THE POTENTIAL PROTECTIVE EFFECTS OF STEARIC ACID AGAINST DIET-INDUCED METABOLIC SYNDROME IN GROWING FRUCTOSE-FED SPRAGUE-DAWLEY RATS

THOBEKILE PRECIOUS DLADLA

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

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DECLARATION

I, **Thobekile Precious Dladla**, certify that this thesis reflects the author's original, unaided work that was completed under the supervision of **Professor Kennedy H. Erlwanger, Dr. Ashwell R. Ndhlala and Dr. Michael T. Madziva.** It has not been submitted in any way to another university for any degree/diploma or award. The study reported in this thesis was conducted at the University of Witwatersrand's Faculty of Health Sciences, School of Physiology, from May 2017 to March 2024 and it is being submitted for the degree of Doctor of Philosophy. The use of other people's work has been recognized and appropriately acknowledged throughout the text.

…Dladla ……

Thobekile Precious Dladla

Monday, 10 February 2025

DEDICATION

I would like to dedicate this work to my late grandparents: Masheshisa Albert "Chamasteshi" Dladla (1936 - 2000) and Nomdududu Fohlinkani "KamTiti" Dladla (1938 - 2019); maternal grandmother: Elizabeth "Gog' obomvu" Meyiwa (1943 - 2006). In addition, I want to dedicate this work to my precious children, Mr Manelisi Praiseworthy Shabalala and Mr Azabongwe Mswazi Hopewell Dladla, for their affection and innocent interruptions. You provided me a purpose to keep continuing with this study even when things were rough. I hope you achieve far more than I will one day.

RESEARCH OUTPUTS

CONFERENCE ATTENDED/PRESENTATIONS

- 47th Conference of the Physiology Society of Southern Africa. 18-21 August 2019. ICC East London. “Hepatic lipid accumulation in Sprague Dawley rats fed a diet enriched with stearic acid.” T.P. Dladla, A. Ndhlala, M.T. Madziva and K.H. Erlwanger.
- Thobekile P. Dladla, Ashwell R. Ndhlala, Michael T. Madziva and Kennedy H. Erlwanger. “Stearic acid prevents the development of non-alcoholic fatty liver in female Sprague Dawley rats fed a high-fructose diet”. The 10th Cross Faculty Postgraduate Symposium. Oral presentation. University of the Witwatersrand, Johannesburg, South Africa, 4 September 2019.
- 2nd International Symposium on Moringa (ISM) 2019. CSIR Convention Centre Pretoria. 10-13 November 2019. “The impact of stearic acid, a major saturated fatty acid of *Moringa oleifera*, on obesity in growing fructose-fed Sprague Dawley rats.” T.P. Dladla, A. Ndhlala, M. Madziva and K. Erlwanger.

ABSTRACT

Consumption of high-fructose and/or high-fat diet during the neonatal development phase increases *de novo* lipogenesis, which leads to the onset of metabolic dysfunction, including visceral obesity, non-alcoholic fatty liver disease and renal impairment. There is strong evidence that stearic acid has hypocholesterolaemia effects and thus its potential nutritional and health beneficial effects on the subsequent development of health outcomes associated with high-fructose and/or high-fat diet induced metabolic dysfunction necessitate further investigation. This interventional study aimed to investigate the impact of a diet enriched with stearic acid alone and/or combined with high-fructose on a) general metabolic health profile, b) non-alcoholic fatty liver disease, c) nephropathy and d) visceral and gastrointestinal tract morphometry, in growing female and male Sprague-Dawley rats.

Eighty, 21-day old rats were randomly allocated to 5 treatment groups of 16 (8 males and 8 females) pups each and fed respective treatments for 13 weeks. Group I, (negative control), receiving standard rat chow and plain water to drink; group II, in which metabolic dysfunction was induced, rats were fed standard rat chow, 20% fructose solution; group III, served to investigate the prophylactic potential of stearic acid, rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; group IV, rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink to investigate the effects of stearic acid alone on metabolic health; group V, (positive control) in which fenofibrate was used for the prevention of metabolic dysfunction, rats were fed standard rat chow, 20% fructose solution to drink and fenofibrate (in gelatine cubes as a vehicle) at 100 mg/kg body mass. Groups I-IV rats all received plain gelatine cubes as vehicle control. Body mass was measured throughout the study. After the 13-week feeding period, rats were terminated and tissue samples collected. Fasting blood glucose, plasma total cholesterol, triglyceride, insulin, HOMA-IR, adiponectin concentrations and kidney-injury molecule-1 (KIM-1) a surrogate biomarker of kidney function were determined. Visceral and epididymal fat masses were measured to assess adiposity. Hepatic lipid content and fatty acid profile were determined. Liver and kidney histomorphometry were also performed.

Feeding the rats with a stearic acid-enriched diet did not negatively affect their growth performance as all rats across dietary groups grew significantly during the feeding period with their ultimate body mass being significantly greater than their initial body mass ($p < 0.0001$).

The high-fructose diet induced hypercholesterolemia, hypertriglyceridemia, hypoadiponectinemia, increased visceral adiposity, increased hepatic lipid content, caused micro-vesicular steatosis and hepatic inflammation and increased KIM-1 ($p < 0.05$) in rats.

Stearic acid significantly increased plasma adiponectin ($p < 0.001$), decreased the high-fructose diet-induced hepatic lipid content (females) ($p < 0.05$), micro-vesicular steatosis (females) ($p < 0.05$), increase polyunsaturated fatty acids (females) ($p < 0.05$) and decrease trans fatty acids (males) ($p < 0.05$) as an intervention. However, stearic acid was ineffective in preventing ($p > 0.05$) high-fructose-induced increased cholesterol, triglycerides, visceral adiposity, hepatic inflammation and kidney damage marker KIM-1. Long bone parameters, fasting blood glucose, plasma insulin and insulin resistance, macro-vesicular steatosis, hypertrophy and kidney histomorphometry were not affected significantly ($p > 0.05$) by the dietary interventions. Furthermore, dietary treatments had no effect on gastrointestinal tract or visceral organ morphometry across treatment groups. Stearic acid alone significantly ($p < 0.05$) lowered cholesterol and triglycerides while increasing adiponectin levels compared to the negative control.

The current data showed sex differences in response to stearic acid and high fructose diets and that stearic acid provided some protective effects against high-fructose-induced metabolic dysfunction, posing as a promising potential natural saturated fat in ameliorating poor metabolic outcomes associated with high-fructose diets. Stearic acid supplementation in the diet should be investigated further as a form of preventive therapy against high-fructose-induced metabolic dysfunction.

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LIST OF ABBREVIATIONS

AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BM	Body Mass
DH_2O	Distilled water
DNA	Deoxyribonucleic acid
EFAs	Essential fatty acids
ELISA	Enzyme-linked immunosorbent assay
FBG	Fasting blood glucose
FFAs	Free fatty acids
FGC	Fenofibrate in gelatine cube
FS	Fructose solution
GIT	Gastrointestinal tract
HDL	High density lipoprotein
HOMA-IR	Homeostatic model of insulin resistance
IGF-1	Insulin growth-like factor 1
IL-1	Interleukin 1
IL-6	Interleukin 6
KIM-1	Kidney injury molecule-1
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
n3-PUFA	Omega-3 polyunsaturated fatty acid
PBS	Phosphate-buffered saline
PGC	Plain gelatine cube
PPARα	Peroxisome proliferator-activated receptor alpha

PW	Plain water
SA	Stearic acid
SD	Standard deviation
SRC	Standard rat chow
SREBP	Sterol regulatory element binding protein
SREBP-1c	Sterol-responsive element binding protein-1c
TC	Total cholesterol
TG	Triglycerides
TMFA	Total monounsaturated fatty acids
TNF	Tumour necrosis factor
TPFA	Total polyunsaturated fatty acids
TSFA	Total saturated fatty acids
w/v	weight/volume
WHO	World Health Organisation
WRAF	Wits Research Animal Facility

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CHAPTER 1

INTRODUCTION

1.1 Preview of the thesis

This thesis is written in a divided block format clustered into seven chapters.

The first chapter outlines the introduction of obesity and metabolic dysfunction as a major problem in both children and adults. Key statistics and an update of the recent research advances and possible interventions to prevent the problem are highlighted. The aims, objectives and hypotheses of the proposed study are then expressed.

The second chapter critically examines the literature in connection with the planned study, describing the concepts, global prevalence and influence on health of visceral obesity, metabolic dysfunction and non-alcoholic fatty liver disease. The metabolism of fructose and saturated fatty acids in the liver is discussed, as well as the pathophysiology of obesity, metabolic dysfunction and non-alcoholic fatty liver disease. There is discussion of potential therapies for preventing, treating and managing metabolic disorders. It also discusses the usage of stearic acid and its role in reducing the risk factors linked with metabolic dysfunction.

The study results are then presented in separate discrete chapters wherein each of these chapters are subdivided into introduction, methods, results and discussion sections much like the format used for research publications.

Chapter three covers the long-term effects of a stearic acid alone and/or combined high-fructose and stearic acid diet fed to growing female and male Sprague-Dawley rats on general metabolic health profile.

Chapter four covers the long-term effects of a stearic acid alone and/or the combined high-fructose and stearic acid on the development of non-alcoholic fatty liver disease in growing female and male Sprague-Dawley rats.

Chapter five constitutes the long-term effects of a stearic acid alone and/or the combined high-fructose and stearic acid on nephropathy in growing female and male Sprague-Dawley rats.

Chapter six contains general supplementary data which highlights the long-term effects of a stearic acid alone and/or the combined high-fructose and stearic acid diet on the gastrointestinal tract and visceral organ morphometry in growing female and male Sprague-Dawley rats.

Chapter seven constitutes the overall conclusions of the major findings from all the chapters and potential use of the knowledge generated from this study. Limitations and recommendations are also included. A list of appendices and references used to backup this study is also included thereafter.

1.2 Introduction

Obesity is a major cause of morbidity and mortality globally in children and adults (Lim et al., 2020). Sedentary lifestyles and the intake of fructose-rich diets has been connected to poor health risk factors including type II diabetes mellitus (de Farias Lelis et al., 2020), atherogenic dyslipidaemia (Benkhaled et al., 2022), hyperglycaemia (Lê and Tappy, 2006), hypertension (Kim et al., 2020, Lê and Tappy, 2006), impaired glucose tolerance (Basciano et al., 2005, Tran et al., 2009), insulin resistance (Ahmed et al., 2020, Softic et al., 2020), non-alcoholic fatty liver disease (Im et al., 2021, Jegatheesan and De Bandt, 2017) and visceral obesity (Wu et al., 2023a, Yu et al., 2015).

High-fructose diets are associated with the development of obesity and metabolic dysfunction (Dobrowolski et al., 2022, Tappy and Lê, 2010). Fructose is absorbed from the gastrointestinal tract and quickly processed by the liver (Muriel et al., 2021, Taskinen et al., 2019). Fructose metabolism bypasses the rate-limiting step in glycolysis, producing glyceraldehyde and dihydroxyacetone phosphate, which can be utilized to synthesize lipids (Dholariya and Orrick, 2022, Lee and Cha, 2018). Therefore, Excessive fructose consumption in neonatal and grownup life leads to increased *de novo* lipogenesis, resulting in the emergence of metabolic dysfunction including dyslipidaemia, hyperglycemia, insulin resistance, non-alcoholic fatty liver disease, and obesity (Basciano et al., 2005, Collino, 2011, Gulati and Misra, 2014, Yadav et al., 2007). Neonatal programming plays a vital function in health outcomes of individuals (Godfrey and Barker, 2001).

Developmental programming is a process whereby progeny are more likely to acquire systemic derangements later in life as a result of nutritional, physiological or pharmacological interventions during the early (gestational, neonatal and infancy) developmental stages (Dearden et al., 2018, Godfrey and Barker, 2001, Wang, 2013). Nutritional interventions in the early developmental stages, when there is enhanced ontogenetic plasticity, might cause temporary or permanent epigenetic modifications (Junien, 2006, Pandey and Pandey, 2017). These epigenetic modifications can either enhance susceptibility or confer resistance to the development of diseases later in life (Greco et al., 2019, Léonhardt et al., 2003). As a result, dietary modifications adopted early in stages of life can contribute to preventing the development of metabolic and developmental disorders later in life such as non-alcoholic fatty liver disease.

Non-alcoholic fatty liver disease, the prevalent cause of chronic liver disease, was considered as the liver expression of metabolic dysfunction (Cheung and Sanyal, 2010). Non-alcoholic fatty liver disease is a wide range of liver pathology that includes non-alcoholic hepatic steatosis, steatohepatitis, fibrosis and cirrhosis (Sattar et al., 2014, Younossi et al., 2019). Non-alcoholic fatty liver disease is caused by increased fat accumulation in the liver that is unrelated to the drinking of alcohol and is linked with insulin resistance (Machado and Cortez-Pinto, 2005, Mitra et al., 2020).

In the adipocytes, despite insulin receptor desensitization, insulin resistance does not decrease the activity of hormone sensitive lipase, an intracellular enzyme that hydrolyzes lipids like triglycerides to glycerol and free fatty acids, resulting in the hydrolysis of triglycerides (Lee et al., 2022, Schreiber et al., 2019). The resultant free fatty acids are released from the adipose tissue into circulation and transported to the liver, where there is an increased synthesis and absorption of free fatty acids from the blood (Kulminskaya and Oberer, 2020, Li et al., 2021). There is also a reduction in lipid release from the liver into the circulation in the form of lipoprotein (Lee et al., 2022, Li et al., 2021). This aforementioned process leads in steatosis and causes fat droplets to build-up within the hepatocytes (Mashek, 2021, Scorletti and Carr, 2022).

The initial stage is hepatic steatosis, the first identifiable stage of non-alcoholic fatty liver disease when fat content surpasses 5% of liver mass (Sattar et al., 2014). The fats in the hepatocytes become prone to breakdown, resulting in cell death that in turn causes hepatic inflammation (Bessone et al., 2019, Pierantonelli and Svegliati-Baroni, 2019, Shojaie et al., 2020). The second stage, often known as non-alcoholic steatohepatitis, is caused by both steatosis and hepatic inflammation (Pierantonelli and Svegliati-Baroni, 2019, Rinaldi et al., 2021). Finally, chronic steatohepatitis can induce stellate cells to generate fibrotic tissue, at which point the illness is categorized as hepatic fibrosis (Rinaldi et al., 2021, Tacke and Weiskirchen, 2021). As the process of hepatic fibrosis proceeds, the liver architecture alters until the illness is called cirrhosis (Sattar et al., 2014, Tacke and Weiskirchen, 2021).

Liver enzymes including aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase leak into the bloodstream when the hepatocytes are being damaged (Jalili et al., 2022, Lala et al., 2023) and can serve as surrogate markers of liver pathology. Recent evidence shows that non-alcoholic fatty liver disease can develop without going through the stages detailed earlier (Powell et al., 2021).

Non-alcoholic fatty liver disease's clinical burden extends beyond liver-related illness and mortality to include extra-hepatic organs. In this setting, non-alcoholic fatty liver disease is linked to a higher risk of cardiovascular disease morbidity and mortality, type II diabetes mellitus (Anstee et al., 2013) and chronic kidney disease (Byrne and Targher, 2020). Non-alcoholic fatty liver disease has been associated to increased fat deposition in various organs, including the heart, kidneys, pancreas, muscles and blood vessels, underlining the elevated cardiometabolic load in these individuals (Gaggini et al., 2013, Koliaki et al., 2019). In terms of pathogenesis, treating type II diabetes mellitus may prevent the development and/or advancement of non-alcoholic fatty liver disease and vice versa (Arrese et al., 2019, Kosmalski et al., 2022, Leite et al., 2014).

