The effects of Quercetin in preventing metabolic dysfunction in male Sprague Dawley rats fed a high fructose diet, post weaning

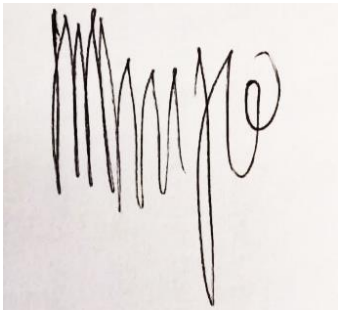
Malehope Churchill Molopo

A dissertation submitted to the Faculty of Health Sciences, University of Witwatersrand, School of Physiology in fulfilment of the requirements for the degree of Master of Science in Medicine

Johannesburg, South Africa, 2019

DECLARATION

I, Malehope Churchill Molopo, hereby declare that this dissertation is my own work, with the assistance of the acknowledged persons. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University. All procedures used in this dissertation were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: **2017/02/07B**).



Molopo M.C

09th day of July 2019 in Johannesburg

DEDICATION

In loving memory of my late father

Raboroko Andries Molopo

(1954-2015)

I hope I make you proud

Robala ka kgotso Motlokwa mmina-nare

CONFERENCE PRESENTATION

The following poster was presented at the **Faculty of Health Sciences Research Day** on the **6th September 2018** hosted by the **University of Witwatersrand (medical campus):**

1. Molopo M.C, Donaldson J. and Erlwanger, K.H. Daily supplementation with the flavonoid quercetin prevents the development of dietary fructose induced hepatic steatosis in growing male Sprague-Dawley rats.

ABSTRACT

Metabolic syndrome (MetS) is a collection of metabolic disorders that predispose to the development of chronic diseases. Risk factors include obesity, insulin resistance, increased blood glucose levels and increased blood triglycerides, leading to the development of non-alcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM). MetS epidemic is a worldwide health concern and it is estimated that a quarter of the world's population has MetS. This high prevalence adds to the economic burden on the health sector. There has been a worldwide increase in the consumption of fructose rich diets, which are associated with MetS development. Management for MetS includes weight loss, increased physical activity, healthy dietary choices and administration of pharmacological agents. Most people rely on medicinal plants which have biologically active phytochemicals. It is important to validate the efficacy and safety of medicinal plants, and their constituent phytochemicals. This study investigated whether the phytochemical quercetin, could prevent the poor metabolic outcomes associated with the consumption of a high fructose diet.

Thirty eight, 21 days old, male Sprague Dawley rats were used and randomly divided into 5 dietary groups and given respective treatments for 10 weeks. Group C (plain water + plain gelatine cubes), group Q (plain water + 20 % quercetin gelatine cubes), group F (20 % fructose solution + plain gelatine cubes), group FQ (20 % fructose solution + 100 mg/kg quercetin gelatine cube), and group FFe (20 % fructose solution + 100 mg/kg fenofibrate gelatine cube). After the 10-week feeding period, all rats were terminated and blood and tissue samples collected. Fructose consumption induced MetS risk factors (insulin resistance, increased glucose blood levels, elevated triglyceride levels) in rats given 20 % fructose solutions. Quercetin was able to significantly ($p < 0.05$) reduce high fructose diet induced hepatic inflammation, and steatosis. Additionally, rats given quercetin showed significantly decreased ($p < 0.01$) insulin levels and significantly increased ($p < 0.0001$) adiponectin compared to rats from the positive control group (FFe). There were no significant changes in clinical markers of general health, lipoprotein profile and bone density of all rat groups ($p > 0.05$).

Dietary supplementation with quercetin should be further explored as a prophylactic intervention in the fight against the poor metabolic outcomes associated high fructose diets.

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8. All my fellow Postgraduate colleagues: Thank you for welcoming me with open arms, assisting with some procedures and giving me clarity throughout my research study. All the best with your own studies.

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LIST OF ABBREVIATIONS

AESC: Animal Ethics Screening Committee

ALP: Alkaline phosphatase

ALT: Alanine aminotransferase

AMPK: Adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

ATP III: Adult treatment panel three

BM: Body mass

BUN/CRE: Blood urea nitrogen/creatinine ratio

BUN: Blood urea nitrogen

C: Negative control rat treatment group

CAS: Central animal service

CRE: Creatinine

F: fructose-only rat treatment group

FBG: Fasting blood glucose

FFe: Fructose-fenofibrate rat treatment group

FQ: Fructose-quercetin rat treatment group

GC: Gelatine cube

IGF-1: Insulin growth-like factor 1

IL-1: Interleukin 1

LDL: Low density lipoprotein

LI: Large intestines

MetS: Metabolic syndrome

mRNA: Messenger ribonucleic acid

n.s: Data in same row not significantly different

NRC: Normal rat chow

PPAR: Peroxisome proliferator activated receptor

Q: Quercetin-only rat treatment group

SD: Standard deviation

SI: Small intestines

TRI: Triglycerides

TW: Tap water

VLDL: Very low density lipoprotein

w/v: weight/volume

WHO: World health organization

CHAPTER 1: LITERATURE REVIEW

1.1. MetS

1.1.1. Prevalence

The metabolic syndrome (MetS) is a collection of several risk factors that predispose to the development of chronic diseases such as Non-alcoholic fatty liver disease (NAFLD), as well as cardiovascular and metabolic complications (Bruce and Hanson, 2010; Kuneš *et al*., 2015; Panchal *et al*., 2012). The MetS epidemic affects a quarter of the world's population and the prevalence is increasing at an alarming rate (Kaur and Meena, 2012; Rodríguez *et al*., 2017).Metabolic syndrome in South Africa varies according to specific region and provinces, with the Western Cape having the highest MetS prevalence, ranging between 42 - 62%, with a prevalence of 5.9% in the Eastern Cape, 22% in Kwa-Zulu Natal and 52% and 40% in rural and urban areas of the Free State province, respectively (Sekgala *et al*., 2018). The South African National Health and Nutrition Examination Survey reported that about 40% of adult females were found to be obese when compared to 10% in males (Naran *et al*., 2018). Obesity is not only a problem seen in adults. Childhood obesity increased from 32 million in 1990 to 42 million in 2013, with the prevalence of obesity in African children increasing from 4 million to 9 million during the same period. It is estimated that 60 million children will be obese by 2020, worldwide [World Health Organisation (WHO)]. This increasing prevalence adds to the economic burden of disease on the health sector.

1.1.2. Cost of management

MetS is a complex and difficult chronic disease to manage or diagnose due to its multi-factorial metabolic dysfunctions, which have to be considered when treating patients (Erasmus *et al*., 2012). The increased prevalence of MetS, along with NAFLD poses a considerable financial burden on the health sector and general public, especially in developing countries where resources are limited (Basaranoglu *et al*, 2015; Motamed *et*

al., 2017) . The complications associated with NAFLD such as insulin resistance and type 2 diabetes might cause severe health consequences within large populations (Smits *et al*., 2013).

MetS is a severe health threat to the global and national public health as it decreases the work productivity of a population, overall life expectancy and health related quality of life (Tremmel *et al*., 2017; Withrow and Alter, 2011). Apart from increased health care expenditure, MetS and obesity imposes costs in the form of productivity loss and compromised economic growth due to lower occupation productivity, lost workdays, permanent disability and mortality (Sander and Bergemann, 2003; Tremmel *et al*., 2017; Withrow and Alter, 2011).

In 2014, it was estimated that obesity, a major risk factor for MetS development, globally costed $2.0 trillion or 2.8 % of the global gross domestic product on the economy, thus the growing MetS epidemic is causing decreased economic productivity and increased health expenses (Müller-Riemenschneider *et al*., 2008; Tremmel *et al*., 2017). A mixed gender cohort study consisting of 216 840 people with an age range of 18 – 86 years which studied the overall cost of NAFLD patients in the United States, found that the total annual cost of NAFLD patients with private insurance was $7,804 (interquartile range: $3,068-$18,688) for a diagnosis, which is very costly for people and the government managing chronic diseases related metabolic dysfunction (Allen et al., 2018). Long-term management of NAFLD per patient was estimated to cost $3,789 per patient and these costs are significantly higher compared to what the average individual can afford (Allen *et al*., 2018; Tremmel *et al*., 2017).

The WHO provided an estimated economic burden cost of diabetes and obesity in sub-Saharan Africa in the year 2000, using data from various sources to estimate the direct and indirect costs, found that 7.02 million cases of diabetes were recorded in 2000, which resulted in an economic loss of $25.51 billion, costing each diabetic patient $11 431.6 annually (Kengne *et al*., 2013; Kruger *et al*., 2005; Tugendhaft *et al*., 2016). In Africa, about 80 % of diabetic and obese patients live in rural areas and have a low to middle class income, thus struggle to keep up with the cost of managing MetS and accessing appropriate treatment (Puoane *et al*., 2005; Erasmus *et al.*, 2012).

1.1.3. Current definition of metabolic syndrome

There have been several criteria used to define MetS over the past years. However for the purposes of this dissertation, the current definition by the American Diabetes Association (ADA) will be highlighted. Diagnosis of MetS is defined by meeting three or more of the following criteria: Increased waist circumference (102 cm for men and 88 cm for women), elevated blood pressure (≥ 140/90 mm Hg), decreased high density lipoprotein (HDL) cholesterol (< 50 mg/dl for men and women), increased triglyceride levels (> 150 mg/dl) and a high blood glucose (> 100 mg/dl) (Ferreira *et al*., 2007; Kuneš *et al.*, 2015; Pierce and Koppe, 2018). According to the National Cholesterol Education Program Adult Treatment Panel-III, it's crucial to diagnose and treat MetS with other related risk factors, especially in high risk populations (Reppert *et al*., 2008; Asrih and Jornayvaz, 2015).

There is a strong association between NAFLD and MetS, as NAFLD patients typically meet the criteria for MetS (Asrih and Jornayvaz, 2015; Jensen *et al*., 2018). This co-existing association between metabolic risk factors is why there is an alarming increase in NAFLD worldwide.

1.2. Non Alcoholic Fatty Liver Disease/Non-Alcoholic Steatohepatitis

1.2.1. Prevalence

About 13.5 % of adults are diagnosed with NAFLD in Africa, but other studies suggest the prevalence is much higher and this prevalence can be attributed to the low diagnosis of population towards NAFLD (Allen *et al*., 2018). It is estimated that between 20 – 30 % of adults in the United States of America are living with NAFLD, and it is estimate that with the disease also developing more in adolescents during recent years (Pierce and Koppe, 2018). Research also shows that 40 – 80 % of diabetic adults and 30 – 90 % of

obese adults worldwide are diagnosed with NAFLD (Bruce and Hanson, 2010; Motamed *et al*., 2017). Recent evidence also shows that NAFLD can develop independently of MetS (Pierce and Koppe, 2018).

1.2.2. NAFLD pathology

Hepatic steatosis is the earliest stage of NAFLD and is classified as a hepatic triglyceride concentration exceeding 55 mg/g of liver tissue (5.5 %), although other criteria use a hepatic triglyceride concentration of ≥ 10 % as the threshold cut-off (Buzzetti *et al*., 2016; Pierce and Koppe, 2018). Over time, NAFLD progresses into non-alcoholic steatohepatitis (NASH), with signs of hepatocyte injury and hepatic inflammation with collagen deposits (Basaranoglu *et al*., 2015).

Hepatic triglyceride accumulation (steatosis) is part of the NAFLD spectrum (Paschos and Paletas, 2009; Verrijiken *et al*., 2011). Steatosis promotes the development of insulin resistance, type 2 diabetes mellitus and hyperglycaemia (glucose intolerance) (Kaur and Meena, 2012). Excess fatty acid accumulation in the liver causes oxidative stress, which promotes pro-inflammatory cytokine expression, resulting in the progression of NAFLD to non-alcoholic steatohepatocellular injury and hepatic fibrosis (Li *et al*., 2016).

Liver biopsy is regarded as the golden standard to stage the progression from steatosis to fibrosis, despite several non-invasive diagnostic markers of NAFLD severity having recently being introduced; (Buzzetti *et al*., 2015; Vilà *et al*., 2011). Liver biopsy also has well known limitations such as risk of sampling errors, invasiveness, intra and inter-observer variability of histopathologists (Buzzetti *et al*., 2016; Jensen *et al.*, 2018). Liver damage is linked to biochemical changes in baseline levels of total serum bilirubin, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase (Cucchetti *et al*., 2011; Hamed *et al.*, 2011) .

Both MetS and NAFLD are strongly associated with the consumption of diets rich in refined sugars including fructose (Schaefer *et al*., 2009).

1.3. Fructose

1.3.1. Global trends in fructose consumption

Due to increasing urbanization globally, there has been a shift towards the consumption of western diets, which are high in fructose (Bruce and Hanson, 2010).

Human beings traditionally consumed little to no sugars as their diets mostly consisted of high proteins and moderate fat with low carbohydrates (Bray *et al*., 2004). Over time, due to widespread popularization of sugar derivatives and new methods of processing foods, there has been an increase in sugar consumption which has been associated with the increased development of non-communicable sugar consumption-related diseases such as diabetes mellitus (figure 1.1) (Johnson *et al.*, 2009; Ferder *et al*., 2010).

