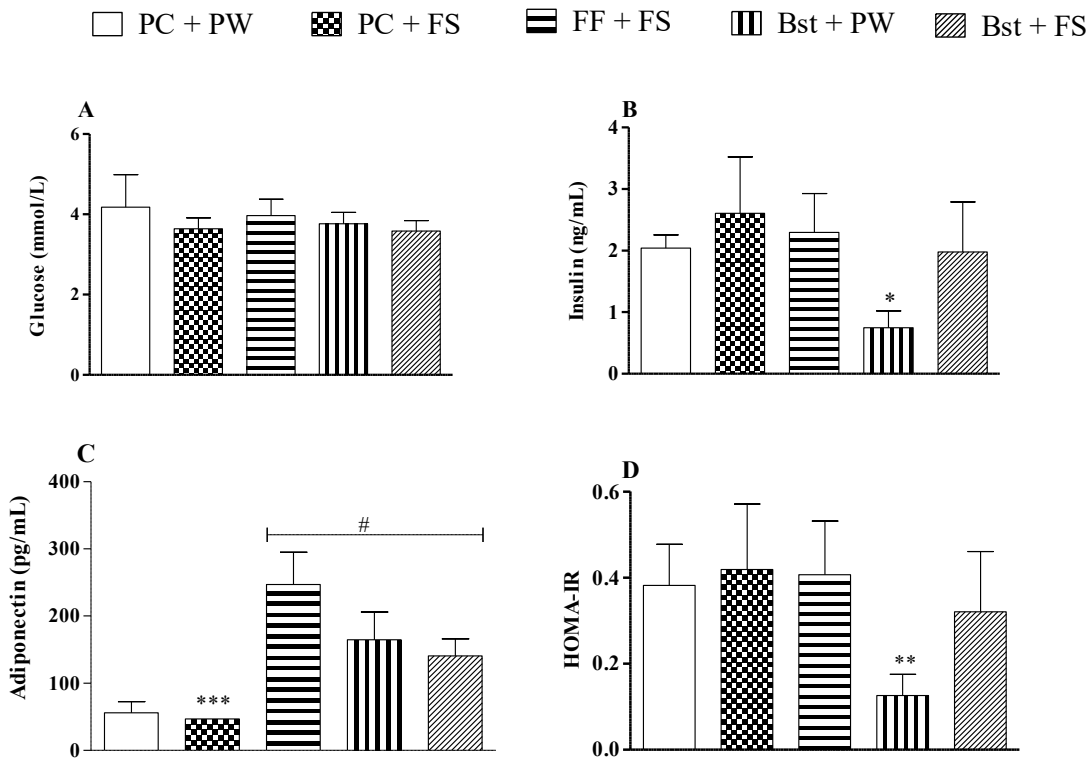

Figure 3.7: Effect of β-sitosterol on glucose (A), insulin (B), adiponectin (C) concentration and HOMA-IR (D) of female high-fructose diet-fed rats.

* $p < 0.05$ when compared to all groups; ** $p < 0.001$ vs PC+ PW, PC + FS and FF + FS; *** $p < 0.0001$ vs FF + FS, Bst + FS and Bst + PW; # $p < 0.0001$ vs control (PC + PW). PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 for all parameters except for plasma insulin concentration (n = 6) per treatment.

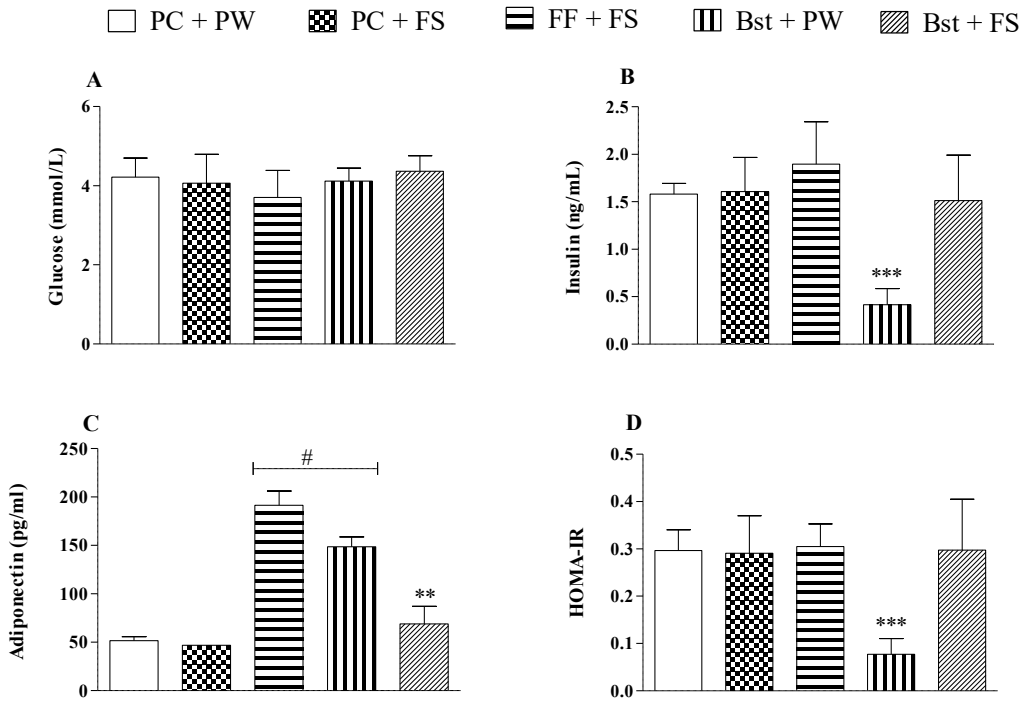

Figure 3.8: Effect of β-sitosterol on glucose (A), insulin (B), adiponectin (C) concentration and HOMA-IR (D) of male high-fructose diet-fed rats.

*** $p < 0.0001$ when compared to all groups; ** $p < 0.001$ vs FF + FS and Bst + PW; # $p < 0.0001$ vs PC + FS and PC + PW. PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 7 for all parameters except for plasma insulin concentration (n = 6) per treatment.

3.4 Discussion

The study aimed to investigate the potential of orally administrated β-sitosterol to protect growing female and male rats against the development of dietary fructose-induced metabolic dysfunction. The consumption of a high-fructose diet for 12-weeks caused visceral obesity, hypoadiponectinaemia and had no effect on body mass gain, plasma glucose, insulin and HOMA-IR in female and male rats (Figures 3.1 to 3.8). The high-fructose diet caused hypertriglyceridaemia only in the female rats (Figure 3.5). β-sitosterol protected against the development of high-fructose diet-induced metabolic dysfunction in female and male rats. Fenofibrate prevented the high-fructose diet-induced visceral obesity and hypoadiponectinaemia in female and male rats. In female rats, β-sitosterol and fenofibrate had no effect on body mass gain, however, both decreased body mass gain in male rats.

In the liver, the metabolism of each molecule of fructose produces three-carbon intermediates, which can enter the glycolytic pathway (Khitan and Kim, 2013). These intermediates may be interconverted or further metabolised to acetyl-CoA (Nomura and Yamanouchi, 2012). Acetyl-CoA can be converted to malonyl-CoA, a metabolite that is utilised in *de novo* triglyceride synthesis (Solinas et al., 2015). The newly synthesised triglycerides are transported to peripheral adipose stores where they may contribute to visceral obesity (Klop et al., 2013; Rizkalla, 2010). Acetyl-CoA can also form HMG-CoA which undergoes a series of reactions to form cholesterol (Siperstein and Guest, 1960). Hypertriglyceridaemia and visceral obesity are key predictors of MetS thus are used to accurately diagnose MetS (He et al., 2013; Ren et al., 2017). In my study, the high-fructose diet caused visceral adiposity in female and male rats (Figures 3.3 and 3.4, respectively). However, the high-fructose diet caused hypertriglyceridaemia only in female rats (Figure 3.5). This finding is similar to that reported by Pektaş et al. (2015) thus suggesting that females might be more susceptible to high-fructose diet-induced MetS than their male counterparts. Oestrogen is reported to cause increase in triglyceride synthesis (Shearer et al., 2000) which may have resulted in the observed sexual dimorphic differences in circulating triglyceride levels.

In the current study, consumption of the high-fructose diet increased ($p > 0.05$) plasma cholesterol by 27% in females and 12% in males (Figures 3.5 and 3.6, respectively). This finding (fructose induced hypercholesterolaemia) might have biological relevance as it suggests that the high-fructose diet might have hypercholesterolemic effects which may predispose to cardiovascular disease.

In the present study β-sitosterol prevented the high-fructose diet-induced visceral obesity in female and male rats (Figures 3.3 and 3.4). Additionally it also prevented the high-fructose diet-induced hypertriglyceridaemia in female rats (Figure 3.5). Hwang et al. (2008) attributed these anti-obesogenic effects of β-sitosterol to its ability to activate AMP-kinase (AMPK) which mediates fatty acid oxidation. I therefore speculate that β-sitosterol prevented the high-fructose diet-induced visceral obesity and hypertriglyceridaemia through AMPK mediated fatty acid oxidation. Another interesting finding in my study was that when β-sitosterol was administered alone (with no dietary insult), it reduced ($p > 0.05$) plasma cholesterol concentration by 12% and 13% in female and male rats, respectively. A 10% decrease in total cholesterol concentration decreases the risk of coronary heart disease-related mortality (Gould et al., 2007). Therefore, my findings could be of biological importance as they suggests that β-sitosterol could be used as a supplement in normal diet with the potential to reduce cholesterol concentration. In the current study, plasma cholesterol concentration increased when β-sitosterol was administered to high-fructose diet-fed female and male rats (Figure 3.3 to 3.6), indicating that β-sitosterol should be used with caution as a dietary supplement if the diet of the individual is not well controlled. *In vitro,* β-sitosterol was reported to decrease cholesterol synthesis by decreasing HMG-CoA reductase activity (Field et al., 1997). Thus, it could be speculated that in the current study the administration of β-sitosterol exacerbated the hypercholesterolemic effects of the high-fructose diet. Another possible explanation could be that β-sitosterol and fructose, together, have adverse synergistic effects which consequently resulted in the hypercholesterolaemia. At the moment the mechanisms involved in these findings are unknown and thus further investigations are needed.

Fenofibrate, an anti-hyperlipidaemic drug, was used as a positive control in the current study. It exerts anti-obesogenic effects by stimulating peroxisome proliferator-activated receptor alpha (PPAR-α) receptor-mediated lipolysis and fatty acid β-oxidation in adipose tissue (Jeong and Yoon, 2009). PPAR-α receptors activate genes that regulate lipoprotein lipase, carnitine palmitoyltransferase and acyl-Co-A oxidase activity in the liver and in adipocytes (Minnich et al., 2001; Schoonjans et al., 1996). Lipoprotein lipase hydrolyses triglycerides into fatty acids and glycerol (Uchida et al., 2011). Carnitine palmitoyltransferase facilitates free fatty acids uptake into the mitochondria. Inside the mitochondria, acyl-Co-A oxidase promotes β-oxidation of non-esterified fatty acids (Hashimoto et al., 1999). In the current study, fenofibrate prevented the high-fructose diet-induced visceral obesity in female and male rats (Figures 3.3 and 3.4, respectively). My findings are in agreement with Jeong and Yoon (2009) who reported that fenofibrate decreased visceral fat mass in high-fat diet-fed obese mice by upregulating triglyceride hydrolysis and fatty acid β-oxidation. It could be speculated that the decrease in visceral fat mass in the current study following fenofibrate administration was due to upregulated triglyceride hydrolysis and fatty acid β-oxidation. In contrast to previous studies, fenofibrate failed to prevent the high-fructose diet-induced dyslipidaemia in female rats (Figure 3.5). Fructose causes dyslipidaemia by increasing *de novo* lipogenesis in the liver (Welsh et al., 2010). Thus it could be speculated that fenofibrate was unable to prevent the high-fructose diet-induced *de novo* lipogenesis in the liver.

Several studies have reported that visceral adiposity decreases the expression of adipokines mainly adiponectin (Balsan et al., 2015; Forny-Germano et al., 2019). Visceral adiposity increases the production of pro-inflammatory adipokines including tumour necrosis factor-α (TNF-α), IL-6, leptin and resistin (Hotamisligil et al., 1995) which in turn inhibit adiponectin gene expression (Maeda et al., 2001). Adiponectin, an anti-inflammatory adipokine, increases insulin sensitivity by stimulating the activity of PPAR-α and AMPK (Kadowaki and Yamauchi, 2005). In the present study, female and male rats fed a high-fructose diet showed undetectable adiponectin concentration (Figures 3.7 and 3.8). This finding suggests that the high-fructose diet caused hypoadiponectinaemia (Figures 3.7 and

3.8). Visceral obesity is known to cause hypoadiponectinaemia (Balsan et al., 2015; Forny-Germano et al., 2019). Indeed, the high-fructose diet caused visceral obesity and thus it could be posited that the high-fructose diet might have led to hypoadiponectinaemia by causing visceral adiposity (Stanhope et al., 2009). Hypoadiponectinaemia is associated with insulin resistance (Yamamoto et al., 2004). In the current study, high-fructose diet had no effect on insulin concentration and HOMA-IR in female and male rats (Figures 3.7 and 3.8). My results thefore showed that the consumption of a high-fructose diet for 12 weeks did not cause insulin-mediated metabolic dysfunction.

In the current study, the oral administration of β-sitosterol alone (Bst + PW) and in combination with fructose (Bst + FS) resulted in increased plasma adiponectin concentrations in female and male rats (Figures 3.7 and 3.8, respectively). β-sitosterol may have exerted its hyperadiponectinaemia effects through the reduction of visceral adiposity. Collins et al. (2007) reported that the hyperadiponectinaemia effect of plant sterols is attributed to their anti-inflammatory activities. The administration of β-sitosterol alone significantly decreased plasma insulin concentration and increased insulin sensitivity in female and male rats (Figures 3.7 and 3.8, respectively). These results are in agreement with those by Radika et al. (2013) who reported reduced insulin concentration and improved insulin sensitivity following a 4 week administration of β-sitosterol to adult Wistar rats fed a high-fat diet. β-sitosterol reduced plasma insulin concentration and HOMA-IR by exerting anti-inflammatory effects and by decreasing the expression of inducible nitric oxide synthase (iNOS) which interferes with insulin signalling (Fujimoto et al., 2005). Therefore it could be speculated that β-sitosterol decreased plasma insulin concentration and increased insulin sensitivity through anti-inflammatory mechanisms and suppressing iNOS-induced insulin signalling interference.

The oral administration of fenofibrate significantly increased plasma adiponectin concentration in female and male rats but had no effect on insulin concentration and HOMA-IR in both female and male rats (Figures 3.7 and 3.8, respectively). Thus it can be speculated that fenofibrate increased adiponectin concentration by decreasing visceral adiposity. Other studies have reported that fenofibrate increases adiponectin concentration

through activation of adipose PPAR-α (Hiuge et al., 2007; Rosenson, 2009) thus I speculate that the observed increase in adiponectin concentration in the current study could have been due to a similar mechanism.

3.5 Conclusion

I have shown that feeding a high-fructose diet to growing rats for 12 weeks causes metabolic dysfunction characterised by visceral adiposity, hypertriglyceridaemia and hypoadiponectinaemia. The high-fructose diet-induced metabolic dysfunction was prevented by oral administration of β-sitosterol. In conclusion, β-sitosterol could potentially be exploited as a prophylactic agent against diet-induced metabolic dysfunction. Importantly when compared to fenofibrate, β-sitosterol exhibited better prophylactic potential against high-fructose diet-induced metabolic dysfunction.

The next chapter (Chapter 4) gives an account on the potential of β-sitosterol to protect against high-fructose diet-induced NAFLD in growing female and male rats.

CHAPTER 4: EFFECTS OF β-SITOSTEROL ON THE PROGRESSION OF HIGH-FRUCTOSE DIET-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE IN GROWING SPRAGUE-DAWLEY RATS

4.0 Introduction

Worldwide, the prevalence of NAFLD is approximately 25% in adults (Younossi et al., 2016) and 3–12% in children (Alisi et al., 2012). Non-alcoholic fatty liver disease is a spectrum of liver disorders characterised by increased hepatic lipid accumulation (5-10 % of liver mass) in the absence of excessive alcohol consumption (Fishbein et al., 2003; Ismail et al., 2014; Tiniakos et al., 2010). The spectrum of NAFLD can range from simple fatty liver to a progressive form of NASH that is characterised by inflammation and hepatocyte degeneration (Kleiner et al., 2005). Non-alcoholic steatohepatitis leads to increased formation of fibrotic tissue in the liver that eventually results in cirrhosis and liver failure (Cohen et al., 2011). Globally, excessive consumption of high-fructose diets has been identified as one of the major drivers of NAFLD in adults and children (Ishimoto et al., 2013). Dietary fructose-induced increased DNL stimulates oxidative stress via the induction of mitochondrial dysfunction and endoplasmic reticulum (ER) stress (Jegatheesan and De Bandt, 2017). Oxidative stress triggers cell damage and a pro-inflammatory hepatic status which contributes to the progression of NAFLD to NASH (Lim et al., 2010).