Non-alcoholic fatty liver disease is thought to predict the formation and progression of non-alcoholic fatty liver disease via metabolic dysfunction components; on the other hand, non-alcoholic fatty liver disease is thought to begin the future development of metabolic dysfunction components. As a result, current research implies that type II diabetes mellitus and metabolic dysfunction have a bi-directional relationship and are causally linked. Regarding the link between type II diabetes mellitus and non-alcoholic fatty liver disease, these two cardio-metabolic diseases share common pathophysiological pathways (Muzica et al., 2020, Tanase et al., 2020) including hyperglycemia, hyperinsulinemia, inflammation, insulin resistance, lipotoxicity and oxidative stress. Subsequently, if these remain untreated, chronic hyperglycaemia leads to the development of macro- and microvascular pathological complications, including, diabetic nephropathy.

Diabetic nephropathy is one of the chronic consequences of diabetes mellitus that occurs more frequently in patients who have had diabetes for a long time (Samsu, 2021). Diabetic nephropathy affects 20- 40% of diabetic people and is the main trigger of end-stage renal disease worldwide (Sagoo and Gnudi, 2020). The sustained chronic hyperglycemia activates the polyol pathway, resulting in increased proteinuria and decreased glomerular filtration rate, which promotes glomerular and proximal tubular damage (Garg and Gupta, 2022, Wu et al., 2023b). Also, chronic continued hyperglycemic state upregulates the hexosamine pathway, resulting in increased glucosamine-6-phosphate formation (Paneque et al., 2023), which increases transcription of the inflammatory cytokines tumor necrosis factor-α and transforming growth factor-1 (Čolak and Pap, 2021, Ganguly et al., 2023). This causes renal cell hypertrophy and an increase in mesangial matrix components (Thomas, 2021, Wu et al., 2023b). Diabetic nephropathy is a significant impediment to the patient's quality of life. Since the burden is

caused by food consumption, which then has a deleterious impact on renal function, lifestyle therapies, such as food calorie restriction and exercise, qualify the first-line treatment approach for the patient who has progressed to diabetic nephropathy (Carson et al., 2014, Kim, 2014). In addition, the management of diabetic nephropathy involves monitoring and controlling blood pressure using antihypertensive agents.

Components of metabolic dysfunction can be treated by employing recognized pharmacological medications that target glucose management and dyslipidemia correction (Dybiec et al., 2023, Vieira et al., 2019, Yamashita et al., 2020). The commercially supplied medications include fenofibrate, statins and metformin (Li et al., 2022a). Lifestyle adjustments, such as eating a nutritious diet and engaging in more physical activity, can also help manage metabolic related complications (Preiss and Sattar, 2008). The use of pharmacological therapeutic agents is limited due to undesirable side effects, low patient compliance and limited accessibility.

Stearic acid is a long chain saturated fatty acid that has been demonstrated to be beneficial in decreasing increased plasma cholesterol levels when added to the diet (Bonanome and Grundy, 1988, Mensink, 2005, Shen et al., 2014). *In vivo* experiments also demonstrated that stearic acid decreased visceral and body fat in athymic nude mice (Shen et al., 2014). Also, *in vitro* studies on the effects of stearic acid on lipid metabolism in a liver hepatocellular cells (HepG2) have found stearic acid to improve hepatic steatosis by *de novo* downregulation of fatty acid production (Kohjima et al., 2009). Furthermore, the reactive oxygen species elimination enzymes play an important role in the upregulation of fatty acid oxidation can be decreased by stearic acid (Kohjima et al., 2009, Sampath and Ntambi, 2005, Wu et al., 2020).

In general, high-fat diets are linked to an increased risk of developing visceral obesity, type II diabetes and cardiovascular diseases (Sampath *et al.*, 2007). While some saturated fatty acids are lipogenic and trigger a protease-activated receptor-2, which is the biomarker for obesity, contributing to inflammation and metabolic dysfunction (Lim *et al.*, 2013), recent evidence advocates the usage of saturated fats in diets (Feinman, 2010; Lawrence, 2013). Interestingly, studies have demonstrated that stearic acid has a good effect on metabolism, especially the decrease of visceral fat and the reduction of blood glucose (Bonanome and Grundy, 1988). Stearic acid has also been strongly recommended as a substitute for trans-fatty acids in foods because it has favourable effects on low density lipoprotein cholesterol (LDL), which is a main goal for addressing cardiovascular related health risks (Hunter *et al.*, 2009b). Therefore, it would be predicted that stearic acid could mitigate hepatotoxicity by reducing cholesterol and

triglyceride synthesis by being incorporated into phospholipids (Pai and Yeh, 1996). Fats are added to diets for a variety of reasons including: they are essential energy source; they are required for growth, development and cell functions and they are crucial for nerve and brain function (Zevenbergen et al., 2009). Fats have a crucial role in cell membrane structure, they transport fat-soluble vitamins and they are needed for the production of hormones, which means fats are required for the formation of steroid hormones that regulate various bodily processes (Lawrence, 2010). Saturated fats, in addition to their other functions, protect vital organs, insulate the body and provide essential fatty acids (Banik and Hossain, 2014).

Children are highly likely to encounter diets high in saturated fats. Given the negative impact of obesity due to combined consumption of foods rich in fat and sugar in growing children, it is important to explore combined effects of high-fructose and high-fat diet on metabolic health in growing children. The study thus explored the *in vivo* effects of high stearic acid intake from an early age to adulthood and also explored the combined effects of high stearic acid and high-fructose diets in growing rats from early age.

1.3 Aims and hypothesis

The overall aim of this project was to investigate the long-term metabolic effects of a stearic acid alone and/or combined high-fructose and stearic acid diet in growing female and male Sprague-Dawley rats.

Therefore, project had two aims:

1. To investigate the long-term metabolic effects of a diet enriched with stearic acid alone in growing Sprague-Dawley rats.

Hypotheses

H_0: Long-term administration of a diet enriched with stearic acid alone to rats has no adverse metabolic effects in growing Sprague-Dawley rats.

H_1: Long-term administration of a diet enriched with stearic acid alone to rats has adverse metabolic effects in growing Sprague-Dawley rats.

2. To investigate whether a combined high-fructose and stearic acid diet fed to growing Sprague-Dawley has no effect, attenuates or exacerbates the metabolic effects of the high-fructose diet in rats.

Hypotheses

H_0: Administration of both high stearic acid and fructose diet to growing Sprague-Dawley rats has no effect, does not attenuate or does not exacerbate the metabolic effects of the high-fructose diet.

H_1: Administration of both high stearic acid and fructose diet to growing Sprague-Dawley rats has an effect on the metabolic effects of the high-fructose diet.

1.4 Specific objectives

The study objectives were grouped into four main aspects that focused on:

A. General health profile

By measuring

1. Growth performance of the rats by determining body mass and assessment of long bone (tibia and femur) indices of growth.
2. Plasma triglyceride and cholesterol concentration.
3. Plasma concentrations of metabolism-regulating hormones, specifically, insulin and adiponectin.
4. Whole-body insulin sensitivity by computing homeostatic model assessment index-IR (HOMA-IR).
5. Visceral fat and epididymal fat masses.

B. Non-alcoholic fatty liver disease (NAFLD)

By measuring/determining

1. Liver absolute mass and hepatosomatic index (weight relative to body mass).
2. Liver lipid content and lipid profiles.
3. Liver histomorphometry and scores of steatosis, lobular inflammation, ballooning and total NAFLD activity score (NAS) as markers of NAFLD.

C. Nephropathy

By assessing

1. Kidney (absolute and relative to body mass) mass.
2. Kidney histomorphometry.
3. The surrogate marker of renal tubular damage (plasma kidney injury molecule-1).

D. General Supplementary information

The gastrointestinal tract is the first point of contact of orally administered substances therefore gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and small and lengths of the small intestine and large intestine were also measured.

E. Sex differences

Despite evidence that nutritional and therapeutic treatments produce sexually dimorphic responses, the majority of interventional research focus on a single sex. Therefore, this study also investigated the effect of the interventions in both male and female rats. The next chapter gives a detailed literature review germane to this research project.

CHAPTER 2

LITERATURE REVIEW AND JUSTIFICATION

2.1 Introduction

Fructose is a hexose monosaccharide sugar. Fruits and vegetables, high-fructose corn syrup, honey and sucrose (glucose-fructose disaccharide) are all good sources of fructose. The intake of fructose in western diets and sedentary lifestyle living is increasing globally (Bidwell, 2017). Obesity, type II diabetes mellitus, non-alcoholic fatty liver disease and metabolic syndrome have all been associated with fructose-rich diets in children and adults. Furthermore, the metabolic derangements have all reached epidemic proportions around the world (Clemente-Suárez et al., 2023, Vancells Lujan et al., 2021).

2.2 Metabolic syndrome

Metabolic syndrome is a pathological metabolic condition characterised by the expression of a combination of risk factors such as the increase in the development of atherosclerosis, cardiovascular disorders, decreased high density lipoprotein, hyperglycaemia, hyperlipidaemia, hypertension, hypertriglyceridemia, increased low density lipoprotein, insulin resistance, type II diabetes mellitus and visceral obesity (Bovolini et al., 2021, Grundy, 2020, Mohamed et al., 2023). This condition has also had a variety of names including deadly quartet, dysmetabolic syndrome, insulin resistance syndrome, syndrome X and syndrome X plus (Lemieux and Després, 2020, Mukhopadhyay and Goswami, 2024). Metabolic syndrome is now recognized as a new non-communicable disease in the 21st century that has become a major health threat (Madan et al., 2023).

Several criteria have been used to diagnose metabolic syndrome over the years. The 1999 World Health Organization report (Alberti and Zimmet, 1998), European Group for the Study of Insulin Resistance (EGIR) (Balkau, 1999) and the definition of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults also known as the Adult Treatment Panel III (ATPIII) (Pasternak, 2003), documented the most frequently accepted and recently utilized criteria in attempts to characterize the metabolic syndrome. A metabolic syndrome diagnosis is given when someone meets three or more of the following criteria, according to the National Cholesterol Education Program Adult Treatment Panel III: abdominal obesity: waist circumference more than 102 cm in men and greater than 88 cm in women; fasting glucose level of ≥ 100 mg/dL (≥ 5.6 mmol/L); high blood pressure of ≥ 130/85 mmHg; low HDL-C: < 40 mg/dL (1.04 mmol/L) in men and < 50 mg/dL (1.29 mmol/L) in women and hypertriglyceridemia: ≥ 150 mg/dL (1.67 mmol/L) (Gigante et al., 2021, Pasternak, 2003, Ramírez-Manent et al., 2023).

The co-occurrence of risk factors and shared responsiveness, metabolic syndrome requires several parameters to be measured in the same way in order to diagnose it and involves several intricate pathways and mechanisms (Bovolini et al., 2021, Fahed et al., 2022).

2.2.1 Prevalence and aetiology of metabolic syndrome

A quarter of the world's population is affected by the metabolic syndrome epidemic and the prevalence continues to increase at an alarming rate (Alshehri, 2023). Previous research has shown that the incidence of metabolic syndrome is generally higher in some developing nations (Bhavadharini et al., 2020), particularly in urban areas and that it usually coincides with the frequency of both obesity and type II diabetes (Li et al., 2022b, Misra and Khurana, 2009). Globally there was about 25% and 19.2% of young adults and children, respectively, who have metabolic syndrome (Bitew et al., 2020) and prevalence was much higher among women than men (Faijer-Westerink et al., 2020, Oliveira et al., 2020). The metabolic syndrome's prevalence in Africa ranges from 0% to 50%, according to the standards employed (ATPIII/AHA/NHLBI) (Okafor, 2012). In Africa, documented cases of metabolic syndrome include 23% of south-western Nigeria (Adejumo et al., 2019), 21.8% of the Eastern Cape in South Africa (Owolabi et al., 2017), 35.73% of Morocco (El Brini et al., 2014) and 25.6% of Kenya (Marbou and Kuete, 2019).

Aetiology: Metabolic syndrome is a combination of risk factors including hyperglycaemia, insulin resistance, obesity, visceral obesity, atherogenic dyslipidaemia, and hypertension, which increases the risk of developing type II diabetes mellitus and cardiovascular complications (Bovolini et al., 2021, Grundy, 2020, Mohamed et al., 2023). Several interrelated factors contribute to the problem of metabolic syndrome. The multiplicity of factors include, genetic predisposition and insufficient physical activity with subsequent weight gain due to poor nutrition (Mohamed et al., 2023, Coronati et al., 2022). Additionally, systemic inflammation (Kunz et al., 2021) and dyslipidaemia with accumulation of dysfunctional adipose tissue and ectopic lipids in abdominal obesity linked to insulin resistance (Benkhaled et al., 2022) are the causes in the development and/or lead to rise in the incidence of metabolic syndrome. The over consumption of western diets rich in processed sugars and fats, significantly increase accumulation of body fat which directly contributes to the global epidemic of metabolic syndrome.

A central feature of metabolic syndrome is visceral obesity (Grundy, 2020, Gunawan et al., 2021, Lemieux and Després, 2020).

2.2.2 Obesity and inflammation

Between 1975 and 2016, the global prevalence of obesity nearly tripled (Lim et al., 2020). Obesity is increasingly recognized as among the most dangerous for public health issues confronting the globe today. Obesity is a disorder that impacts individuals of all ages and socioeconomic backgrounds and it is defined by the abnormal buildup of fat that can be harmful to one's health (Mohajan and Mohajan, 2023, Rakhra et al., 2020). Over the last few decades, the prevalence of childhood obesity has expanded rapidly, globally (Faienza et al., 2020, Lim et al., 2020). Around 175 million (38% of the world's population) children and adolescents aged 5 to 19 years were considered obese globally in 2020 (Orsini et al., 2023). This proportion is expected to climb to more than 383 million (51%) by 2035 (Keaver et al., 2020). Around 813 million (32%) adults aged 20 years and older globally were deemed obese in 2020 (Keaver et al., 2020). This figure is predicted to rise to more than 1.5 billion by 2035 (Koliaki et al., 2023).

South Africa is also affected by a high obesity prevalence. Obese adults account for 31% of men, 68% of women and 13% of children under the age of five in South Africa (Boachie et al., 2022, Ratshikombo et al., 2021). It is projected that by 2030, South Africa will have 36.7% obesity among adults, with 50% of women (11 million) and 23% of men (4.7 million) (Lobstein et al., 2022). Between 2010 and 2030, the annual average increase in adult and child obesity is 1.9% (medium) and 8.2% (extremely high), respectively (Lobstein et al., 2022). In South Africa, it is expected that obesity among children aged 5 to 9 years will be 28% in 2030, with children aged 10 to 19 years being 27% (Lobstein et al., 2022). This rise in prevalence is linked to a combination of hereditary factors, sedentary lifestyle and excessive consumption of high-fructose diets (Clemente-Suárez et al., 2023, Johnson et al., 2023).

Obesity is linked to a number of challenges, including cardiovascular (hypertension, dyslipidaemia and left ventricular hypertrophy) (Perone et al., 2023), endocrine (metabolic syndrome, diabetes, hyperlipidaemia and hypercholesterolemia) (Korac et al., 2021, Milewska et al., 2022), gastrointestinal (non-alcoholic acute steatohepatitis) (Cabré et al., 2020), neuropsychological (pseudo-tumour cerebri and depression) (Lentoor, 2022), orthopaedic (slipped femoral epiphysis) (Chatziravdeli et al., 2023, Winston et al., 2022), renal and respiratory (asthma, bronchial hyper-reactivity and obstructive sleep apnoea) (Arismendi et al., 2020, Hall et al., 2021). Indeed, obesity has far-reaching repercussions; therefore, additional research is needed to develop efficient and conveniently accessible remedies.