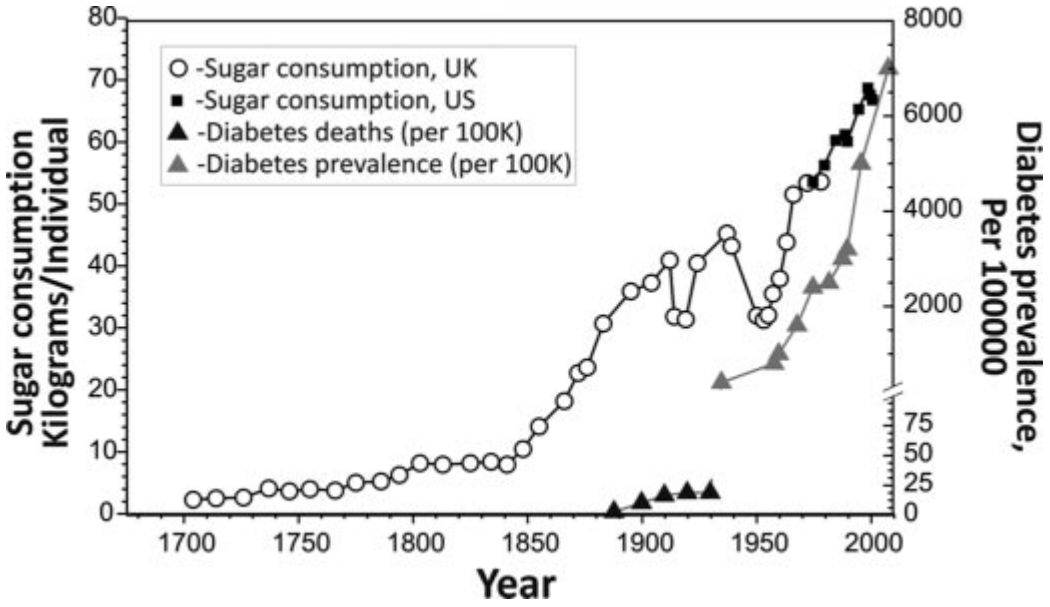

Figure 1.1: Sugar consumption (kg/individual) in the United Kingdom (UK) and the United States (US) in parallel with diabetes prevalence and mortality (per 100000). Source: (Johnson *et al*., 2009)

High sugar consumption from 1850 resulted in increased diabetes deaths in the early 1900s (figure 1.1), and the prevalence of diabetes has been on the rise in both UK and US, even to this day (Johnson *et al.*, 2009; Asrih and Jornayvaz, 2015). This trend has also been demonstrated in other parts of the world (Tappy and Le, 2010; Weeratunga *et al*., 2014). The industrial age and ever-increasing population in the 1970s brought about the increased use of sweetened sugars such as fructose, high fructose corn syrup (HFCS), maltose and sucrose in the food industry which were used as sweeteners and preservatives (Bray *et al*., 2004; Survey and Services, 2015). In the 1980s, there was a 50% noticeable shift from sucrose to HFCS consumption, mostly consumed in carbonated beverages (Rizkalla, 2010; Tappy and Le, 2010). Although recent studies indicate sugar consumption has been on the decrease, the prevalence of diet related non-communicable diseases has been on the increase worldwide due to its co-existing risk factors, which can be linked to early life sugar consumption, which through epigenetic mechanisms can impact the health of subsequent generations (Johnson *et al*., 2009; Lambertz *et al*., 2017).

1.3.2. Fructose metabolism

Fructose is used to sweeten processed foods worldwide and is found in most processed food products (Catak *et al*., 2014). Compared to glucose, fructose is more lipogenic as it does not stimulate insulin and leptin production, which are key hormones involved in long-term homeostatic energy regulation (Dekker *et al*., 2010a; Dulloo, 2008). Fructose (10 % of drinking water) administration for 14 days to Sprague Dawley male rats caused increased lipogenesis and decreased hepatic fatty acid β-oxidation (Vilà *et al*., 2011). High consumption of fructose in humans acts as an unregulated source of acetyl-CoA, which increases *de novo* lipogenesis, which affects intracellular macronutrient metabolism and alters signalling and inflammatory processes (Nakagawa *et al.*, 2006; Sanchez-Lozada *et al.*, 2006).

De novo lipogenesis primarily occurs in the liver and results in the conversion of excess carbohydrates into fatty acids that are then esterified to form triacylglycerols (Sánchez-

Lozada *et al*., 2007; Enos *et al*., 2016). Elevated hepatic fatty acid accumulation is associated with NAFLD (Rivera *et al*., 2008; Wang *et al*., 2012). Excessive fructose consumption results in an overflow of free fatty acids to the liver which can result in endoplasmic reticulum stress and mitochondrial dysfunction and consequently, activation of inflammatory cytokines (Buzzetti *et al*., 2016). High fructose diet consumption in humans during early life is associated with leptin/insulin resistance, hyperinsulinemia and a higher risk of developing cardiovascular complications during adult life (Dekker *et al*., 2010; Bruce and Hanson, 2010; Liu *et al*., 2012). Due to its complicated aetiology and complementary risk factors, which often require drastic lifestyle changes, MetS is a challenging disorder to treat and live with (Zimmet *et al*., 2007; Bruce and Hanson, 2010).

1.4. Management of MetS and NAFLD

Management of MetS includes weight loss, increased physical activity, healthy dietary choices and the administration of pharmacological agents (Reyes *et al*., 2015). Due to the multiple pathological processes associated with MetS, therapeutic interventions with pharmacological agents target specific risk factors. The pharmacological agents used in the treatment of MetS risk factors include thiazolidinediones, metformin, statins and fenofibrate (Takahashi, 2003; Zhang *et al*., 2009; Catak *et al*., 2014).

1.4.1. Pharmacological agents for the management of MetS

Thiazolidines (TZDs) are antidiabetic agents that bind and activate the gamma isoform of peroxisome proliferator-activated receptor gamma (PPARγ), mainly present in adipose tissue for regulating fatty-acid storage and glucose metabolism (Hauner, 2002; Lomonaco *et al*., 2012). TDZs have been shown to improve insulin resistance in both human and animal studies (Kahn *et al*., 2000; Hauner, 2002; Lomonaco *et al.*, 2012).

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase which regulates cholesterol synthesis (Magan-Fernandez *et al*., 2018; Vecchione *et al*., 2007). Statins are mostly used for treating hypercholesterolemia (Stancu and Sima, 2001; Vecchione *et al*., 2007), since they reduce endogenous cholesterol synthesis by inhibiting the principal cholesterol-regulating enzyme 3-Hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) (Vecchione *et al.*, 2007; Magan-Fernandez *et al.*, 2018).

Metformin is derived from guanidine, and is mostly used to treat type 2 diabetes mellitus as it lowers glucose levels and increases insulin sensitivity (Pernicova and Korbonits, 2014; Van Poelje *et al*., 2006). Metformin acts on the mitochondrion by reducing gluconeogenesis, halting the glucagon mediated signalling within the liver and to some extent, increasing glucose uptake in skeletal muscles (Nandhini *et al*., 2005; Nolan *et al*., 2017; Pernicova and Korbonits, 2014). Metformin is often used with a combination of other drugs, such as fenofibrate, in order to ensure greater effectivity, to target multiple sites and thus alleviate MetS risk factors (Forcheron *et al*., 2009; Li *et al*., 2011). Fenofibrate was used in the current study as the positive control intervention, thus it warrants a bit more discussion.

1.4.2. Fenofibrate

Fenofibrate ($C_{20}H_{21}ClO_4$) is a biopharmaceutics classification system class II drug with an aqueous solubility of less than 0.1mg/ml, which gives it a poor oral bioavailability (Belfort *et al*., 2010; Yousaf *et al*., 2015). Fenofibrate belongs to the fibrate class of amphiphathic carboxylic acids and acts on the peroxisome proliferator-activated receptor alpha (PPARα), which regulates hepatic fatty acids by controlling the proliferation and differentiation of adipose cells (Luquet *et al.*, 2005; Pawlak *et al*., 2015). This activation of PPARα also results in transcriptional changes of multiple genes for triglyceride synthesis and fatty acid catabolism in different cells (Uchida *et al.*, 2011; Pawlak *et al*., 2015). Fenofibrate modifies lipid parameters by altering low-density lipoprotein (LDL)

particle morphology, increasing high-density lipoprotein (HDL) cholesterol and decreasing triglyceride synthesis (Takahashi, 2003; Egert *et al*., 2010).

The drug fenofibrate, may also improve insulin sensitivity as it limits lipid accumulation in various tissues such as the liver and muscles (Luquet *et al*., 2005; Tsuchida *et al*., 2005).

Studies have shown that fenofibrate prevents and/or improves liver steatosis in animal models of NAFLD, as it decreases hepatic lipogenesis (Harano *et al*., 2006; Tanoue *et al*., 2011). There is also supporting evidence showing fenofibrate to possess anti-inflammatory and anti-thrombotic properties which improve endothelial function in patients suffering from MetS and type 2 diabetes (Kostapanos *et al*., 2013; Buzzetti *et al*., 2016). These pharmacological treatments are not available or too expensive for the general population (Weeratunga *et al*., 2014).

1.5. Use of Traditional Medicines

About 60 % of the world's population rely on traditional medicine, including plants, due to the easy accessibility and presumed limited side effects of traditional medicines (Modak *et al*., 2007a; Umashanker and Shruti, 2011). A study in Nigeria which evaluated why patients prefer traditional medicinal treatments as opposed to pharmacological treatments found that 40 % use of the traditional medicine is attributed to traditional beliefs, 35 % for efficacy and 20 % for their low costs, with 5 % being attributed towards a phobia and distrust of synthetic manufactured pharmacological drugs (Dièye *et al*., 2008).

Within the past two decades in developed countries, medicinal plant use has significantly increased as 40 % of adult Americans now use herbal medicines and remedies, with similar trends occurring in European countries, Australia and Canada, with 80 % of the world population in developing countries relying on medicinal plant extracts for primary health care needs (Du Plooy *et al*., 2002; J Kuneš *et al*., 2015;

Modak *et al*., 2007a). Africa has the highest use of medicinal plants, with 90 % of the population utilizing traditional medicines and remedies (Dhanalakshmi *et al*., 2017).

Most of the medicinal plants contain several biologically active phytochemicals, the medicinal efficacy of which, have not yet been scientifically validated. It is important to validate the efficacy and safety of medicinal plants, for clearer insight into the medicinal properties of their constituent phytochemicals; a scientific approach demands that individual phytochemicals be screened for their biological activity (Modak *et al.*, 2007; Zhang *et al*, 2009; Li *et al.*, 2016). My study focused on quercetin, a phytochemical found in various foods and which has medicinal properties (Coskun *et al.*, 2005; Modak *et al.*, 2007b; Panchal *et al*, 2012).

1.5.1. Quercetin

Quercetin (figure 1.2) (3,3´,4´,5,7pentahydroxyflavone) is a polyphenol flavonoid

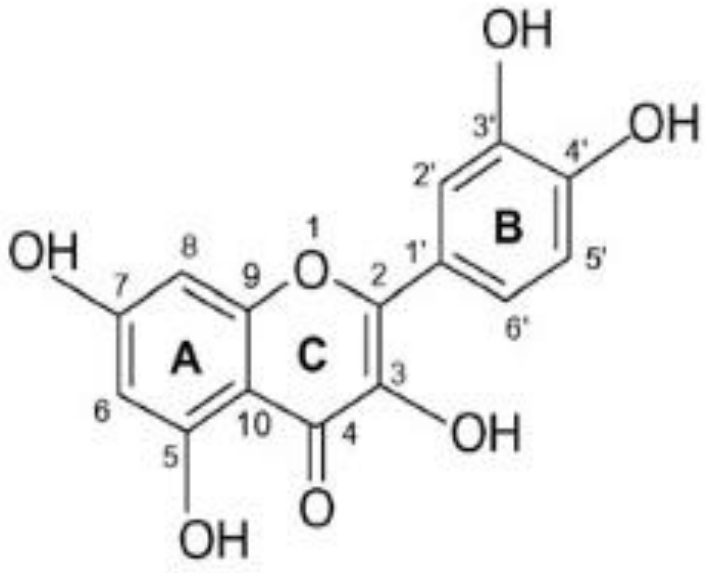

Figure 1.2: Structure of quercetin (3,3',4',5,7-pentahydroxyflavone), Source: (Bukhari *et al*., 2009).

responsible for the colour in many fruits, flowers and vegetables (Li *et al*., 2016). Diet sources rich in quercetin include red onions, unpeeled apples, citrus fruits, berries, tea (*Camellia sinensis*) and red wine (Kaur and Meena, 2012; Hossain *et al*., 2016).

Quercetin bioavailability is generally poor and highly variable as different conjugated compounds bound to quercetin affects its permeability (Graefe *et al*., 2001; Wiczkowski *et al*., 2008). Quercetin aqueous solubility is 1.53 - 12.5 mg/l at gastrointestinal pH (Pool *et al*., 2013) and its absolute bioavailability was found to be 16 % in rats when administered in an aqueous solution (Kaşıkcı and Bağdatlıoğlu, 2016). Flavonoids have been shown to protect biological membranes against free radical-induced oxidative damage by scavenging reactive oxygen species, restricting free-radical induced membrane lipid oxidation and inhibiting LDL oxidation (Kitagawa *et al*., 2004).

Quercetin reduced serum triglyceride levels and cholesterol concentrations in 16 weeks old, adult male Wistar rats on a high carbohydrate, high fat diet in which 0.8 g/kg quercetin was mixed in the feed for 8 weeks following an earlier 8 weeks on the high carbohydrate, high fat diet (Panchal *et al*.,2012). Quercetin administration, together with a high fat diet in mice, was shown to increase energy expenditure and reduce plasma concentrations of inflammatory markers without any changes in food consumption, physical activity or body composition (Panchal *et al*., 2012; Hossain *et al.*, 2016). A study by Zhang *et al*., (2009) in obese Zucker rats, using 10 mg/kg dose of quercetin in the feed for 8 weeks showed a decrease in dyslipidaemia, hyperinsulinemia and demonstrated anti-inflammatory effects.