Although certain aspects of NAFLD and its complications can be managed by synthetic pharmaceutical agents inclusive of fibrates (Lim et al., 2010), their high cost, limited accessibility and the deleterious side effects they elicit have triggered increased interest in the use of natural plant-derived phytochemicals which are deemed safe. β-sitosterol, a naturally occurring phytosterol (Ulbricht, 2016), has been shown to have hepatoprotective properties in carbon tetrachloride-induced liver damage of adult rats (Mondal et al., 2011). A recent study showed that β-sitosterol protected against high-fat diet-induced NAFLD in a mouse experimental model (Feng et al., 2018b). *In vitro,* β-sitosterol was demonstrated to reduce triglyceride and cholesterol content in myotubes through displacement of cholesterol from micelles (Hwang et al., 2008). β-sitosterol exerts anti-obesogenic effects through upregulating cellular AMP-protein kinase (AMPK) activity (Hwang et al., 2008) that results in increased fatty acid β-oxidation. Using an *in vitro* bile system model, β-sitosterol was shown to reduce cholesterol absorption by decreasing cholesterol solubilisation in intestinal micelles (Jesch and Carr, 2006). Despite the reported anti-

obesogenic properties of β-sitosterol, mainly *in vitro*, there are no studies, to my knowledge, that have interrogated its potential to protect against high-fructose diet-induced NAFLD in growing rats modelling children consuming unhealthy fructose-rich diets. This study evaluated the potential prophylactic effects of β-sitosterol on diet-induced NAFLD parameters (lipid content, liver steatosis, hypertrophy and lobular inflammation) of growing Sprague-Dawley rats fed a high-fructose diet.

4.1 Material and methods

4.1.1 Ethical approval

Details of ethical approval and care of experimental animals are as described in Chapter 3, subheading 3.1.1 page 28.

4.1.2 Chemicals and reagents

Chemical and reagents used were similar to those described in Chapter 3, subheading 3.1.2 page 28.

4.1.3 Animals, housing and general care

Housing and care of the rats were as described in Chapter 3, subheading 3.1.3, page 28.

4.1.4 Experimental design

The experimental design was as described in chapter 3, subheading 3.1.4, page 29.

4.1.5 Terminal procedures

Following 12-weeks of the administration of the treatment regimens, the rats were fasted overnight and euthanised with an intraperitoneal injection of sodium pentobarbitone (Eutha-naze, Centaur labs, Johannesburg, South Africa) at 200 mg/kg body mass. Blood was then collected via cardiac puncture into heparinised blood collection tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France), centrifuged (SorvallRT ®6000B, Pegasus Scientific Inc., Rockville USA) and plasma harvested and stored at −20°C pending the determination of surrogate markers of liver function. The liver was

carefully dissected out and weighed. A sample of the liver was taken from the medial lobe and preserved in 10% phosphate-buffered formalin for histology. The remainder of the liver was placed in sealed ziplock bags and stored in a freezer (Bosch, Stuttgart, Germany) at -20˚C pending the determination of total liver lipid content.

4.1.6 Determination of total liver lipid content

Liver lipids were extracted as described by Bligh and Dyer (1959). Briefly, each frozen liver sample (5-7g) was thawed to reach room temperature and then steeped in 50ml of 2:1 chloroform-methanol (Minema Chemicals, Johannesburg, South Africa) overnight at 4°C. The mixture was filtered through filter paper (Whatmann®, No 1, size 185 mm, pore size 7-11µm, England) into a 250ml separating funnel where 30ml of 0.9% saline was added to the filtrate from a dispensing bottle and then left at 4°C overnight to separate into two layers. The bottom layer (chloroform) was collected into a round-bottomed flask (Scott Duran, Germany) and the lipids were recovered by evaporating the solvent at 37˚C using a rotor evaporator (Labocon (Pty) Ltd, Krugersdorp, Transvaal, South Africa). The lipid extract was then dissolved in 20ml chloroform. Two millilitres of the lipid extract was aliquoted into a pre-weighed dry vial, dried at 50°C and then the vial containing the lipid was reweighed. The total liver lipid content was then expressed as a percentage of the original weight of the liver sample.

4.1.7 Liver histological analyses

The preserved liver samples were routinely processed, embedded in paraffin wax, sectioned (5 µm) and stained with haematoxylin and eosin (H&E) (Reyes-Gordillo et al., 2007). To assess the hepatocellular changes, three random fields per slide were viewed under a light microscope at low and high-power magnification. The semi-quantitative NAFLD activity score (NAS) method was used to assess the progression and severity of the NAFLD (Kleiner et al., 2005; Liang et al., 2014). NAS criteria: *steatosis grade* scoring 0: 0-5%; 1: 5-33%; 2: 33-66%; 3; > 66%; *foci of lobular inflammation scoring* (at low magnification) 0: none; 1: 1-2; 2: >2; hypertrophy/ballooning scoring 0: none; 1: 1-2; 2: >2; *total NAS* score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

4.1.8 Determination of surrogate markers of liver health

Plasma samples that had previously been frozen were thawed, warmed to room temperature and gently mixed to ensure homogeneity of the sample prior to assaying. The plasma activities of alanine transaminase (ALT) and aspartate transaminase (AST) were determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions.

4.2 Statistical analysis

GraphPad Prism 6.0 software (Graph-pad Software Inc. San Diego, USA) was used to analyse data. Parametric data are expressed as mean ± standard deviation (SD) and non-parametric data (NAS) are expressed as median and range (min, max). A one-way ANOVA, followed by a Tukey *post-hoc* test, was used to analyse parametric multiple-group data. The Kruskal-Wallis test was used to analyse multiple-group NAS data followed by a multiple-comparisons Dunn's *post hoc* test. Significance was accepted when $p < 0.05$.

4.3 Results

4.3.1 Liver masses

Tables 4.1 and 4.2 present the liver masses of the female and male rats following their respective treatments. The high-fructose diet had no effect ($p > 0.05$) on liver masses (absolute and relative to body mass) in female and male rats (Figures 4.1 and 4.2). The female rats fed the high-fructose diet and administered fenofibrate (FF + FS) had significantly greater ($p < 0.05$) liver masses (absolute and relative to body mass) when compared to all counterparts (Table 4.1). The male rats fed the high-fructose diet and administered fenofibrate (FF + FS) had significantly greater ($p < 0.0001$ when compared to PC + PW; $p = 0.0068$ when compared to PC + FS, respectively) liver masses (relative to body mass) (Table 4.2). In female rats β-sitosterol alone (Bst + PW) significantly ($p = 0.0145$ when compared to FF + FS; $p < 0.0001$ when compared to Bst + FS) decreased liver masses (Figure 4.1).

Table 4.1: The effect of β-sitosterol on absolute and relative (to body mass) liver masses of growing female rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Liver (g)	6.53 ± 0.91^{ab}	7.35 ± 0.54^{abc}	8.08 ± 0.67^{c}	6.26 ± 0.54^{a}	6.99 ± 0.48^{a}	*
Liver (%BM)	2.64 ± 0.27^{ab}	2.84 ± 0.14^{ab}	3.26 ± 0.31^{c}	2.50 ± 0.18^{a}	2.82 ± 0.24^{a}	*

[abc]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Data presented as mean ± SD; n = 7-9 per treatment regimen. %BM = relative to body mass.

Table 4.2: Effects of β-sitosterol on absolute and relative (to body mass) liver masses of growing male rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Liver (g)	11.58 ± 1.29^{a}	11.94 ± 1.56^{a}	12.27 ± 1.24^{a}	11.10 ± 1.13^{a}	12.14 ± 1.08^{a}	ns
Liver (%BM)	2.62 ± 0.17^{ab}	2.88 ± 0.25^{abc}	2.40 ± 0.53^{c}	2.63 ± 0.21^{a}	3.11 ± 0.13^{c}	**

[abc]Within row means with different superscripts are significantly different at $p < 0.05$.** $p < 0.001$. ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Data presented as mean ± SD; n = 7-9 per treatment regimen. %BM = relative to body mass.

4.3.2 Liver lipid content

Figures 4.1 and 4.2 show the total liver lipid content of female and male rats following respective treatments. The high-fructose diet (PC + FS) fed female rats had a 36% increase in total liver lipid content compared to their control counterparts (Figure 4.1). The high-fructose diet (PC + FS) fed male rats had significantly increased ($p = 0.0197$) total liver lipid content compared to their control counterparts (Figure 4.2). β-sitosterol (Bst + FS) and fenofibrate (FF + FS) failed to prevent ($p > 0.05$) the high-fructose diet-induced hepatic lipid accumulation in female and male rats (Figures 4.1 and 4.2). β-sitosterol alone (Bst + PW) did not affect ($p > 0.05$) total liver lipid content in female and male rats (Figures 4.1 and 4.2).

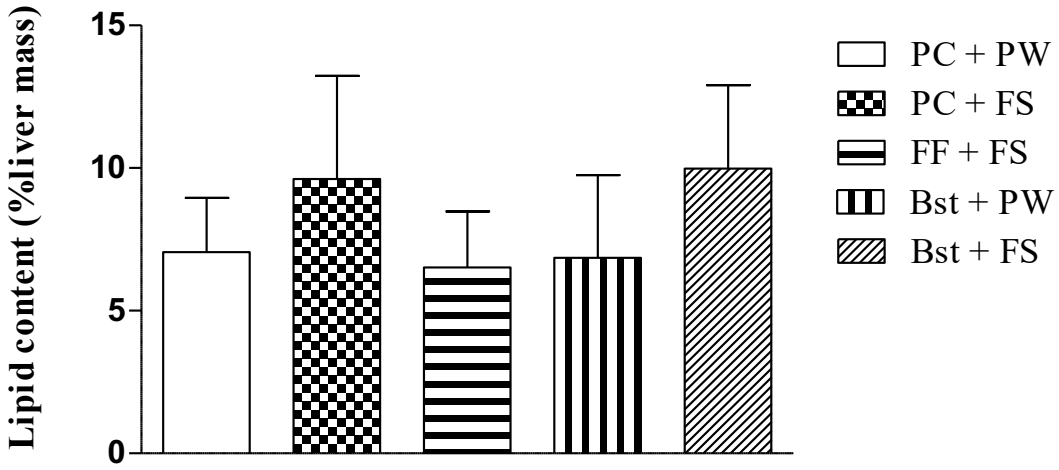

Figure 4.1: Effect of β-sitosterol on the liver lipid content of high-fructose diet-fed growing female rats.

PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Data presented as mean ± SD; n = 7 per treatment regimen.

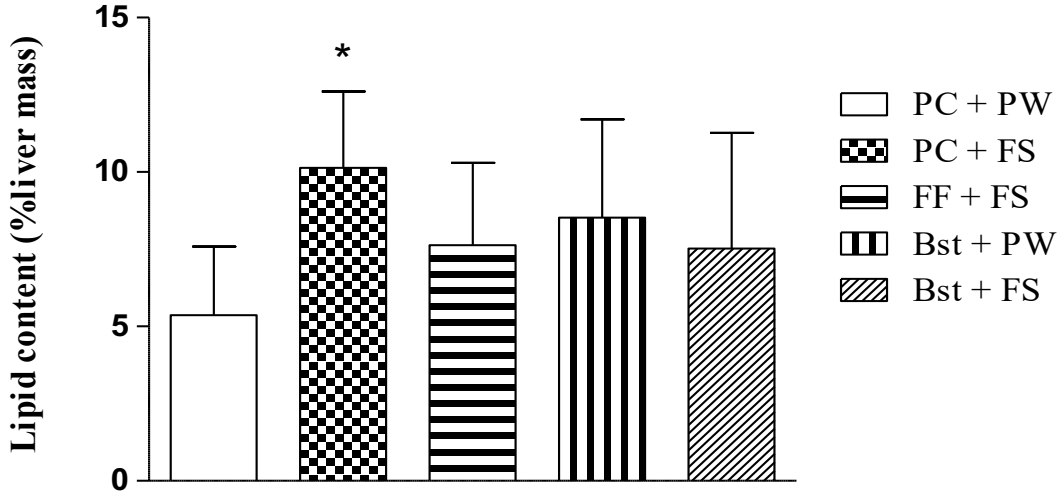

Figure 4.2: Effect of β-sitosterol on the liver lipid content of high-fructose diet-fed growing male rats.

* $p < 0.05$ vs control (PC + PW). PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Data presented as mean ± SD; n = 7 per treatment regimen.

4.3.3 Liver histomorphometry

Figures 4.3 and 4.4 show the representative liver histology photo sections (H and E staining, 400 X magnification) of female and male rats from the different treatment groups. The high-fructose diet (PC + FS) resulted in micro- and macro-vesicular hepatic steatosis in female and male rats (Figures 4.3 and 4.4). β-sitosterol (Bst + FS) and fenofibrate (FF + FS) prevented the high-fructose diet-induced macro-vesicular hepatic steatosis in female and male rats (Figures 4.3 and 4.4).

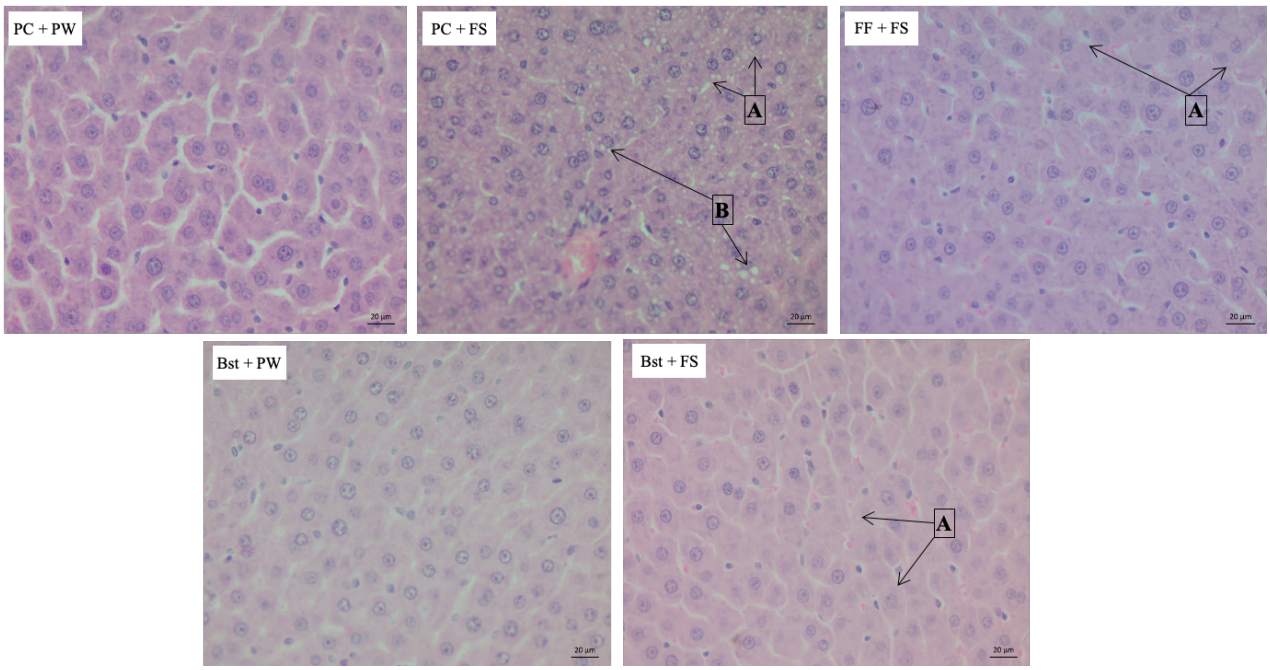

Figure 4.3: Representative photos showing the hepatic histology (H and E staining, 400 X magnification) of female rats in the different treatment groups.

Arrows A point to hepatic micro-vesicular steatosis, arrows B point to macro-vesicular steatosis. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Scale bar = 20 μm in the H&E stain sections.

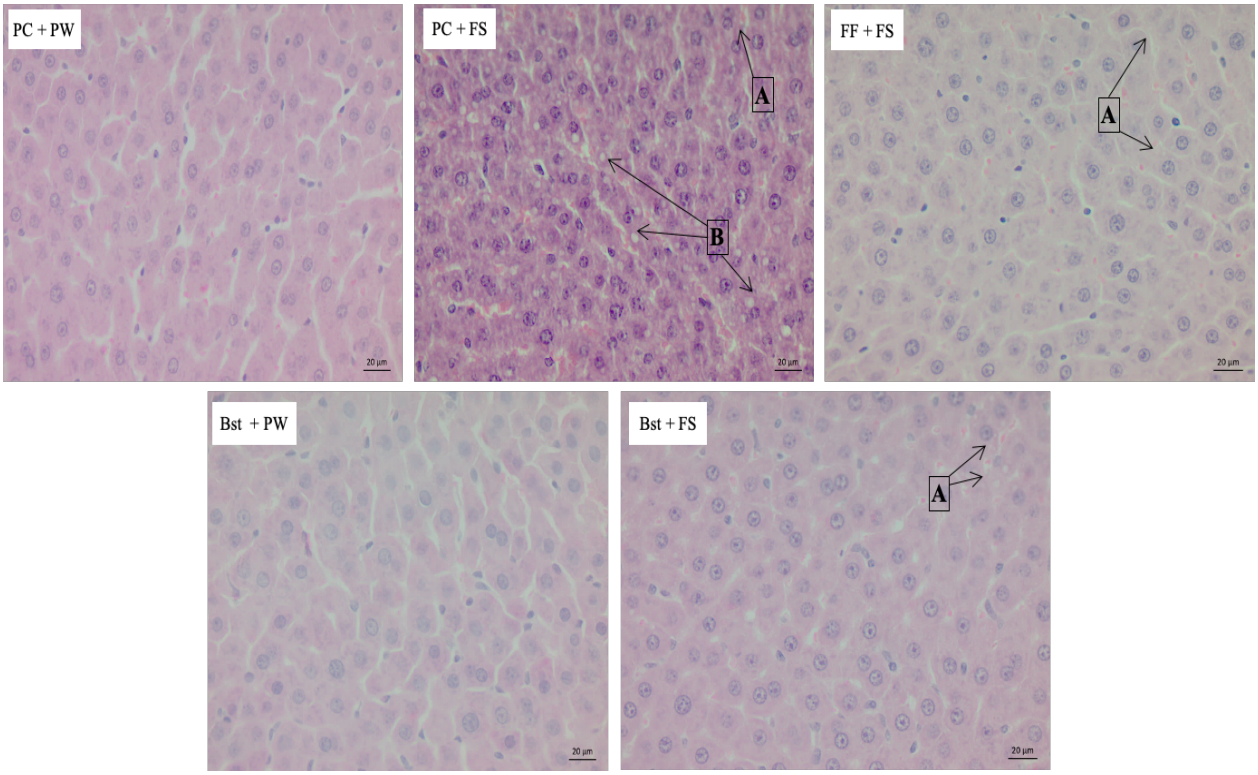

Figure 4. 4: Representative photos showing the hepatic histology (H and E staining, 400 X magnification) of male rats in the different treatment groups.