Visceral fat accumulation, in particular, is an important aspect of obesity because it is associated with systemic inflammation (Kunz et al., 2021), resulting in the production of local and systematic biologically active substances adipokines (leptin and retinol-binding protein) and pro-inflammatory cytokines (cc-chemokine ligand- 2) (Kunz et al., 2021, Rajesh and Sarkar, 2021, Sengupta and Avtanski, 2023). These, in turn, stimulate systemic inflammation and the advancement of metabolic dysfunction associated with obesity (Zorena et al., 2020). In addition, pro-inflammatory cytokines inhibit the development of GLUT-4 receptors and lead to insulin resistance (Al-Mansoori et al., 2022, Zorena et al., 2020). Spillover from larger adipose tissue causes ectopic fat deposition in the heart, liver, pancreas, and muscle, resulting in tissue lipotoxicity (Chait and Den Hartigh, 2020, Rajesh and Sarkar, 2021, Verhaegen and Van Gaal, 2022). Insulin resistance is also a characteristic of obesity.

A characteristic of metabolic syndrome is an increase in circulating pro-inflammatory markers such as C-reactive protein, interleukin-1, interleukin-6 and tumour necrosis factor-alpha (Kunz et al., 2021, Rajesh and Sarkar, 2021, Sengupta and Avtanski, 2023). During acute inflammation, monocytes secrete both interleukin-6 and tumour necrosis factor-alpha, whereas interleukin-6 action on the gene responsible for C-reactive protein transcription primarily induces hepatic synthesis of the protein (Kunz et al., 2021, Rajesh and Sarkar, 2021, Sengupta and Avtanski, 2023). Increased visceral adiposity stimulates the production of these pro-inflammatory cytokines by the adipose tissue (Chait and Den Hartigh, 2020, Rajesh and Sarkar, 2021. Pro-inflammatory cytokines suppress the formation of glucose transporter type 4 receptors and inhibit insulin receptor substrate-1 signalling pathway , consequently, resulting in hyperglycaemia and insulin resistance in obesity (Al-Mansoori et al., 2022, Zorena et al., 2020). These metabolic signalling outcomes further exacerbate the pathology caused by the earlier mentioned cytokines derived from the visceral fat.

2.2.3 Insulin resistance

Insulin is a hormone secreted by pancreatic islet cells. Its role is to maintain adequate glucose absorption and lipid metabolism in peripheral tissues (Rahman et al., 2021). This is performed via the activation of complex signalling pathways including phosphoinositide 3-kinase/protein kinase-B and Ras-mitogen activated protein kinase (Sinha and Haque, 2022a). Insulin also interacts with transcription factors including forkhead box O1 (FOXO1) and peroxisome proliferator-activated receptor gamma (PPAR-γ) (Benchoula et al., 2021, Zhang et al., 2020).

Insulin resistance is a pathological illness defined by cells or the body's failure to respond and use insulin efficiently to reduce glucose and lipid levels (Gaggini et al., 2013, Lee et al., 2022). Consequently, insulin resistance results in decreased uptake of glucose, decreased glycogenesis and lipogenesis and therefore blood glucose levels stay persistently raised, precipitating the establishment of type II diabetes mellitus or hyperglycaemia (Ahmed et al., 2021, Tanase et al., 2020). Hyperglycaemia and dyslipidaemia generate oxidative stress and initiate the inflammatory response, inducing cell and/or organ damage (Raut and Khullar, 2023). Chronically high insulin levels promote fat accumulation in muscles, liver and blood (as triglycerides), resulting in long-term medical conditions including cardiovascular disease, dyslipidaemia, non-alcoholic fatty liver disease and visceral obesity (Ahmed et al., 2021, Gaggini et al., 2013, Pal and Méndez-Sánchez, 2023).

2.2.4 Dyslipidaemia

Dyslipidaemia is defined by the build-up of free fatty acids in the liver (Heeren and Scheja, 2021), as well as insulin-induced lipogenesis (Dybiec et al., 2023, Julius, 2003), increased triglyceride production and release (Boren et al., 2022), increased hepatic uptake and renal clearance of high-density lipoprotein cholesterol (Kon et al., 2021, Tao et al., 2020) and thus the dysregulated lipid profile of low high-density lipoprotein cholesterol and high triglyceride levels seen in metabolic syndrome (Hajian-Tilaki et al., 2020, Kosmas et al., 2023). Atherogenic dyslipidaemia is associated to an increased risk of cardiovascular illnesses (Lorenzatti and Toth, 2020) and is defined by increased non-esterified free fatty acids in the circulation (Heeren and Scheja, 2021, Onuchukwu et al., 2022).

Non-esterified circulating free fatty acids bind to receptors on the heart, liver, pancreas and muscle, amplifying inflammatory signalling through the c-Jun N-terminal kinase pathways and nuclear factor kappa-light-chain-enhancer of activated-B cells and resulting in an inflammatory vicious cycle (da Silva Rosa et al., 2020, Zbinden Foncea, 2013). Furthermore, the build-up of non-esterified free fatty acids in the circulation promotes mitochondrial dysfunction by encouraging increased oxidant production, that results in the synthesis of reactive oxygen species, which grades in oxidative stress and cell damage (Bourebaba and Marycz, 2021, Hafizi Abu Bakar et al., 2015). Increased amounts of reactive oxygen species also disrupt insulin signalling and GLUT4 translocation (Ayer et al., 2022, Bourebaba and Marycz, 2021).

Glucotoxicity and lipotoxicity both cause pancreatic β-cell malfunction, this results in insulin resistance and hyperinsulinemia (Benito-Vicente et al., 2021, da Silva Rosa et al., 2020).

Hyperinsulinemia and insulin resistance both contribute to hypertension development (Sinha and Haque, 2022b). Type II diabetes mellitus is caused by atherogenic dyslipidemia, hyperglycemia, insulin resistance, and an increase in the production of inflammatory cytokines (Kunz et al., 2021, Poznyak et al., 2020, Rajesh and Sarkar, 2021). The expression of all of these risk factors together culminates in metabolic syndrome. This syndrome is well studied using different experimental models.

2.3 Experimental models of metabolic syndrome

Researchers have established a number of animal models that investigate metabolic disorders because of the impact that metabolic ailments place on the health-care system. Some of the approaches often used in research to induce metabolic disorders in animal models include dietary modification, genetic alterations and chemical alterations (Preguica et al., 2020, Suman et al., 2016).

2.3.1 Genetically-induced

Given that metabolic diseases affecting individuals are typically multifactorial, genetic animal models are well suited to investigating the pathophysiology of diet-induced metabolic disorders in humans with genetic aetiology. The most extensively utilized animal genetic-induced models of metabolic diseases include leptin receptor-deficient (db/db) mice (Deng et al., 2020), leptin deficient (ob/ob) mice (Zhao et al., 2020) and Zucker diabetic fatty rats (Capcarova and Kalafova, 2020). These metabolic syndrome models produced by a single gene are valuable for understanding metabolic diseases.

2.3.2 Pharmacologically-induced

Drugs or chemical compounds such as alloxan (glucose analogue), antipsychotics, glucocorticoids and streptozotocin can be used to establish metabolic syndrome models (Gunawan et al., 2021). Glucocorticoids are steroid hormones that are released by the adrenal gland in reaction to stress and are commonly used to treat inflammatory and autoimmune disorders (Chourpiliadis and Aeddula, 2020, Reichardt et al., 2021). Glucocorticoids serve to regulate glucose metabolism in the adipose tissue, liver, pancreas and skeletal muscle (Reichardt et al., 2021). Glucocorticoids control the expression of major gluconeogenic enzymes in the liver, including phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and tyrosine aminotransferase (Akalestou et al., 2020, Reichardt et al., 2021). Oral feeding, intraperitoneal injections, or surgical implantation of glucocorticoid pellets are all options for

administering glucocorticoids to experimental animals (Gunawan et al., 2021, Xavier et al., 2021). The aforementioned processes will result in almost identical metabolic syndrome components including abdominal fat build-up, dyslipidaemia, glucose intolerance, hyperglycaemia, hypertension, insulin resistance and weight increase.

Additionally, several animal research studies have found that antipsychotics, particularly second-generation antipsychotics, may contribute to glucose intolerance, increased visceral fat, insulin resistance, lipid abnormalities and weight gain (Sneller et al., 2021). As a result, they contribute to the development of metabolic syndrome and insulin resistance (Akinola et al., 2023). The GLUT2 transporters allow alloxan and streptozotocin to enter pancreatic β-cells (Berger and Zdzieblo, 2020). Once in the bloodstream, these glucose analogues cause pancreatic β-cell damage, decreasing or impeding the secretion and production of insulin (Berger and Zdzieblo, 2020). Having stated a few abovementioned examples, research on insulin resistance, type II diabetes mellitus and the effects of the metabolic syndrome are best conducted using chemically generated animal models.

2.3.3 Diet-induced

Diet is crucial in the development of metabolic syndrome clinical symptoms. Metabolic syndrome, non-alcoholic fatty liver disease, obesity and type II diabetes mellitus have all been linked to the intake of unhealthy obesogenic diets rich in carbohydrates and lipids, which affect metabolism and body homeostasis via glucose metabolic pathways, hormones and lipid metabolism (Arslanbulut and Çiftçi, 2023, Bovolini et al., 2021, Clemente-Suárez et al., 2022, Kolderup and Svihus, 2015). As a result, there is an increasing demand for research into the pathology and pathophysiology of diet-induced metabolic diseases.

Several studies on metabolic disorders have been undertaken using animal models including Sprague-Dawley rats (Donaldson et al., 2019, Preguica et al., 2020). The most extensively utilized animal diet-induced models of metabolic diseases include the high-fructose diet (Yustisia et al., 2022), the high-sucrose diet, the high-fat diet or the respective combinations of a high-fructose high-fat diet (Abdelmoneim et al., 2021) or a high-sucrose high-fat diet or high sodium chloride high fructose diet (Gunawan et al., 2021, Suman et al., 2016).

Commonly associated with metabolic syndrome and obesity is non-alcoholic fatty liver disease.

2.4 Non-alcoholic fatty liver disease

2.4.1 From a healthy liver to the onset of non-alcoholic fatty liver disease and its progression and diagnosis

The liver is an essential metabolic organ that is in charge of a variety of processes such as bile generation, glucose metabolism, drug and toxin degradation, iron storage and metabolism, plasma protein synthesis and vitamin storage and modification (Hodson et al., 2020, Muriel et al., 2021). Insulin and other metabolic hormones strictly regulate the metabolic activity of the liver (Pal and Méndez-Sánchez, 2023, Rahman et al., 2021). The liver stores fat from circulating fatty acids, dietary fat and *de novo* lipogenesis (Geidl-Flueck and Gerber, 2023, Softic et al., 2016). However, when this total fat intake surpasses the total quantity of fat burned or exported as lipoproteins, non-alcoholic fatty liver disease develops (Fernando et al., 2019, Gaggini et al., 2013, Rinaldi et al., 2021).

Non-alcoholic fatty liver disease has been related to an increase in obesity prevalence owing to excessive fructose consumption (Arslanbulut and Çiftçi, 2023, Jegatheesan and De Bandt, 2017). Non-alcoholic fatty liver disease has a wide histopathological variety in which the liver has accumulated lipids that exceed its mass by 5% (hepatic steatosis) in the absence of any secondary causes (alcohol consumption, infections, medications, etc.) and progresses to non-alcoholic steatohepatitis (DiStefano, 2020, Pierantonelli and Svegliati-Baroni, 2019). Hepatic inflammation and hepatocyte ballooning degeneration with or without fibrosis define non-alcoholic steatohepatitis (Pierantonelli and Svegliati-Baroni, 2019, Tacke and Weiskirchen, 2021). When non-alcoholic fatty liver disease is not treated, it worsens, increasing the risk of cardiac arrest and liver failure, as illustrated in figure 2.1 (Anstee et al., 2013, Chalasani et al., 2018). Consequently, non-alcoholic fatty liver disease became known as a prevalent root of chronic liver disease (Arslanbulut and Çiftçi, 2023).

Histological alterations are frequently reversible in the early stages of the disease. However, as cirrhosis progresses, the possibility of reversibility decreases, as shown in figure 2.1 (Anstee et al., 2013). Severe hepatocyte damage results in the production of liver enzymes such as aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase, which leak into the blood and can be utilized as surrogate markers for liver disease (Jalili et al., 2022, Lala et al., 2023, Nishitani et al., 2007). Some important variables influencing the rate of recurrence of non-alcoholic fatty liver disease include abdominal obesity caused by diet composition

(fructose rich diets) and other medical conditions such as hypopituitarism, hypothyroidism, sleep deprivation and type II diabetes mellitus (Arrese et al., 2019, Hydes et al., 2021).

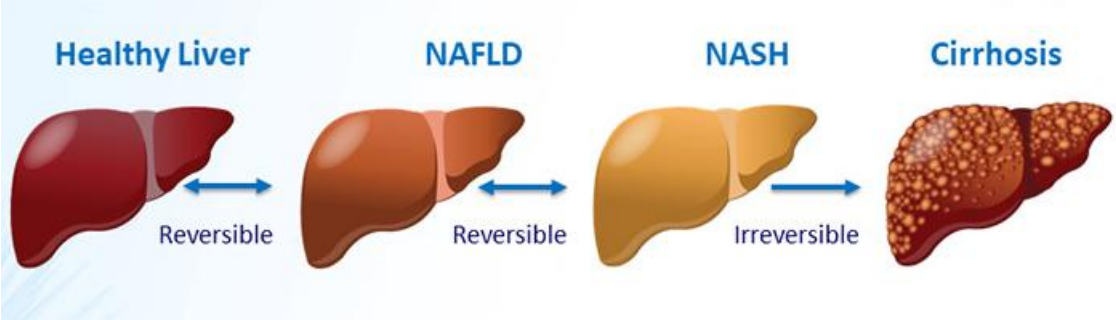


Image obtained from (Chalasani et al., 2018).

Figure 2.1. Schematic representation of progression of non-alcoholic fatty liver disease. A healthy liver has normal fat droplets, nucleus and hepatocyte size. Big fat droplets, swollen hepatocytes and a misplaced nucleus characterize non-alcoholic fatty liver. Non-alcoholic steatohepatitis (NASH): collagen fibers and irritated dying hepatocytes are present. Cirrhosis: scarring caused by excess fibrous tissue, irritated liver and dead cells.

Non-alcoholic fatty liver disease can be diagnosed according to clinical history, radiographic examinations and laboratory tests, which are supplemented by histomorphometric analysis and it can be scored using the non-alcoholic fatty liver disease activity score (NAS) or Brunt's steatosis criteria (Brunt et al., 1999, Kleiner et al., 2005). Histopathological assessment and evaluation of morphological alterations are significant in the diagnosis and treatment of liver diseases (Kleiner et al., 2005). A liver biopsy is considered to be the pinnacle in the evaluation of medical liver disorders (Barazesh et al., 2024). When the aetiology of liver illness has already been identified, examining morphological changes may offer additional information valuable for therapeutic care (Barazesh et al., 2024, Cotter and Rinella, 2020).

Non-alcoholic fatty liver disease histological alterations include fatty change described as macro-vesicular steatosis and micro-vesicular steatosis (Pierantonelli and Svegliati-Baroni, 2019, Scorletti and Carr, 2022, Tacke and Weiskirchen, 2021). Other histological abnormalities observed in non-alcoholic fatty liver disease include hepatocellular hypertrophy, inflammatory infiltration, hepatic degenerative changes (such as ballooning, bile stasis), fibrosis and liver cell death (Mashek, 2021, Pierantonelli and Svegliati-Baroni, 2019).