Quercetin has also been shown to lower blood glucose levels in genetically obese Zucker rats (de Oliveira *et al*., 2012; Kawser Hossain *et al*., 2016). Its effect on glucose uptake is thought to be due to its action on the glucose transporter GLUT4 and it is known to alter the ERK1/2 pathway that plays a significant role in the regulation of glucose-induced insulin secretion (Coskun *et al*., 2005); Ghasemzadeh and Ghasemzadeh, 2011). On the contrary, a study on 4 week old mice on a 40 % high fat diet and a low dose of quercetin (0.02 %) for 16 weeks showed quercetin to have no impact on MetS risk factors such as insulin resistance, adiposity or in the attenuation of hepatic steatosis and body weight gain (Enos *et al*., 2016). Thus, it seems like the type of diet used to induce the metabolic dysfunction may influence the impact of quercetin on the risk factors for MetS. With high fructose diets being culpable in the increasing prevalence of MetS in children, it is important to investigate the potential prophylactic effects of quercetin in growing animals on a high fructose diet. There is still controversy

about the cause and development of MetS, However the prevention of metabolic risk factors associated with MetS is a key component in combating the development of MetS. The development of MetS is highly linked with alterations in hormonal functions responsible for energy balance such as insulin and adiponectin (Buzzetti *et al*, 2016).

1.6. Rationale for Study

The development of obesity in adulthood, amongst several other factors, has been linked to malnutrition and stressors during childhood. The current burden of obesity and fatty liver disease is thus, not confined to adults. There is an increasing incidence of metabolic disorders in children. Interventions during childhood can have long-term health benefits. Previous studies have used quercetin as a treatment for high fat-diet induced obesity. High fructose diets are culpable in the global increase in obesity and MetS in children and thus there is a need to investigate the effects of quercetin in a high fructose diet-induced experimental model of metabolic dysfunction in growing animals.

Previous studies have focussed on adult rats thus not factoring in the early onset of dietary influences on childhood obesity and its complications. Therefore in the current study, early post-weaning rats were used in this study so as to mimic the early onset (childhood) of dietary exposure to obesogenic diets. Dietary interventions can influence growth performance especially in actively growing children. Thus, measures of growth performance and the general impact of the interventions on the general health of individuals need to be assessed when validating the medicinal potential of phytochemicals. For orally consumed substances, the gastrointestinal tract (GIT) is the first point of contact with the body and consequently the GIT is prone to structural and functional changes. The impact of phytochemicals on the GIT, which is also an important source of peptides and compounds regulating metabolism, thus also needs to be considered in the scientific validation of phytochemicals affecting metabolism.

1.7. Study Objectives

Study Aim

The main aim of the current study is to investigate whether quercetin prevents the development of metabolic dysfunction in growing Sprague Dawley male rats on a high-fructose diet.

***Specific objectives*:**

To determine the effects of dietary quercetin supplementation in rats on a high fructose diet, on:

- The growth performance of the rats, through the assessment of body mass gain and linear growth as determined by the length, mass and relative density (Seedor indices) of the tibia and femur.
- The development of metabolic dysfunction through the assessment of general impairment of metabolism by determining:
    - circulating levels of metabolites (glucose, triglycerides, and cholesterol).
    - adiposity (visceral and epididymal fat pad).
    - the concentrations of hormones involved in metabolic regulation (insulin and adiponectin).
    - Serum haematocrit and haemoglobin concentration
- The development of metabolic dysfunction through the assessment of the liver for Non-alcoholic fatty liver disease, specifically considering:
    - Hepatic storage of lipids and the development of fatty liver by histological scores of hepatic steatosis.
- The general health profile of the rats through the assessment of:

- surrogate markers of liver function [alanine transaminase (ALT) and total bilirubin]
- renal function (creatinine (CRE) and blood urea nitrogen (BUN)).

- The morphology and morphometry of the gastrointestinal tract (and accessory organs) through the assessment of:
  - the lengths and masses of visceral organs.

Hypothesis

H_0: Quercetin administration to growing Sprague Dawley male rats on a high fructose diet does not prevent them from developing metabolic dysfunction, including negative outcomes on general health and changes in morphology of the rats' gastrointestinal tract (GIT).

H_1: Quercetin administration to growing Sprague Dawley male rats on a high fructose diet prevents them from developing metabolic dysfunction, sustains good health and prevents changes in the morphology of the GIT.

CHAPTER 2: METHODS AND MATERIALS

2.1. Methods and Materials

2.1.1. Ethical clearance

Ethical clearance for the study was obtained from the Animal Ethics Screening Committee of the University of the Witwatersrand (Certificate number: AESC no. **2017/02/07B**). Approval for the modifications and extensions to the study were also sought and granted (See Appendix 1, 2 and 3 for copies of relevant certificates).

2.1.2. Animals and housing

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Thirty eight (N = 38), weaned 21-day old Sprague Dawley male rats were used in the study. The rats were selected from litters of Sprague Dawley dams (*Rattus norvegicus*), each with between 8-12 (average 10) rats and at least 5 males (one to be allocated to each treatment group).

The rats were individually housed at the Central Animal Service unit of the University of the Witwatersrand in perspex cages lined with wood shavings as bedding and shredded paper for environmental enrichment. Rats from each litter were assigned to each of the different groups to avoid dam-effect bias. Ambient temperature was maintained at 26 ± 2°C. Twelve-hour light and dark cycles were maintained (lights on at 07.00 hours) and adequate ventilation was provided at all times.

2.1.3. Study design

As shown in figure 2.1, on postnatal day 21, the rats were randomly divided into five different experimental groups, with 7 - 8 rats in each group.

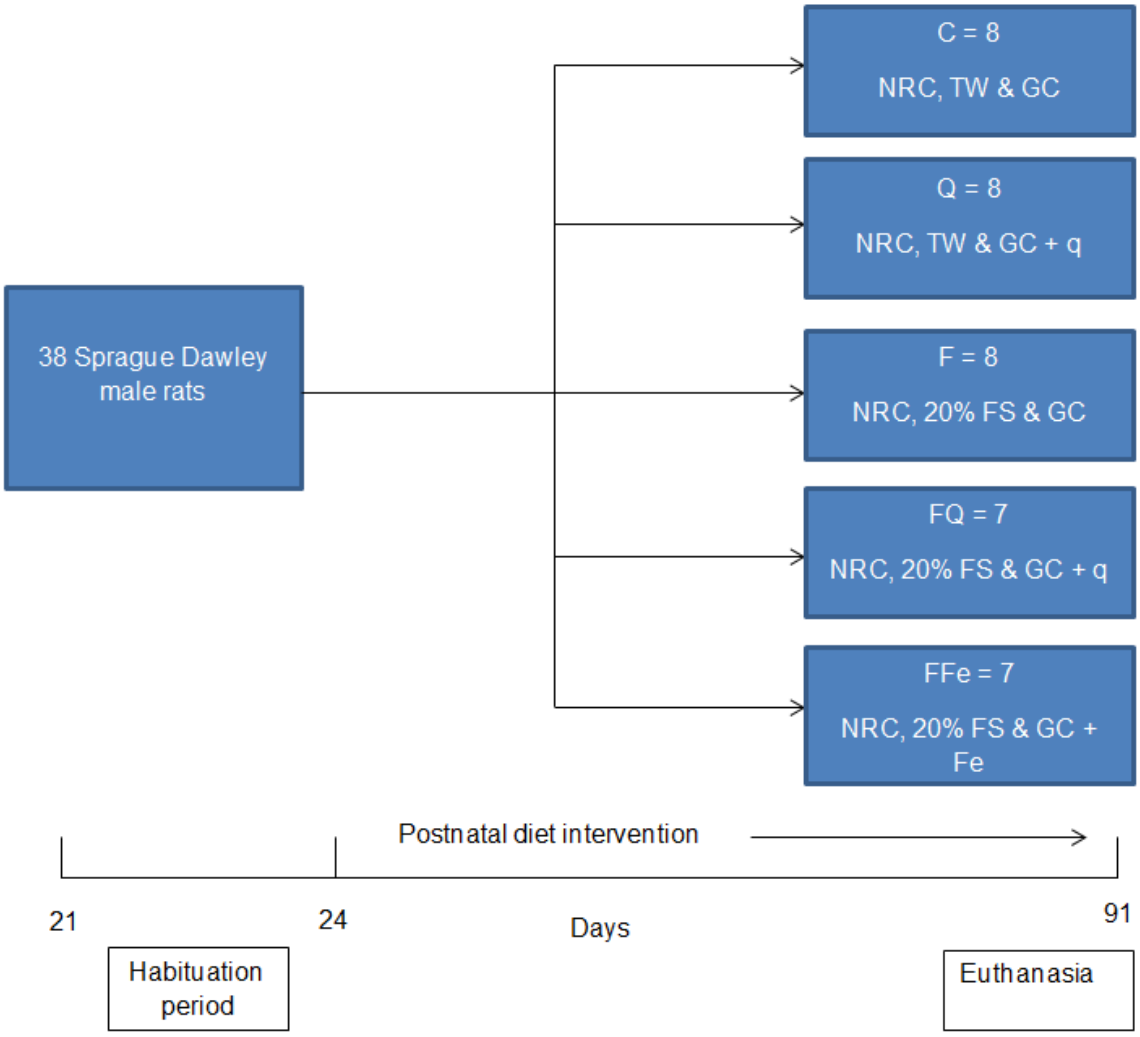

Figure 2.1: Study design for the effects of quercetin in preventing the development of outcomes associated with metabolic dysfunction in male Sprague Dawley rats fed a high fructose diet, post weaning following a 10 week treatment period.

During the 3-day habituation (adaptation) period, rats were given plain gelatin cubes with normal rat chow for acclimatization and thereafter the 10-week feeding period commenced, during which the rats were fed their respective diets.

All rats were weighed three times a week using an electronic balance scale (Clover Scales (Pty) Ltd, Johannesburg, South Africa) for the duration of the experiment. The weighing was in order to monitor growth performance and to adjust the amount of

pharmacological agents administered so as to maintain a constant dosage in relation to body weight.

Feed and solution intake for each rat was measured for one week before euthanasia. After the 10-week feeding period, rats were terminated on postnatal day 91 using anaesthetic overdose.

Group 1 (C) which served as the negative control group received normal rat chow (NRC) to eat, tap water (TW) to drink and plain gelatine cubes (GC) daily. Group 2 (Q) received NRC, TW and a gelatine cube containing quercetin (GC + q) in order to investigate the effects of quercetin alone. Group 3 (F) received NRC, a 20 % fructose solution (20 % FS) to drink and a plain gelatine cube daily in order to induce metabolic dysfunction. Group 4 (FQ) received NRC, the 20 % FS to drink and a gelatine cube containing quercetin (GC + q) daily in order to investigate whether quercetin can prevent the fructose-induced metabolic dysfunction. Group 5 (FFe) which served as a positive control was given NRC, 20 % FS and a gelatine cube containing fenofibrate (GC + Fe) daily.

2.1.4. Preparation of dietary interventions

The standard gelatine stock solution for cube preparation contained 8 g brown sugar, 8 g gelatine and 2 ml bovril (Pioneer Foods Pty, Ltd, South Africa) for enhanced taste and an appetizing aroma. The fructose solution was prepared by adding 800 g of fructose into 4 L of tap water and fresh solutions were prepared every 3 – 4 days. The gelatine cubes (2 ml volume) were formulated to administer quercetin at a dose of 100 mg/kg body weight daily. The selected dose of quercetin was used previously in other studies (Chan *et al*., 2014). The positive control drug fenofibrate (Hong *et al*., 2007) was also administered in the gelatine cubes at a dose of 100 mg/kg body weight daily for treatment group FFe. A fructose dose solution of 20 % was found to induce MetS risk factors when given to rats (Dai and McNeill, 1995). All rats were fed individually and were observed to consume their gelatine cubes immediately after they were placed on a piece of paper inside the rat cage.

2.2. Termination

2.2.1. Terminal procedures

Rats were fasted overnight (no feed with access to normal drinking water). Fasted blood glucose, haemoglobin and haematocrit were determined using a glucometer (Bayer (Pty) Ltd, Kempton Park, South Africa) and Hct meter (Woodley Equipment Company Ltd, Bolton, England), respectively. Blood for these measurements was collected via a pin prick to the tail vein of the rats. The rats were then euthanized using an overdose of sodium pentobarbital (Euthapent, Kyron laboratories, Johannesburg, South Africa) at 200 mg/kg body mass i.p. Tissue and blood samples were collected for further analysis.

2.2.2. Blood sample collection

Blood samples were collected by cardiac puncture using 20G hypodermic needles on 10ml syringes into plain tubes (Lasec (Pty) Ltd, Cape Town, South Africa). The blood was then centrifuged (1500g for 20 minutes) in order to obtain serum (plain tubes) which was used for the clinical biochemistry and hormone measurements.

2.2.3. Blood parameters

2.2.3.1. Insulin and adiponectin concentrations

An Enzyme linked Immune-Sorbent Assay (ELISA) was used for measuring fasting insulin and adiponectin levels (Arhoghro *et al*., 2009).

2.2.3.2. HDL/LDL cholesterol

Serum blood samples collected from rats were used to measure the HDL and LDL cholesterol concentrations using a HDL/LDL cholesterol kit (Elabscience (Pty) Ltd, rat HDL/LDL ELISA kit, Wuhan, China).

2.2.3.3. Clinical biochemistry assay

Serum triglyceride, surrogate markers of liver function (alanine transaminase (ALT) total bilirubin and albumin), renal function (creatinine and blood urea nitrogen) and clinical metabolite markers (total protein, calcium, phosphate, amylase and globulin) were measured using the IDEXX VetTest Chemistry Analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., USA) as per manufacturer's instructions.

2.2.4. Tissue parameters

2.2.4.1. Markers of obesity

Visceral and epididymal fat deposition were analysed by dissecting out and then weighing the mesenteric, epididymal and retroperitoneal fat pads. The retroperitoneal and mesenteric fat were weighed together and reported as visceral fat.