Arrows A point to hepatic micro-vesicular steatosis, arrows B point to macro-vesicular steatosis. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v). Scale bar = 20 µm in the H&E stain sections.

4.3.4 Non-alcoholic fatty liver disease activity score

Tables 4.3 and 4.4 depicts the micro- and macro-steatosis, hypertrophy, lobular inflammation scores and total NAS score of the female and male rats, respectively. The high-fructose diet (PC + FS) resulted in micro- and macro-vesicular hepatic steatosis in female and male rats when compared to PC + PW (Tables 4.3 and 4.4). The high-fructose diet (PC + FS) resulted in inflammation and total NAS > 5 in male rats when compared to their control counterparts PC +PW (Table 4.4). β-sitosterol (Bst + FS) and fenofibrate (FF + FS) attenuated the high-fructose diet-induced micro-steatosis and prevented ($p < 0.01$) macro-vesicular hepatic steatosis in female ($p = 0.0063$) and male ($p = 0.0061$) rats when compared to PC + FS (Tables 4.3 and 4.4).

Table 4.3: Effect of β-sitosterol on non-alcoholic fatty liver disease activity score in the liver of female rats fed a high-fructose diet

Criteria	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Micro-steatosis	0 (0, 0)^{a}	2 (1, 2)^{b}	1 (0, 1)^{ab}	0 (0, 0)^{a}	1 (1, 1)^{ab}	*
Macro-steatosis	0 (0, 0)^{a}	2 (1, 3)^{b}	0 (0, 0)^{ab}	0 (0, 0)^{a}	0 (0, 0)^{a}	**
Hypertrophy	0 (0, 1)^{a}	2 (0, 2)^{a}	0 (0, 1)^{a}	0 (0, 0)^{a}	0 (0, 1)^{a}	ns
Inflammation	0.5 (0, 1)^{a}	2 (1, 2)^{a}	0 (0, 1)^{a}	0.5 (0, 1)^{a}	1 (0, 1)^{a}	ns
Total NAS	0.5 (0, 2)^{a}	3.5 (2, 6)^{a}	1.5 (0, 3)^{a}	0.5 (0, 1)^{a}	2 (2, 2)^{a}	ns

^{ab} Within row means with different superscripts are significantly different at $p < 0.05$.*$p < 0.05$; **$p < 0.001$. ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS =

gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); NAS = non-alcoholic fatty liver disease activity score. Data presented as median and range (min, max). n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

Table 4.4: Effect of β-sitosterol on non-alcoholic fatty liver disease activity score in the liver of male rats fed a high-fructose diet

Criteria	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Micro-steatosis	0 (0, 0)[a]	3 (1, 3)[b]	1 (0, 1)[ab]	0 (0, 0)[b]	1 (0, 1)[ab]	* *
Macro-steatosis	0 (0, 0)[a]	1 (1, 2)[b]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	* *
Hypertrophy	0 (0, 0)[a]	0 (0, 1)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 1)[a]	ns
Inflammation	0 (0, 0)[a]	2 (1, 2)[b]	1 (1, 1)[ab]	0 (0, 1)[b]	1 (1, 1)[ab]	*
Total NAS	0 (0, 0)[a]	5 (4, 5)[b]	2 (1, 2)[ab]	0 (0, 1)[a]	2.5 (2, 3)[ab]	* *

[ab] Within row means with different superscripts are significantly different at $p < 0.05$.* $p < 0.05$; ** $p < 0.001$. ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); NAS = non-alcoholic fatty liver disease activity score. Data presented as median and range (min, max). n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

4.3.5 Non-alcoholic fatty-liver disease frequency

Tables 4.5 and 4.6 depicts the hepatic micro- and macro-vesicular steatosis, hypertrophy, lobular inflammation scores and total NAS of the rats, respectively. Hepatic micro- and macro-vesicular steatosis was observed in 100% of the sampled high-fructose diet-fed (PC + FS) female and male rats (Tables 4.6 and 4.7). Inflammation and total NAS > 5 was observed in 100% of the sampled high-fructose diet-fed (PC + FS) male rats (Table 4.6). β-sitosterol (Bst + FS) and fenofibrate (FF + FS) attenuated the high-fructose diet-induced micro-vesicular steatosis and prevented ($p < 0.01$) macro-vesicular steatosis in 100% of the sampled female and male rats (Tables 4.5 and 4.6).

Table 4.5: Non-alcoholic fatty liver disease activity score criteria frequency of high-fructose diet-fed female rats

Criteria	Steatosis								Hypertrophy				Inflammation				Total NAS		
	Microvesicular				Macrovesicular														
Score	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*<2*	*3-4*	*≥ 5*
PC + PW	100	0	0	0	100	0	0	0	75	25	0	0	50	50	0	0	100	0	0
PC + FS	0	25	75	0	0	25	50	25	50	25	25	0	0	25	75	0	50	0	50
FF + FS	25	75	0	0	100	0	0	0	75	25	0	0	25	75	0	0	75	25	0
Bst + PW	100	0	0	0	100	0	0	0	100	0	0	0	50	50	0	0	100	0	0
Bst + FS	0	100	0	0	100	0	0	0	75	25	0	0	25	75	0	0	100	0	0

PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v);. n = 4 per treatment. NAS criteria: *steatosis grade* scoring 0: 0-5%; 1: 5-33%; 2: 33-66%; 3; > 66%; *foci of lobular inflammation scoring* 0: none; 1: 1-2; 2: >2. Hypertrophy/ballooning scoring 0: none; 1: 1-2; 2: >2. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis

Table 4.6: Non-alcoholic fatty liver disease activity score criteria frequency of high-fructose diet-fed male rats.

Criteria	Steatosis								Hypertrophy				Inflammation				Total NAS		
	Microvesicular				Macrovesicular														
Score	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*<2*	*3-4*	*≥ 5*
PC + PW	100	0	0	0	100	0	0	0	100	0	0	0	100	0	0	0	100%	0%	0%
PC + FS	0	0	25	75	0	75	25	0	50	50	0	0	0	25	75	0	0%	25%	75%
FF + FS	25	75	0	0	100	0	0	0	100	0	0	0	0	100	0	0	100%	0%	0%
Bst + PW	100	0	0	0	100	0	0	0	100	0	0	0	75	25	0	0	100%	0%	0%
Bst + FS	0	100	0	0	100	0	0	0	50	50	0	0	0	100	0	0	50%	50%	0%

PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v);. n = 4 per treatment. NAS criteria: *steatosis grade* scoring 0: 0-5%; 1: 5-33%; 2: 33-66%; 3; > 66%; *foci of lobular inflammation scoring* 0: none; 1: 1-2; 2: >2. Hypertrophy/ballooning scoring 0: none; 1: 1-2; 2: >2. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

4.3.6 Surrogate markers of liver function

Tables 4.7 and 4.8 show plasma concentration of alanine aminotransferase (ALT) and aspartate transaminase (AST) in high-fructose diet-fed female and male rats, respectively. The plasma AST and ALT concentration were similar across treatment regimens (Tables 4.7 and 4.8).

Table 4.7: Effect of β-sitosterol on plasma aspartate aminotransferase and alanine aminotransferase activity in growing female rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
ALT (U/L)	45.85 ± 11.09[a]	46.71 ± 9.44[a]	63.57 ± 16.46[a]	57.66 ± 10.13[a]	63.14 ± 22.45[a]	ns
AST (U/L)	157.14 ± 51.88[a]	172 00 ± 22.12[a]	183.14 ± 23.78[a]	172.16 ± 41.02[a]	189.57 ± 50.93[a]	ns

[a]Within row means are similar at $p > 0.05$; ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day (Bst) + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); AST = aspartate aminotransferase; ALT = alanine aminotransferase. Data presented as mean ± SD; n = 7-9 per treatment.

Table 4.8: Effect of β-sitosterol on plasma aspartate aminotransferase and alanine aminotransferase activity in growing male rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
ALT (U/L)	77.57 ± 27.21[a]	64.14 ± 24.12[a]	85.33 ± 33.61[a]	64.42 ± 11.39[a]	63.28 ± 18.65[a]	ns
AST (U/L)	201.85 ± 27.59[a]	177 ± 33.55[a]	211.5 ± 49.48[a]	171.28 ± 23.16[a]	157.28 ± 45.30[a]	ns

[a]Within row means are similar at $p > 0.05$; ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day (Bst) + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); AST = aspartate aminotransferase; ALT = alanine aminotransferase. Data presented as mean ± SD; n = 7-9 per treatment.

4.4 Discussion

The study aimed to investigate the potential of orally administrated β-sitosterol to protect growing female and male rats against the development of high-fructose diet-induced NAFLD. Findings from the current study showed that male rats administered the high-fructose diet had significantly higher liver lipid content compared to that of rats administered the control (PW + PC) but similar to that of the other groups. In female rats there was no significant difference in the liver lipid content across treatment regimens. Despite this similarity the liver lipid content of the female rats administered PC + FS had a 25% increase in liver lipid content. However, rats across treatment regimens had a liver lipid content within the 5-10% criteria cut-off for diagnosis of NAFLD (Ismail et al., 2014). In female rats, the NAFLD was characterised by micro- and macro-vesicular hepatic steatosis whereas in male rats it was typified by increased hepatic lipid accretion, micro- and macro-vesicular steatosis and hepatic inflammation. Orally administered β-sitosterol and fenofibrate attenuated the high-fructose diet-induced macro-vesicular hepatic steatosis in female and male rats. In female rats fenofibrate caused hepatomegaly without compromising liver function. The high-fructose diet and or β-sitosterol had no impact on the liver mass and did not compromise the liver function of the rats.

Dietary fructose results in increased hepatic *de novo* lipid synthesis and storage as well as decreased β-oxidation (Basciano et al., 2010; Collison et al., 2009). This increase in hepatic *de novo* lipid synthesis and accretion, typically, manifests in steatosis. In the current study, the consumption of 20% dietary fructose solution for 12-weeks resulted in a significant increase in hepatic lipid accretion only in male rats (Figure 4.2). Importantly, histo-morphological examination of the liver sections, which is considered as the gold standard for assessing NAFLD, revealed that the high-fructose diet caused micro- and macro-vesicular steatosis in female and male rats as well as hepatic inflammation in male rats (Figures 4.3 and 4.4). Non-alcoholic fatty liver disease activity score (NAS) is used to evaluate the progression and severity of NAFLD (Jegatheesan and De Bandt, 2017). An increase in total NAS (≥5) is indicative of NASH. My results show that consumption of dietary fructose caused the total NAS to be >5 in male rats only (Tables 4.4 to 4.6),

suggesting that the dietary-fructose caused the male rats to develop NASH. My findings are in agreement with previous studies that have shown that high-fructose diets cause NAFLD that can progress to NASH (Armiliato, 2015; Jensen et al., 2018). Importantly, my results also suggest that male rats are likely to be more susceptible than female rats to high-fructose diet-induced NAFLD. My findings on the susceptibility of male rats to develop NAFLD are consistent with several other studies which have shown that males have a higher prevalence of NAFLD than females (Caballería et al., 2010; Torres et al., 2012; Williams et al., 2011). These observed sex differences were considered to be due to the role of sex steroid hormones in the pathogenesis of NAFLD. Oestrogen activation is reported to protect against the development and progression of NAFLD (Ballestri et al., 2017; Summart et al., 2017). Other findings show that males are more suspectible due to lower plasma adiponectin levels, lower expression of M2 genes in the liver which are responsible in aggravation of pro-inflammatory response (Matsushita et al., 2017). However, there still is a need to further interrogate and identify the patho-physiological mechanisms driving the sexually dimorphic responses reported in the current study.

In the present study, orally administered β-sitosterol prevented the rats from developing high-fructose diet-induced macro-vesicular hepatic steatosis and attenuated the severity of fructose diet-induced micro-vesicular steatosis and hepatic inflammation in female and male rats (Tables 4.3 to 4.6, respectively). However β-sitosterol failed to prevent the high-fructose diet-induced increase in total liver lipid accretion seen in the male rats. It has been shown that macro-vesicular hepatic steatosis is associated with increased hepatic inflammation (Tandra et al., 2011). Indeed, female and male rats that were fed the high-fructose diet with β-sitosterol, as an intervention, had lower lobular inflammation scores compared to their counterparts that were fed only the high-fructose diet (Tables 4.3 to 4.6, respectively). This finding suggests that β-sitosterol attenuated the high-fructose diet-induced macro-vesicular hepatic steatosis by reducing hepatic inflammation. In human and animal-based studies β-sitosterol has been shown to possess anti-inflammatory properties (Backhouse et al., 2008). Furthermore, studies have reported that β-sitosterol regulates glucose and lipid metabolism in a muscle cell line through AMP-activated protein kinase

(Hwang et al., 2008). Hwang et al. (2008) reported reduced intracellular concentrations of triglycerides and cholesterol in L6 myotube cells following treatment with β-sitosterol. Thus β-sitosterol may have also prevented the high-fructose diet-induced macro-vesicular hepatic steatosis by reducing the expression of lipogenic genes in the liver (Feng et al., 2018a). Nonetheless, the mechanism by which β-sitosterol exerted its health beneficial effects needs to be further investigated using molecular techniques. Another important finding from my study was that the total NAS of male rats which were fed the high-fructose diet with the administration of β-sitosterol as an intervention was <5 (Table 4.4 to 4.6). My findings suggest that β-sitosterol attenuated the progression of high-fructose diet-induced NAFLD to NASH in male rats.

Fenofibrate, which was used as a positive control in the current study, failed to prevent the diet-induced hepatic lipid accretion, micro-vesicular hepatic steatosis and inflammation but, like β-sitosterol, it prevented macro-vesicular hepatic steatosis in female and male rats (Tables 4.3 to 4.6, respectively). However, it is of importance to note that in the current study the high-fructose diet-fed rats that had fenofibrate administered as an intervention had mean total liver lipid content that was below the clinically significant level of 10% liver mass in both female and male rats (Figures 4.1 and 4.2, respectively). Although from a statistical point of view findings from my current study contradict those by Muhammad et al. (2019), it is important to note that in my study the administration of fenofibrate as an intervention reduced the liver lipid content of the rats to below 10% suggesting that it mitigated liver lipid accretion.

The liver is a key site for metabolite storage and processing (Weickert and Pfeiffer, 2006). Liver mass can be impacted by the quantity of metabolites stored and inflammation (Özen, 2007; Schwarz et al., 2015; Stanhope et al., 2009). Though in the current study, the high-fructose diet increased total liver lipid accretion (Figures 4.1 and 4.2) and caused inflammation, it did not increase the liver mass of the female and male rats (Table 4.1 to 4.2, respectively). My findings suggest that the consumption of a high-fructose diet in the form of a 20% fructose solution for 12-weeks does not have an impact on liver mass. Similarly, the administration of β-sitosterol in female and male rats had no effect on the

liver mass (Tables 4.1 and 4.2, respectively). The administration of fenofibrate increased liver masses in female and male rats in the present study (Table 4.1). The increase in liver mass seen in the current study is in agreement with Hong et al. (2007) who reported that fenofibrate aggravated the high-fat diet-induced hepatomegaly. Therefore, the adverse outcomes associated with fenofibrate prompt that its use, as an intervention for NAFLD, should be done with careful consideration as it may exert trophic effects on organs including the liver.

To further investigate the potential hepatotoxic effects of the treatments, I determined their effects on plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), surrogate markers of liver function. Plasma AST and ALT activities are indicative of liver parenchyma cell damage (Botezelli et al., 2012). However the release of AST and ALT from damaged hepatocytes is dependent on the severity of liver damage (Giannini, 2005). My results revealed that feeding a high-fructose diet to growing rats caused fatty liver disease that progressed to NASH in male rats only (Tables 4.4 and 4.6). It, however, did not compromise the rats' liver function as depicted by the plasma ALT and AST activities not being elevated across treatments (Tables 4.7). This could possibly be due to the high-fructose diet-induced fatty liver disease being in its early stages of development. In its initial stages fatty liver disease does not cause significant hepatocyte damage (Palekar et al., 2006). Oral administration of interventions inclusive of synthetic pharmaceutical agents and phytochemicals has the potential to cause adverse outcomes on liver function (Chan et al., 2015). In the current study, β-sitosterol and or fenofibrate had no effect on plasma AST and ALT activities in female and male rats (Table 4.7 and 4.8, respectively) suggesting that these interventions did not compromise the liver function.

4.5 Conclusion

This study has demonstrated that feeding a high-fructose diet caused NAFLD which progressed to NASH only in the male rats. β-sitosterol and or fenofibrate prevented the high-fructose diet-induced NAFLD in the rats and prevented it from progressing to NASH

in the male rats. Therefore β-sitosterol can prospectively be used to mitigate diet-induced NAFLD.

Diabetic nephropathy is another complication that can arise from high fructose consumption. The next chapter (Chapter 5) gives an account of potential protective effects of β-sitosterol against high-fructose diet-induced renal dysfunction in growing female and male rats.