2.4.2 Epidemiology and prevalence

Non-alcoholic fatty liver disease currently impacts roughly 37.8% of the global population (Manikat et al., 2023). The largest regional prevalence of non-alcoholic fatty liver disease was shown to be 64.6% in America (Fattahi et al., 2016) and 31.8% in the Middle East (Juanola et al., 2021, Ofosu et al., 2018), with estimates ranging from 23.4% in Asia to 23.7% in Europe (Fan et al., 2017), 30.45% in South America (Younossi et al., 2016) and 13% in Africa (Mitra et al., 2020). Unfortunately, data on the prevalence of non-alcoholic fatty liver disease in Africa are few, and the majority of studies on non-alcoholic fatty liver disease epidemiology have come from the United States and North America, where the prevalence rate is 21-24.7% (Mitra et al., 2020). The average incidence of non-alcoholic fatty liver disease in the overall population in Western nations is higher among obese people (80-90%), accounting for 30-50% of diabetics and 90% of dyslipidaemia patients (Manikat et al., 2023). Currently, the non-alcoholic fatty liver disease global incidence is estimated to be 47 cases per 1,000 people, with men having a greater prevalence than women. In addition, among adults, estimated global prevalence of non-alcoholic fatty liver disease is 32%, with males having a higher rate (40%) than females (26%) (Teng et al., 2023). It is also predicted that by 2030, non-alcoholic fatty liver disease would be the primary cause of liver transplantation. Sedentary lifestyles and a diet consisting of obesogenic diets contribute to an increasing in the rate of non-alcoholic fatty liver disease in both children and adults.

2.4.3 Aetiology

A number of variables, including changes in gut microbiota composition, environment, epigenetic changes, nutrition consisting of obesogenic diets and sedentary lifestyles are causative in the development and/or lead to a rise in the incidence of non-alcoholic fatty liver disease. The key danger elements in the aetiology and progression of non-alcoholic fatty liver disease are triglyceride retention and insulin resistance (Gaggini et al., 2013, Pal and Méndez-Sánchez, 2023). Insulin resistance may develop in a variety of organs, including adipocytes, hepatocytes, kidney cells, muscle cells and pancreatic β-cells (Ahmed et al., 2021), resulting in increased visceral adipose tissue lipolysis and dyslipidaemia, which contributes to increased hepatic lipid infiltration and deposition (da Silva Rosa et al., 2020, Tanase et al., 2020).

2.5 The effect of high-fructose and high-fat diets in the development of metabolic diseases

2.5.1 High-fructose diet metabolism

Fructose metabolism by the liver increases total fat circulation, which directly favours the formation of triglyceride-rich lipoproteins, resulting in a reduced lipid profile (Basciano et al., 2005, Bidwell, 2017, Dholariya and Orrick, 2022). Higher intake of fructose-rich diets has increased the prevalence of non-alcoholic fatty liver disease, as has the risk of developing metabolic syndrome and obesity (Coronati et al., 2022, Geidl-Flueck and Gerber, 2023).

Fructose is absorbed from the intestinal lumen to the intestinal epithelium cell first through the glucose transporters 5 (Coronati et al., 2022, Dholariya and Orrick, 2022). Once within the epithelium cell, it is transported to the circulation by glucose transporters 2 and delivered to the liver (Hannou et al., 2018). Fructose metabolism involves fructokinase (enzyme specific for fructose, it is a ketohexokinase), fructose-bisphosphate aldolase B (a member of the glycolysis sequence of intermediates) and adenosine triphosphate (ATP) dependent dihydroxyacetone kinase enzymes (Dholariya and Orrick, 2022, Jegatheesan and De Bandt, 2017). In liver, hepatocytes take in fructose through GLUT2 which is insulin-independent. That is, GLUT2 only absorbs fructose when plasma fructose concentrations are greatly increased. GLUT2 are only present in the liver, pancreas and intestines (Merino et al., 2019, Softic et al., 2020).

Fructokinase is an enzyme controlled by the carbohydrate response element binding protein (ChREBP) that catalyses the conversion of fructose to fructose1-phosphate (Dholariya and Orrick, 2022). Aldolase B cleaves the fructose-1-phosphate generated and converts it into dihydroxyacetone phosphate and glyceraldehyde, which are both 3-carbon molecules. (Jegatheesan and De Bandt, 2017). The generated glyceraldehyde is in turn converted to glyceraldehyde-3-phosphate catalysed by ATP dependent dihydroxyacetone kinase (also known as triose kinase). Dihydroxyacetone phosphate is also processed to glyceraldehyde-3-phosphate by triose phosphate isomerase. The resultant glyceraldehyde-3-phosphate goes through the rest of the glycolytic pathway (Dholariya and Orrick, 2022). As a result, there are no rate-limiting stages in fructose metabolism and an increased substrate leads to metabolic processes such as glycolysis, glycogenesis, gluconeogenesis, lipogenesis and fatty acid esterification from triose phosphate (Dholariya and Orrick, 2022, Hannou et al., 2018,

Jegatheesan and De Bandt, 2017). This elicits obesity, metabolic syndrome and eventually, non-alcoholic fatty liver disease.

In addition to the above listed health risks, excessive dietary fructose consumption has been demonstrated to cause impaired kidney function (Byrne and Targher, 2020, Hall et al., 2021). Its use stimulates the content of uric acid, which activates pro-inflammatory processes by increasing the creation of monocyte chemotactic protein-1 in proximal renal tubular cells (Johnson et al., 2013, Russo et al., 2021). This finally causes progressive harm to the kidneys.

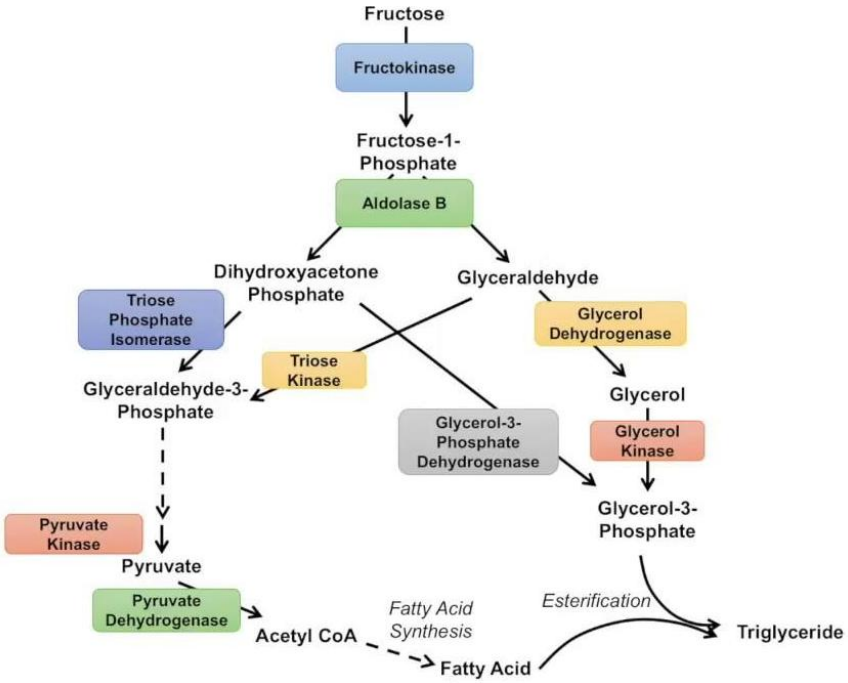


Figure 2.2. The liver's fructose metabolism illustrated schematically.
Fructokinase catalyzes the synthesis of fructose-1-phosphate from fructose. Aldolase B in the liver converts fructose-1-phosphate to glyceraldehyde and dihydroxyacetone phosphate. The ATP-dependent dihydroxyacetone kinase catalyzes the phosphorylation of glyceraldehyde to produce glyceraldehyde-3-phosphate and other glycolytic pathway intermediates (Merino et al., 2019).

Unlike fructose, glucose is carried into intestinal endothelium cells by the only independent glucose transporter 1 and it employs GLUT2 like fructose when transported out to the circulation (Merino et al., 2019). Glucose metabolism in the liver activates rate-limiting phosphofructokinase activity, which limits triglyceride production, enhances glycogen creation from glucose and stimulates insulin secretion, in contrast to fructose metabolism (Merino et al., 2019). During the triose phosphate stage, the fructose and glucose metabolism routes in the liver converge and their metabolism remains comparable (Poznyak et al., 2020).

2.5.2 Saturated fat and/or high-fat diet metabolism

Lipids are a class of organic substances composed of fats, diglycerides, fat-soluble vitamins, monoglycerides, oils, phospholipids, steroid hormones and waxes. Bile acids and bile salts emulsify lipids to tiny fat droplets in the small intestines (di Gregorio et al., 2021, Salih, 2021). Pancreatic lipases then breakdown the fat droplets to generate free fatty acids and monoglycerides (di Gregorio et al., 2021, Rajan et al., 2020, Yadav and Paul, 2023). Bile salts subsequently bundle the free fatty acids into micelles, which are then passively absorbed by the enterocytes of the small intestine (di Gregorio et al., 2021). Once within the enterocyte, free fatty acids and monoglycerides are resynthesized into triglycerides by the endoplasmic reticulum (Yadav and Paul, 2023). The triglycerides are subsequently packed into chylomicrons, which diffuse out of the enterocyte's basolateral membrane into the lacteal system (Salih, 2021, Yadav and Paul, 2023). The lacteal system transports lipids into the circulation, where they are delivered to numerous tissues throughout the body, including adipose tissue, liver and muscle where they are stored (Salih, 2021). Consequently, consuming saturated fat-rich meals can elicits obesity, metabolic syndrome and eventually, non-alcoholic fatty liver disease.

2.6 Therapeutic interventions for preventing and managing metabolic syndrome and non-alcoholic fatty liver disease

2.6.1 Exercising and switching to a healthy dietary lifestyle

The most commonly used therapies for treating and controlling metabolic syndrome deal with individual risk factors such as correction of dyslipidaemia, hypertension and type II diabetes mellitus (Grundy, 2020, Lemieux and Després, 2020). The favourable benefits of exercise and dietary changes in the prevention of metabolic syndrome and its related risk factors are well established. Physical inactivity and sedentary lifestyle are the primary underlying contributors to the development of metabolic syndrome, therefore, exercising and switching to a healthy dietary lifestyle in an attempt to lose weight can help prevent the ill risk factors while also restoring your body's ability to recognize insulin and greatly reducing the risk of exacerbating metabolic syndrome. Diets high in whole grains, fruits, vegetables and nuts have been demonstrated to lower the risk of developing metabolic dysfunction likely this diet mediates an increase in high density lipoprotein-cholesterol concentration, which transports cholesterol

from the blood to the liver, as well as by lowering elevated blood glucose, triglycerides and low-density lipoprotein-cholesterol.

2.6.2 Conventional pharmacological interventions

Bezafibrate, which lowers cholesterol and triglycerides while raising high density lipoproteins and decreasing low density lipoproteins (Bodaghi et al., 2023, León-Martínez et al., 2021), is one of several well-known pharmacological drugs with lipid-lowering and hypoglycaemic qualities that are now in use. Fenofibrate is used to treat hypercholesterolemia, mixed dyslipidaemia and severe hypertriglyceridemia (Jin et al., 2023, Miceli et al., 2021), whereas Metformin is used to treat diabetes (Feingold and Grunfeld, 2023). Niacin is a vitamin B derivative that is used to treat vitamin deficiencies, as well as hyperlipidaemia, dyslipidaemia and hypertriglyceridemia (Aguilera-Méndez et al., 2022, Nagarthna et al., 2020). Statins, which include lovastatin, rosuvastatin, mevastatin, GFT14, FM-VP4, fluvastatin, pitavastatin, atorvastatin and pravastatin, are lipid-lowering drugs used to reduce abnormal cholesterol and lipid concentrations by suppressing endogenous cholesterol synthesis in the liver, thereby reducing the possibility of development of cardiovascular disease (Abdul-Rahman et al., 2022, Fiorentino and Chiarelli, 2023, Liao, 2002). Sulfonylureas are all authorized medications for the treatment of metabolic syndrome (Kang et al., 2023, Tomlinson et al., 2022). All conventional pharmacological medications listed above have been authorized and in use as therapies to reduce and/or manage individual risk factors in the management of metabolic syndrome and non-alcoholic fatty liver disease.

Recently, there has been a surge of interest in the impacts of novel generation of anti-diabetic drugs, such as dipeptidyl peptidase 4 inhibitors (DPP-4i), glucagon-like peptide-1 receptor agonists (GLP-1 RAs), monoclonal antibodies and sodium glucose cotransporter 2 inhibitors (SGLT2i) on non-alcoholic fatty liver disease (Ding et al., 2022, Filipovic et al., 2021, Meier, 2012, Ranjbar et al., 2019, Seko et al., 2017).

2.6.2.1 Dipeptidyl peptidase 4 inhibitors (DPP-4i)

These medications work largely by inhibiting the enzyme DPP-4, which participates in the breakdown of incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Chen et al., 2020, dos Santos et al., 2021).

2.6.2.2 Sodium glucose cotransporter 2 inhibitors (SGLT2i)

SGLT2i, a novel hypoglycaemic agent, inhibits glucose reabsorption in the proximal tubule, which causes glycosuria and reducing plasma glucose levels (Ding et al., 2022, Vallon, 2015). Furthermore, SGLT2i has a number of cardiorenal and metabolic advantages, including weight loss via glycosuria-induced caloric loss (Ranjbar et al., 2019). In non-alcoholic fatty liver disease patients, SGLT2i appear to be viable therapeutic agents as they improve liver function irrespective of changes in the body weight (Komiya et al., 2016, Ranjbar et al., 2019).

2.6.2.3 Glucagon-like peptide-1 receptor agonists (GLP-1 RAs)

The L cells of the distal ileum and proximal colon release GLP-1, an incretin hormone (Ding et al., 2022). It is important to note that glucagon-like peptide-1 receptor agonists have been proven to increase insulin sensitivity and have glucose-lowering properties (Drucker and Nauck, 2006, Meier, 2012). They also stimulate weight reduction, which highlights their potential involvement in the treatment of non-alcoholic fatty liver disease (Ding et al., 2022, Vilsbøll et al., 2012). Furthermore, they appear to have a direct influence on hepatocytes via lipid metabolism, lowering hepatic steatosis (Ding et al., 2022, Seko et al., 2017). Furthermore, GLP-1 agonists protect hepatocytes by inhibiting the malfunctioning endoplasmic reticulum stress response in connection to fatty acid-related mortality (Ranjbar et al., 2019, Seko et al., 2017).

2.7 The role of fats in health and disease

Fats are the fundamental cellular membrane's structural elements. They also provide cellular energy. The varieties of fats ingested in the diet are linked to the development of chronic metabolic diseases (Moszak et al., 2020). Dietary fats are classified into four types: monounsaturated fats, polyunsaturated fats, saturated fats and trans fats.

Unsaturated fats are found in fish, numerous plant-derived oils, nuts and seeds (Rizzo et al., 2023a). Animal products have the largest concentration of saturated fats (López-Pedrouso et al., 2021). Trans fats are present in minor amounts in animal products but are also found in meals, mostly as a result of the processing of vegetable oils (Bajželj et al., 2021). Unsaturated fats, among the previously described forms of dietary fats, are related with lower common metabolic risk factors and mortality risks (Kapoor et al., 2021, Rizzo et al., 2023a). Trans fats and saturated fats, on the other hand, have been linked to detrimental health effects and increased mortality risk.