2.2.4.2. Liver lipid extraction

Liver lipids were solvent extracted and measured via standard procedures using Chloroform:methanol (Bligh and Dyer, 1959). Firstly, 5g of each rat liver sample was cut and steeped in a 150 ml of 2:1 chloroform: methanol solution, inside a flat bottomed 250 ml flask covered with foil refrigerated at 4°C. The next day the mixture from each beaker

was filtered through a filter paper (Whatmann, filter paper, No 1, size 185 mm, pore size 7 – 11 µm, England) into a 250 ml separating funnel. After 24 hours, 30 ml of 0.9 % saline solution was added to each filtrate, mixed and allowed to stand refrigerated at 4°C overnight for the two mixtures to separate. The bottom phase (Chloroform) was collected and reduced to dryness under vacuum at 37°C, using a rota-evaporator (Labex, Krugersdorp, South Africa) and thereafter made up to 20 ml with chloroform. An aliquot of 2ml of the extract was then placed into a dried, pre-weighed vial, and dried at 50°C for 30-min in an oven (Salvis, Salvis Lab, Switzerland) and then cooled in a desiccator and then reweighed to determine the lipid content. The liver lipid content was computed on the basis of the dry liver weight using the formula:

% liver lipids = total mass of the lipids/mass of the sample x 100.

2.2.4.3. Morphology and morphometry of the gastro-intestinal tract, accessory organs and kidneys.

The liver, kidneys, pancreas, stomach, large and small intestines were weighed. The small and large intestines lengths were measured using a mounted ruler on a dissection board. All organ masses were expressed as absolute masses and relative masses, which were calculated using the formula [terminal rat mass (g)/ absolute organ mass (g)].

2.2.4.4. Liver histology

A segment of liver was dissected and preserved in 10 % buffered formalin, processed (Leica Biosystems Instruments (Pty), Ltd, model RM 2125, Wetzlar, Germany) sectioned and then stained (Masson trichrome for fibrosis, haematoxylin eosin for steatosis) for histological examination by a histopathologist using a light compound microscope (OLYMPUS BX50) provided with CCD camera and image capture software for further analysis. The liver histology slides were scored for steatosis as described by Brunt *et al*., 2011.

2.2.4.5. Bone density and long bone growth determinations

The femoral attachment of the left hind leg was excised from each rat carcass, defleshed and then disarticulated from the tibia. The bones were dried using an oven (Salvis ®, Salvis Lab, Switzerland) at 50 °C for ten days and then weighed. Thereafter the tibia (between tibia head medial malleolus) and femur (between distal femora articular surface to the greater trochanter) lengths were measured using a pair of Vernier calipers (Hi-impact, Dejuca, South Africa). Bone density was estimated using the following formula:

Bone Density = mass of bone (mg) /length of bone (mm)

For further bone density evaluation of the tibia and femora, radiographs were taken using a Fuji film x-ray machine (Industrial X-ray flim FR, Fuji Photo Co, ltd, Tokyo, Japan) with settings at 61kV and 2.8 mAs. The plate was set at a distance of 1 metre from the source of the radiation beam. The images were captured digitally.

2.3. Data Analysis

The data was expressed as means ± standard deviation unless otherwise stated. Graphpad prism version 7 software (Graph-pad Software Inc., San Diego, USA.) was used for data analysis. Values between dietary groups were compared using a one way ANOVA, (except for changes in body mass which were assessed using a two way ANOVA) with a Bonferroni post hoc test and the differences were considered statistically significant if $p \leq 0.05$. A non-parametric Kruskal-Wallis test, followed by a Dunn's post hoc test was performed for the liver histology scores.

CHAPTER 3: RESULTS

3.1. Feed and Fluid Intake

The table (table 3.1) below shows the average feed and fluid intake of Sprague Dawley male rats in the last 7 days of the 10-week treatment period. Rats in the fructose-only group (F) drank significantly greater ($p < 0.0001$) fluid than those in the quercetin-only group (Q) and fructose-fenofibrate group (FFe). Rats in the fructose-quercetin group (FQ) drank significantly greater fluid than those in the negative control group (C) ($p < 0.01$), quercetin-only group (Q) ($p < 0.0001$) and the fructose-fenofibrate group (FFe) ($p < 0.0001$). Rats in the fructose-quercetin group (FQ) consumed significantly greater ($p < 0.01$) feed than those in the negative control group (C) and quercetin only group (Q). Rats in the fructose-only group (F) consumed significantly less ($p < 0.0001$) feed than those in the negative control group (C) and quercetin-only group (Q).

Table 3.1: The average daily feed and fluid intake of Sprague Dawley rats following a 10-week treatment period.

Intake	**TREATMENT GROUP**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
Fluid (mL/day)	82.5 ± 7.2^{ab}	73.2 ± 6.1^{a}	106.0 ± 18.8^{bc}	108.0 ± 16.6^{c}	77.6 ± 19.4^{a}	*
Feed (g/day)	21.4 ± 2.4^{a}	21.4 ± 2.3^{b}	14.3 ± 3.2^{ac}	16.3 ± 1.2^{abc}	17.6 ± 2.3^{abc}	**

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; * = $p < 0.05$; ** = $p < 0.01$; ^{abc} = data in the same row with different superscripts are significantly different.

Data expressed as mean ± SD; n = 6 (F & FQ); n = 7 (C); n = 5 (FFe).

3.2. Growth Performance

3.2.1. Body mass

The figures (figure 3.1 & 3.2) below show the effects of quercetin on body mass and percentage body mass gain of Sprague Dawley male rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) between the induction masses between groups. Rats in all treatment groups displayed a significantly greater ($p < 0.0001$) terminal mass compared to their induction masses. Rats in the fructose-only group (F) had significantly lower terminal masses ($p < 0.05$) than rats in the quercetin-only group (Q) and rats from the fructose-quercetin group (FQ). Rats in the fructose-fenofibrate group (FFe) had a significantly lower ($p < 0.0001$) terminal mass than the rest of the rat treatment groups.

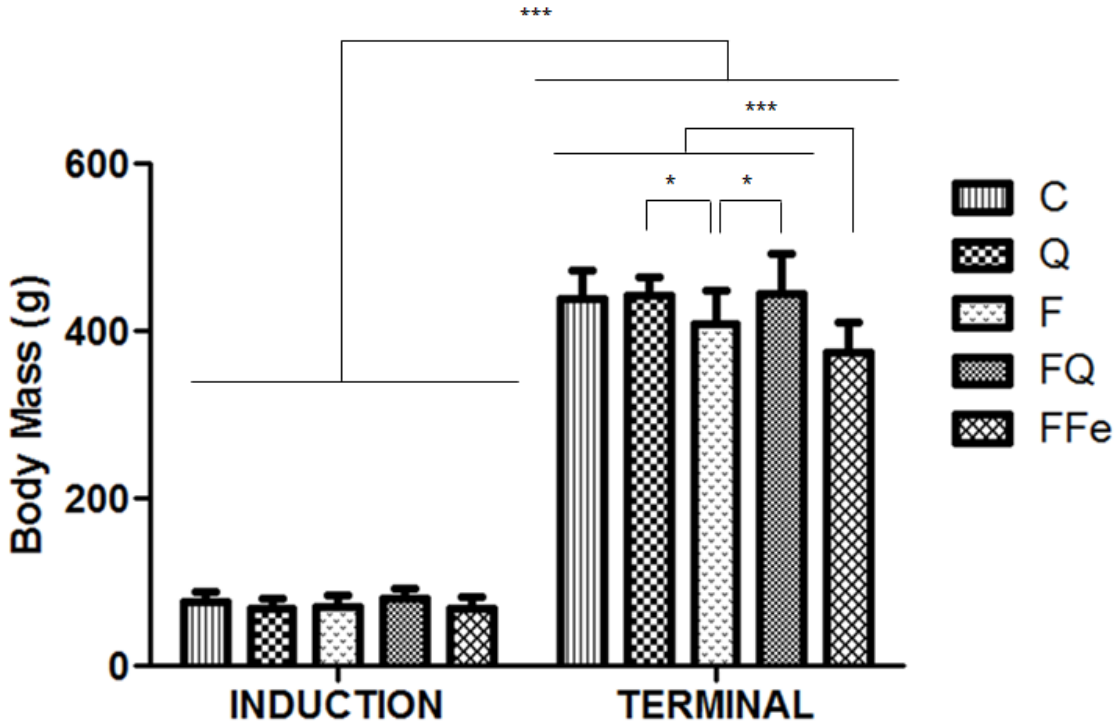

Figure 3.1: The effects of quercetin on the initial and terminal body masses of Sprague Dawley male rats following a 10-week treatment period.

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; * = $p < 0.05$; *** = $p < 0.0001$.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.2.2. Percentage body mass gain

The figure below (figure 3.2) shows the percentage body mass gain of Sprague Dawley male rats in different diet treatment groups following a 10-week treatment period. There was no significant difference ($p > 0.05$) in percentage body mass gain between rats from all different diet treatment groups.

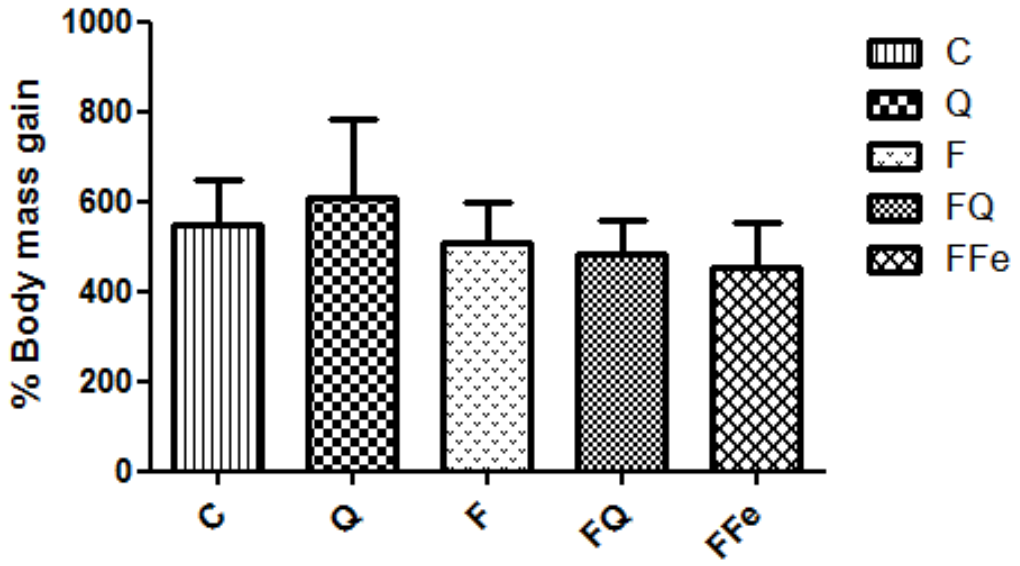

Figure 3.2: The effects of quercetin on the percentage body mass gain of Sprague Dawley male rats following a 10-week treatment period.

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.2.3. Linear growth

The table (table 3.2) and figure (figure 3.3) below show the effects of quercetin on the masses, lengths and densities of the femora and tibiae of male Sprague Dawley rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) between all treatment groups in terms of the femora mass and density. Rats from the Fructose-fenofibrate group (FFe) had significantly shorter ($p < 0.05$) femora than rats in the control group (C). There was no significant difference ($p > 0.05$) in the masses, length and bone density of the rats' tibiae across all treatment groups.

Table 3.2: The effects of quercetin on the linear growth (Bone Density) of Sprague Dawley male rats following a 10-week treatment period.

Bone Parameters	Treatment Groups					p-value
	C	**Q**	**F**	**FQ**	**FFe**	
Femora Mass (mg)	697.0 ± 135.9	632.5 ± 83.3	669.9 ± 148.5	684.3 ± 148.5	591.4 ± 114.8	n.s
Femora Length (mm)	35.0 ± 1.7^{a}	34.6 ± 0.9^{ab}	33.8 ± 1.3^{ab}	34.7 ± 1.9^{ab}	32.4 ± 1.5^{b}	*
femora Density (mg)/(mm)	19.8 ± 3.0	18.2 ± 2.0	19.7 ± 3.7	19.6 ± 3.2	18.2 ± 3.2	n.s
Tibiae Mass (mg)	535.7 ± 123.7	535.7 ± 101.3	506.7 ± 89.4	507.1 ± 100.3	482.0 ± 111.7	n.s
Tibiae Length (mm)	34.9 ± 1.8	34.6 ± 1.0	34.0 ± 0.7	34.7 ± 1.9	32.3 ± 2.7	n.s
Tibiae Density (mg)/(mm)	13.6 ± 2.9	12.9 ± 2.0	12.2 ± 2.6	12.5 ± 2.0	12.4 ± 3.3	n.s

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; ^{ab} = data in the same row with different superscripts are significantly different; n.s = data in same row not significantly different; * = $p < 0.05$.

Data expressed as mean ± SD; n = 7 (C, Q, F & FQ) n = 5 (FFe).