CHAPTER 5: EFFECTS OF β-SITOSTEROL ON HIGH-FRUCTOSE DIET-INDUCED RENAL DERANGEMENTS IN GROWING SPRAGUE-DAWLEY RATS

5.0 Introduction

According to the World Health Organisation, worldwide, renal diseases cause 5–10 million deaths annually ("WHO | The global burden of kidney disease and the sustainable development goals," 2018). It is estimated that in 2015, 1.2 million people died from kidney failure, an increase of 32% since 2005 (Wang et al., 2016). Additionally, each year, approximately 1.7 million people die from acute kidney injury (Mehta et al., 2015). In South Africa, the cost of sustaining a patient on dialysis is approximately R200 000 annually (Wearne et al., 2019) which translates into increased burden on the health delivery system. Diabetic nephropathy is a microvascular complication of diabetes mellitus and has become the main cause of chronic kidney disease (Cao and Cooper, 2011). Research suggests that obesity and MetS play a critical role in the development of renal disease (Chen et al., 2004). A key feature of MetS is insulin resistance that leads to hyperinsulinaemia, glucose intolerance and dyslipidaemia (Ormazabal et al., 2018; Reaven, 2012). Fructose consumption is linked with renal injury (Nakayama et al., 2010). In the small intestines, fructose is absorbed unchanged and then transported to the liver where it is metabolised (Hallfrisch, 1990). High-fructose diets result in increased fructose outflow from the liver to the systemic circulation and into the kidney (Gaby, 2005) contributing to the early onset of renal damage (Kizhner and Werman, 2002; Tasevska et al., 2005). Fructose metabolism in the liver results in increased uric acid production coupled with a reduction in its excretion through the kidneys (Bratoeva et al., 2017). Increased uric acid causes glomerular injury and tubulointerstitial fibrosis (Johnson et al., 2013). In rats, high-fructose diets have been shown to induce MetS and accelerate chronic renal disease (Johnson et al., 2010; Nakayama et al., 2010). High-fructose diet-induced renal damage has also been reported in humans (Johnson et al., 2018, 2010). Long term consumption of fructose is reported to cause proteinuria, kidney enlargement, focal tubulointerstitial injury and glomerulosclerosis (Kizhner and Werman, 2002; Zaoui et al., 1999). The reported kidney damage following fructose consumption has been attributed to decreased renal excretion of uric acid (Johnson et al., 2007). Increased uric acid results in afferent arteriolopathy leading to glomerular hypertension (Sánchez-Lozada et al., 2008).

Additionally high-fructose diets have been shown to stimulate pro-inflammatory mediators such as monocyte chemotactic protein-1 (MCP-1) in the proximal tubular cells which injure the tubules and contributes to progression of renal injury (Cirillo et al., 2009). Kidney-injury molecule-1 (KIM) plays an important role in the proliferation and regeneration processes of renal tissue (Ichimura et al., 2012). Thus, following tubular injury, the kidney cells secrete KIM, which is considered a specific biomarker for assessing tubular injury (Moresco et al., 2018).

Lifestyle modification and the use of synthetic pharmaceutical drugs can mitigate the onset and progression of diet-induced renal disease (James et al., 2010). However, synthetic pharmaceutical drugs are associated with side effects (Olaiya et al., 2015) hence the growing interest in medicinal-plant derived phytochemicals. Medicinal-plant derived phytochemicals such as phytosterols possess anti-oxidant activities that have been demonstrated to ameliorate the oxidative stress-induced kidney damage (Rafieian-Kopaei, 2013). β-sitosterol, a phytosterol of interest to the current study, is reported to reduce plasma creatinine and blood urea nitrogen (BUN) concentration in rats with hypertension-induced renal damage (Olaiya et al., 2015). Furthermore, in chemically-induced acute nephrotoxicity rats, oral administration of β-sitosterol improved renal function by improving the anti-oxidant status (Sharmila et al., 2016). Despite the reported renoprotective properties of β-sitosterol, there are no studies that have interrogated the potential reno-protective effects of β-sitosterol in high-fructose diet-fed growing female and male rats. Oestrogens are reported to retard the progression of chronic renal disease in women (Baylis, 2009; Neugarten et al., 2000) while androgens exacerbate kidney damage (Baylis, 2009). This therefore justifies the need to evaluate any potential prophylactic agent in both females and males due to possible sexual dimorphism.

5.1 Material and methods

5.1.1 Ethical approval

Details of ethical approval and care of experimental animals are as described in Chapter 3, subheading 3.1.1 page 28.

5.1.2 Chemicals and reagents

Chemical and reagents used are similar to those described in Chapter 3, subheading 3.1.2 page 28.

5.1.3 Animals, housing and general care

Housing and care of the animals is as described in Chapter 3, subheading 3.1.3, page 28.

5.1.4 Experimental design

The experimental design was as described in chapter 2, subheading 3.1.4, page 29.

5.1.5 Terminal procedures and sample collection

Following 12 weeks of the administration of the treatments, the rats were fasted overnight but allowed access to plain drinking water. The rats were euthanised with an intraperitoneal injection of sodium pentobarbitone at 200 mg/kg body mass. Following euthanasia, a blood sample was collected via cardiac puncture using 18G needles and 10ml syringes, into heparinised blood tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The blood samples were centrifuged at 20°C for 15 min at 5000 g in a Sorvall RT® 6000B centrifuge (Pegasus Scientific Inc., Rockville USA). The harvested plasma was stored in microtubes at -20°C pending determination of creatinine, urea and kidney injury molecule-1 concentration.

The abdomen of each rat was opened via a midline incision and the kidneys were dissected out and weighed. The kidney mass relative to body mass (%BM) was computed according to the formula:

Relative kidney mass = [kidney mass (g) /terminal body mass (g)] x 100.

5.1.6 Kidney histological analyses

The preserved kidney samples were routinely processed using an automatic tissue processor (Micron STP 120, Thermo Fischer Scientific, USA), embedded in paraffin wax, sectioned (5 μm) and stained with haematoxylin and eosin (H&E) (Reyes-Gordillo et al., 2007). Triplicate digital images of the kidney sections were obtained from different fields using a camera (AxioCam ERc 5s Rev.2; ZEISS, Germany) connected to a light microscope. Bowman's space area, glomerular tuft and glomerular density were obtained using a computerised morphometric analysis system (ImageJ 1.47v, Java 1.8.0_191; LOCI, University of Wisconsin). Urinary space and glomerular density were computed according to the following formulas:

urinary space = Bowman's space area (μm^2) - glomerular tuft area (μm^2) (Demircin et al., 2009).

glomerular density = number of glomerular corpuscles in a section (N)/ total area of the section (μm^2).

5.1.7 Determination of surrogate markers of kidney health

The plasma creatinine and urea concentration were determined using a colorimetric-based clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per the manufacturer's instructions. Plasma kidney injury molecule-1 (pg/mL) concentration was measured using a kidney injury molecule-1 (ELISA) kit for rats (Elabscience Biotechnology Co, Wuhan, Hubei Province, China). A detailed description of the protocol used is attached in appendix VI. A standard curve was constructed using known concentrations. The concentrations of kidney injury molecule-1 in the samples were then determined from the constructed standard curve.

5.2 Statistical analysis

GraphPad Prism 6.0 (Graph-pad Software Inc. San Diego, USA) software was used to analyse data. Data are expressed as mean ± SD. Data on weekly body mass was analysed using a repeated-measures analysis of variance (ANOVA). Multiple-group data were

analysed using one-way ANOVA, followed by Tukey's *post-hoc* test. Significance was accepted when $p < 0.05$.

5.3. Results

5.3.1. Kidney masses

Tables 5.1 and 5.2 present the kidney masses of the female and male rats following their respective treatments. The high-fructose diet (PC + FS), β-sitosterol (Bst + FS) and fenofibrate (FF + FS) had no effect on kidney masses (absolute and relative to body mass) in female and male rats (Tables 5.1 and 5.2).

Table 5.1: Effect of β-sitosterol on kidney masses of growing female rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Kidney (g)	1.43 ± 0.12[a]	1.60 ± 0.08[a]	1.56 ± 0.17[a]	1.47 ± 0.12[a]	1.44 ± 0.21[a]	ns
Kidney (%BM)	0.58 ± 0.05[a]	0.62 ± 0.03[a]	0.63 ± 0.01[a]	0.59 ± 0.05[a]	0.58 ± 0.08[a]	ns

[a]Within row means are similar at $p > 0.05$; ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); BM = body mass. Data presented as mean ± SD; n = 7- 9 per treatment regimen.

Table 5.2: Effect of β-sitosterol on kidney masses of growing male rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Kidney (g)	2.60 ± 0.17^{a}	2.37 ± 0.17^{ab}	2.30 ± 0.16^{b}	2.41 ± 0.17^{ab}	2.29 ± 0.20^{b}	*
Kidney (% BM)	0.59 ± 0.02^{ab}	0.57 ± 0.02^{a}	0.64 ± 0.10^{b}	0.58 ± 0.02^{a}	0.59 ± 0.03^{ab}	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v);. BM = body mass. Data presented as mean ± SD; n = 7-9 per treatment regimen.

5.3.2. Surrogate markers of kidney function and damage

The plasma concentration of creatinine, urea and kidney injury molecule-1 (KIM-1) of female and male rats are shown in Tables 5.3 and 5.4, respectively. The high-fructose diet increased ($p < 0.0001$) plasma concentration of KIM-1 in female and male rats (Tables 5.3 and 5.4). β-sitosterol (Bst + FS) and fenofibrate (FF + FS) failed to protect against the high-fructose diet-induced increase in KIM-1 in female and male rats ($p > 0.05$; Tables 5.3 and 5.4).The high-fructose diet (PC + FS) and β-sitosterol (Bst + FS) had no effect on plasma creatinine and urea concentration in female and male rats (Tables 5.3 and 5.4). In male rats, fenofibrate increased ($p = 0.0012$ when compared to PC+ FS; $p = 0.0110$ when compared to Bst + FS, respectively) (Table 5.4) but had no effect in female rats (Table 5.3).

Table 5.3: Effect of β-sitosterol on creatinine, urea and kidney injury molecule-1 in growing female rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Creatinine (U/L)	20.37 ± 4.22^{a}	23.99 ± 4.31^{a}	25.26 ± 3.34^{a}	24.66 ± 4.56^{a}	23.99 ± 4.31^{a}	ns
Urea (U/L)	6.78 ± 1.03^{a}	6.32 ± 1.18^{a}	6.88 ± 1.23^{a}	6.55 ± 0.86^{a}	5.92 ± 1.22^{a}	ns
KIM-1 (pg/mL)	nd^{a}	143.55 ± 69.31^{b}	162.35 ± 52.97^{b}	180.91 ± 0.55^{b}	180.55 ± 0.24^{b}	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$; ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day (Bst) + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); nd = not detected; KIM-1 = kidney injury molecule-1. Data presented as mean ± SD; n = 6-7 per treatment.

Table 5.4: Effect of β-sitosterol on creatinine, urea and kidney injury molecule-1 in growing male rats fed a high-fructose diet

Parameter	PC + PW	PC + FS	FF + FS	Bst + PW	Bst + FS	Significance
Creatinine (U/L)	26.52 ± 0.01^{a}	26.52 ± 6.25^{a}	22.98 ± 4.84^{a}	23.99 ± 4.31^{a}	22.98 ± 7.91^{a}	ns
Urea (U/L)	7.49 ± 0.77^{ab}	6.32 ± 1.03^{a}	8.45 ± 0.70^{b}	7.74 ± 0.75^{ab}	6.73 ± 1.01^{ac}	*
KIM-1 (pg/mL)	nd^{a}	180.89 ± 0.64^{b}	181.13 ± 0.22^{b}	181.31 ± 0.25^{b}	181.33 ± 0.07^{b}	*

[abc]Within row means with different superscripts are significantly different at $p < 0.05$.* $p < 0.05$; ns = not significant, $p > 0.05$. PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day (Bst) + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v);. nd = not detected. KIM-1= kidney injury molecule-1. Data presented as mean ± SD; n = 6-7 per treatment.

5.3.3 Renal histology outcomes

The Bowman's capsule area (A), glomerular tuft area (B), urinary space area (C) and glomerular density (D) of female and male rats are shown in Tables 5.1 and 5.2. The high-fructose diet decreased ($p = 0.0013$ and $p < 0.0001$ when compared to PC + PW respectively; glomerular density in female and male rats (Figures 5.1 and 5.2). However, the high-fructose diet reduced ($p < 0.05$ when compared to PC + PW) Bowman's capsule area and urinary space area only in female rats (Figure 5.1). β-sitosterol (Bst + FS) protected ($p < 0.05$; compared to PC + FS) against the high-fructose diet-induced decrease in glomerular density in female rats but not in male rats (Figures 5.1 and 5.2). Fenofibrate (FF + FS) failed to protect ($p > 0.05$) against the high-fructose diet-induced renal histological changes in female and male rats (Figures 5.1 and 5.2). β-sitosterol (Bst + PW) alone increased ($p < 0.05$; when compared to PC + PW; Figure 5.2) Bowman's capsule area and glomerular tuft only in male rats.

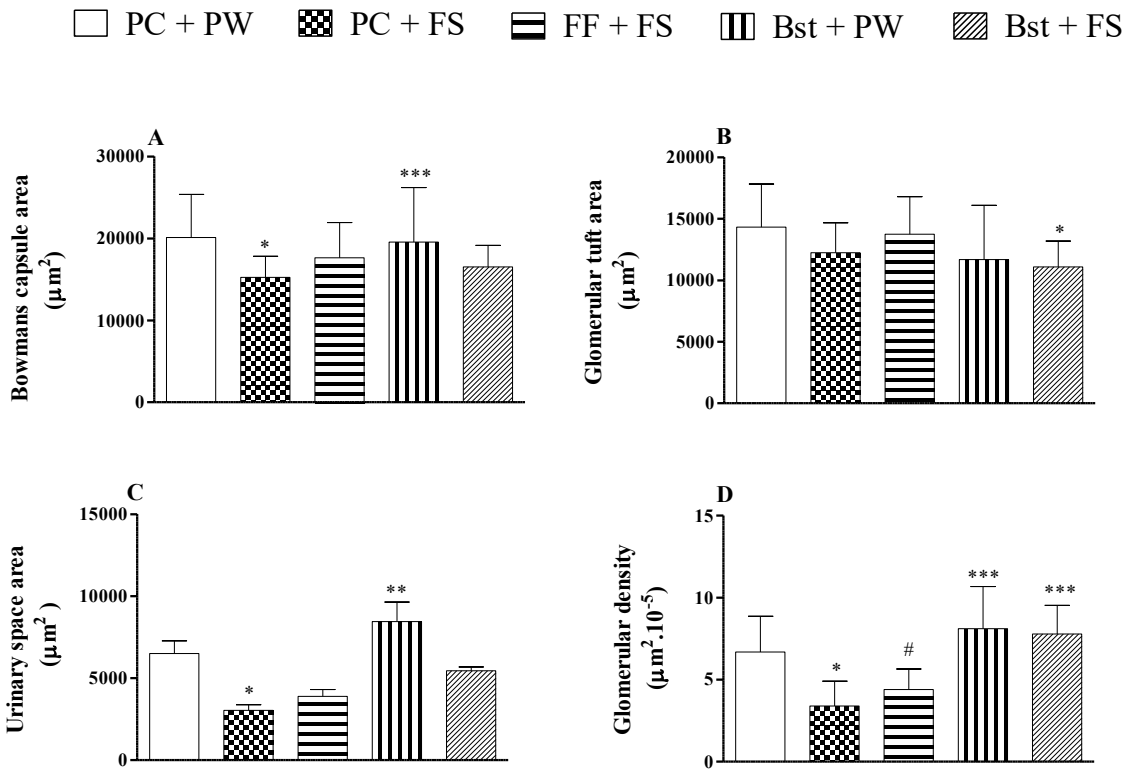

Figure 5.1: The effect of β-sitosterol on Bowman's capsule area (A), glomerular tuft area (B), urinary space area (C) and glomerular density (D) on high-fructose diet-fed female rats.

* $p < 0.05$ when compared to control PC + PW; **$p < 0.001$ when compared to PC + FS, FF + FS and Bst + FS; *** $p < 0.0001$ when compared to PC + FS; # $p < 0.05$ when compared to Bst + FS and Bst + PW. PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 4 per treatment.

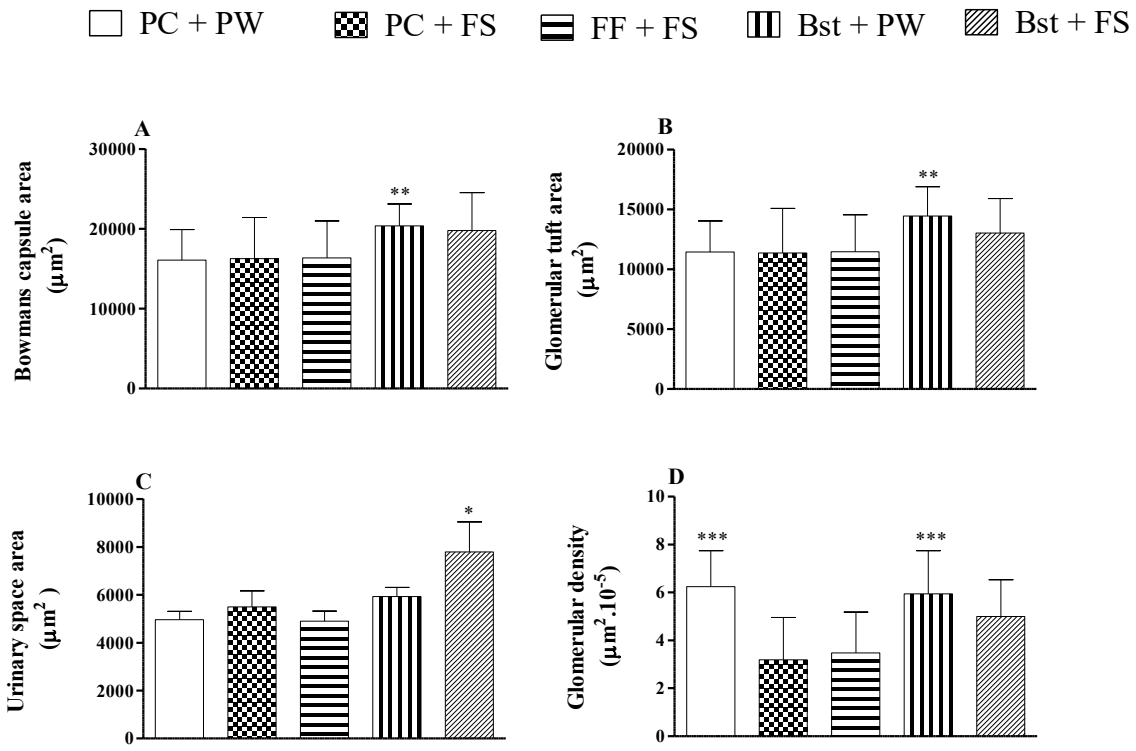

Figure 5.2: The effect of β-sitosterol on Bowman's capsule area (A), glomerular tuft area (B), urinary space area (C) and glomerular density (D) on high-fructose diet-fed male rats.