Polyunsaturated fats in liquid vegetable oils, nuts and seeds are examples of healthy fats that can help preclude metabolic syndrome and non-alcoholic fatty liver disease (Kapoor et al., 2021). Omega-3 and omega-6 polyunsaturated fatty acids are necessary for optimal body growth, cognitive function and reproduction, they are classified as essential fatty acids (Djuricic and Calder, 2021). However, because the body does not synthesize them, they must be derived from dietary sources. Omega-3 fatty acids are classified into three types: α-linolenic acid, docosahexaenoic acid and eicosapentaenoic acid (Djuricic and Calder, 2021). They have all been extensively researched for their potential health advantages, with research indicating that when included in the diet, enough omega-3s throughout pregnancy and the early neonatal growth period are critical for the child's development (Khalid et al., 2022, Sherzai et al., 2023). The intake of omega 3 has been linked to fighting several autoimmune diseases, improving intelligence, lowering the risk of many heart diseases, improving several metabolic syndrome risk factors, improving systemic insulin resistance, potentially lowering the risk of osteoporosis and arthritis by improving bone strength and joint health and lowering liver fat in people with non-alcoholic fatty liver disease (Fauzy et al., 2023, Khalid et al., 2022, Watanabe and Tatsuno, 2021).

The richest source of unsaturated fat is extra-virgin olive oil and a diet supplemented with it lowered the frequency of fatal heart illnesses in individuals with metabolic syndrome risk factors (Enas and Dharmarajan, 2020). According to new research, including healthy fats in your diet might enhance your health (Agregán et al., 2022, Enas and Dharmarajan, 2020). There is a relationship between dietary fat and some malignancies (Steck and Murphy, 2020). However, clinical research is seeking to uncover conclusive evidence of the link between dietary fat and cancer incidence (Bose et al., 2020). In a study looking at the influence of a low-fat diet on the development of breast and colon cancer, adult females consuming a low-fat diet and those on a fixed diet had identical risks of breast and colon cancer (Bojková et al., 2020, Dladla, 2015, Pan et al., 2021). Previous research suggests that diets heavy in animal fat and saturated fat may increase the risk of prostate cancer (Bojková et al., 2020). The precise relationship between dietary fat and prostate cancer in men is unknown, more study, however, is needed to understand and validate any potential links between dietary fat and prostate cancer.

There is increasing interest in research on the impact of dietary lipids on illnesses including osteoporosis, multiple sclerosis, infertility and other chronic disorders exists (Amelianchik et al., 2022, Samadi et al., 2021). The World Health Organization recommends balancing calorie intake, minimizing saturated and trans fats and increasing consumption of unsaturated fats

(Organization, 2019). It is currently unavoidable that saturated fats will be added to food. While focus tends to be on cholesterol, there is need for alternative dietary additives can be employed as long-term preventive approaches against diet-induced metabolic illness. The current study focussed on stearic acid.

2.8 Stearic acid

2.8.1 Description and chemical composition

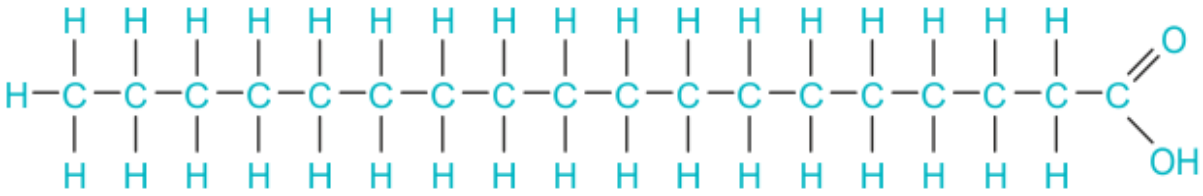


Figure 2.3. Chemical structure and natural sources of stearic acid

Stearic acid ($C_{18}H_{36}O_2$) also known as octadecanoic acid), has the appearance of a waxy and/or crystalline solidity (Acid, 1987). It is a saturated fatty acid that occurs naturally in fats and oils derived from vegetable oils and a variety of animal lipids (Acid, 1987). The food and drug administration usually considers it safe (Fiume et al., 2012).

It functions as a plant metabolite, a human metabolite and an algal metabolite (Kumari et al., 2013, Tvrzicka et al., 2011).

2.8.2 Industrial uses

It is used in the production of cosmetics, candles and plastics, as well as hardening soaps and softening plastics (Aydin et al., 2005, Fiume et al., 2012, Hartwig et al., 2003).

2.8.3 Therapeutic uses of stearic acid

Stearic acid has been shown to have some health benefits in that it lowers low-density lipoprotein cholesterol, often known as "bad cholesterol," and does not raise the risk of atherosclerosis (Bonanome and Grundy, 1988, Thijssen and Mensink, 2005).

Stearic acid has anti-inflammatory properties (Nishitani et al., 2007, Pan et al., 2010). This saturated fatty acid has a wide range of impacts on liver metabolism (Sampath and Ntambi, 2005). Previous research was aimed at the effects of stearic acid on hepatocyte transplantation indicators in rats with acetaminophen-induced liver injury (Fernandez-Checa et al., 2021, Goradel et al., 2016). The studies reported that the 10-day medication was administered to

Wistar rats at random and the rats with acetaminophen-induced liver damage were given stearic acid. In addition, rats were given the isolated liver cells intraperitoneally and blood samples were collected to assess changes in serum liver enzymes. It was found that the levels of serum liver enzymes of rats with acetaminophen-induced liver damage rose considerably and reverted to normal values following stearic acid treatment (Goradel et al., 2016). The most important finding from the study was that a stearic acid-rich diet coupled with cell therapy hastened the recovery of hepatic dysfunction in a rat model of hepatic damage (Goradel et al., 2016). Stearic acid also has additional protective properties. It was shown that it possesses a neuroprotective effect against oxidative stress via the phosphatidylinositol 3-kinase pathway (Wang et al., 2021, Wang et al., 2006). Stearic acid also protected brain slices (cortical or hippocampus) against injury induced by oxygen-glucose deprivation or glutamate. Its neuroprotective action might be achieved mostly through the activation of peroxisome proliferator-activated receptor-γ (Berger and Moller, 2002, Singh et al., 2023).

Furthermore, contrary to other saturated fatty acids, the health benefits of stearic acid might be linked to the inhibitory action of 5-alpha reductase (Kabara, 2000), which inhibits the β-hydroxy-β-methylglutaryl-CoA reductase in the cholesterol biosynthesis pathway (Igwe et al., 2015, Vadivel and Gopalakrishnan, 2011). β -hydroxy- β-methylglutaryl-CoA is a target of the statin pharmaceuticals mentioned earlier.

2.8.4 Stearic acid and gastrointestinal function

The bacteria found in the gastrointestinal tract are involved in the digestion and absorption of several nutrients, as well as the development of metabolic disorders (Gill et al., 2021, Iwasaki et al., 2019). As a result, forthcoming advancements in metabolic dysfunction therapy will likely target gut bacteria (Agus et al., 2020, Singh et al., 2021, Vernocchi et al., 2020). The authors first demonstrated that a high stearic acid diet exacerbates acute graft-versus-host disease by enriching *A. muciniphila*, implying that gut microbiota modulation may represent novel therapeutic targets for the prevention and treatment of acute graft-versus-host disease (Gao et al., 2023, Yang et al., 2021). Stearic acid also prevented alcohol-induced liver damage by strengthening intestinal tight junctions and regulating intestinal microorganisms (Nie et al., 2022).

2.9 Justification of the study

There is a global need to encourage and implement positive health beneficial interventions- in order to reduce or eliminate the health-harming effects of obesity, type II diabetes mellitus, non-alcoholic fatty liver disease and metabolic syndrome, as well as their associated ill-risk components. Saturated fatty acids are often incorporated into diets and they can contribute to poor metabolic health.

Stearic acid was of special interest in this study. Unlike other saturated fatty acids, which exhibited negative health effects when consumed, stearic acid has been shown to have hypoglycaemic, hypolipidaemic and hypocholesterolaemic activities (Bonanome and Grundy, 1988, Grundy, 1994, Pan et al., 2010), implying that it may have health-beneficial properties against the development of obesity, type II diabetes mellitus, non-alcoholic fatty liver disease and metabolic syndrome, with no negative health effects. In the literature, there is a dearth of long-term animal studies involving the administration of stearic acid to growing rats. In this work, long-term stearic acid therapy of developing rats will provide a significant model that matches growing children with increased passive plasticity and may perhaps produce temporary epigenetic modifications and resistance to the development of metabolic diseases later in life (Hochberg et al., 2011, Rajamoorthi et al., 2022).

There is a need to expand the body of evidence on the potential combined diets in preventing long-term diet-induced metabolic syndrome. As a result, the search for bioactive replacement chemicals is critical, particularly those that improve diet-induced obesity and metabolic syndrome and its components. Given its pharmacological qualities and nutritional value status, stearic acid is among the most promising substances for the treatment of diet-induced metabolic syndrome.

The present chapter examined relevant literature to the topic at hand. Obesity, metabolic syndrome and other health risks related with excessive consumption of high-fructose and saturated fat were discussed.

The following chapter (chapter 3) discusses the first part of this study, which looks at the influence of stearic acid on health if it is included in the diet instead of cholesterol or other obesogenic fats. The study sought to answer the question ‘does stearic acid consumption during the early post-weaning development period have long-term health consequences in female and male Sprague-Dawley rats fed a high-fructose diet?’ This study also aimed to investigate the combined effect of high stearic acid and high-fructose diets on developing female and male

Sprague-Dawley rats, simulating children given an obesogenic diet and continuing on the diets throughout adulthood.

CHAPTER 3

THE COMBINED EFFECT OF A DIET ENRICHED WITH STEARIC ACID AND HIGH FRUCTOSE ON GENERAL METABOLIC HEALTH PROFILE IN GROWING FEMALE AND MALE SPRAGUE-DAWLEY RATS

3.1 Introduction

3.1.1 Epidemiology

Estimates of the prevalence of obesity worldwide indicate that by 2035, more than 4 billion individuals may be affected (Kalra et al., 2023). Obesity is projected to experience fastest rises from 14% to 24% of the population affecting approximately 2 billion in adults, children and adolescents by 2035 (Chooi et al., 2019, Mohajan and Mohajan, 2023). Obesity is characterized as an abnormal build-up of body fat that can lead to negative health implications. Poor westernized dietary choices rich in lipids, particularly saturated and trans fatty acids, refined cereals and soft drinks and sedentary lifestyles have all been strongly linked to metabolic dysfunction, non-alcoholic fatty liver disease and obesity (Iguacel et al., 2021, Vancells Lujan et al., 2021).

3.1.2 Understanding the impact of high-fructose on metabolic diseases.

Evidence from human and animal research has revealed that an increased intake of high-fructose and/or high-fat diets and/or high-cholesterol, in addition to sedentary behaviour is associated with manifestation of adverse metabolic dysfunction including hypercholesterolaemia (Donaldson et al., 2019), resistance to insulin (Lee et al., 2022, Softic et al., 2020), increased blood sugar levels (Savva et al., 2022), lipogenesis (Geidl-Flueck and Gerber, 2023), visceral obesity (Yu et al., 2015) and subsequently the development non-alcoholic fatty liver disease and impaired kidney functions (Im et al., 2021, Jegatheesan and De Bandt, 2017, Wu et al., 2023b). While lipids can be found in a variety of animal food products like meat, dairy products and eggs, fructose is a common sweetener that is employed in a variety of foods and beverages (Ventura et al., 2011).

Fructose consumption as early as in childhood has been linked to changes in caloric regulation and adverse health effects (Rippe et al., 2017, Tappy and Lê, 2010), particularly metabolic dysfunction that may lay the groundwork for the onset of non-alcoholic fatty liver disease and impaired kidney function later in adulthood. After intake of a high-fructose diet, the glucose transporter 5 passively transports approximately 30% of the fructose from the intestinal lumen into intestinal epithelial cells (Iametti et al., 2022). It then passes into the bloodstream across the basolateral membrane through glucose transporter 2 (Iametti et al., 2022). The liver is the primary site for fructose uptake and metabolism, after it (fructose) enters the bloodstream (Dholariya and Orrick, 2022, Grattagliano et al., 2008).

Fructokinase converts fructose in the liver to fructose-1-phosphate, which is subsequently cleaved by aldolase B into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Dholariya and Orrick, 2022). The final product of fructolysis is pyruvate, which is further processed by pyruvate dehydrogenase to generate acetyl CoA (Hannou et al., 2018). Because there is little to no regulation of fructose metabolism, there is a high degree of Acetyl CoA formation, resulting in fatty acid synthesis (Henry et al., 1991, Jegatheesan and De Bandt, 2017). Triglycerides can be formed through esterification of fatty acids and glyceraldehyde 3-phosphate. As a result, unlike glucose, fructose metabolism bypasses the critical regulating phase of glycolysis, resulting in quick *de novo* lipogenesis (Henry et al., 1991), hepatic lipid accumulation and visceral obesity (Tappy and Lê, 2010). Prolonged uncontrolled triglyceride synthesis can lead to poor glycaemic control, hypercholesterolaemia, hyperlipidaemia, hypertriglyceridemia and impaired insulin sensitivity and visceral obesity (Tappy and Lê, 2010), all which have been recognized as reliable predictors of adverse metabolic dysfunction (Kolderup and Svihus, 2015).

3.1.3 Therapeutic approaches for metabolic diseases and stearic acid's potential ability to improve metabolic health.

Currently, South Africa carries the largest burden of obesity at 28.3% (Hashan et al., 2021). Therefore, obesity prevention, management and treatment must now be prioritized throughout the lifespan. Some aspects of metabolic dysfunction can be treated or managed with synthetic pharmacological drugs like fenofibrate (Abdelmoneim et al., 2021). However, it is a life-long treatment and its long-term side effects such as hepatotoxicity and nephrotoxicity are of significant concern. Since there is no specific drug for all predictors of metabolic dysfunction and obesity, only a combination of drugs is used. There is a global market demand for the use of safe supplementary compounds. Stearic acid is one of the most plentiful and widely used saturated fatty acid found in fats and oils of plants and in animal products (Grundy, 1994). Despite being known that the majority of saturated fatty acids are obesogenic (promote excessive weight gain) (Deol et al., 2015, Zhou et al., 2020), there is strong suggestive evidence that stearic acid possesses hypocholesterolaemia effects (Bonanome and Grundy, 1988, Mensink, 2005) and thus has potential to benefit metabolic health, non-alcoholic fatty liver disease and kidney function impairment induced by high-fructose and/or high-fat diet.

There is a lack of evidence in the literature on the long-term intake of stearic acid-enriched diets, particularly when introduced into the diet early in infancy. Long-term stearic acid

administration to growing rats in this study will provide a significant model that mimics growing children with increased passive plasticity and could potentially confer temporary epigenetic changes and resistance to the development of metabolic diseases later in life.

In this study, we first investigate the impact on health of stearic acid if it is to be included in the diet in place of cholesterol or other obesogenic fats. We pose the question 'Does stearic acid intake in the early post-weaning growth phase have long lasting preventive, ameliorative or exacerbate the health effects in growing female and male Sprague-Dawley rats on high-fructose diet?' This study, also, sought to interrogate the combined effect of high stearic acid and high-fructose diets in growing female and male Sprague-Dawley rats thereby mimicking children fed obesogenic diets and continuing on the diets through to adulthood.

3.2 Aim and Objectives

This study aimed to investigate the long-term effects of a stearic acid alone and/or combined high-fructose and stearic acid diet fed to growing female and male Sprague-Dawley rats on general metabolic health profile and growth performance.

Hypotheses

H_0: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has no effect on metabolic health.

H_1: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has no effect on metabolic health.

The specific objectives of this part of the study focused on:

3.2.1 Growth performance

3.2.1.1 By specifically determining the changes in body mass and

3.2.1.2 Assessment of long bone (tibia and femur) indices of growth.

3.2.2 Visceral obesity

3.2.2.1 By measuring visceral and epididymal (in male rats) fat mass

3.2.3 Metabolic health profile

By assessing the metabolic impact of the combined effect of a diet enriched with stearic acid and high fructose on:

3.2.3.1 plasma or blood fasting metabolic substrates including glucose, triglycerides and cholesterol.