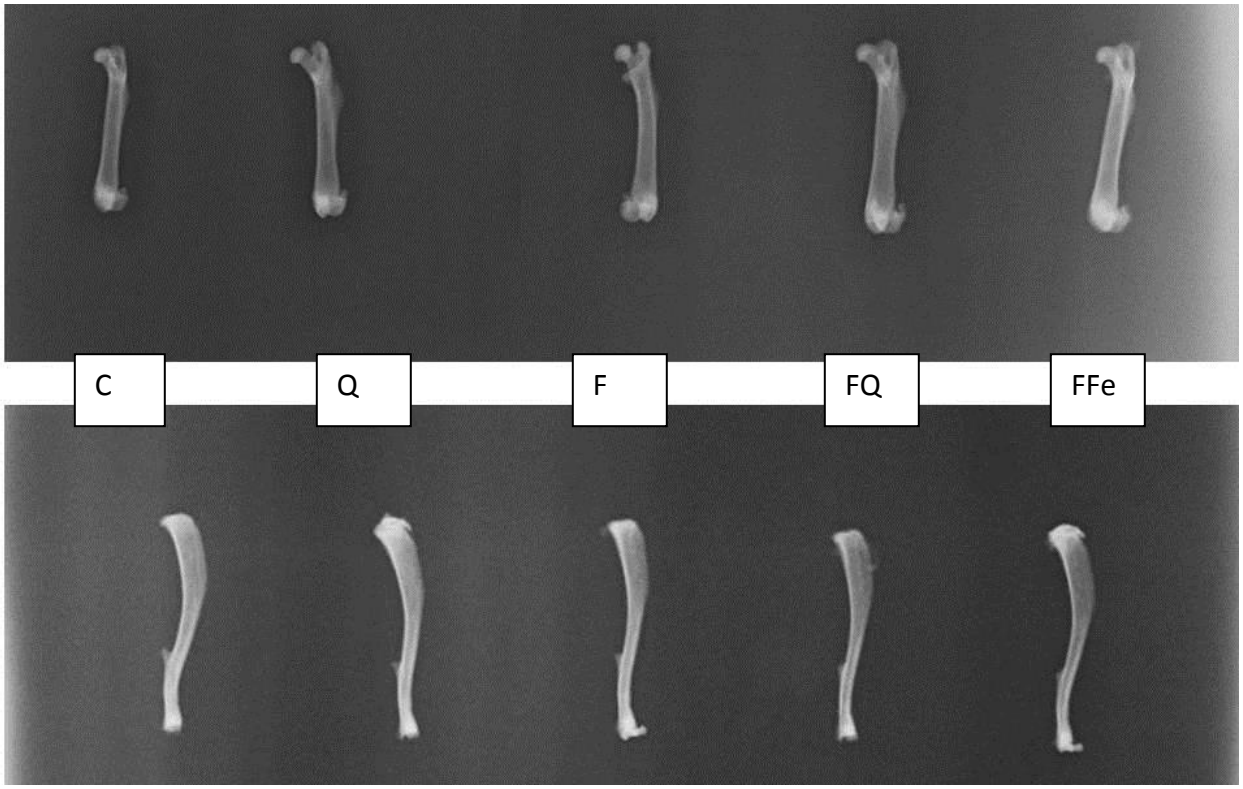

Figure 3.3: Representative radiographs of femora (top panel) and tibiae (bottom panel) from Sprague Dawley male rats of different treatment groups following a 10-week treatment period.

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid.

3.3. Blood Parameters

3.3.1. Haematocrit and haemoglobin

Haematocrit and haemoglobin concentrations were not significantly different ($p > 0.05$) between rats from all treatment groups (table 3.3).

Table 3.3: The effects of quercetin on haematocrit and haemoglobin concentrations of Sprague Dawley male rats following a 10-week treatment period.

	Treatment Groups					p-value
	C	**Q**	**F**	**FQ**	**FFe**	
Haematocrit (%)	51.7 ± 1.5	53.0 ± 1.5	50.6 ± 1.5	53.7 ± 2.6	51.3 ± 6.9	n.s
Haemoglobin (g/dl)	17.2 ± 0.5	17.7 ± 0.5	16.8 ± 0.5	17.9 ± 0.9	17.1 ± 2.2	n.s

C = Plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = Plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100mg/kg b.w quercetin + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; n.s = data in same row not significantly different.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.3.2. Insulin and adiponectin concentration

The table below (table 3.4) shows the effects of quercetin on insulin and adiponectin concentration in Sprague Dawley male rats following a 10-week treatment period**.** The rats in the fructose-only group (F), fructose-quercetin group (FQ) and the fructose-fenofibrate group (FFe) had significantly greater ($p < 0.01$) insulin concentrations than those in the negative control group (C) and those in the quercetin-only group (Q). Rats in the fructose-quercetin group (FQ) and the fructose-fenofibrate group (FFe) had significantly greater ($p < 0.0001$) adiponectin concentrations compared to those in the negative control group (C). Rats in the quercetin-only group (Q) had a significantly lower ($p < 0.0001$) adiponectin concentrations than the rats in the fructose-quercetin (FQ) and the rats in the fructose-fenofibrate group (FFe). The rats in the fructose-fenofibrate group (FFe) had significantly higher adiponectin concentrations when compared with the rats in the fructose-only group (F) ($p < 0.0001$) and the rats in the fructose-quercetin group (FQ) ($p < 0.01$).

Table 3.4: The effects of quercetin on insulin and adiponectin concentration of Sprague Dawley rats following a 10-week treatment period.

Hormone	**Treatment Group**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
Insulin (ng/ml)	2.2 ± 1.0^{a}	2.9 ± 1.0^{a}	4.8 ± 1.2^{b}	5.0 ± 0.6^{b}	5.7 ± 0.7^{b}	**
Adiponectin (pg/ml)	26.0 ± 6.4^{a}	42.0 ± 19.1^{a}	72.1 ± 24.0^{ab}	102.8 ± 20.5^{b}	169.3 ± 66.3^{c}	***

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance ** = $p < 0.01$; *** = $p < 0.0001$; ^{abc} = data in the same row with different superscripts are significantly different.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.3.3. Lipoprotein profile

The table (table 3.5) below shows the HDL and LDL concentrations of Sprague Dawley male rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) in HDL concentrations between all rat treatment groups. Rats receiving the fructose-fenofibrate (FFe) treatment had significantly greater ($p < 0.05$) LDL concentrations than rats receiving the negative control treatment (C)**.**

Table 3.5: The effects of quercetin on HDL and LDL concentrations in Sprague Dawley male rats following a 10-week treatment period.

Lipoprotein	**Treatment Group**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
HDL	6.8 ± 3.0	6.0 ± 0.6	6.0 ± 1.5	5.3 ± 0.6	5.7 ± 2.5	n.s
LDL	1.8 ± 0.4^{a}	2.0 ± 1.3^{ab}	2.2 ± 0.7^{ab}	1.9 ± 0.9^{ab}	3.6 ± 1.9^{b}	*

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; HDL = high density lipoprotein; LDL = low density lipoprotein; * = $p < 0.05$; ^{ab} = data in the same row with different superscripts are significantly different; n.s = data in same row not significantly different.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.3.4. Clinical metabolic markers

The table (table 3.6) below shows the effects of quercetin on the clinical metabolic markers of Sprague Dawley male rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) in the fasting blood glucose (FBG), blood urea nitrogen (BUN), triglyceride (TRI), alanine amino-transferase (ALT), creatinine (CRE) and the BUN/CRE ratios from all rat treatment groups.

Table 3.6: The effects of quercetin on clinical metabolic markers in Sprague Dawley male rats following a 10-week treatment period.

Metabolic Markers	**Treatment Groups**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
FBG (mmol/L)	4.1 ± 0.4	4.0 ± 0.5	4.0 ± 0.4	4.4 ± 0.4	4.5 ± 0.4	n.s
BUN (mg/dL)	20.0 ± 3.9	19.9 ± 2.9	16.1 ± 3.2	17.4 ± 3.6	20.3 ± 5.1	n.s
TRI (mg/dL)	66.9 ± 15.7	65.3 ± 6.8	65.0 ± 28.5	73.0 ± 21.6	79.1 ± 34.0	n.s
ALT (mg/dL)	61.4 ± 12.8	75.4 ± 32.6	53.4 ± 16.2	79.1 ± 30.4	92.4 ± 45.7	n.s
CRE (mg/dL)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	n.s
BUN/CRE Ratio	73.5 ± 24.3	78.7 ± 18.9	68.3 ± 22.1	60.8 ± 11.5	93.3 ± 22.3	n.s

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; FBG = Fasting blood glucose; BUN = Blood Urea Nitrogen; TRI = Triglycerides; ALT = Alanine transaminase; CRE = Creatinine; BUN/CRE = blood urea nitrogen/ creatinine ratio; n.s = data in same row not significantly different.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.4. Tissue Parameters

3.4.1. Morphometry and morphology of the gastro-intestinal tract, accessory organs and kidneys

The table (table 3.7) below shows the effects of quercetin on the gastro-intestinal tract, accessory organs and kidneys of Sprague Dawley male rats following a 10-week treatment period. Rats from the quercetin-only group (Q) had significantly lower absolute small intestine masses than rats from the fructose-only group (F) ($p < 0.05$). The relative masses of the small intestine from both the fructose-only (F) and fructose-fenofibrate (FFe) treatment groups were significantly greater ($p < 0.01$) than those in the control group (C) and quercetin-only group (Q). The small intestine lengths from all rats were not significantly different ($p > 0.05$) for all treatment groups. The large intestines' absolute masses, relative masses and lengths were not significantly different ($p > 0.05$) between treatment groups.

Absolute liver masses from rats receiving the fructose-fenofibrate treatment (FFe) were significantly greater ($p < 0.0001$) than those from all other rat groups. Relative liver masses from rats in the fructose-only group (F) ($p < 0.05$), fructose-quercetin group (FQ) ($p < 0.05$) and fructose-fenofibrate group (FFe) ($p < 0.0001$) were significantly greater than relative liver masses from rats in the control group (C). The rats' relative liver masses from the fructose-fenofibrate treatment group (FFe) were significantly greater ($p < 0.0001$) than the relative liver masses from the quercetin-only group (Q), fructose-only group (F) and rats in the fructose-quercetin group (FQ). Absolute kidney masses were not significantly different ($p > 0.05$) between treatment groups. Rats in the fructose-fenofibrate treatment group (FFe) had significantly greater relative kidney masses than rats in the control group (C) ($p < 0.0001$), quercetin-only group (Q) ($p < 0.0001$), fructose-only group (F) ($p < 0.01$) and the fructose and quercetin group (FQ) ($p < 0.0001$).

The absolute and relative pancreas masses were not significantly different ($p > 0.05$) between rats from all treatment groups. Absolute caecum masses from the fructose-fenofibrate treatment group (FFe) were significantly greater ($p < 0.05$) than those in the

negative control treatment group (C). There was no significant difference ($p > 0.05$) in the relative caecum masses from all rat treatment groups. The absolute and relative stomach masses in all rat treatment groups were not significantly different ($p > 0.05$).

Table 3.7: The effects of quercetin on the gastro-intestinal tract, accessory organs and kidneys of Sprague Dawley rats following a 10-week treatment period.

Organ	Treatment Groups					p-value
	C	Q	F	FQ	FFe	
S.I mass (g)	9.1 ± 1.0^{ab}	8.5 ± 1.0^{b}	9.9 ± 0.6^{a}	9.8 ± 1.3^{ab}	8.9 ± 0.6^{ab}	*
%BM S.I mass	2.1 ± 0.2^{a}	2.1 ± 0.2^{a}	2.5 ± 0.2^{b}	2.3 ± 0.2^{ab}	2.6 ± 0.2^{bc}	**
S.I length (mm)	1345 ± 118.8	1326.0 ± 95.5	1334 ± 162.9	1387.0 ± 61.0	1360.0 ± 54.5	n.s
L.I mass (g)	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	1.5 ± 0.2	n.s
%BM LI mass	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	n.s
L.I length (mm)	215.0 ± 18.5	211.9 ± 11.3	207.5 ± 16.7	210.7 ± 16.4	196.4 ± 13.8	n.s
Liver (g)	11.4± 1.6^{a}	11.1 ± 1.4^{a}	11.9 ± 1.4^{a}	12.5 ± 1.1^{a}	17.0 ± 1.4^{b}	***
%BM liver	2.6 ± 0.2^{a}	2.7 ± 0.2^{ab}	3.0 ± 0.2^{b}	3.0 ± 0.5^{b}	4.9 ± 0.3^{c}	***
Kidneys (g)	2.5 ± 0.3	2.4 ± 0.2	2.5 ± 0.3	2.4 ± 0.3	2.5 ± 0.4	n.s
%BM kidneys	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.6 ± 0.1^{a}	0.7 ± 0.1^{b}	***
Pancreas (g)	1.3 ± 0.3	1.3 ± 0.3	1.5 ± 0.3	1.4 ± 0.1	1.2 ± 0.2	n.s
%BM pancreas	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	n.s
Caecum (g)	1.6 ± 0.2^{ab}	1.5 ± 0.2^{a}	1.2 ± 0.2^{ab}	1.4 ± 0.2^{ab}	1.3 ± 0.3^{b}	*
%BM caecum	0.4 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	n.s
Stomach (g)	1.7 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	n.s
%BM stomach	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	n.s

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; %BM = Relative organ mass; S.I = Small intestines; L.I = Large intestines; ^{abc} = data in the same row with different superscripts are significantly different; n.s = data in same row not significantly different; ^{abc} = data in the same row with different superscripts are significantly different; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.0001$.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.4.2. Markers of obesity

The table (table 3.8) below shows the markers of obesity (abdominal and epididymal fat) in Sprague Dawley rats following a 10-week treatment period. The absolute and relative abdominal fat pad masses for rats in all treatment groups were not significantly different ($p > 0.05$). The absolute epididymal fat pad masses from rats in the fructose-fenofibrate group (FFe) were significantly less ($p < 0.0001$) than those of the negative control group (C). There was no significant difference ($p > 0.05$) in the relative epididymal fat pad masses from all rat treatment groups.

Table 3.8: The effects of quercetin on markers of obesity in Sprague Dawley male rats following a 10-week treatment period.