* $p < 0.05$ when compared to PC + PW and FF + FS; **$p < 0.001$ when compared to PC + PW, PC + FS and FF + FS; ***$p < 0.0001$ when compared to control PC + FS and FF + FS. PC + PW= plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + 20% (w/v) fructose solution to drink. Data presented as mean ± SD; n = 4 per treatment

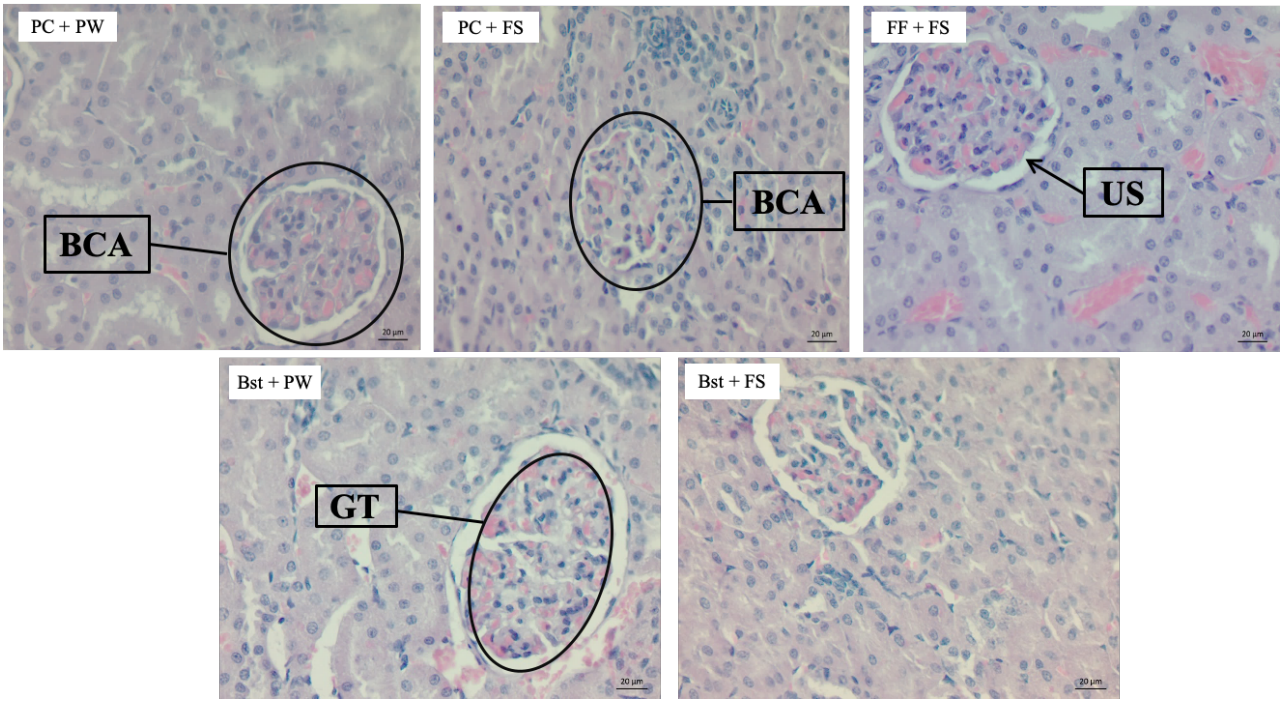

Figure 5.3: Representative photos showing kidney histology (H and E staining, 400 X magnification) of female rats in the different treatment groups.

PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v); BCA = Bowman's capsule area; GT = glomerular tuft; US = Urinary space. Scale bar = 20 μm in the H&E stain sections.

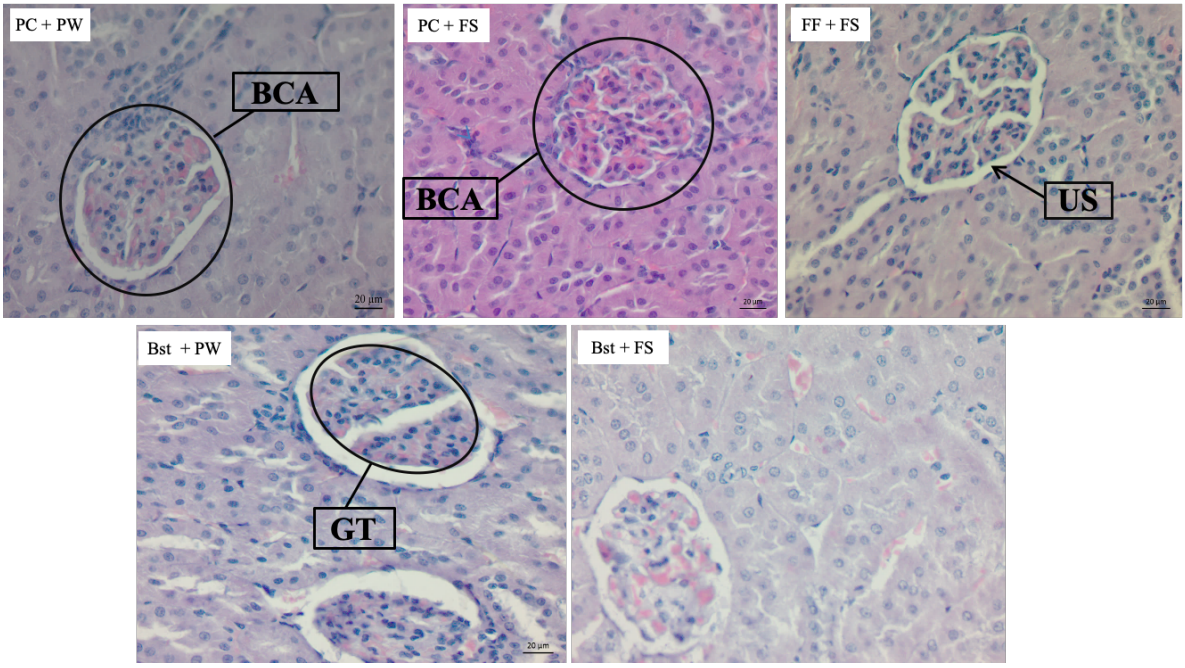

Figure 5.4: Representative photos showing kidney histology (H and E staining, 400 X magnification) of male rats in the different treatment groups.

PC + PW = plain gelatine cubes + plain tap water to drink; PC + FS = plain gelatine cubes + 20% (w/v) fructose solution to drink; FF + FS = gelatine cube containing fenofibrate at a dose of 100 mg/kg body mass per day + fructose solution to drink. Bst + PW = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + plain tap water to drink; Bst + FS = gelatine cube containing β-sitosterol at a dose of 20 mg/kg body mass per day + fructose solution to drink (w/v);. BCA = Bowman's capsule area; GT = glomerular tuft; US = Urinary space. Scale bar = 20 μm in the H&E stain sections.

5.4 Discussion

The study aimed to investigate the potential renoprotective effect of orally administrated β-sitosterol in high-fructose diet-fed growing female and male rats. In the present study, the high-fructose diet decreased glomerular density, increased kidney injury molecule-1 (KIM-1) but had no effect on kidney mass and urea and creatinine (surrogate markers of kidney function) in female and male rats. Additionally, the high-fructose diet decreased Bowman's capsule area and urinary space in female rats but not in male rats. β-sitosterol (Bst + FS) protected the female rats against the high-fructose diet-induced decrease in glomerular density but failed to protect the rats against the high-fructose diet-induced increase in KIM-1. Although in male rats β-sitosterol increased absolute kidney mass, it did not compromise kidney function in female and male rats. Fenofibrate failed to protect the rats against the high-fructose diet-induced renal abnormalities and increased kidney mass and plasma urea concentration which was above the normal range of (3.21- 7.5 mmol/L) in male rats. When β-sitosterol (Bst + PW) was administered alone, it increased KIM-1 in female and male rats as well as the Bowman's capsule area and glomerular tuft area in male rats.

Fructose consumption has been reported to cause kidney hypertrophy through increased uric acid concentration (Sánchez-Lozada et al., 2007, 2005). In the present study, the high-fructose diet had no impact on the kidney mass in female and male rats (Table 5.1 and 5.2, respectively), suggesting that the fructose did not result in kidney damage. However, kidney mass is an inaccurate surrogate marker of kidney damage. It is therefore important to assess renal cellular changes through histological analysis. Kidney histological analyses from the present study revealed that in female and male rats the high-fructose diet decreased glomerular density and reduced Bowman's capsule area and urinary space area in female rats (Figures 5.1 to 5.4, respectively). A decrease in glomerular density is associated with renal vasoconstriction (Rule et al., 2011) and damage (Sánchez-Lozada et al., 2007). Therefore, my finding suggests that the fructose diet caused renal damage. Furthermore, MetS and NAFLD are associated with renal damage (Chen et al., 2004; Ipsen et al., 2018). Indeed, in the current study, the high-fructose diet caused MetS and NAFLD (reported in Chapters 3 and 4). In the present study, the high-fructose diet decreased Bowman's capsule

and urinary space areas in female rats (Figures 5.1 to 5.4, respectively) thus suggesting that dietary fructose caused renal vasoconstriction in a sex-dependent manner. Plasma and urinary KIM-1 are reported to have high sensitivity to renal tubular damage (Han et al., 2002). To further assess renal damage in the rats, plasma KIM-1 was determined in the present study. The consumption of 20% fructose diet for 12 weeks increased plasma KIM-1 concentration in female and male rats suggesting possible fructose-induced tubular damage (Table 5.1 to 5.4, respectively). My results are consistent with previous research that documented that dietary fructose causes renal tubule damage (Cirillo et al., 2009; Sánchez-Lozada et al., 2007). Despite, the high-fructose diet causing renal abnormalities, it did not compromise kidney function as indicated by the normal plasma creatinine and urea concentration (Table 5.3 and 5.4). According to Nisha et al. (2017), renal failure increases plasma creatinine and urea concentration only when glomerular filtration rate (GFR) is reduced to ~ 50%. Thus, I speculate that despite the high-fructose diet causing renal tubular injury, it did not reduce GFR below 50% and hence the normal plasma creatinine and urea concentration.

The present study reports that β-sitosterol protected only the female rats against the high-fructose diet-induced decrease in glomerular density (Figure 5.1). This finding suggests that β-sitosterol may have exerted its reno-protective effects in a sexually dimorphic manner. The recorded sexually dimorphic effects of β-sitosterol could be attributed to the reported higher absorption of β-sitosterol in females compared to male (Sanders et al., 2000). Additionally, β-sitosterol was unable to protect against the fructose-induced proximal tubular damage as indicated by elevated plasma KIM-1 concentration in Bst + FS female and male rats (Table 5.3 and 5.4, respectively). The unexpected finding from my study was that β-sitosterol alone (Bst + PW), resulted in elevated plasma KIM-1 concentration. However, KIM-1 can also increase as a result of increased renal proximal tubular epithelial cell regeneration (Zhang and Cai, 2016). At the moment it is unclear whether β-sitosterol increased KIM-1 by causing nephrotoxicity or by stimulating proximal tubular epithelial cell regeneration. Thus, further investigations are required.

Fenofibrate is known to decrease kidney function by increasing serum creatinine and blood urea nitrogen concentrations (Ansquer et al., 2008).The current study found that fenofibrate did not protect against fructose-induced renal abnormalities in female and male rats. Importantly in the present study, fenofibrate increased kidney masses and plasma urea concentration in the present study (Table 5.4) suggesting that it may cause renal damage. The observed heavier kidneys and increased plasma urea concentration seen in the current study suggest that fenofibrate might have elicited hyperplasia of the kidney cells and affected kidney function.

5.5 Conclusion

My findings support that the consumption of a high-fructose diet for 12 weeks may cause renal abnormalities in female and male rats. Moreover, my study revealed that orally administered β-sitosterol protected only the female rats against the high-fructose diet-induced decrease in glomerular density. My findings also suggest that β-sitosterol like fenofibrate may cause renal disturbances and thus should be used with careful consideration of the possible risks.

Having shown that daily chronic intake/administration of Bst from weaning to adulthood, had positive health benefits in high fructose fed rats, the next chapter (Chapter 6) presents the second experiment which sought to determine whether strategic neonatal orally administered β-sitosterol programmes for protection against high-fructose diet-induced metabolic derangements in adulthood.

CHAPTER 6: THE POTENTIAL OF β-SITOSTEROL TO PROGRAMME FOR PROTECTION AGAINST DIET-INDUCED METABOLIC DERANGEMENTS IN SPRAGUE-DAWLEY RATS

6.0 Introduction

According to the developmental origins of health and diseases (DoHaD), biological and environmental events during the early critical developmental plastic periods of life can have long-term effects on the physiological development and function of an organism (Devaskar and Thamotharan, 2007; Léonhardt et al., 2003). These long-term effects can predispose or protect the organism to developing diseases in later life (Devaskar and Thamotharan, 2007; Léonhardt et al., 2003). On the other hand, metabolic programming is a phenomenon whereby an organism has increased or decreased risk of developing metabolic disease in early-life (childhood) or in later-life (adulthood) due to dietary events that occurred in the early developmental periods of life (Gluckman and Hanson, 2004; Zambrano et al., 2005). When comparing the two above mentioned concepts, the DoHaD concept does not only result in disease but also good health. Furthermore, the DoHaD concept can occur in any of the developmental stages in life.

According to the 'multiple-hit' hypothesis, various dietary and or environmental suboptimal stimuli during the various developmental phases such as the gestation and neonatal phase are necessary for the onset of metabolic disease (Tamashiro and Moran, 2010). Several studies have shown that the consumption of suboptimal diets during the neonatal period and during the subsequent developmental stages increases the risk of developing metabolic disorders (Tarry-Adkins and Ozanne, 2011; Wang, 2013). Importantly, pre-clinical and clinical studies have demonstrated that the intake of high-fructose diet during the perinatal periods and in adulthood results in the manifestation of obesity, NAFLD, MetS and T-II-DM (Bray et al., 2004; Lozano et al., 2016; Rebolledo et al., 2008).

The neonatal period is typified by increased developmental plasticity (Tarry-Adkins and Ozanne, 2011). Research has revealed that the administration of agents with antioxidant, antiobesity and antidiabetic activities can programme long-term protection against high-fructose diet-induced metabolic derangements (Ching et al., 2011). In view of the increasing prevalence of metabolic disorders, there is a need to develop preventative

interventions against diet-induced metabolic disease. As a result, the suckling period represents a potential period of opportunity where anti-obesogenic, anti-inflammatory and anti-diabetic substances can be orally administered as prophylaxis against obesity, MetS and NAFLD (Guilloteau et al., 2009; Vickers et al., 2005).

Neonatal (PND 6-20) oral administration of s-allyl cysteine, an organosulphur found in garlic, to rats has been shown to attenuate high-fructose diet-induced fatty liver disease in adulthood (Lembede et al., 2018). Lembede et al. (2018) speculated that the mechanisms of his findings could be accounted for by the reported modification of the expression of genes that are involved in lipogenesis and β-oxidation of free fatty acids following fructose consumption. Ibrahim et al. (2019) reported that neonatal orally administered (PND 6-21) curcumin protected against dietary fructose-induced NASH in adult rats by suppressing hepatic TNF-α and AMPKα expression. Nyakudya et al. (2018) reported that neonatal (PND7-21) oral administration of oleanolic acid protected against the development of fructose diet-induced metabolic dysfunction and NAFLD in adult rats.

β-sitosterol possesses anti-obesogenic, anti-inflammatory and anti-diabetic properties (Gupta et al., 2011; Vivancos and Moreno, 2005). In Chapters 3, 4 and 5, my findings revealed that β-sitosterol when taken daily, protected against high-fructose diet-induced metabolic disorders. Lifelong daily intake of dietary supplements is limited by accessibility, compliance and costs. Therefore, based on the health-beneficial effects of β-sitosterol that I have reported, it can potentially be administered neonatally to protect against high-fructose diet-induced adverse metabolic programming outcomes in adulthood. To my knowledge, there are no studies that have investigated the potential of neonatal orally administered β-sitosterol to programme for protection against metabolic disorders induced by long-term high-fructose diet intake in adulthood. The current study, therefore, investigated, in rats, the effect of neonatal orally administered β-sitosterol to programme for protection against long-term high-fructose diet-induced metabolic derangements in adulthood.

6.1 Material and methods

6.1.1 Ethical approval

Details of ethical approval and care of experimental animals are as described in Chapter 3, subheading 3.1.1 page 28.

6.1.2 Chemicals and reagents

Th pups were housed and the environment controlled as described in Chapter 3, subheading 3.1.3, page 28.