3.2.3.2 key hormones regulating metabolism: serum insulin (hormone used as a marker of diabetes) and adiponectin (hormones produced by fat cells used as a marker of obesity) as well as computation of the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR).

3.3 Materials and methods

3.3.1 Ethical clearance for the use of animals and study site

Ethical clearance to undertake the animal studies was granted by the University of the Witwatersrand - Animal Research Ethics Committee (AREC) with AREC clearance number: 2018/01/03/B. A copy of the certificate is included in Appendix 1. The Wits Research Animal Facility (WRAF), University of the Witwatersrand provided and housed the rats for the study. The National Research Council of the National Academies (2011) Guide for the Care and Use of Laboratory Animals was complied with when undertaking this study. Furthermore, the study was in accordance with South African National Standard (SANS document 10386:2008) recommendations.

3.3.2 Chemicals and reagents

Analytical grade chemicals and reagents were used in the study. Stearic acid (≥ 95%, FCC, FG), fenofibrate and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). From Nature's Choice in Randvaal, South Africa, fructose was bought.

3.3.3 Preparation of normal diet as well as a diet enriched with 3% stearic acid

Rat chow was commercially sourced from (Lab Chef, Stellenbosch, South Africa). The standard rat chow had the following nutrient composition (stated by the manufacturer): crude protein 200 g/kg, crude oils and fats 50 g/kg, Linoleic acid 12 g/kg, crude fibre 40 g/kg, crude ash 70 g/kg, calcium 12 g/kg, phosphorus 7.5 g/kg and Vitamin A, D and E 16 000, 2 000 lU/kg and 100 mg/kg.

A hammer mill was used to grind the rat chow pellets into a coarse powder. The powder was then used to formulate experimental diets (standard rat chow and stearic acid enriched). For the standard rat chow diet, water was added to form a thick paste from which balls were manually moulded, placed in a tray and allowed to dry in a convection oven overnight at 37°C.

For the 3% stearic acid enriched feed, 300 g of stearic acid was thoroughly mixed with 10 kg of the ground feed and then 3 litres of water to form a thick paste from which balls were formed and allowed to dry in a convection oven overnight at 37°C. These experimental diets were separately placed in tightly sealed containers and stored in a dark cupboard at room temperature pending use. The 3% stearic acid dose used in this study has been previously used by (Shen *et al.*, 2014) in metabolic studies.

3.3.4 Preparation of 20% fructose solution and gelatine cubes for administration of treatment

The 20% fructose solution (w/v) was prepared by adding 1000 g of GMO-free granulated fructose (Nature's Choice, Highbury, Randvaal, South Africa) into 5 litres of plain tap water in a plastic container. Three drops of red food colouring (Robertson's, Libstar Operations Pty Ltd, Dunkeld, South Africa) were then added into the fructose solution for identification and differentiation purposes. To the rats receiving plain drinking water, three drops of blue food colouring (Robertson's, Libstar Operations Pty Ltd, Dunkeld, South Africa) were added to each five litres of tap water.

Cubes of fenofibrate gelatine and plain gelatine were made independently. Fenofibrate gelatine cubes preparation: 8g gelatine, 8g sugar and 5ml Bovril were combined in 100 ml boiling water. Due to the poor aqueous solubility of fenofibrate, it was first dissolved in a 5% DMSO solution before being added into the gelatine stock solution (resulting in a final 0.5% DMSO concentration). This gelatine stock solution containing fenofibrate was poured into ice cube trays and allowed to chill in a refrigerator to form cubes prior being diced into one-millilitre cubes and delivered to the rats accordingly. As a positive control, fenofibrate was employed because it is known to enhance metabolic disease features such as insulin sensitivity and lower hyperglycaemia. The current study's fenofibrate dose (100mg/kg) was likewise based on concentrations utilized in previous metabolic investigations (Al-Snafi, 2017).

Plain gelatine cubes preparation: 8g gelatine, 8g sugar and 5ml Bovril were combined in 100 ml boiling water. This gelatine stock solution was poured into ice cube trays and allowed to chill in a refrigerator to form cubes prior being diced into one-millilitre cubes and delivered to the rats. The control groups' plain gelatine cubes were made as well with 0.5% DMSO. The gelatine cubes were made with modifications to the method published by (Kamerman et al., 2004). Instead of 16 g of brown sugar, 8 g of brown sugar (Huletts, Tongaat, South Africa Ltd) were used to make the cubes palatable while avoiding an additional dietary load with sucrose.

3.3.5 Animals, housing and feeding

Animals: A total of eighty weaned 21-day old Sprague-Dawley rats (*Rattus novergicus*) females (n = 40) and males (n = 40) were used in the experiment which ran from postnatal day 21 to 116. Sprague Dawley rats serve as efficient experimental models for studying *in vivo* physiological, metabolic and cellular events in controlled interventional studies.

Housing: The rats were housed individually in Perspex cages with stainless steel mesh lids enabling the rats to visualise each other socially. The cages were lined with wood shavings for bedding, plastic toys and shredded paper were included for environmental enrichment. The bedding was changed twice weekly. A 12 h light: 12 h dark cycle was maintained with lights on from 7 am to 7 pm. Room temperature was maintained at 26 ± 2° C.

Feeding: Depending on treatment regimen, the rats had *ad libitum* access to either a reconstituted standard rat chow (LabChef, Stellenbosch, South Africa) or standard rat chow enriched with 3% (w/w) stearic acid. Also, depending on the treatment regimen, the rats had *ad libitum* access to drinking solution (water or 20% fructose solution) throughout the experiment as described earlier in 3.3.4. The rats drinking solutions were changed after 3 days if unfinished. Gelatine was used as a stress-free route of oral administration, thus treatments using fenofibrate were prepared into gelatine cubes while other treatment groups rats received plain gelatine cubes. Feeding and changing of fluids was done daily between 08h00-10h00.

Rats were given a two-day habituation period to become acquainted with handling and housing (postnatal days 21 and 22). Rats were weighed individually using an electronic balance scale (Clover scales, Pty Ltd, Johannesburg, South Africa) twice a week to assess body mass gain of the rats and for welfare monitoring purposes.

3.3.6 Experimental design and treatments

On post-natal day 23, the eighty rat pups (from dams with a litter size of 6-12) were in an interventional study, randomly divided (with each litter represented across groups to avoid sibling bias in particular groups) into five treatment groups of 16 (8 males and 8 females) pups each and fed the following treatment regimens for 13 weeks:

group I, served as the negative control, receiving standard rat chow (SRC), plain water to drink (PW) and plain gelatine cubes as the vehicle control (PGC);

group II, in which metabolic dysfunction was induced, rats were fed standard rat chow (SRC), 20% fructose solution to drink (FS) and plain gelatine cubes (PGC);

group III, served to investigate the prophylactic potential of stearic acid, rats were fed standard rat chow enriched with 3% stearic acid (SA), 20% fructose solution to drink (FS) and plain gelatine cubes (PGC);

group IV, rats were fed standard rat chow enriched with 3% stearic acid (SA), plain water to drink (PW) and plain gelatine cubes (PGC) to investigate the effects of stearic acid alone on metabolic health;

group V, served as the positive control in which fenofibrate was used for the prevention of metabolic dysfunction, rats were fed standard rat chow (SRC), 20% fructose solution to drink (FS) and fenofibrate in gelatine cubes at 100 mg/kg body mass (FGC).

The rats were fed their respective treatment regimens daily for the duration of the study from postnatal day 23 to 116 (13 weeks).

The researcher was not blinded to the groups during feeding. This is because the researcher was solely responsible for feeding of the rats.

3.3.7 Terminal procedures and sample collection

Prior to termination, the rats had access to plain drinking water overnight, however, they were fasted when their feed was removed at 22h00 after the 13-week intervention. On termination day (postnatal day 116), the rats were weighed (Clover scales, Pty Ltd, Johannesburg, South Africa) and fasting blood glucose and triglyceride concentrations were measured using blood collected following a sterile needle puncture of the tail vein. A calibrated glucose meter (Contour Plus Meter, Bayer, Isando-Johannesburg, South Africa) and a calibrated Accutrend triglyceride meter (Roche, Mannheim, Germany) were used to detect blood glucose and triglyceride concentrations respectively.

The rats were then euthanized with a sodium pentobarbitone overdose (200 mg/kg body mass, intraperitoneally). Cardiac blood was drawn using 18G needles and 10ml syringes into heparinized blood tubes (Becton Dickinson Vacutainer Systems Europe, Meylan Cedex, France). The blood samples were centrifuged at 20°C for 15 minutes at 5000 x g in a Pegasus Scientific Inc., Rockville, USA Sorvall RT® 6000B centrifuge. The plasma was removed and kept in microtubes at -20°C and later used to test for metabolic hormones (insulin and adiponectin) and biochemical markers of health.

Each rat's abdominal viscera were accessed via an incision at the linea alba and removed. Thereafter, each rat’s empty carcass was weighed (Presica 310 M, Presica Instruments, Dietikon, Switzerland) and used for further investigation provided in the current chapter as a measure for growth performance.

The visceral fat pad and epididymal fat in males was weighed (Presica 310 M, Presica Instruments, Dietikon, Switzerland). The visceral and epididymal fat masses relative to body mass (%BM) were calculated as follows:

$$\text{Relative visceral fat mass} = \frac{\text{visceral fat mass (g)}}{\text{fasted terminal body mass (g)}} \times 100.$$

$$\text{Relative epididymal fat mass} = \frac{\text{epididymal fat mass (g)}}{\text{fasted terminal body mass (g)}} \times 100.$$

The rest of the abdominal tissues were weighed (Presica 310 M, Presica Instruments, Dietikon, Switzerland) and collected for hepatic histology, nephropathy, general supplementary data on GIT and visceral morphometrical examination, respectively. The details for additional research on the obtained tissues are supplied in this thesis's chapters 4 (liver), 5 (nephropathy) and 6 (general supplemental information on GIT and visceral morphometrical analysis).
Before storage, the tissues were prepared as follows:

Liver: After weighing, each rat's liver was divided into three parts. One part was stored at -20ºC for assessing the dietary stearic acid and fructose effects on liver lipid content. Another part of the liver was stored in sealed ziplock plastic bags at -20ºC for liver fatty acid profiling. A sample from the right lobe of each liver was preserved in 10% phosphate buffered formalin (Merck, Johannesburg, South Africa) for liver histomorphometry and hepatic steatosis, lobular inflammation, ballooning analysis and total NAFLD activity score (NAS).

Kidney: The kidneys were weighed (Presica 310 M, Presica Instruments, Dietikon, Switzerland) for assessing the stearic acid effects on renal health by measuring kidney (absolute and relative to body mass) mass. One kidney was preserved in 10% phosphate buffered formalin for assessment of kidney histomorphometry.

Visceral organ morphometry: Measurements were performed on the small and large intestines, stomach and caecum which had been carefully dissected out. The small and large intestines were gently stretched out on a dissection board and their lengths were measured using a ruler mounted on the dissection board. The contents of the small and large intestines were gently emptied after which the GIT visceral organs were weighed on an electronic balance (Presica 310M, Presica Instruments AG, Switzerland).

3.3.8 Determination of growth performance and long bone parameters

In addition to the body mass measurements made during the study, in order to observe changes in the long bone parameters of the rats as a measure of linear growth, the right hind leg (femur with the attached tibia) was extracted from each rat carcass. The femur and tibia were then defleshed and disarticulated. The bones were then dried for at least 7 days in an oven (Salvis ®, Salvis Lab, Switzerland) at 40°C until the masses remained unchanged, thereafter allowed to cool at room temperature. An electronic balance (Presica 310M, Presica Instruments AG, Switzerland) was used to determine the dry tibiae and femur dry masses. Digital vernier calipers (Major Tech (Pty) Ltd, KTV 150 digital caliper, Elandsfontein, South Africa) were used to measure the lengths of the tibiae and femurs. Briefly, the length of the tibia was determined by measuring the distance from the centre of the lateral condylar surface to the centre of the distal articular surface. The femur length was determined by measuring the distance from the most proximal point on the femur head to the line connecting the two distal condyles (most distal point) of the femur. The mass to length ratio was used to calculate the density of the tibiae and femurs (Seedor et al., 1991).

$$\text{Bone mass to bone length} = \frac{\text{bone mass (mg)}}{\text{bone length (mm)}}$$

For bone radiographs, a Fujifilm digital X-ray system (Industrial X-ray film FR; Fuji Photo Film Co., Ltd, Tokyo, Japan) was utilized to allow subjective assessment of bone densities. The bones were put on a photographic plate 100 cm away from the X-ray radiation source. The light source was set at 52 kVp and 0.80 mA, with a fifteen-second exposure time.

3.3.9 Determination of fasting lipids: plasma triglycerides and total-cholesterol

In accordance with the manufacturer's instructions, an Enzyme-Linked Immunosorbent Assay (ELISA) kit was used to measure serum fasting total cholesterol and total triacylglycerol concentrations. The protocols utilized are described in detail in the appendix section. A spectrophotometer (DU 720, General purpose UV Spectrophotometer, Beckman Coulter) was utilized to determine the absorbencies of the samples at 510 nm.

3.3.10 Determination of hormones associated with development of metabolic dysfunction: changes in plasma insulin and adiponectin and computation of HOMA-IR

The fasting insulin (ng/mL) and adiponectin (pg/mL) concentrations in the plasma were calculated using rat insulin and adiponectin ELISA kits with detection ranges of 0.3-20 ng/mL

and 46.88-3000 pg/mL, respectively (Elabscience Biotechnology Co, Wuhan, Hubei Province, China). Appendices offer an extensive overview of the protocol employed. Using calibrator concentrations, a standard curve for each hormone was created. The insulin and adiponectin concentrations in the collected samples were then calculated using the standard curves that had been generated. Insulin resistance was estimated by computing the homeostatic model assessment index, HOMA-IR, using the fasting blood glucose and insulin concentration using the equation:

$$\text{HOMA-IR} = \frac{\text{insulin (ng/mL) x blood glucose (nmol/L)}}{22.5}$$ (Divi et al., 2012).

3.3.11 Statistical analysis

All data are expressed as mean ± standard deviation. Data analysis was done using GraphPad Prism 7 software (Graph-pad Software Inc., San Diego, USA). Group means for comparisons between different dietary treatment groups were analysed using one-way Analysis of variances (ANOVA) and mean comparison using a Tukey post hoc test with statistical significance of $p < 0.05$ considered. The Tukey test was selected instead of the Scheffe post-hoc test which has a low statistical power and ability to identify actual differences between groups. For the data presented in the table, the statistical level of significance (p-value) is that of the overall group differences before applying post hoc test.

The induction and terminal body masses across the interventions was compared using a two-way ANOVA test with statistical significance of $p < 0.05$ considered.

3.4 Results

Mortality and morbidity of the animals: Two rats were excluded from the analysis. Specifically, one male rat allocated to group I was removed due to its failure to thrive resulting in a reduced mass gain relative to its peers. Therefore, group I rats resulted in n = eight female and n = seven males. Also, one rat allocated to group III females in which the combined effect of stearic acid and high-fructose were tested, lagged by more than 15% (prescribed as a study terminal end point by the Animal Research Ethics Committee) growth rate when compared with the control group rats hence was eliminated from the study. Therefore n = seven for the group. There were no post mortems done on the rats and hence the causes of the illthrift were not identified.

The rest of the animals retained in the study showed no signs of ill health.