Fat	**Treatment Groups**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
Abdominal fat (g)	11.5 ± 4.3	11.0 ± 2.8	11.6 ± 3.3	12.9 ± 3.3	8.7 ± 1.9	n.s
%BW	2.6 ± 0.8	2.7 ± 0.6	2.9 ± 0.7	3.0 ± 0.6	2.5 ± 0.4	n.s
Epididymal fat (g)	3.7 ± 1.0[a]	3.2 ± 0.6[ab]	2.9 ± 1.1[ab]	3.3 ± 0.9[ab]	2.2 ± 0.8[b]	*
%BW	0.9 ± 0.2	0.8 ± 0.1	0.7 ± 0.3	0.8 ± 0.1	0.6 ± 0.2	n.s

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; %BW = relative organ mass; n.s = data in same row not significantly different; * = $p < 0.05$.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 7 (FQ & FFe).

3.4.3. Liver lipid content

The figure (figure 3.4) below shows the effects of quercetin on the liver lipid content of Sprague Dawley male rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) in the liver lipid content between rats from all treatment groups.

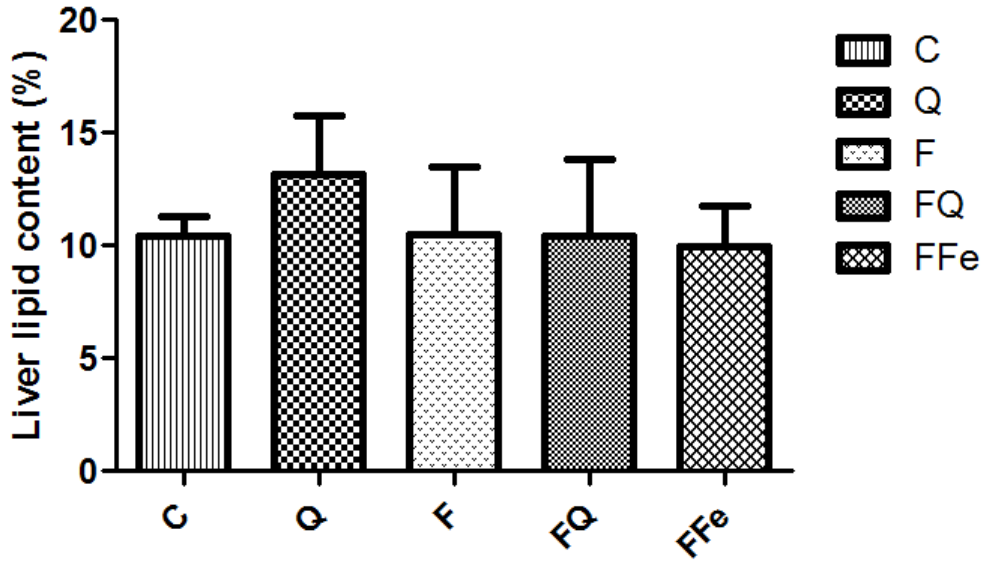

Figure 3.4: The effects of quercetin on the liver lipid concentrations in Sprague Dawley male rats following a 10-week treatment period.

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg quercetin b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid.

Data expressed as mean ± SD; n = 8 (C, Q & F); n = 6 (FQ & FFe).

3.4.4. Liver histology

The figure (figure 3.5, A-E) shows representative photos of sections from livers of rats in the different treatment groups.

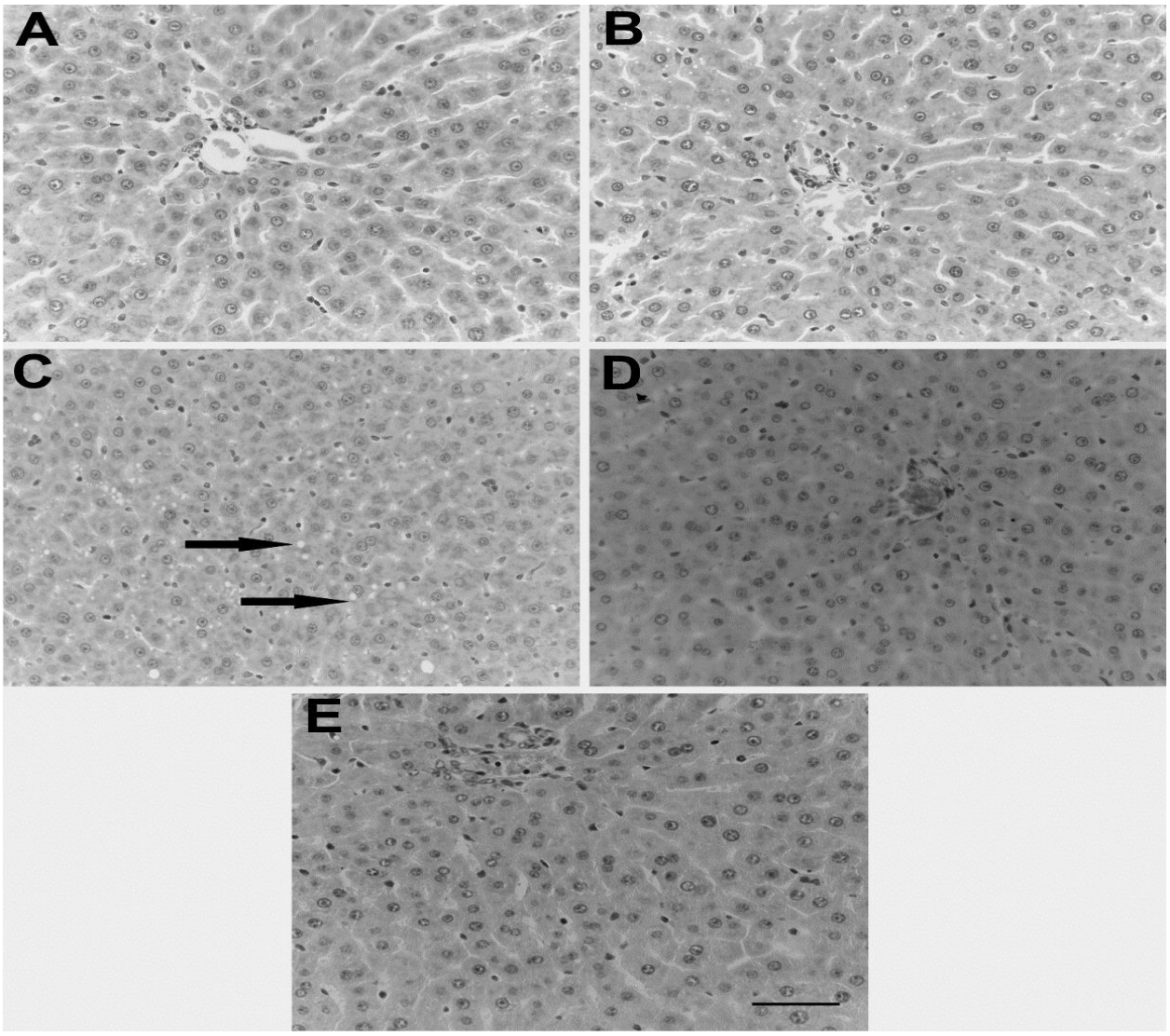

Figure 3.5: Representative histology images of the effects of quercetin on the liver histology of Sprague Dawley male rats from different treatment groups.

A = Control group (plain gelatine cubes + tap water to drink); B = Quercetin only group (100 mg/kg b.w quercetin + tap water to drink); C = Fructose only group (plain gelatine cubes + 20 % fructose solution as drinking fluid); D = Fructose and quercetin group (100 mg/kg b.w + 20 % fructose solution as drinking fluid); E = Fructose and fenofibrate group(100 mg/kg b.w fenofibrate + 20 % fructose solution as drinking fluid); _ = 50 µm scale, applies to all images; → = Micro-steatosis.

The table (table 3.9) below shows the effects of quercetin on the liver histology scores of Sprague Dawley male rats following a 10-week treatment period. There was no significant difference ($p > 0.05$) in the macro-steatosis and hypertrophy scores in the liver histology from all rat treatment groups. Rats from the fructose-only group (F) showed significantly greater ($p < 0.01$) micro-steatosis scores when compared to rats from the quercetin-only group (Q), fructose-quercetin group (FQ) and the fructose-fenofibrate group (FFe). Liver histology from rats in the fructose-only group (F) showed significantly greater ($p < 0.05$) inflammation scores than rats in the quercetin-only group (Q) and rats in the fructose-fenofibrate group (FFe).

Table 3.9: The effects of quercetin on the liver histology score for Sprague Dawley rats following a 10-week treatment period.

Histology	**Treatment Groups**					**p-value**
	C	**Q**	**F**	**FQ**	**FFe**	
Macro-steatosis	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.s
Micro-steatosis	0.0 (0.0-1.0)^{ab}	0.0 (0.0-0.0)^{a}	2.0 (0.5-0.2)^{b}	0.0 (0.0-0.0)^{a}	0.0 (0.0-0.0)^{a}	**
Infla-mmation	0.0 (0.0-1.0)^{abc}	0.0 (0.0-0.0)^{ab}	1.0 (1.0-1.0)^{c}	1.0 (0.0-1.0)^{ac}	0.0 (0.0-0.0)^{ab}	*
Hyper-trophy	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.s

C = plain gelatine cubes + tap water to drink; Q = 100 mg/kg b.w quercetin + tap water to drink; F = plain gelatine cubes + 20 % fructose solution as drinking fluid; FQ = 100 mg/kg b.w + 20 % fructose solution as drinking fluid; FFe = 100 mg/kg fenofibrate + 20 % fructose solution as drinking fluid; p-value = group significance; ^{abc} = data in the same row with different superscripts are significantly different; n.s = data in same row not significantly different; * = $p < 0.05$; ** = $p < 0.01$.

CHAPTER 4: DISCUSSION

4.1. Overview

This study was done in order to investigate whether quercetin prevents the development of metabolic dysfunction in growing Sprague Dawley male rats on a high-fructose diet (20 % fructose solution).

Fructose administration for 10-weeks resulted in a decreased feed intake; plasma elevated insulin and adiponectin concentrations and, an increase in liver masses. The dietary intervention with fructose also induced hepatic pathology characterised by micro-steatosis and inflammation. Fructose consumption had no significant effect on the growth performance (body mass and linear growth), haemoglobin concentration, clinical metabolite markers, and markers of obesity and organ morphometry.

Quercetin administration alone and with 20 % fructose solution resulted in decreased insulin and adiponectin concentrations, decreased small intestines and liver masses, and showed hepatoprotective properties by reducing liver micro-steatosis.

Fenofibrate, which was the positive control, resulted in a significant decrease in rats' body masses and femora lengths, increased insulin and adiponectin concentrations, increased LDL levels, increased relative small intestinal masses, increased absolute and relative liver masses, increased relative kidney masses, increased absolute caecum masses, decreased absolute epididymal fat and a decreased micro-steatosis and inflammation score.

These findings will now be discussed further.

4.2. Body Mass

Body mass is used to measure health and growth performance (Elliott *et al*., 2002a; Roberts and Rosenberg, 2006). Body mass is influenced by biological (e.g. physiology, genetic makeup and age) and individual factors (e.g. physical activity level and diet) (Management *et al*., 2004). There are inconsistent findings on the impact of high

fructose diets on body mass. Some research shows that high fructose intake is associated with increased body mass, whereas other studies report no significant impact of fructose on body mass (Hwang *et al*., 1988; Jürgens *et al*., 2005). Rats fed fructose did not have a significantly increased body mass gain compared to the other groups in the present study. It was notable that the rats given fructose consumed less feed, probably due to fructose being highly palatable (Lê and Tappy, 2006) and so it was preferentially consumed. It was expected that the increased consumption of fructose would result in an increased body mass gain as a result of increased fat deposition (Jürgens *et al*., 2005). Fructose does not stimulate insulin secretion from pancreatic beta cells, which decreases leptin levels and causes increased food (fructose) consumption (Basciano *et al*., 2005; Elliott *et al*., 2002b). Unlike glucose, fructose is not negatively controlled by phosphofructokinase and it continuously enters the glycolytic pathway which produces excess energy substrates (glucose, glycogen, lactate, and pyruvate) that initiate triglyceride production (Basciano *et al*., 2005). The lack of increase in body mass gain of the rats on the fructose diets when compared to rats not fed fructose could be attributed to several factors including age of the rats and the duration of feeding (Jensen *et al*., 2018; Patel and Srinivasan, 2010). It has also been reported that significant body mass changes in rats due to fructose consumption occur only after 100 days of treatment (Patel and Srinivasan, 2010). During the early life rapid growth phases, energy expenditure is utilized for developmental growth thus less energy is available for fat storage, at an older age, excess energy is stored as fat explaining why body mass changes mostly show after 100 days of fructose consumption (Girard *et al*., 2006; Patel and Srinivasan, 2010).

In the present study, quercetin administration did not significantly affect the body mass of rats compared to the other groups. Quercetin is known to reduce body weight by stimulating mitochondrial oxidative pathways and increasing transcriptional factor, nuclear erythroid 2-related factor 2 (Nrf-2), and HO-1 protein of lipid-laden hepatocytes and the liver lipid metabolism (Wattel *et al*., 2004; Kim *et al*., 2015) in the obese rats. In the current study the rats were not obese and so quercetin may not have activated these processes.

The rats which were fed the combination of fructose and fenofibrate had significantly lower ($p < 0.0001$) terminal masses than all the other treatment groups. This reduced body mass is likely due to the pharmacological action of the positive control supplement, fenofibrate which has been shown to reduce body mass gain (Lee *et al*., 2002) by restricting insulin secretion thereby increasing mitochondrial β-oxidation in the liver, decreasing subcutaneous fat and visceral fat (Lee *et al*., 2002).