6.1.3 Animals, housing and general care

Seventy 4-day old, female and male pups from eight Sprague-Dawley dams (litter size: between 8 and 12 pups), were used in the study. The study was conducted in two stages: the pre-weaning (postnatal day 7–20) and post-weaning (postnatal day 21–125) stages. In the pre-weaning phase, the pups were kept with their respective dams and housed in Perspex cages lined with hardwood shavings in the Central Animal Services (CAS), University of the Witwatersrand. The pups were allowed to nurse freely until weaning on postnatal day 21. A twelve-hour light-dark cycle was adhered to with lights on from 7 am to 7 pm. Room temperature was maintained at 26 ± 2°C. The dams had *ad libitum* access to a standard rat chow (Nutritionhub (PTY) LTD, Stellenbosch, South Africa) and plain drinking water throughout the pre-weaning stage. On PND 21, the pups were weaned from their dams which were then returned to stock. Throughout the post-weaning stage, the weaned rats were housed individually in Perspex cages and had *ad libitum* access to drinking water or fructose solution to drink and were maintained under similar environmental conditions as described above.

6.1.4 Experimental design

In the pre-weaning (first) experimental stage, the rat dams with their litters were received on PND 4 and the pups were acclimatised to handling for two days (postnatal days 5 and 6). Thereafter, the seventy 4-day old rat pups (34 female; 36 male) were, in an interventional

study, randomly distributed to and administered one of the following four treatment regimens from PND 7-20:
group I: DMSO + PW (7 female; 10 male) = gavaged daily with 10 mL/kg body mass 0.5% v/v dimethyl sulfoxide (DMSO) at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning,

group II: FS + FS (10 female; 8 male) = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) in 0.5% v/v DMSO at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning,

group III: Bst + PW (7 female; 10 male) = gavaged daily with 20 mg/kg body mass β-sitosterol in 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning and,

group IV: Bst + FS (10 female; 8 male) = gavaged daily with a bolus of 20 mg/kg β-sitosterol dissolved in 0.5% v/v DMSO plus 10 mL/kg body mass of 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning.

In the pre-weaning experimental stage, the pups were weighed daily in order to ensure the maintenance of a constant dose of treatment and to monitor growth performance. The pups were weaned on PND 21. In the post-weaning stage (PND 21-125), group I and III rats were fed SRC + plain drinking water, whereas group II and IV were fed SRC and had 20% fructose solution to drink. On PND 125 the rats from each group were fasted overnight prior to being euthanised on PND 126 with an intraperitoneal injection of sodium pentobarbitone at 200 mg/kg body mass.

The β-sitosterol doses used were similar to those previously described by Baskar et al. (2010). Due to the poor aqueous solubility of Bst, dimethyl sulphoxide [(DMSO); 0.5%] was used as a vehicle to solubilise β-sitosterol (Baskar et al., 2010; McCormack et al., 2015). The study design is summarised in Figure 6.1 below.

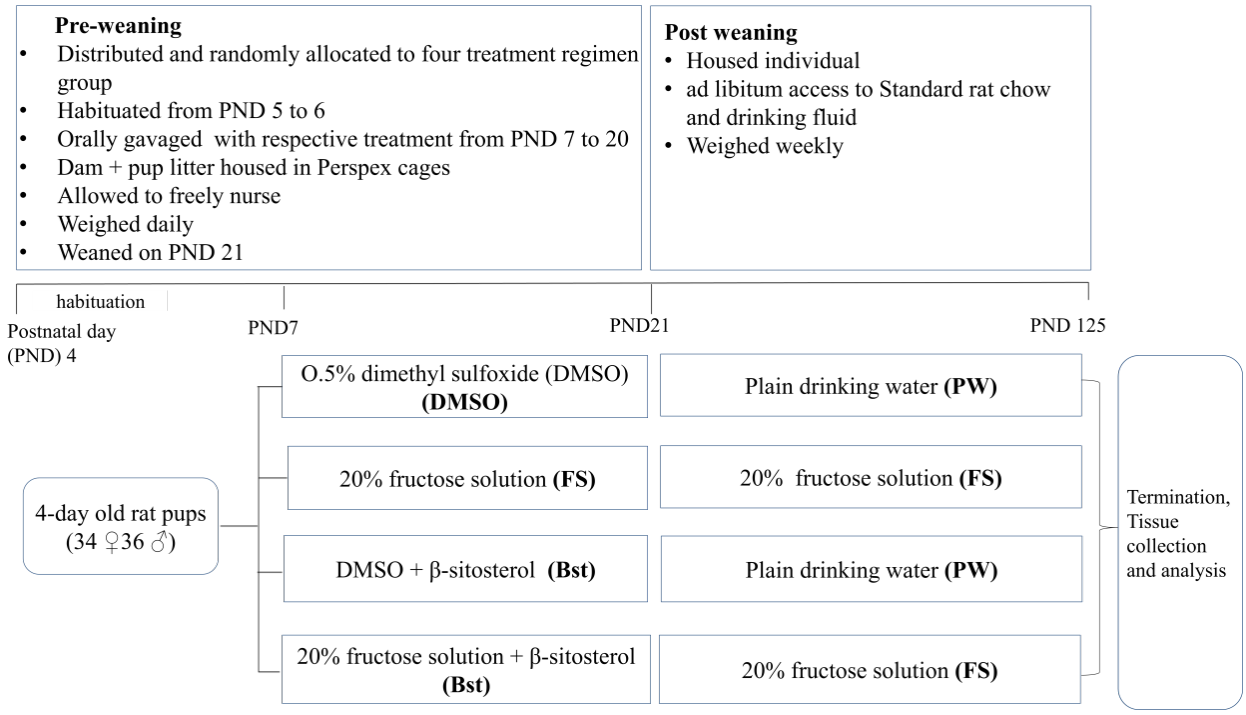

Figure 6.1: Flow diagram illustrating the study design

6.1.5 Terminal procedures and tissue collection

Prior to euthanasia on PND 126, fasting blood glucose and triglyceride concentrations of each rat were determined from drops of blood obtained from the tail vein following a pin prick using a hypodermic needle (Ndong et al., 2007). A calibrated Accutrend triglyceride meter (Roche, Mannheim, Germany) and a calibrated glucose meter (Contour Plus Meter, Bayer, Isando-Johannesburg, South Africa) were used to determine triglyceride and blood glucose concentrations, respectively. Following euthanasia, the abdomen of each rat was opened via a midline incision and the visceral fat pad (retroperitoneal, perirenal mesenteric) and liver were dissected out and weighed. The relative visceral fat pad and liver masses were computed according to the formula:

Relative organ mass = [organ mass (g) /terminal body mass (g)] x 100.

6.1.6 Liver histological analyses

Liver histological analyses was carried out as described in chapter 4, subheading 4.1.7, page 50.

6.2 Statistical analysis

GraphPad Prism 6.0 (Graph-pad Software Inc. San Diego, USA) software was used to analyse data. Parametric data are expressed as mean ± SD. Within-group weekly body mass data were analysed using repeated-measures analysis of variance (ANOVA). Multiple-group data were analysed using one-way ANOVA followed by Tukey's *post-hoc* test to compare the means. Non-parametric data are expressed as median and range (min, max). The Kruskal-Wallis test was used to analyse multiple groups NAS data followed by multiple comparisons Dunns's post-hoc test to compare medians. Significance was accepted when $p < 0.05$.

6.3 Results

6.3.1 Body mass

Figures 6.2 and 6.3 below show the induction, weaning, and terminal body masses of female and male rats (Figures 6.2 and 6.3). The female and male rats from all experimental treatment groups grew significantly ($p < 0.05$) from induction to weaning and from weaning to termination (Figures 6.2 and 6.3). The high-fructose diet had no effect on terminal body mass in female and male rats (Figures 6.2 and 6.3). While neonatal β-sitosterol (Bst + FS) increased ($p = 0.0261$ when compared to DMSO + PW) terminal body mass in high-fructose diet-fed female rats and it decreased ($p = 0.0250$ when compared to Bst + PW) terminal body mass in male rats (Figure 6.2).

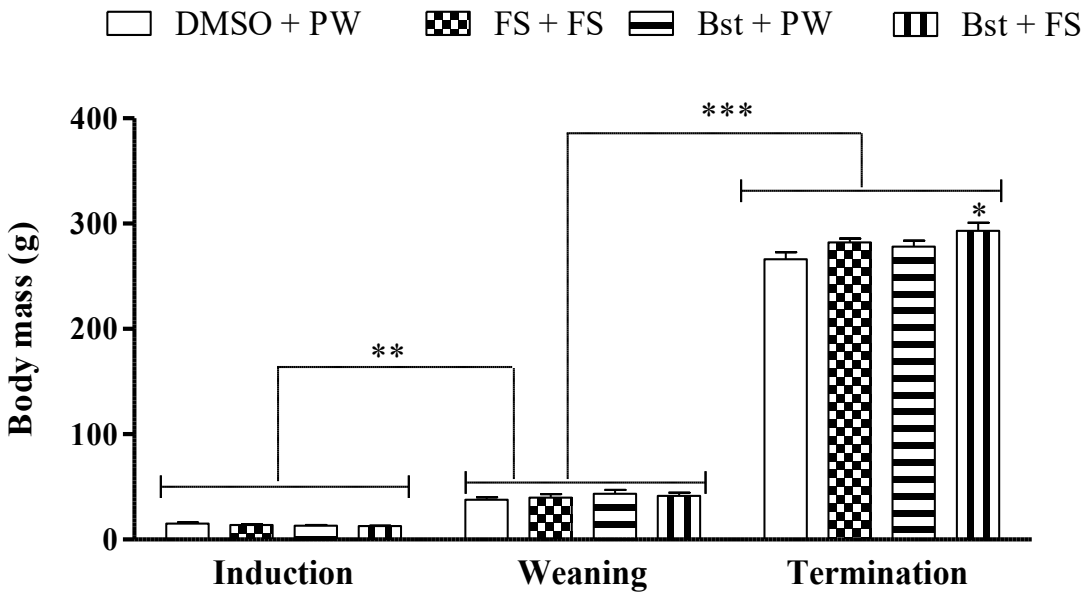

Figure 6.2: Induction, weaning and terminal body masses of the female rats.
*** $p < 0.0001$ when weaning was compared to termination.** $p < 0.001$ when induction was compared to weaning. * $p < 0.05$ when compared to control (DMSO + PW) terminal masses. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Data presented as mean ± SD; n = 7-10 per treatment.

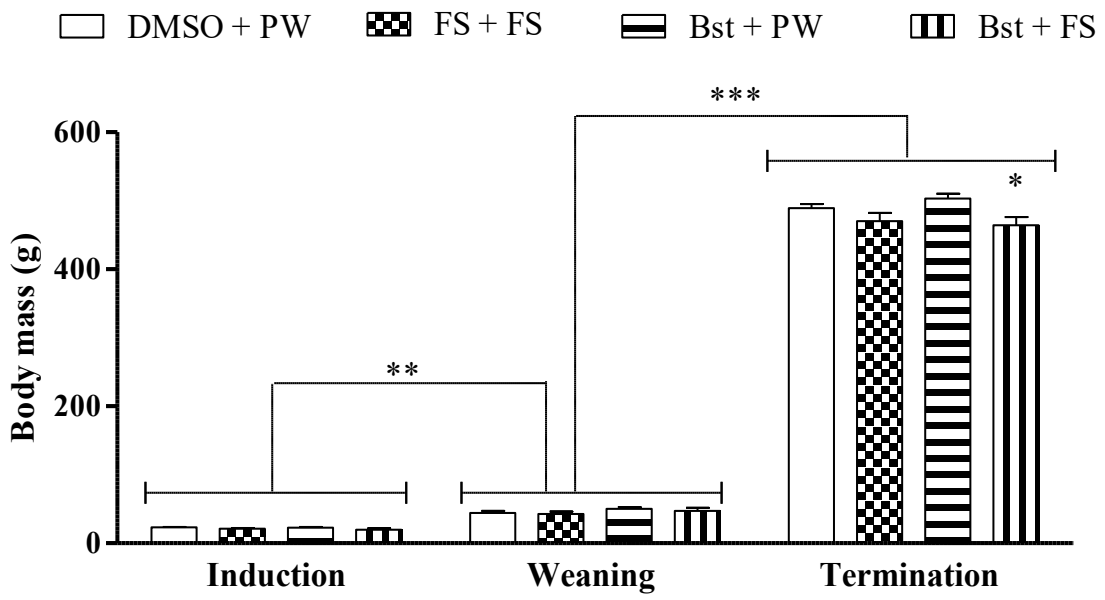

Figure 6.3: Induction, weaning and terminal body masses of the male rats.

*** $p < 0.0001$ when weaning was compared to termination.** $p < 0.001$ when induction was compared to weaning. * $p < 0.05$ when terminal mass was compared to Bst + PW. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Data presented as mean ± SD; n = 8-10 per treatment.

6.3.2 Visceral obesity

Tables 6.1 and 6.2 show the visceral fat pad masses of female and male rats, respectively. Long-term high-fructose diet consumption (FS + FS) significantly increased ($p = 0.0046$) visceral fat mass (absolute and relative to body mass) in adulthood in female rats but not in male rats (Table 6.1 and 6.2). Neonatal orally administered β-sitosterol (Bst + FS) failed to prevent ($p > 0.05$) the long-term high-fructose diet-induced visceral obesity in female rats (Table 6.1). Additonally neonatal orally administered β-sitosterol (Bst + PW) alone had no effect on visceral fat mass in female and male rats (Tables 6.1 and 6.2).

Table 6.1: Effect of neonatal orally administered β-sitosterol on visceral obesity of long-term high-fructose diet-fed adult female rats

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Visceral fat (g)	10.79 ± 0.21^{a}	16.85 ± 3.26^{b}	14.27 ± 1.87^{ab}	15.73 ± 3.70^{b}	*
Visceral fat (%BM)	4.05 ± 0.99^{a}	6.13 ± 1.17^{b}	5.14 ± 0.72^{ab}	5.82 ± 1.52^{b}	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. BM = body mass. Data presented as mean ± SD; n = 7-10 per treatment.

Table 6.2: Effect of neonatal orally administered β-sitosterol on visceral obesity of long-term high-fructose diet-fed adult male rats

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Visceral fat (g)	15.43 ± 1.44[a]	16.61 ± 3.36[a]	15.15 ± 2.23[a]	15.97 ± 3.85[a]	ns
Visceral fat (%BM)	3.33 ± 0.58[a]	3.53 ± 0.59[a]	3.01 ± 6.41[a]	3.58 ± 0.70[a]	ns

[a]Within row means are similar at $p > 0.05$; ns = not significant, $p > 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. BM = body mass. Data presented as mean ± SD; n = 8-10 per treatment.

6.3.3 Liver masses

Tables 6.3 and 6.4 present the liver masses of the female and male rats following their respective treatments. Long-term high-fructose diet intake (FS + FS) had no effect on liver masses in female and male rats (Figures 6.3 and 6.4). Neonatal orally administered β-sitosterol (Bst + FS) significantly increased ($p = 0.0439$; $p = 0.0499$, respectively) liver masses (absolute and relative to body mass) in adult female rats fed a high-fructose diet during the pre-weaning and post-weaning stages (Table 6.3). However, neonatal orally administered β-sitosterol alone (Bst + PW) had no effect ($p > 0.05$) on liver masses in female and male rats in adulthood (Tables 6.3 and 6.4).

Table 6.3: Effect of neonatal orally administered β-sitosterol on liver masses of long-term high-fructose diet-fed adult female rats

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Liver (g)	7.37 ± 0.41^{a}	8.17 ± 0.69^{a}	7.22 ± 0.77^{a}	9.32 ± 1.42^{b}	*
Liver (%BM)	2.77 ± 0.16^{a}	2.89 ± 0.17^{a}	2.59 ± 0.19^{a}	3.17 ± 0.30^{b}	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. BM = body mass. Data presented as mean ± SD; n = 7-10 per treatment.

Table 6.4: Effect of neonatal orally administered β-sitosterol on liver masses of long-term high-fructose diet-fed adult male rats

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Liver (g)	13.16 ± 0.84[a]	13.31 ± 1.67[a]	13.61 ± 1.11[a]	13.48 ± 1.75[a]	ns
Liver (%BM)	2.69 ± 0.14[a]	2.82 ± 0.23[a]	2.71 ± 0.15[a]	2.71 ± 0.59[a]	ns

[a]Within row means are similar at $p > 0.05$; ns = not significant, $p > 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. BM = body mass. Data presented as mean ± SD; n = 8-10 per treatment.

6.3.4 Triglyceride and glucose concentration

Table 6.5 and 6.6 show the plasma triglyceride and blood glucose concentrations of female and male rats, respectively. There were no significant differences in the triglyceride concentration across all treatment groups in female and male rats ($p > 0.05$; Tables 6.5 and 6.6). The long-term high-fructose diet intake (FS + FS) had no effect on blood glucose concentrations in female and male rat pups ($p > 0.05$; Tables 6.5 and 6.6). In long-term high-fructose diet-fed rats, neonatal orally administered β-sitosterol (Bst + FS), increased ($p = 0.0362$) blood glucose concentration in females but decreased ($p = 0.0288$) plasma glucose concentration in males (Table 6.5 and 6.6). Neonatal orally administered β-sitosterol alone (Bst + PW) had no effect on plasma triglyceride and glucose concentration in female and male rats (Table 6.5 and 6.6).

Table 6.5: Effect of long-term high-fructose diet on plasma triglyceride and glucose concentration of adult female rats orally administered β-sitosterol during suckling

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Triglycerides (mmol/L)	1.38 ± 0.13^{a}	1.59 ± 0.35^{a}	1.36 ± 0.26^{a}	1.82 ± 0.49^{a}	ns
Glucose (mmol/L)	4.00 ± 0.26^{ab}	3.96 ± 0.30^{a}	4.24 ± 0.46^{ab}	4.61 ± 0.70^{b}	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$; ns = not significant, $p > 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Data presented as mean ± SD; n = 7-10 per treatment.