3.4.1 The effect of oral administration of stearic acid on growth performance in growing female and male Sprague-Dawley rats fed a high-fructose diet

3.4.1.1 Changes in the body mass of female and male rats

Body masses of both female and male rats were recorded in this study to monitor growth performance and to adjust the concentration of the treatments of the rats, that is, the stearic acid and fenofibrate, since they are body mass dependent so that a constant dose delivery was maintained as the rats grew. Figure 3.1 shows the induction (postnatal day 21) and terminal (postnatal day 116) body masses of female (A) and male (B) rats following the 13-week treatment interventions. Figure 3.2 shows empty (eviscerated) carcass masses of female (A) and male (B) rats following 13 weeks' treatment interventions.

There was no significant difference ($p = 0.9851$, one-way ANOVA, $F_{(4, 34)} = 0.0896$) observed in the induction body masses of the female rats across the five treatment regimens (Figure 3.1 A). However, the females across treatment regimens grew significantly ($p < 0.0001$, two-way ANOVA, $F_{(1, 68)} = 5994$). During the course of the trial, the body masses of the female rats subjected to a control; high-fructose; combined stearic acid and high-fructose; stearic acid alone; combined fenofibrate and high-fructose diets increased by 487 %; 479 %; 487 %; 506 % and 489 %, respectively. While female rats grew significantly, their terminal body masses across the five-treatment interventions were similar ($p = 0.6575$, one-way ANOVA, $F_{(4, 34)} = 0.6111$) as shown in figure 3.1 A.

There were insignificant differences ($p = 0.7156$, one-way ANOVA, $F_{(4, 34)} = 0.528$) in the induction body masses of the male rats across the five treatment regimens (Figure 3.1 B).

Following the 13-week treatment intervention, the male rats across treatment regimens grew significantly in body mass ($p < 0.0001$, two-way ANOVA, $F_{(1, 58)} = 5588$). The male rats had an increase in body mass of 763 % for the control group; 813 % for the high-fructose group; 825 % for the combined stearic acid and high-fructose group; 837 % for the stearic acid alone group; 769 % for the combined fenofibrate and high-fructose group. While the terminal body masses of the male rats fed a combined fenofibrate and high-fructose diet were significantly lower ($p = 0.0128$) compared to the terminal masses of the male rats in the control group, the terminal body masses of the male rats from other treatment interventions were similar ($p = 0.226$, one-way ANOVA, $F_{(4, 34)} = 1.494$) as shown in figure 3.1 B.

Following the 13-week treatment intervention, the empty carcass masses of female rats across different treatment interventions were similar ($p = 0.2096$, one-way ANOVA, $F_{(4, 34)} = 1.551$) as shown in figure 3.2 A. The empty carcass masses of all male rats across different treatment interventions were similar ($p = 0.1320$, one-way ANOVA, $F_{(4, 34)} = 1.906$) as shown in figure 3.2 B.

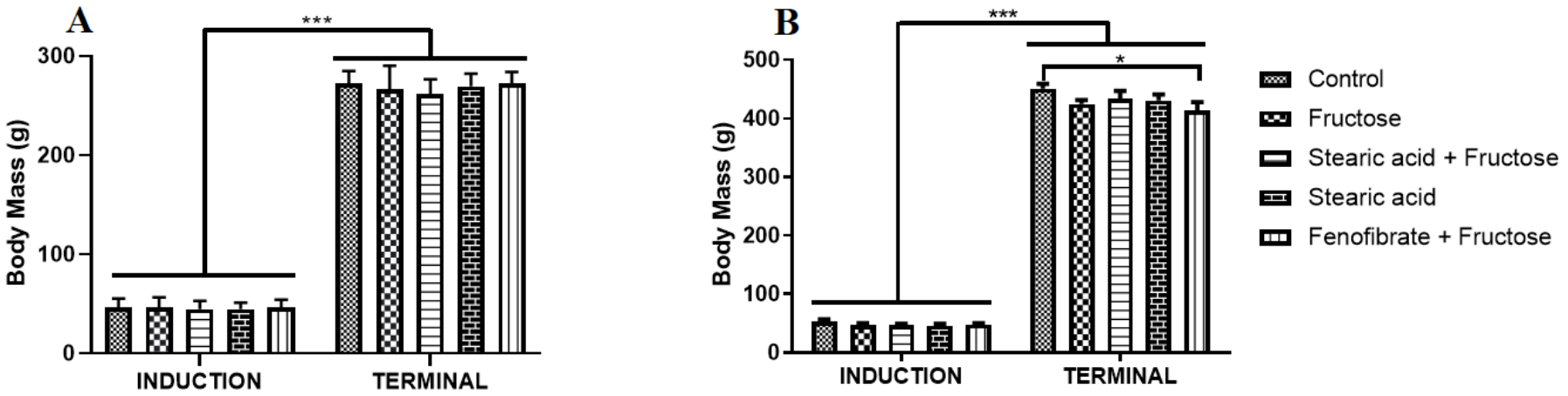


Figure 3.1 Effect of stearic acid on terminal body masses of female (A) and male (B) rats fed a high-fructose diet.

Data presented as mean ± standard deviation. A two-way ANOVA test was used to analyse induction and terminal body masses of the rats across treatment interventions. * = significantly different at $p < 0.05$ and *** = significantly different at $p < 0.0001$. Control group is the negative control in which rats received standard rat chow and plain water to drink (n = 7 for males and n = 8 for females); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8 for males and n = 7 for females); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8) and the fenofibrate and fructose group rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

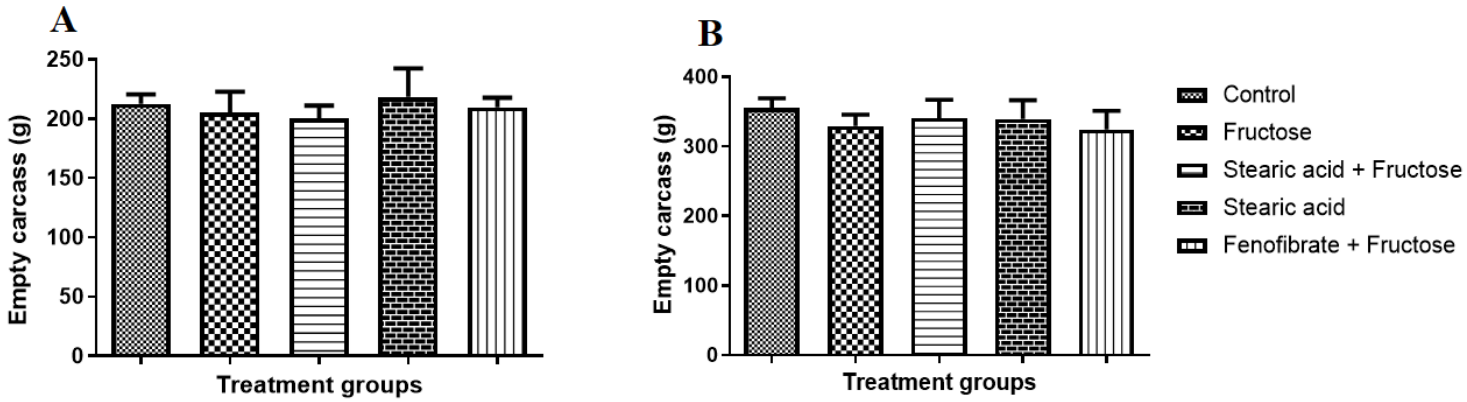


Figure 3.2 Effect of stearic acid on the empty carcass masses of female (A) and male (B) rats fed a high-fructose diet.

Data presented as mean ± standard deviation. One-way ANOVA was used to analyse the empty carcass masses of the rats across treatment interventions. Control group is the negative control in which rats received standard rat chow and plain water to drink (n = 7 for males and n = 8 for females); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8 for males and n = 7 for females); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8) and the fenofibrate and fructose group rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

3.4.1.2 Changes in the long bone parameters of growing female and male Sprague-Dawley rats fed a high-fructose diet

The assessment of long bone parameters, that is, tibia and femur masses, lengths and bone densities, is an important factor as indicators of growth. Table 3.1 (A) and table 3.1 (B) show the morphometric measurements of tibial and femoral bone masses, lengths and bone densities in growing female and male rats fed a high-fructose diet and/or stearic acid for 13 weeks.

In females, there were insignificant differences ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 1.450$) in the tibial mass of the rats across the different treatment groups. The femur masses of the female rats were not significantly different ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.39$) across the treatment groups. An insignificant difference was observed in terms of tibial ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.99$) and femur ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.29$) lengths of the rats across the different treatment groups. Both the bone indexes of the tibial ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.99$) and femoral ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.96$) of the female rats were similar across the treatment groups (Table 3.1: A).

Table 3.1 (B) show that in male rats, administration of a diet enriched with stearic acid and/or high-fructose to rats resulted in no significant differences across the dietary treatment groups in terms of the tibial ($p > 0.05$, one-way ANOVA $F_{(4, 34)} = 0.86$) and femoral ($p > 0.05$, one-way ANOVA $F_{(4, 34)} = 0.63$) bone masses, tibial ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.78$) and femur ($p > 0.05$, one-way ANOVA $F_{(4, 34)} = 1.73$) lengths; and bone indexes of the tibial ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.50$) and femur ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.50$) across different treatment groups (Table 3.1 B).

Long bones (tibiae and femora) representative radiographs of female and male rats fed different diets following a 13-week treatment period are shown in Figures 3.3 A and B respectively. The assessment of the radiographs was done subjectively and did not show any visible differences in the densities of the long bones from both female and male rats fed a high-fructose diet following a 13-week treatment period.

Table 3.1 A. The effects of stearic acid on tibial and femoral bone masses, lengths and bone densities in growing Sprague-Dawley female rats fed a high-fructose diet and/or stearic acid for 13 weeks.

Bone Parameters	**Treatment Groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Tibia						
mass (mg)	453.8 ± 70.1[a]	422.5 ± 84.5 [a]	382.9 ± 54.1 [a]	440.0 ± 50.7 [a]	416.3 ± 29.3 [a]	ns (p = 0.2389)
length (mm)	35.1 ± 1.2 [a]	33.7 ± 3.0 [a]	32.3 ± 3.3 [a]	33.6 ± 3.5 [a]	34.1 ± 2.7 [a]	ns (p = 0.4383)
Density (mg/mm)	1.3 ± 0.2 [a]	1.3 ± 0.2 [a]	1.2 ± 0.2 [a]	1.3 ± 0.1 [a]	1.2 ± 0.1 [a]	ns (p = 0.4237)
Femur						
mass (mg)	588.8 ± 127.0 [a]	552.5 ± 116.5 [a]	527.1 ± 114.0 [a]	570.0 ± 46.9 [a]	571.30 ± 87.1 [a]	ns (p = 0.8165)
length (mm)	29.0 ± 3.0 [a]	28.7 ± 2.9 [a]	28.8 ± 2.8 [a]	27.5 ± 3.5 [a]	28.4 ± 3.0 [a]	ns (p = 0.8797)
Density (mg/mm)	20.20 ± 3.04 [a]	19.2 ± 3.5 [a]	18.3 ± 3.1 [a]	20.88 ± 2.0 [a]	20.2 ± 2.2 [a]	ns (p = 0.4449)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8). [a] = Within rows means with similar superscripts are not significantly different at $p > 0.05$.

Table 3.1 B. The effects of stearic acid on tibial and femoral bone masses, lengths and bone densities in growing Sprague-Dawley male rats fed a high-fructose diet and/or stearic acid for 13 weeks.

Bone Parameters	**Treatment Groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Tibia						
mass (mg)	524.3 ± 55.3 [a]	510.0 ± 54.5 [a]	528.8 ± 55.4 [a]	517.5 ± 58.9 [a]	493.8 ± 50.1 [a]	ns (p = 0.4988)
length (mm)	36.2 ± 3.4 [a]	36.9 ± 2.4 [a]	38.1 ± 1.0 [a]	37.9 ± 1.0 [a]	36.5 ± 1.8 [a]	ns (p =0.5464)
Density (mg/mm)	1.4 ± 0.2 [a]	1.4 ± 0.1 [a]	1.4 ± 0.1 [a]	1.4 ± 0.1 [a]	1.4 ± 0.1 [a]	ns (p =0.7352)
Femur						
mass (mg)	685.7 ± 97.8 [a]	713.8 ± 136.4 [a]	676.3 ± 106.9 [a]	697.5 ± 139.0 [a]	630.0 ± 72.3 [a]	ns (p =0.6436)
length (mm)	30.9 ± 2.9 [a]	32.0 ± 2.5 [a]	32.8 ± 2.3 [a]	33.0 ± 2.4 [a]	30.6 ± 1.9 [a]	ns (p =0.1665)
Density (mg/mm)	22.4 ± 4.1 [a]	21.3 ± 3.0 [a]	20.6 ± 2.7 [a]	21.11 ± 3.5 [a]	20.7 ± 3.1 [a]	ns (p =0.7354)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8). [a] = Within rows means with similar superscripts are not significantly different at $p > 0.05$.

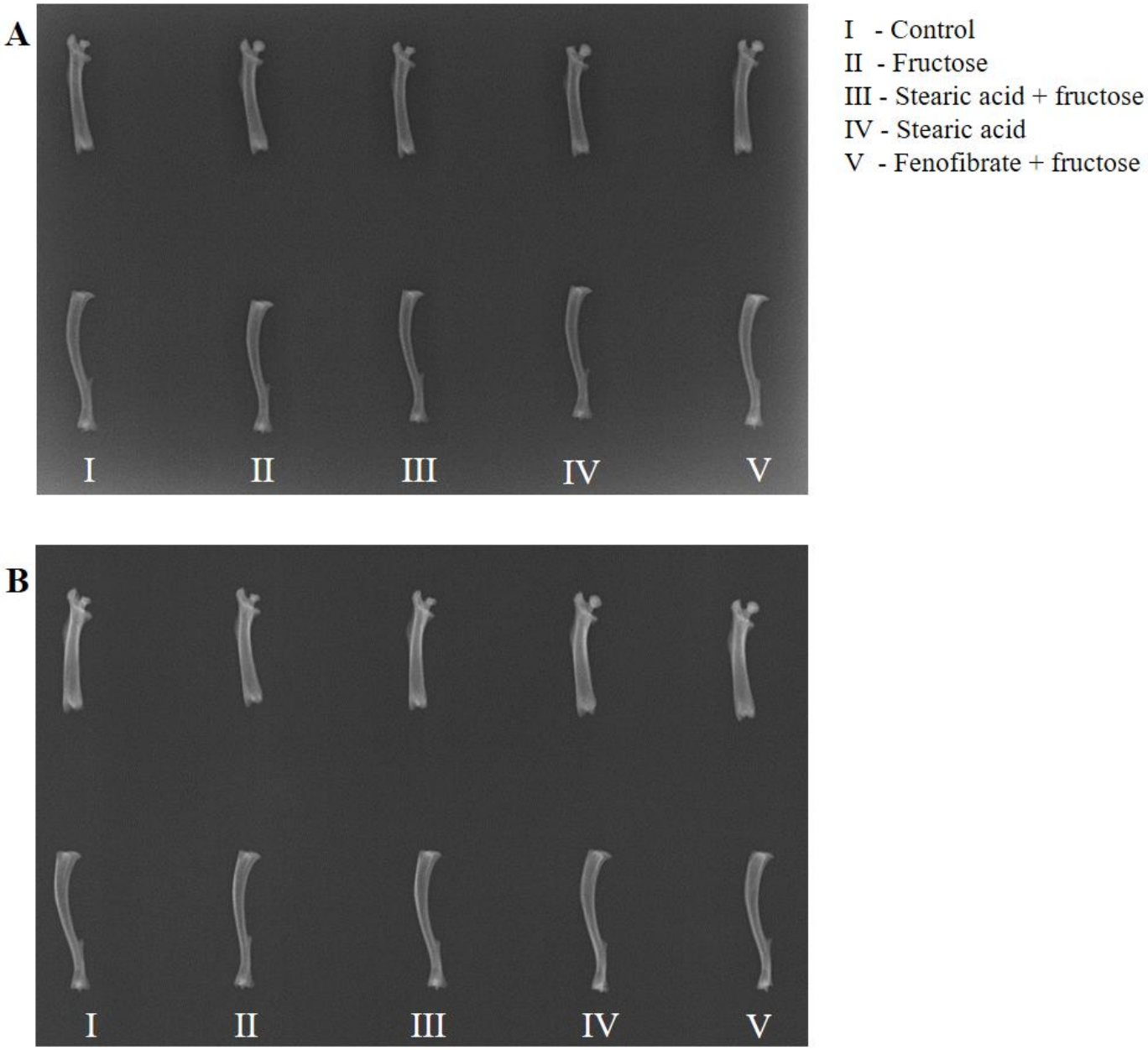


Figure 3.3. Radiograph images of femora and tibiae of female (A) and male (B) Sprague Dawley rats. The top row shows the femora and the bottom row shows the tibiae radiographs of representative rats from each of the different treatment groups. Control group (I) is the negative control which received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; fenofibrate and fructose group (V) is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink.