Fenofibrate also plays a critical role in lipid metabolism as an agonist for peroxisome proliferator activated receptor alpha (PPARa), a protein responsible for cell membrane uptake of fatty acids, fatty acid oxidation and lipoprotein assembly (Shi *et al*., 2017). Fenofibrate may thus have affected the body mass via activation of PPARa that increases lipolysis and reduces lipid building components such as Apolipoprotein C-III, which increase high density lipoproteins (HDL) (Vosper *et al*., 2002; Fruchart, 2009).

There are multiple factors which affect body mass, some are long term (e.g. age, metabolism) and others that cause sudden body mass changes such as gut filling, hydration status and excretion, which makes body weight an unreliable measure of growth performance (Tappy and Le, 2010). Linear growth as measured by the assessment of long bones is therefore a better measure of growth performance.

4.3. Linear Growth

Unlike body weight, the factors affecting linear growth affect bone after a relatively prolonged time, thus it is a better predictor of growth performance when compared with body mass (Management *et al*., 2004). Linear growth can be affected by endogenous factors (hormones and age) as well as exogenous factors (diet and physical activity) (Paz *et al*., 2006).

In this study, fructose administration did not affect the length and masses of the femora and tibiae. It has been reported that sugars may indirectly affect bone metabolism by stimulating the secretion of insulin, which promotes bone formation (Shapiro *et al*., 2008). In other studies, rats given glucose showed decreased levels of phosphate and

calcium intake and elevated urinary calcium excretion than rats on fructose, indicating bone turnover to not be directly linked to the consumption of fructose (Tsanzi *et al*., 2008; Tappy *et al*., 2010). This could be explained by the low potency of fructose to stimulate insulin secretion as discussed earlier.

Despite the reduced (solid) feed intake in the rats drinking the fructose solution, the findings on bone parameters suggest that there was adequate mineral supply from the feed even though they consumed different feed amounts. In the present study, quercetin did not alter linear growth and bone density. The findings in other studies on the effects of quercetin on bone are varied. An *in vivo* study found quercetin to promote peak bone mass in six - nine weeks old male mice given 2 - 10 mg/kg quercetin (Yamaguchi and Weitzmann, 2011). A contradicting study showed quercetin to decrease bone masses and increase bone minerals using 4 months old, 30- inbred male Wistar albino rats given 15 mg/kg b.w quercetin for four weeks (Coskun *et al*., 2005). Further research shows quercetin exerts inhibitory effects in *in-vitro* bone resorption activity of mature osteoclasts by inducing apoptosis (Wattel *et al*., 2004). Quercetin was also found to reverse the decreasing serum osteocalcin, ALP activity, biomechanical quality and impaired micro-architecture of the femurs in rats (Luo *et al*., 2011). The study by (Wattel *et al*., 2004) showed bone improvement at a low quercetin dose daily, and it was further shown that at low concentrations quercetin inhibits RANKL- induced NFkB and AP-1 activation to decrease bone formation (Wattel *et al*., 2004; Wattel *et al*., 2003). Measurement of markers for bone turnover and structural characteristics is thus recommended for future studies.

Rats given fenofibrate had significantly shorter tibiae than those in some of the other groups. This was most likely due to the known effects of fenofibrate on bone. A study using high-fat-diet induced type 2 diabetes mellitus mouse model was given 100 mg/kg fenofibrate and after four weeks there was a significant decrease in bone quality, expression of collagen I and osteocalcin in bone tissue (Shi *et al*., 2017). This is a result of fenofibrate down regulating Runt-related transcription factor 2 (Runx2), which is a transcriptional factor for osteoblast differentiation (Shi *et al*., 2017).

Having discussed body mass and linear growth, it is important to consider that it is the extent of obesity which has a bearing on metabolic health.

4.4. Markers of Obesity

In metabolic studies it is important to assess visceral adiposity as, adipose tissue is considered to also be an endocrine organ that regulates lipid and energy metabolism (Kim *et al*., 2012). Markers of obesity were assessed by direct measurement of masses of the epididymal and visceral fat pads of the rats. Our study showed no significant difference in the masses of these tissues from rats given fructose and or quercetin, but the rats receiving fenofibrate with fructose had a significantly lower absolute epididymal fat pad mass than the negative control group. It is likely that previously reported changes due to fructose intake were absent as the rats were still in their rapid growth phase at the end of the treatment period thus energy was utilized for growth (Patel and Srinivasan, 2010; Sheludiakova *et al*., 2012).

Kobori *et al.*, (2011) showed that quercetin decreased lipid accumulation by improving adiponectin secretion, suppressed adipogenesis by inhibiting adipocytes and increased mitochondrial breakdown of lipids. These changes occurred only after 20 weeks thus in future a longer treatment period is required in order to not only assess the lipid accumulation induced by fructose but the potential impact of a dietary supplementation with quercetin (Krauss *et al*., 2009; Kobori *et al*., 2011). Rats given fenofibrate and fructose had significantly decreased absolute epididymal fat pad mass compared to rats in the negative control group most likely due to the action of fenofibrate which is as an agonist for PPARa and increases lipid oxidation (Oosterveer *et al*., 2009; Stunes *et al*., 2011). However when expressed relative to body mass, there was no difference compared to the other rats. Therefore the observed difference in absolute mass may not be biologically significant.

4.5. Lipoproteins

Studies have demonstrated that fructose consumption can increase lipoprotein synthesis in the liver (Krauss *et al*., 2009). Fructose bypasses phosphofructokinase during glycolysis and is in turn activated by pyruvate dehydrogenase and esterification modifications, increasing very low density lipoproteins (VLDL) (Basciano *et al*., 2005; Schaefer *et al*., 2009). Increased VLDL serves as a component for further lipoprotein lipid production including low density lipoprotein (LDL) and high density lipoprotein (HDL) (Basciano *et al*., 2005; Krauss *et al*., 2009). Increased insulin secretion, which is an indirect consequence of fructose consumption, controls the expression of hepatic sterol regulatory element binding protein (SREBP), a crucial transcription factor for cholesterol biosynthesis (Basciano et al., 2005; Lewis and Rader, 2005). Fructose also results in increased fatty acid synthase (FAS) mRNA concentrations, which accelerates fatty acid synthesis (Srivastava *et al*., 2006; Schaefer *et al*., 2009).

In the current study, fructose consumption did not cause any significant change in HDL levels compared to the other rat groups. This could be due to the duration of feeding and or the age of the animals, which both can influence lipid profiles (Patel and Srinivasan, 2010). During the early post-weaning period, the HDL is especially utilized for growth and development of vital organelles and it cannot accumulate as much as LDL (McCracken and Monaghan, 2018). Research shows that quercetin suppresses expression of PPARa and SREBP-1 while targeting the hepatic synthesis of fatty acids and induces LDL expression (Yamamoto *et al*., 1999; Moon *et al*., 2012). Quercetin decreases LDL oxidation through inhibition of neutrophil myelo-peroxidase (Bischoff, 2008). Quercetin also scavenges for free radicals (nitric oxide, peroxynitrite) that are responsible for LDL oxidation (Dias *et al*., 2005). Despite these previously reported actions, the rats in the current study given quercetin with or without fructose did not show any significant differences in the lipoproteins compared to the other rat groups. The lack of significance between rats given quercetin alone and those in the negative control demonstrate that quercetin did not affect normal lipoprotein concentrations during the treatment period.

Quercetin did not significantly affect LDL or HDL profile as the levels of lipoproteins were unaffected, as was the case with fructose alone. Rats given fructose and fenofibrate had significantly greater LDL levels than other treatment groups. As described previously, fenofibrate activates PPARa to regulate lipid levels and inhibit inflammatory reactions (Yang *et al*., 2004). PPARa targets lipoprotein lipase and alipoprotein C-111(ApoC-III), which is used for plasma triglyceride hydrolysis and β-oxidation (Yoon *et al*., 2003). It has been shown that long term fenofibrate use can increase LDL size and composition, this change in size could also account for the positive health outcomes of fenofibrate use (Vakkilainen *et al*., 2002).

4.6. Fasting Blood Glucose

Glucose is a monosaccharide serving as the main energy source for cellular respiration (Schalkwijk *et al*., 2004). After fasting, the liver becomes the main organ for glucose release, with the kidneys producing minor portions (Dirlewanger *et al*., 2000).

Fasting blood glucose was assessed after 12 hours of fasting and we expected long term fructose consumption to induce hyperglycemia through diet induced insulin resistance in the rats but fasting blood glucose concentrations were not significantly different between rat groups. The lack of significance in fasting blood glucose between rat groups may be a due to increased insulin secretion as a result of fructose intake, which maintains normal blood levels (Schalkwijk *et al*., 2004; Lê and Tappy, 2006). The impact of the interventions on insulin levels is discussed later.

Studies have shown quercetin to inhibit α-glucosidase activity and decrease fasting hyperglycemia in STZ-treated rats given 50 mg/kg quercetin for 6-weeks (Jeong *et al*., 2012). Quercetin was also found to increase glucose uptake and inhibition of fructose 1,6-Bisphosphatase, decreasing endogenous glucose synthesis (Van Poelje *et al*., 2006; Fang *et al*., 2008). In the current study, quercetin supplementation did not affect blood glucose levels. This could be attributable to differences in the animal experimental model used. Rats on fenofibrate and fructose treatment also did not have abnormal blood glucose concentrations. Fenofibrate activates adenosine monophosphate-

activated protein kinase (AMPK) in vascular endothelial cells, which protects against metabolic abnormalities caused by glucose (Field, 2005; Tomizawa *et al*., 2011).

Fructose breakdown does not respond to the glycolytic negative feedback mechanisms and can produce excess lipoproteins and triglycerides (Wang *et al*., 2014).

4.7. Triglycerides

Breakdown of fructose produces triglyceride and fatty acids that can be used for either energy stores or catabolized through β-oxidation (Basaranoglu *et al*., 2015). In our study, there were no significant differences in triglyceride concentrations between rat groups. This is probably a result of the rats' utilizing the additional calories from fructose for growth rather than converting them for storage.

Rats given quercetin did not show any differences in triglyceride levels compared to the other groups.

Fenofibrate administration did not affect the levels of triglyceride concentrations. Fenofibrate inhibits triglyceride accumulation by activating AMPK in cells, causing an increase in nitric oxide (NO) and inhibiting apoptosis (Tomizawa *et al*., 2011). Fenofibrate has been shown to reduce blood triglycerides by activating PPARa and decrease lipogenesis (Shi *et al*., 2017). In the current study with the absence of diet induced hypertriglyceridaemia, it is likely that the administered fenofibrate did not have an effect as the triglycerides were already at normal levels and homeostatic mechanisms would have been in effect to prevent hypolipidaemia.

The triglyceride levels present in plasma does not represent the level of accumulated fatty acids within the liver, as most triglycerides in the body are stored as fat (Wang and Russell, 1999). Excess accumulation of triglycerides within the liver causes steatosis and inflammation (Day and James, 1998).

4.8. NAFLD/ NASH

The progression of NAFLD is characterized by hepatic inflammation and/or steatosis with liver fibrosis and cirrhosis during severe progression, during this stage it is classified as non-alcoholic steatohepatitis (NASH) (Hift, 2005; Steatohepatitis *et al*., 2012). After hepatic injury, mediators such as transforming growth factor β and platelet-derived growth factors (PDGF) enhance collagen formation, which promotes fibrosis (Steatohepatitis *et al*., 2012; Buzzetti *et al*., 2016).

Hepatic fatty acid synthesis is highly dependent upon the amount of available triglycerides (Dekker *et al*., 2010b). Fructose increases lipogenesis by directly producing triglycerides after fatty acid degradation (Dirlewanger *et al*., 2000). Elevated triglycerides increase lipid synthesis by increasing de novo lipogenesis and increasing malonyl-CoA in the liver, resulting in inhibition of fatty acid oxidation by restricting fatty acid transport into the mitochondria (Van Poelje *et al*., 2006; Dekker *et al*., 2010b).

The development and progression of NAFLD involves complex and multifactorial mechanisms, making it difficult to diagnose and manage (Buzzetti *et al*., 2016; Porras *et al*., 2017). Increased hepatic lipid concentration is associated with the increased production of reactive oxygen species (ROS), which are known to cause inflammation (Nakagawa *et al*., 2006b; Buzzetti *et al*., 2016). A liver lipid extraction was done in order to determine the pooled global differences in hepatic lipid concentration between treatment groups. There was no significant difference in liver lipid levels between groups. By determining the pooled average lipid content, the sample population distribution per group is not accurately reflected therefore histological assessment is advised.

Liver biopsy remains the gold standard for diagnosing of NAFLD to NASH (Buzzetti *et al*., 2016). We did a liver histology score assessment to determine the presence of NAFLD development and the severity of damage to the liver. Although no macro-steatosis was observed, micro-steatosis was present in the fructose-fed rats. Micro-steatosis can progress to NASH, independently of macro-steatosis (Buzzetti *et al*., 2016; Jensen *et al*., 2018).

During hepatic steatosis, inflammatory cell infiltration and increased pro-inflammatory cytokine production, can leads to liver damage (Steatohepatitis *et al*., 2012).

Quercetin supplementation in the rats resulted in a significant decrease in inflammation and micro-steatosis score compared with those given fructose alone for 10 weeks. Quercetin has been shown to upregulate genes associated with mitochondrial biogenesis and oxidative metabolism in lipid-laden hepatocytes, which limits hepatic lipid accumulation (Kim *et al*., 2015). In addition, quercetin is responsible for promoting Heme-oxygenase-1 (HO-1) and activating nuclear factor erythroid 2–related factor 2 (Nrf-2) for regulating hepatic mitochondrial oxidative capacity in obese conditions (Kim *et al*., 2015); Sorrentino *et al*., 2018). The negative control group inflammation score was equal to the quercetin-only group, indicating that quercetin did not negatively affect the liver.