Table 6.6: Effect of long-term high-fructose diet on plasma triglyceride and glucose concentration of adult male rats orally administered β-sitosterol during suckling

Parameter	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Triglycerides (mmol/L)	1.33 ± 0.14	1.52 ± 0.21	1.30 ± 0.23	1.56 ± 0.25	ns
Glucose (mmol/L)	4.04 ± 0.30^{ab}	4.47 ± 0.57^{a}	4.03 ± 0.41^{ab}	3.76 ± 0.55^{b}	*

^{ab}Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$; ns = not significant, $p > 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Data presented as mean ± SD; n = 8-10 per treatment.

6.3.5 Liver histomorphometry

Figures 6.4 and 6.5 show the representative liver histology photo sections (H and E staining, 400 X magnification) of female and male rats from the different treatment groups. Long-term high-fructose diet intake (FS + FS) resulted in the development of micro- and macro-vesicular hepatic steatosis in female and male rats (Figures 6.4 and 6.5). Neonatal orally administered β-sitosterol (Bst + FS) attenuated the long-term high-fructose diet-induced micro-steatosis in female and male rats and mitigated ($p = 0.0103$; $p = 0.0106$) macro-vesicular hepatic steatosis in female and male rats (Figures 6.4 and 6.5). Neonatal orally administered β-sitosterol alone (Bst + PW) did not cause micro- and macro-vesicular hepatic steatosis in female and male rats (Figures 6.4 and 6.5).

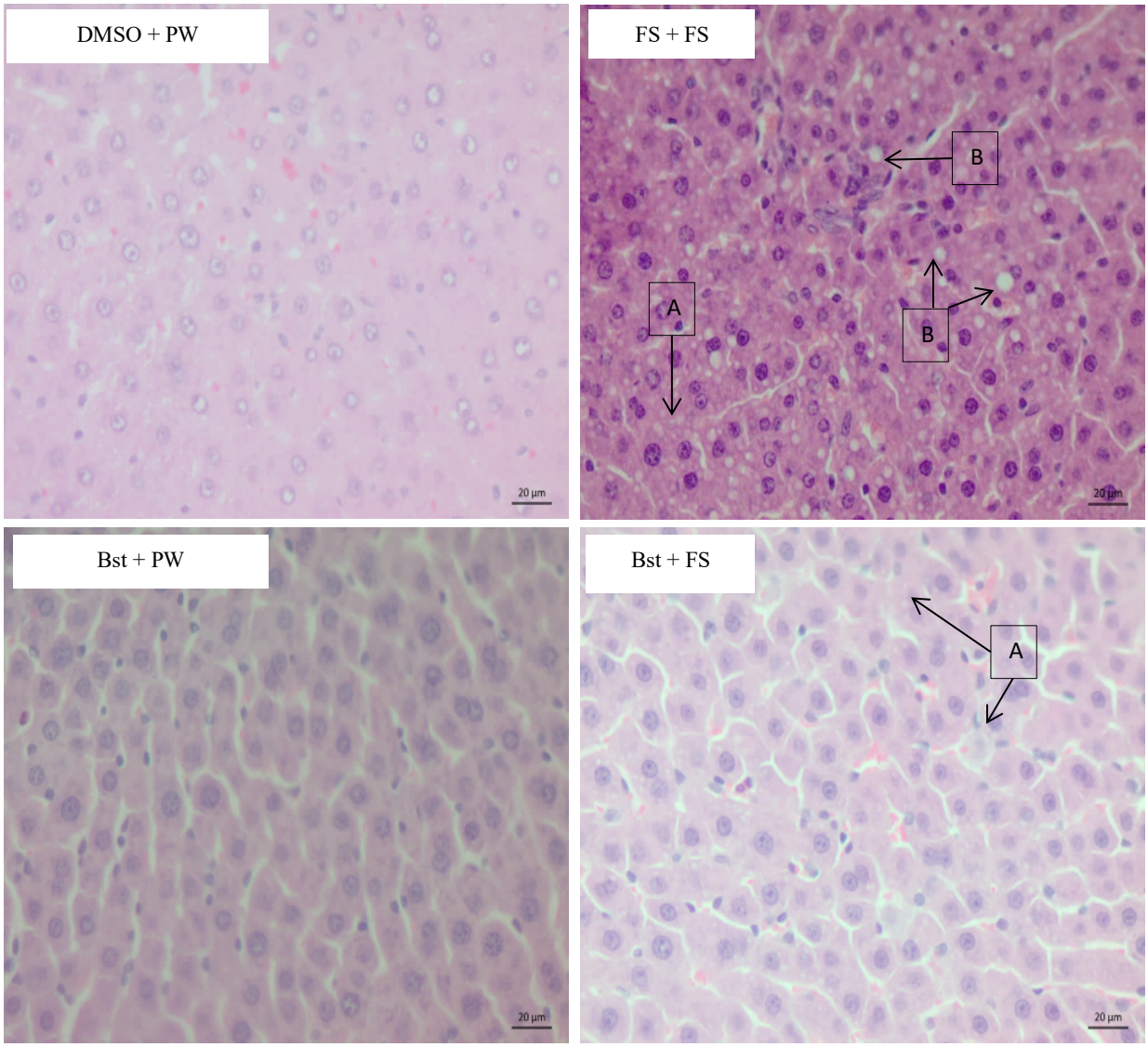

Figure 6.4: Representative photos showing the hepatic histology (H and E staining, 400 X magnification) of female rats in the different treatment groups.

Arrows A point to hepatic micro-vesicular steatosis, arrows B point to macro-vesicular steatosis. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Scale bar = 20 µm in the H&E stain sections.

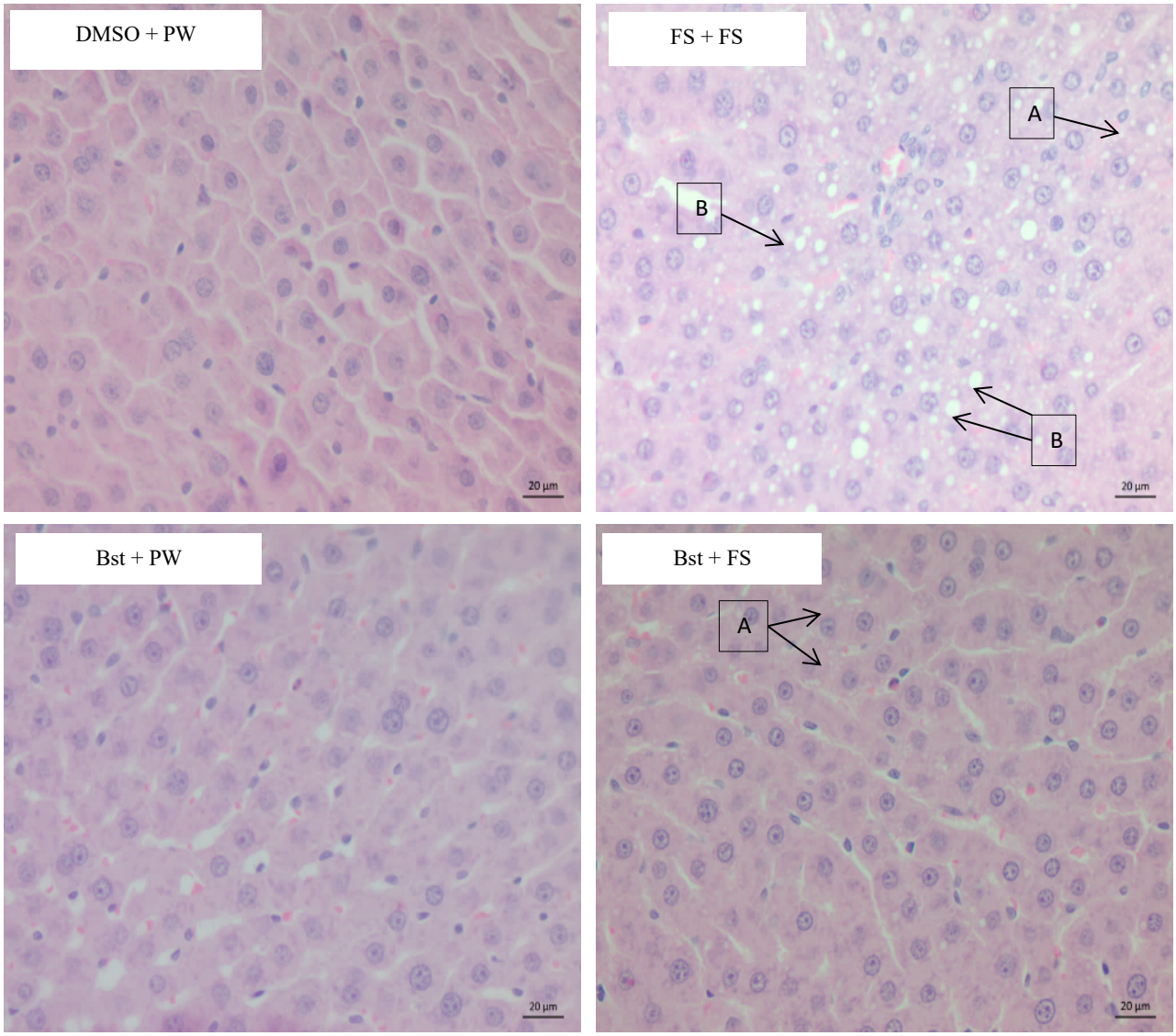

Figure 6.5: Representative photos showing the hepatic histology (H and E staining, 400 X magnification) of male rats in the different treatment groups.

Arrows A point to hepatic micro-vesicular steatosis, arrows B point to macro-vesicular steatosis. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning. Scale bar = 20 µm in the H&E stain sections.

6.3.6 Non-alcoholic fatty liver disease activity score

Table 6.7 and 6.8 depict the micro- and macro-steatosis, hypertrophy, lobular inflammation scores and total NAS of the female and male rats, respectively. Long term high-fructose diet intake (FS + FS) resulted in the development of micro- and macro-vesicular hepatic steatosis in female and male rats (Tables 6.7and 6.8). In male rats, the long term high-fructose diet intake (FS + FS) resulted in increased ($p= 0.0042$; $p= 0.0282$; $p= 0.0050$, respectively) inflammation, hypertrophy and total NAS (Table 6.8). Neonatal orally administered β-sitosterol (Bst + FS) attenuated the high-fructose diet-induced micro-steatosis and mitigated ($p = 0.0103$; $p = 0.0106$) macro-vesicular hepatic steatosis in female and male rats (Figures 6.7 and 6.8) in adulthood. However, neonatal orally administered β-sitosterol (Bst + PW) did not result in micro- and macro-vesicular hepatic steatosis and inflammation in adult female and male rats (Figures 6.7 and 6.8).

Table 6.7: Effect of neonatal orally administered β-sitosterol on non-alcoholic fatty liver disease activity score in long-term high-fructose diet-fed adult female rats

Criteria	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Micro-steatosis	0 (0, 0)[a]	3 (3, 3)[b]	0 (0, 0)[a]	1 (1,)[ab]	*
Macro-steatosis	0 (0, 0)[a]	3 (2, 3)[b]	0 (0, 0)[a]	0 (0, 0)[a]	*
Hypertrophy	0 (0, 1)[ab]	1.5 (1, 2)[a]	0 (0, 0)[b]	1 (1, 1)[ab]	*
Inflammation	0 (0, 1)[a]	1 (0, 1)[a]	0.5 (0, 1)[a]	1 (1, 2)[a]	ns
Total NAS	0 (0, 2)[a]	5.5 (4, 6)[b]	0.5 (0, 1)[ab]	3 (3, 3)[a]	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$. * $p < 0.05$; ns = not significant, $p > 0.05$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; NAS = non-alcoholic fatty liver disease activity score . Data presented as median and range (min, max). n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

Table 6.8: Effect of neonatal orally administered β-sitosterol on non-alcoholic fatty liver disease activity score in long-term high-fructose diet-fed adult male rats

Criteria	DMSO + PW	FS + FS	Bst + PW	Bst + FS	Significance
Micro-steatosis	0 (0, 0)[a]	3 (3, 3)[b]	0 (0, 0)[a]	1 (1, 2)[ab]	* *
Macro-steatosis	0 (0, 0)[a]	2.5 (2, 3)[b]	0 (0, 0)[a]	0 (0, 0)[a]	*
Hypertrophy	0 (0, 0)[a]	1 (1, 1)[b]	0 (0, 0)[a]	0.5 (0, 1)[ab]	*
Inflammation	0 (0, 1)[a]	2 (2, 2)[b]	1 (0, 1)[ab]	1.5 (1, 2)[ab]	*
Total NAS	0 (0, 1)[a]	6 (6, 6)[b]	1 (0, 1)[ab]	2.5 (2, 4)[a]	*

[ab]Within row means with different superscripts are significantly different at $p < 0.05$; * $p < 0.05$; ** $p < 0.001$. DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; NAS = non-alcoholic fatty liver disease activity score. Data presented as median and range (min, max); n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

6.3.7 Non-alcoholic fatty-liver disease frequency table

Table 6.9 and 6.10 depict the hepatic micro- and macro-vesicular steatosis, hypertrophy, lobular inflammation scores and total NAS frequencies of female and male rats, respectively. Hepatic micro- and macro-vesicular steatosis was observed in 100% of the sampled long-term high-fructose diet-fed (FS + FS) female and male rats (Tables 6.9 and 6.10). Moreover, inflammation, hypertrophy and total NAS > 5 was observed in 100% of the sampled high-fructose diet-fed (FS + FS) male rats (Table 6.10). Neonatal orally administered β-sitosterol attenuated the high-fructose diet-induced micro-steatosis and significantly reduced ($p = 0.0103$; $p = 0.0106$) macro-vesicular hepatic steatosis in 100% of the sampled Bst + FS female and male rats (Figures 6.9and 6.10).

Table 6.9: Non-alcoholic fatty liver disease activity score criteria frequency of adult female rats

Criteria	Steatosis								Hypertrophy				Inflammation				Total NAS		
	Microvesicular				Macrovesicular														
Score	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*<2*	*3-4*	*≥ 5*
DMSO + PW	100	0	0	0	100	0	0	0	75	25	0	0	75	25	0	0	100	0	0
FS + FS	0	0	0	100	0	0	25	75	0	50	50	0	25	75	0	0	0	25	75
Bst + PW	100	0	0	0	100	0	0	0	100	0	0	0	50	50	0	0	100	0	0
Bst + FS	0	100	0	0	100	0	0	0	0	100	0	0	0	100	0	0	0	100	0

DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; NAS = non-alcoholic fatty liver disease activity score. Data presented as median and range (min, max). n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

Table 6.10: Non-alcoholic fatty liver disease activity score criteria frequency of adult male rats

Criteria	Steatosis								Hypertrophy				Inflammation				Total NAS		
	Microvesicular				Macrovesicular														
Score	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*	*0*	*1*	*2*	3	*<2*	*3-4*	*≥ 5*
DMSO + PW	100	0	0	0	100	0	0	0	100	0	0	0	75	25	0	0	100	0	0
FS + FS	0	0	0	100	0	0	50	50	0	100	0	0	0	0	100	0	0	0	100
Bst + PW	100	0	0	0	100	0	0	0	100	0	0	0	25	75	0	0	100	0	0
Bst + FS	0	75	25	0	100	0	0	0	50	50	0	0	0	100	0	0	50	50	0

DMSO + PW = gavaged daily with 10 mL/kg body mass 0.5% v/v DMSO at pre-weaning + *ad libitum* access to plain drinking water at post-weaning; FS + FS = gavaged daily with 10 mL/kg body mass 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking water at post-weaning; Bst + PW = gavaged daily with 20 mg/kg body mass β-sitosterol at pre-weaning + *ad libitum* access to plain drinking fluid at post-weaning; Bst + FS = gavaged daily with 10 mL/kg body mass of β-sitosterol (20 mg/kg) and 20% fructose solution (w/v) at pre-weaning + *ad libitum* access to 20% fructose solution (w/v) as drinking fluid at post-weaning; NAS = non-alcoholic fatty liver disease activity score. Data presented as median and range (min, max). n = 4 per treatment. Total NAS score interpretation: <2 = not steatohepatitis; 3–4 = uncertain; ≥ 5 = steatohepatitis.

6.4 Discussion

This study was designed to investigate whether neonatal orally administered β-sitosterol protects against metabolic derangements induced by long-term high-fructose diet-feeding in adult rats. Long-term high-fructose diet feeding caused visceral obesity in female rats as well as NAFLD in both female and male rats. The NAFLD progressed to NASH only in male rats. Neonatal β-sitosterol (Bst + FS) protected the female and male rats against the high-fructose diet-induced NAFLD but was unable to prevent the fructose-induced visceral adiposity in female rats.

Neonatal dietary intervention can have short- and long-term effects on body mass (Elliott et al., 2002; Huynh et al., 2008). Body mass gain is an important indirect indicator of an animal's health status, growth and proxy for adiposity (Caan et al., 2018). Fructose consumption increases *de novo* triglyceride synthesis which subsequently leads to hypertriglyceridaemia and visceral obesity (Klop et al., 2013; Rizkalla, 2010). In the present study, neonatal fructose and or β-sitosterol did not have an effect on body mass in female and male rats (Figures 6.2 and 6.3, respectively). Ramos et al. (2017) reported that due to the high lipogenic nature of fructose, its long-term consumption can result in visceral adiposity without increasing body mass if accompanied with reduction in lean tissue mass gain. Therefore it is important to not only consider the body mass but also measure visceral fat accumulation.

In the present study long-term fructose consumption caused visceral adiposity in female rats (Table 6.1) which was not observed in the male rats (Table 6.2). Therefore, the observed visceral obesity in the long-term fructose-fed female rats is likely due to the high lipogenic turnover caused by the fructose diet. Similar findings were reported by Nyakudya et al. (2018b) who also observed sexual dimorphic effects. The observed sexual dimorphism observed in this study may be attributed to the fact that androgens promote cellular glucose uptake and energy utilisation in skeletal muscles and liver therefore reducing the accumulation of total body fat in males (Navarro et al., 2015) .

In the present study neonatal β-sitosterol (Bst + FS) did not protect the female rats against the long-term fructose diet-induced visceral adiposity (Table 6.1). Additionally, neonatal orally administered β-sitosterol (Bst + PW) alone did not compromise body mass gain of the rats and did not impact on visceral adiposity of the rats. These findings suggest that neonatal orally administered β-sitosterol did not negatively impact the rats' health and welfare.

Visceral fat is considered the main depository for triglycerides (Jensen, 2008). When visceral fat is oversaturated, it is unable to uptake and store excess triglycerides and this consequently results in hypertriglyceridaemia (Horst, 2017). Hypertriglyceridaemia and visceral obesity lead to glucose homeostasis-related disturbances (Lanaspa et al., 2013). In the present study, although long-term fructose diet caused visceral adiposity in female rats, it had no effect on plasma triglyceride and glucose concentration in adulthood of female and male rats (Tables 6.5 and 6.6). My findings suggest that long-term fructose (FS + FS) did not cause the rats, male and female, to develop hypertriglyceridaemia and hyperglycaemia in adulthood. In the present study long-term fructose diet had no effect on plasma glucose concentration (Tables 6.5 and 6.6, respectively). Similar findings were observed by Lembede et al. (2018) who reported no changes in the blood glucose concentration of Wistar rats administered a 20% fructose solution during suckling and in adulthood. Neonatal orally administered β-sitosterol alone (Bst + PW) did not impact on the blood triglyceride concentration in female and male rats (Tables 6.5 and 6.6). Interestingly, neonatal β-sitosterol (Bst + FS) increased plasma glucose concentration in long-term fructose diet-fed female rats but decreased plasma glucose concentration in the male counterparts (Tables 6.5 and 6.6, respectively). According to the WHO diagnostic criteria, a normal fasting plasma glucose should be < 5.6 mmol/L and plasma glucose above 7.1 mmol/L increases the risk of microvascular and cardiovascular complications (WHO, 2006). In my study despite the differences in the blood glucose concentration in female and male rats across treatment regimens, the observed blood glucose concentrations were within normal range. Gupta et al. (2011) reported that β-sitosterol decreased blood glucose concentration in STZ-diabetic adult rats by increasing anti-oxidant activity and insulin secretion. Since neonatal orally administered β-sitosterol alone (Bst + PW) had no

effect on plasma glucose concentration of female and male rats, my findings suggest that the intake of β-sitosterol in combination with fructose (Bst + FS) may have synergistic effects which negatively interfere with mechanisms that regulate glucose homeostasis.

Dietary fructose has been shown to stimulate *de novo* hepatic lipid synthesis and accretion thus resulting in increased liver mass (Rippe and Angelopoulos, 2013). In the current study, long-term fructose (FS + FS) diet caused fatty liver disease but had no effect on liver masses of female and male rats (Tables 6.3 and 6.4). My findings are similar to those from other studies that have demonstrated that dietary fructose intake during critical windows of developmental cellular plasticity results in increased risks for NAFLD later in adulthood (Ibrahim et al., 2019; Lembede et al., 2018; Nyakudya et al., 2018a). Furthermore, the sexually dimorphic effects of long-term fructose (FS + FS) on the liver fatty disease that were noted in the current study are consistent with several studies which reported that males are more susceptible to NAFLD than females (Caballería et al., 2010; Williams et al., 2011). Amongst several factors, in humans, this is likely due to males having less oestrogen and differences in lifestyle when compared to females (Bhatia et al., 2012; Kander et al., 2017; Torres et al., 2012; Williams et al., 2011). While the former is applicable to rats the latter is unlikely due to similar housing conditions and feeding regimens. Neonatal orally administered β-sitosterol attenuated the high-fructose diet-induced micro-steatosis and protected the rats against high-fructose diet-induced macro-vesicular hepatic steatosis (Figures 6.4 and 6.5). Furthermore, neonatal orally administered β-sitosterol (Bst + FS) prevented the progression of the long-term fructose-induced NAFLD to NASH in adult female and male rats (Tables 6.7 to 6.10). The liver is a major site of metabolism and stores metabolites such as glycogen, lipids, minerals and vitamins (Vdoviaková et al., 2016). Thus liver mass can be influenced by the amount of metabolites stored and hepatic inflammation (Senaphan et al., 2015). Although neonatal orally administered β-sitosterol (Bst + PW) alone did not impact the liver masses of rats, when neonatal orally administered β-sitosterol (Bst + FS) was administered in combination with fructose, liver masses increased in female rats (Table 6.3). My finding suggests that β-sitosterol and fructose (Bst + FS) may have synergistic effects that increased liver mass in female rats.The observed increase in liver

mass can be attributed to possible increased hepatic *de novo* lipid synthesis (Rippe and Angelopoulos, 2013). The increase in liver mass seen in the current study could also be the mechansim involved in the observed effect of neonatal orally administered β-sitosterol on NAFLD. However, it's important to further interrogate the mechanisms involved in this finding.

6.5 Conclusion

This study demonstrated that long-term fructose diet intake (from the pre-weaning stage to the post-weaning stage) caused fatty liver disease in adult female and male rats and visceral adiposity only in female rats. Neonatal orally administered β-sitosterol protected against the development of NAFLD in long-term fructose-fed female and male rats. Nonetheless, neonatal orally administered β-sitosterol was unsuccessful at protecting against visceral adiposity in female rats fed the long-term fructose-diet. In conclusion, β-sitosterol programmed for protection against high-fructose diet-induced NAFLD. Furthermore my findings support that β-sitosterol can be strategically administered during the neonatal period as a prophylactic against long-term fructose diet-induced metabolic derangements.

The next chapter (Chapter 7) begins by outlining the major conclusions drawn from my study. Thereafter, limitations of my study are highlighted and finally recommendations for future studies are suggested.

CHAPTER 7: CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

The overall aim of my study was to determine whether β-sitosterol can protect growing and adult rats against high-fructose diet-induced metabolic derangements. I carried out my study in two main experiments.

In the first experiment I found that feeding a high-fructose diet to growing female and male rats for 12 weeks caused metabolic dysfunction, NAFLD and renal derangements. Whereas in female rats the metabolic dysfunction was characterised by visceral adiposity, hypertriglyceridaemia and hypoadiponectinaemia, in male rats it was typified by visceral obesity and hypoadiponectinaemia. The high-fructose diet-induced NAFLD was characterised by micro- and macro-vesicular hepatic steatosis in female rats and typified by increased hepatic lipid accretion, micro- and macro-vesicular steatosis and hepatic inflammation in male rats. Orally administered β-sitosterol protected the male and female rats against the high-fructose diet-induced metabolic dysfunction, NAFLD and renal abnormalities. It is noteworthy that when β-sitosterol was orally administered alone (without the fructose insult), it decreased plasma insulin concentration and increased plasma adiponectin concentration and insulin sensitivity in female and male rats. Additionally, I found that β-sitosterol did not adversely impact liver and kidney morphometry and function. Overall, I have shown that β-sitosterol has a protective effect against the development of metabolic dysfunction and NAFLD. In the fight against MetS and NAFLD scourge, I therefore encourage the use of foods rich in β-sitosterol. Due to the limitations, cost and side effects associated with synthetic pharmacological agents, results from the present study show that β-sitosterol would be a useful supplement in managing the components of MetS and NAFLD.

In the first experiment plasma cholesterol concentration increased when β-sitosterol was administered to high-fructose diet-fed female and male rats, indicating that β-sitosterol should be used with caution as a dietary supplement if the diet of the individual is not well controlled. To provide some clarification future studies should in addition to determination of total cholesterol, as was done in the current study, also assess LDL-C and HDL-C in order to fully characterise the intervention's effects on the different types of cholesterol. The high-fructose diet caused increased liver lipid content. However, to clarify the

increased liver lipid content, future studies should look at activity and expression of hepatic lipogenic enzymes, proteins and genes in order to understand the mechanisms involved in the pathogenesis of NAFLD.

Findings from my second experiment revealed that a long-term fructose dietary intake (from pre-weaning through to adulthood) caused fatty liver disease in adult female and male rats and visceral adiposity in female rats. Neonatal orally administered β-sitosterol protected against the development of NAFLD in long-term fructose-fed female and male rats but failed to prevent high fructose diet-induced visceral adiposity in female rats. Neonatal orally administered β-sitosterol prevented the high-fructose diet-induced macro-vesicular hepatic steatosis and attenuated the severity of micro-vesicular hepatic steatosis, inflammation and prevented against the high-fructose diet-induced progression of NAFLD to NASH in the first and second experiment. To my knowledge, this is the first study to report on the effects of neonatal administration of β-sitosterol to programme against diet-induced NAFLD later in life. The mechanism by which β-sitosterol may have prevented the high-fructose diet-induced macro-vesicular hepatic steatosis could be by reducing the expression of lipogenic genes in the liver. I therefore recommend that future studies should consider using cellular and molecular techniques to elucidate the possible mechanisms through which β-sitosterol exerted its beneficial effects. Hormones such as insulin and adiponectin which play an important role in the regulation of metabolism should also be measured.

Overall, I conclude that β-sitosterol has the potential to programme for protection against the development of high fructose-induced metabolic derangement in later life. The second experiment shed light on the importance of intervention in the neonatal period for prophylaxis. β-sitosterol protected the rats against the high-fructose diet-induced metabolic derangements and NAFLD in a sexually dimorphic manner thus highlighting the importance for future studies to assess potential prophylactic agents in males and females especially in this era of precision medicine. I also recommend that further studies on the kidney function should also include direct function tests such as determination of glomerular filtration rate which is the gold standard.

CHAPTER 8:REFERENCES

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APPENDICES

Appendix I: Animal ethics clearance certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/08/55/B

APPLICANT: Miss N Gumede

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The potential effects of B-sitosterol to protect and to programme for protection against diet-induced metabolic derangements in Sprague-Dawley rats

Number and Species

16X female nursing dams Sprague Dawley rats, 100X male and female 21-day old rat pups Sprague Dawley rats and 160X male and Female 7-day old rat pups Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/08/29. This approval remains valid until 2019/09/20.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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:

Signed: _____ (Chairperson, AESC) Date: 21 September 2017

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: 21 September 2017

cc: Supervisor: Professor E Chivandi, Prof K Erlwanger and Prof R Brooksbank
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix II: Modification of the ethics clearance

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Nontobeko Gumede (904306)

b. School and email address: School of Physiology. Nontobeko.gumede@wits.aca.za

c. Experiment to be modified / extended

			AESC NO
Original AESC number	2017	08	55b
Other M&Es :			nil

d. Project Title: The potential effects of β-sitosterol to protect and to programme for protection against diet-induced metabolic derangements in Sprague-Dawley rats

		No.	Species
e.	Number and species of animals originally approved:	260	Sprague Dawley
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	172	
h.	Number of animals used to date:	100	

i. Specific modification / extension requested:

To refine the study by modifying the design of experiment 2, currently approved on this clearance.

To include Lihle Goba (1628097) and Kgomotso Maotshoane (831228) as co-workers.

j. Motivation for modification / extension:

Based on the findings from the first experiment which I have just concluded, it has become necessary that I refine the study by modifying the design of the approved experiment 2.

In my first experiment I investigated whether the administration of *β-sitosterol* to weaned pups through to adulthood could prevent the development of high fructose diet induced metabolic dysfunction (see figure 1 in appendix for study design). Having established the potential of *β-sitosterol* the second experiment (approved by the AESC) was designed to investigate whether administration of *β-sitosterol* to neonatal pups could programme for good health outcomes later in life of rats on a high fructose diet. The experiment was characterised by three phases. First rat pups were to be given treatments pre-weaning, then from weaning to postnatal day 60 there would be no intervention and then from postnatal day 60 to 125 half of the rats would be given access to a high fructose diet (see figure 2 in appendix).

I now wish to modify the study such that there is no longer a period without intervention (see figure 3 in appendix). The change in the design of experiment 2 will allow me to align the

question(s) asked in experiment 1 and the question(s) asked in experiment 2. Additionally, the proposed changes to the design of experiment 2 will result in reducing the numbers of animals used in the study. In the proposed design for experiment 2 eighty (80) rat pups will be used instead of the currently approved one hundred and sixty (160) pups. The new design for experiment 2 will be as outlined below. The neonatal interventions will remain unchanged (group allocation, doses of fructose and *β-sitosterol* etc) other than a reduction in number of rats viz:

Eighty (instead of 160) rat pups will be randomly distributed to one of four treatment regimen groups: Group 1: DMSO (0.5%); Group 2: 20% fructose solution in DMSO; Group 3: β-sitosterol in DMSO (20 mg.kg-1 per day); Group 4: 20% fructose + β-sitosterol in DMSO (20 mg.kg-1 per day). The rats will be gavaged with the respective treatment regimens from postnatal day 7 to postnatal 20. The rats will be weaned on PND 21, then housed in individual Perspex cages and allowed to grow to PND 125. Post weaning (postnatal day 20 and onwards), rats from group 1 and group 2 will be fed SRC + plain drinking water until PND 121. The other (Group 2 and 4) will be continued on 20% fructose drinking water + SRC until PND 121. On PND 121-125, the animals will be subjected to terminal procedures as previously approved by the AESC.

Ms Lihle Goba and Kgomotso Maotshoane will assist with animal husbandry and laboratory assays as part of their Masters studies.

Date:10/04/18 Signature: *N.Gumede*

RECOMMENDATIONS

- Modification of experiment 2 design as stated above
- Addition of Lihle Goba and Kgomotso Maotshoane as a co-workers.

Condition: The co-workers must attend the first time user's course facilitated by CAS before working with animals.

Date:17/04/18 Signature:

Chairman, AESC PP

Appendix III: Rat insulin enzyme-linked immunosorbent assay (ELISA) kit protocol

6th Edition, revised in June, 2015

Assay procedure

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

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

Important Note:

1. **ELISA Plate**: The just opened ELISA Plate may appear water-like substance, which is normal and will not have any impact on the experimental results.
2. **Add Sample**: The interval of sample adding between the first well and the last well should not be too long, otherwise will cause different pre-incubation time, which will significantly affect the experiment's accuracy and repeatability. For each step in the procedure, total dispensing time for

addition of reagents or samples to the assay plate should not exceed 10 minutes. Parallelmeasurement is recommended.

3. **Incubation**: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Do not let the strips dry at any time during the assay. Strict compliance with the given incubation time and temperature.
4. **Washing**: The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings. Residual liquid in the reaction wells should be patted dry against absorbent paper in the washing process. But don't put absorbent paper into reaction wells directly. Note that clear the residual liquid and fingerprint in the bottom before measurement, so as not to affect the micro-titer plate reader.
5. **Reagent Preparation**: As the volume of Concentrated BiotinylatedDetection Ab and Concentrated HRP Conjugate is very small, liquid may adhere to the tube wall or tube cap when being transported. You better hand-throw it or centrifugal it for 1 minute at 1000rpm. Please pipette the solution for 4-5 times before pippeting. Please carefully reconstitute Standards, working solutions of Detection Ab and HRP Conjugate according to the instructions. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated. It is recommended to suck more than 10μL for once pipetting. Do not reuse standard solution, working solution of Detection Ab and HRP Conjugate, which have been diluted. If you need to use standard repeatedly, you can divide the standard into a small pack according to the amount of each assay, keep them at -20～-80℃ and avoid repeated freezing and thawing.
6. **Reaction Time Control:** Please control reaction time strictly following this product description!
7. **Substrate:** Substrate Solution is easily contaminated. Please protect it from light.
8. **Stop Solution:** As it is an acid solution, please pay attention to the protection of your eyes, hands, face and clothes when using this solution.
9. **Mixing:** You'd better use micro-oscillator at the lowest frequency, as sufficient and gentle mixing is particularly important to reaction result. If there is no micro-oscillator available, you can knock the ELISA plate frame gently with your finger before reaction.
10. **Security:** Please wear lab coats and latex gloves for protection. Especially detecting samples of blood or other body fluid, please perform following the national security columns of biological laboratories.
11. Do not use components from different batches of kit(washing buffer and stop solution can be an exception).
12. To avoid cross-contamination, change pipette tips between adding of each standard level, between sample adding, and between reagent adding. Also, use separate reservoirs for each reagent. **Otherwise, the results will be inaccurate!**

Appendix IV: Rat adiponectin enzyme-linked immunosorbent assay (ELISA) kit protocol

Assay procedure (A brief assay procedure is on the 11th page)

1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 µL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Add 50 µL of **Stop Solution** to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

Appendix VI: Rat kidney injury molecule 1 enzyme-linked immunosorbent assay (ELISA) kit protocol

Assay procedure (A brief assay procedure is on the 11th page)

1. Add the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Add the samples to the other wells (100 uL for each well). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37°C. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Remove the liquid out of each well, do not wash. Immediately add 100 µL of **Biotinylated Detection Ab working solution** to each well. Cover with the Plate sealer. Gently mix up. Incubate for 1 hour at 37°C.
3. Aspirate or decant the solution from each well, add 350 uL of **wash buffer** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
4. Add 100 µL of **HRP Conjugate working solution** to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. Add 90 µL of **Substrate Reagent** to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Add 50 µL of **Stop Solution** to each well. Note: Adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.