Our findings were supported by a study showing quercetin to decrease micro-vascular steatosis and hepatocellular ballooning in male mice given a methionine- and choline-deficient diet (MCD) for 4 weeks by halting lipid peroxidation and reducing AST and ALT (Marcolin *et al*., 2013). Quercetin was successful in reducing inflammation and micro-steatosis in rats given 20 % fructose solution.

Fenofibrate supplementation significantly decreased both inflammation and micro-steatosis in the rat livers. Treatment with fenofibrate has been proven to reduce hepatic triglycerides through expression of genes involved in fatty acid turnover (Tanoue *et al*., 2011). Fenofibrate also caused a reduction in lipid peroxidation by improving steatosis and inducing catalase (Tanoue *et al*., 2011; Gross *et al*., 2016).

High fructose diets can affect lipid accumulation which is also associated with decreased insulin sensitivity and increased adipokine release from adipose tissue which can affect metabolism (Buzzetti *et al*., 2016).

4.9. Insulin and Adiponectin

Rats given fructose had a significantly greater insulin concentration than the negative control group. Excessive fructose intake has been strongly linked with the development of insulin resistance (Hwang *et al*., 1988).

Fructose is absorbed in the jejunum through glucose transporter 5 (GLUT5) and fructose is transported to the liver, but since fructose is converted to lipids and is not regulated, excess fructose causes overproduction of triglycerides (Bezerra *et al*., 2000; Basciano *et al*., 2005). Increased fructose consumption causes activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and increases reactive oxygen species (ROS) that results in β-cell destruction and signaling, leading to the reduction of insulin sensitivity and an increased insulin resistance (Basciano *et al*., 2005; Dandona *et al*., 2004). Adipose tissue also produces pro-inflammatory molecules implicated in insulin resistance development (Rayssiguier *et al*., 2006).

Although we did not grossly observe obesity as a result of the high fructose diet, insulin resistance can develop in the absence of gross obesity (Dandona *et al*., 2004). In the current study, the fructose-fed rats had significantly increased insulin concentrations as compared to those drinking plain water. Quercetin administration did not alter insulin concentration when compared with the negative control group. Therefore quercetin did not affect or alter insulin concentrations in our study.

Adiponectin is a hormone produced in adipocytes, which is involved in regulation of lipids and carbohydrates and enhances normal insulin function (Havel, 2014). Adiponectin action involves stimulating nitric oxide (NO) production via activating the adenosine 5’-monophosphate-activated protein kinase to increase fatty acid oxidation and glucose uptake (Meier and Gressner, 2004; Metais *et al*., 2008). Low levels of adiponectin concentrations are linked with insulin resistance as adiponectin increases insulin action (Meeprom *et al*., 2011). Therefore it was necessary to assess adiponectin concentrations as it plays a pivotal part in energy metabolism.

Rats given fructose to drink had significantly higher adiponectin concentrations compared to those in the negative control group. This is a result of an elevated insulin

secretion, which stimulates adiponectin secretion to increase insulin action (Sharabi *et al*., 2007); Havel, 2014). This increase in adiponectin concentrations counteracts the effects of fructose by increasing insulin sensitivity (Hamed *et al*., 2011; Kim *et al*., 2012).

Supporting evidence suggests that adiponectin's insulin sensitizing effects, involving AMP kinase activation, is one of the metabolic pathways quercetin acts on to increase insulin action, therefore increasing the degree of adiponectin effects (Carvalho *et al*., 2010). Quercetin also increased adiponectin concentrations in our study as shown by others (Kim *et al*., 2012; Porras *et al*., 2017).

4.10. Liver Mass

The liver is one of the most vulnerable organs as it receives 50 % of its blood supply from the splanchnic district, which leaves the liver prone to modulation by substances absorbed from the gut (Starzl *et al*., 1973; Harper and Chandler, 2016).

Fructose is lipogenic and produces pro-inflammatory effects that result in the upregulation of TNF-α, oxidative stress and increased liver mass in a rat study (Buzzetti *et al*., 2016; Tanoue *et al*., 2011). In the current study, all rat groups receiving fructose had a significant increase in relative liver masses compared to the rat groups receiving the control diet (Elliott *et al*., 2002b; Nakagawa *et al*., 2006a). Fructose consumption results in altered mitochondrial function that causes accumulation of lipids in the liver (Abdelmalek *et al*., 2010; Bruce and Hanson, 2010).

The rats given fructose with quercetin had increased liver masses, indicating that quercetin failed to protect the liver from the hepatic fructose effects. Although the quercetin prevented micro-steatosis and inflammation, these were probably not responsible for the increased liver mass. The hepatomegaly could be due to an increase in hepatocyte size or number. However, neither the hepatocyte number nor the other stromal components which could increase liver mass were quantified. The lack of impact on liver size by the quercetin could be either due to dose effects or lack of innate protective capacity of the quercetin.

The relative and absolute liver masses for rats given fructose with fenofibrate were significantly greater than the livers on all other treatment groups. Previous studies have shown fenofibrate treatment to cause hepatomegaly (Ji *et al*., 2005; Kostapanos *et al*., 2013).

4.11. Gastro-Intestinal tract and accessory organ morphometry

The gastro-intestinal tract is the first point of contact with the supplemented quercetin treatment thus it was assessed. Dietary components which have adverse effects on the GIT can cause a reduction in the length of various segments and the mass due to cell loss (Montagner *et al*., 2016).

Rats given quercetin-only treatment had a significantly lower absolute small intestine mass than those in the fructose-only group. However when the small intestinal mass was considered relative to body mass, this observation was no longer seen and instead it was noted that the rats on fructose only or fructose with fenofibrate had heavier small intestines compared to the other groups.

A study showed fenofibrate to alter intestinal gene expression, causing reduction in lipid absorption and increasing the small intestines' villi height, which could explain why rats given fenofibrate had significantly higher relative small intestine masses when compared to all other groups (Uchida *et al*., 2011).

Caecum mass was also assessed as this organ is a major site for fermentation and provision of short chain fatty acids which contribute to the energy extracted from fibrous dietary components (Zacchetti *et al*., 2007). Increasing fibre in the diet increases the mass of the caecum (Egawa and Tsugaki, 2005). There was no significant difference in caeca masses between all rat groups thus the diet treatments had no significant effects on the caecum.

The stomach and pancreas masses were not significantly difference between treatment groups, suggesting a lack of toxicity to these two organs.

General health of the rats was further assessed through clinical biochemistry markers, haematocrit and haemoglobin concentration.

4.12. Clinical biochemistry, Haematocrit and Haemoglobin

Fasting blood glucose (FBG), serum triglycerides (TRI), Markers of liver (ALT) and kidney function (BUN and CRE) as well as the red blood cell parameters were not significantly different between treatment groups. Thus although fructose has been associated with diabetic nephropathy, abnormalities in red blood cells and hepatic abnormalities, the surrogate markers of organ function were not yet significantly elevated. Generally there is need for more than 50 % damage to these tissues before significant elevations of the clinical markers are noted (Muhammad, 2017; Sciences, 2015).

CHAPTER 5: CONCLUSION AND RECOMMENDATION

5.1. Conclusion

The current study aimed to determine whether quercetin can reduce or prevent the development of metabolic dysfunction in Sprague Dawley male rats given 20% fructose for 10-weeks. At the end of the treatment period, to an extent quercetin was able to reduce metabolic dysfunction induced by fructose, whereby the most affected organ was the liver. In addition it was notable that all rats given fructose had a significantly higher insulin concentration as opposed to the negative control and quercetin-only rat groups, suggesting the onset of insulin resistance. However quercetin was able to counteract the effects of fructose on insulin concentrations in rats given fructose.

The liver was the organ observed to be most affected by the fructose, since rats given fructose developed hepatic inflammation and micro-steatosis, but importantly those given quercetin and fenofibrate showed significantly lower histopathology scores, indicating the treatments were able to protect the liver from the fructose-induced NASH. Although the interventions did not affect the hepatic lipid content of the rats, our study confirms recent findings in other studies which show that inflammation and steatosis can develop without an increase in hepatic lipid content. NASH, which in itself can progress to further liver cirrhosis and carcinoma is increasing the burden of health care.

Thus the findings of the current study in which we have shown that quercetin was able to prevent the development of NASH contribute new knowledge to the potential prophylactic use of phytochemicals in the fight against increasing health care costs associated with management of non-communicable diseases.

5.2. Limitations and Recommendations

A mixed sex study design should also be considered in future studies to investigate the effects of quercetin on both male and female rats as there is evidence of sexually dimorphic metabolic responses to dietary interventions. Since studies have shown fructose to only affect body mass gain after 12 weeks, a prolonged treatment period of 12 weeks or more might prove insightful. Fructose and quercetin groups with two

different doses should also be implemented to see the effects of fructose with different quercetin doses. Molecular studies for the mechanisms involved in the protective effect of quercetin should be performed.

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APPENDICES

Appendix 1: Ethics Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2017/02/07/B

APPLICANT: Dr J Donaldson

SCHOOL: School of Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups

Number and Species

192 each male and female postnatal day 7, 36 female postnatal day 21 and 40 female nursing adult Sprague Dawley rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2017/02/28. This approval remains valid until 2019/03/15.
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 additionall conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 22/03/2017

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: 19/03/17

cc: Supervisor: N/A
Director: CAS

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Appendix 2: First Modification to the Ethics Clearance

AESC 2017

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2017/02/07/B**

Other M&E's : N/A

d. Project Title: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups.

e.	Number and species of animals **originally approved**:		192 male and 192 female Sprague Dawley rat pups-postnatal day 7; 36 female Sprague Dawley rat pups-postnatal day 21; 40 female nursing adult Sprague Dawley rats
f.	Number of additional animals **previously allocated on M&Es**:		N/A
g.	Total number of animals **allocated** to the experiment to date:		N/A
h.	Number of animals **used** to date:		N/A

i. Specific modification / extension requested:

- I would like to add my newly registered Master's student (Malehope Churchill Molopo) to the ethics application.
- I would also like to include Fenofibrate at a dose of 100 mg/kg/day (Liu et al., 2011; Legendre et al., 2002) as my positive control substance instead of Metformin. Fenofibrate will be administered via oral gavage (Experiments 1 and 2) or in gelatine cubes (Experiments 2 and 3).
- All other procedures will remain the same.

j. Motivation for modification / extension:

- Malehope Churchill Molopo is a Master's student (Student no: 1164995) registered in the School of Physiology with myself (Dr Janine Donaldson) as his supervisor. He will be working on some of the projects for which the ethics was approved, for his master's studies.

1 of 2

- Fenofibrate is commonly used to lower blood cholesterol and triglyceride levels (Legendre et al., 2002) and thus will be a more appropriate than metformin as a positive control in our proposed studies. Our study includes the use of added cholesterol.

References:

Liu, S; Liu, Q; Li, L; Huan, Y; Sun, S and Shen, Z (2011). Long-term fenofibrate treatment impaired glucose-stimulated insulin secretion and up-regulated pancreatic NF-kappa B and iNOS expression in monosodium glutamate-induced obese rats: Is that a latent disadvantage? *Journal of Translational Medicine* 9: 176-186.

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Signature: Dr Janine Donaldson Date: 28/03/2017

RECOMMENDATIONS: Approved. I. Inclusion of Malehope Churchill Molopo as a co-worker. II. Use of fenofibrate as detailed above.

CONDITION(S): Mr Molopo should attend a first time user's session facilitated by the CAS before working with the rats.

Signature: ..... Chairman, AESC Date: 4/4/2017

Appendix 3: Second Modification to the Ethics Clearance

AESC 2017

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr Janine Donaldson

b. Department: School of Physiology

c. Experiment to be modified / extended **AESC NO: 2017/02/07/B**

Other M&E's : 1

d. Project Title: Quercetin exposure pre- and post-weaning in the prevention and treatment of diet-induced metabolic syndrome in growing Sprague Dawley rat pups.

e.	Number and species of animals **originally approved**:		192 male and 192 female Sprague Dawley rat pups-postnatal day 7; 36 female Sprague Dawley rat pups-postnatal day 21; 40 female nursing adult Sprague Dawley rats
f.	Number of additional animals **previously allocated on M&Es**:		N/A
g.	Total number of animals **allocated** to the experiment to date:		36 female Sprague Dawley rat pups-postnatal day 21
h.	Number of animals **used** to date:		36 female Sprague Dawley rat pups-postnatal day 21

i. Specific modification / extension requested:

- We would like to use 60 male Sprague Dawley rat pups – postnatal day 21 (after weaning); thus there will be no gavaging of the rat pups prior to weaning and the study interventions will commence on postnatal day 21.
- We would also like to use both a low dose (75 mg/kg) and a high dose of Quercetin (100mg/kg; Hu et al., 2009) and only make use of one positive control Fenofibrate group (instead of two). Thus we will have 6 experimental groups with 10 rats in each group which will be fed their respective diets for a period of 10 weeks (please see Figure 1 below, detailing groups and experimental timeline).

group and thus no extra rats are required.

Mr. Malehope Churchill Molopo (student no: 1164995) will use the data for his masters project.

REFERENCES:
Hu, Q-H; Wang, C; Li, J-M; Zhang, D-M and Kong, L-D (2009). Allopurinol, rutin, and quercetin attenuate hyperuricemia and renal dysfunction in rats induced by fructose intake: renal organic ion transporter involvement. *Am J Physiol Renal Physiol* **297**: 1080-1091.

Signature:

Date: 03/07/2017

Dr Janine Donaldson

RECOMMENDATIONS: Approved. i. Change in age for intervention (21 days old) in 60 male rats. ii Use of a higher dose of quercetin. iii. Grouping of rats as detailed above. iv. Inclusion of Mr. Malehope Churchill Molopo (student no: 1164995) as a co-investigator on the study. He will use data from the study for purposes of his higher degree (Masters).

Signature: ..... Chairman, AESC

Date: 7/7/2017

AESC NO: 2017/02/07/B