3.4.2 The effect of stearic acid on the masses of the abdominal visceral fat pad and epididymal fat (in male rats) masses in both female and male growing Sprague-Dawley rats fed a high-fructose diet.

3.4.2.1 Changes in the visceral fat masses of the female rats

The visceral fat mass (absolute and relative to body mass) of the female rats following the 13-week treatment period are shown in figure 3.4 A (I) and (II) respectively.

The overall differences observed in the absolute mass of visceral fat across treatment groups were statistically significant ($p = 0.0010$, one-way ANOVA, $F_{(4, 34)} = 8.09$). As shown in figure 3.4 A (I), the female rats fed a high-fructose diet ($p = 0.0002$) and their counterparts whose diet was a combined fenofibrate and high-fructose ($p = 0.0146$) had significantly increased ($p < 0.05$) absolute visceral fat mass when compared to the control rats fed a standard. However, the female rats fed a combined stearic acid and high-fructose ($p > 0.05$) and counterparts fed stearic acid alone ($p > 0.05$) had similar absolute mass of visceral fat compared to the control rats fed a standard diet. As shown in figure 3.4 A (I), stearic acid alone female rats had similar absolute mass of visceral fat compared to the negative control rats fed a standard diet ($p > 0.05$) and counterpart fed a combined stearic acid and high-fructose ($p > 0.05$) as well as rats fed a combined fenofibrate and high-fructose diet ($p > 0.05$) but lower than those whose diet was high-fructose ($p = 0.0013$).

In summary, a high-fructose diet resulted in increased visceral obesity which stearic acid and fenofibrate interventions were unable to prevent. Stearic alone diet resulted in lower visceral fat compared to the group fed a high-fructose diet.

The overall differences observed in the visceral fat relative (to body mass) across treatment groups were statistically significant ($p = 0.038$, $F_{(4, 34)} = 2.87$). The high-fructose diet fed ($p = 0.0001$); combined diet of stearic acid and high-fructose ($p = 0.0479$) and the fenofibrate and high-fructose ($p = 0.01$) fed female rats had significantly increased visceral fat mass relative (to body mass) compared to their control rats that were fed standard rat chow. However, female rats whose diet had combined stearic acid and high-fructose ($p = 0.1635$) as well as those fed a combined fenofibrate and high-fructose ($p = 0.3554$) has similar visceral fat relative (to body mass) compared to rats fed the high-fructose only diet ($p > 0.05$). Stearic acid alone as a dietary supplement had similar ($p > 0.05$) visceral fat relative (to body mass) compared to negative control ($p = 0.9536$), combined stearic acid and high-fructose ($p = 0.1985$) as well as combined

fenofibrate and high-fructose ($p = 0.0560$) but significantly lower than high-fructose only fed rats as shown in figure 3.4 A (II).

In summary, a high-fructose diet resulted in an increased visceral fat relative (to body mass) which both stearic acid and fenofibrate intervention were unable to prevent.

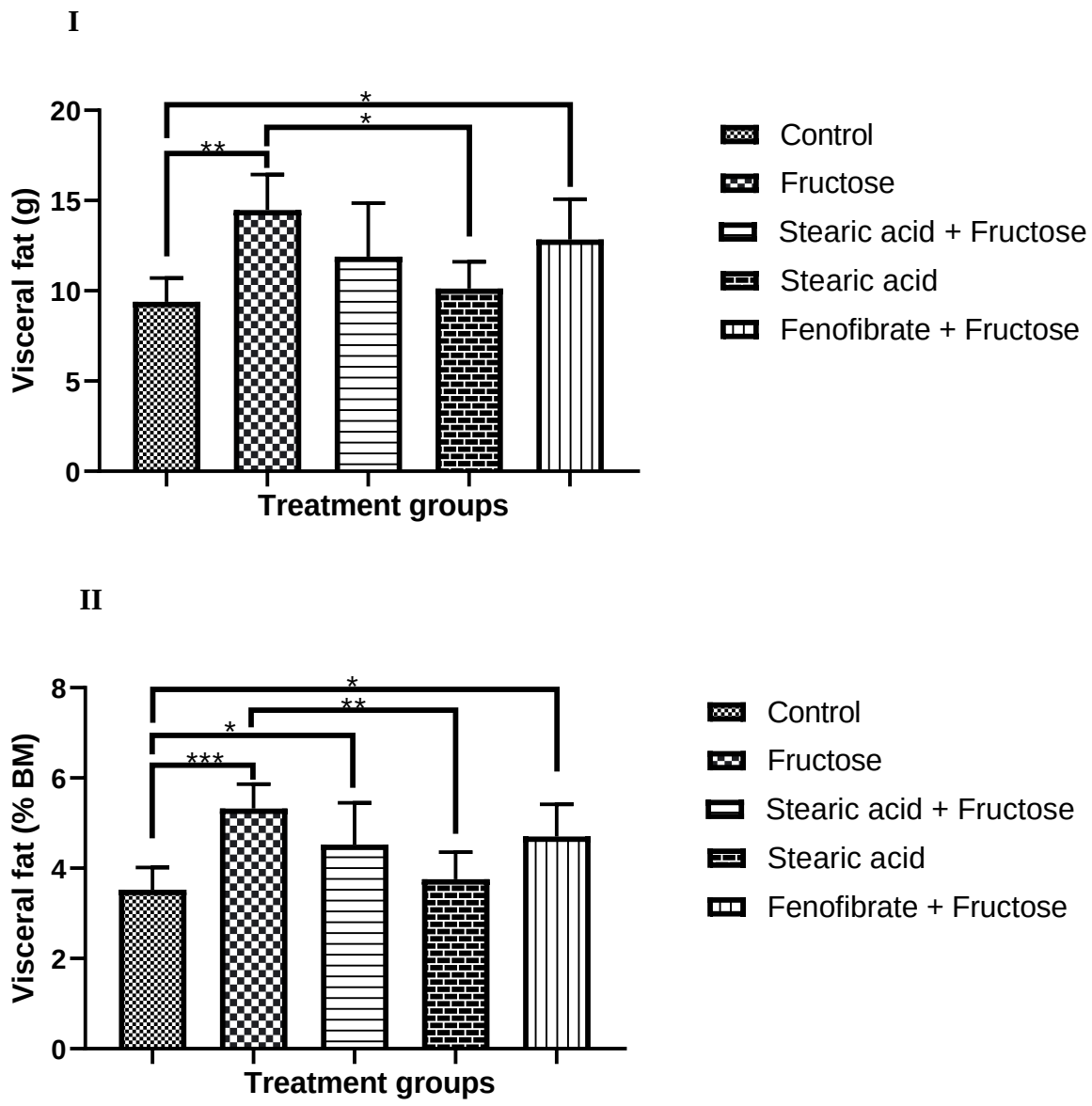


Figure 3.4 A. Effect of stearic acid on absolute (I) and relative (II) visceral fat pad mass of female rats fed a high-fructose diet. Data presented as mean ± standard deviation. Control group is the negative control in which rats received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8) and the fenofibrate and fructose group rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8) * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$.

3.4.2.2 Changes in the visceral fat masses of the male rats

The absolute masses of visceral and visceral fat relative (to body mass) of the male rats following the 13-week treatment period are shown in figure 3.4 B (I and II).

In male rats, the overall differences observed in the absolute ($p = 0.0010$, $F_{(4, 34)} = 5.957$) and relative to body mass ($p = 0.0323$, $F_{(4, 34)} = 2.99$) of visceral fat across treatment groups were statistically significant. The male rats that were fed a combined high-fructose and stearic acid diet had significantly increased ($p = 0.0032$) absolute visceral fat mass when compared to their negative control rats fed standard rat chow. However, the male rats whose diet had high-fructose only ($p = 0.4535$), stearic acid alone ($p = 0.2531$) and those fed a combined fenofibrate and high-fructose ($p = 0.9998$) had similar visceral fat ($p > 0.05$) compared to the negative control fed a standard diet.

In summary, a high-fructose did not increase abdominal visceral fat in male rats. Only rats fed combined stearic acid and high-fructose had an increased visceral fat pad mass.

The absolute masses of epididymal and epididymal fat relative to body mass of the male rats following the 13-week treatment are shown in figure 3.4 B (III and IV). In male rats, the epididymal fat masses across treatment groups were not significantly different ($p = 0.0736$, one-way ANOVA, $F_{(4, 34)} = 2.359$). No significant differences were observed across treatment groups on the epididymal relative (to body mass) fat masses of the rats ($p = 0.4384$, one-way ANOVA, $F_{(4, 34)} = 0.97$) as shown in figures 3.4 B (III and IV).

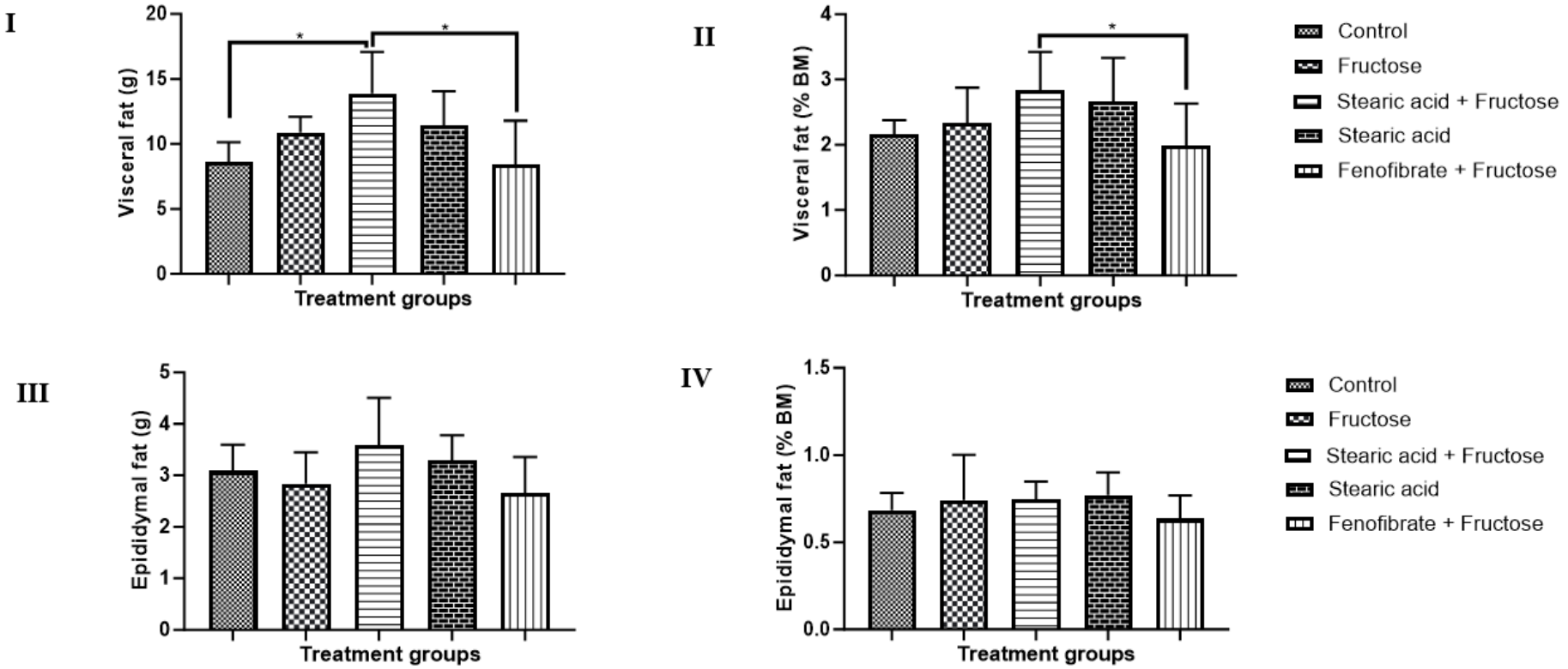


Figure 3.4 B. Effect of stearic acid on absolute (I); relative (II) visceral fat pad masses; absolute (III) and relative (IV) epididymal fat pad masses of male rats fed a high-fructose diet. Data presented as mean ± standard deviation. Control group is the negative control in which rats received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8) and the fenofibrate and fructose group rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8). * $p < 0.05$

3.4.3 The effect of oral administration of stearic acid on general clinical biochemistry health profiles, hormones regulating metabolism in growing female and male Sprague - Dawley rats fed a high-fructose diet

3.4.3.1 Changes in plasma metabolic substrates

Total cholesterol and triglycerides are the metabolic substrates used to monitor the impact of a treatment intervention on the development of metabolic dysfunction. Obese individuals are usually characterized by abnormalities in lipid metabolism, which include elevated triglycerides, phospholipids and cholesterol concentrations. The effect of stearic acid on plasma cholesterol and triglyceride concentrations of female and male rats fed a high-fructose diet are shown below in table 3.2 A and B., respectively.

In female rats, overall total cholesterol concentrations across treatment groups showed significant differences ($p < 0.0001$, one-way ANOVA, $F_{(4, 34)} = 7.83$). The female rats that were fed high-fructose alone in their diet had significantly increased total cholesterol concentration ($p < 0.01$) when compared to the negative control rats. Similarly, female rats that received a treatment of combined fenofibrate and high-fructose in their diet had significantly higher ($p < 0.01$) total cholesterol concentrations in comparison to the negative control rats. However, the female rats whose diet was supplemented with high-fructose only and their counterparts whose diet had combined fenofibrate and high-fructose had similar plasma cholesterol concentration ($p > 0.05$). Stearic acid alone had similar cholesterol concentration ($p > 0.05$) compared to the control but lower than high-fructose ($p < 0.0001$), combined stearic acid and high-fructose ($p < 0.05$) and combined fenofibrate and high-fructose ($p < 0.0001$).

In summary, a high-fructose diet increased female rats' cholesterol concentrations, however stearic acid and fenofibrate intervention did not prevent this increase. Nonetheless, using stearic acid alone as a dietary supplement reduced cholesterol concentration.

In females, overall triglyceride concentrations across treatment groups showed significant differences ($p = 0.0003$, one-way ANOVA, $F_{(4, 34)} = 7.132$). The female rats that were fed high-fructose alone in their diet had significantly increased ($p < 0.05$) plasma triglycerides concentration when compared to the negative control rats. However, the female rats whose diet was combined high-fructose and stearic acid, stearic acid alone and combined fenofibrate and high-fructose had similar triglyceride concentration ($p > 0.05$) when compared to the negative control rats. Stearic acid alone had similar triglyceride concentration ($p > 0.05$) compared to

the control but significantly lower than high-fructose ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.05$).

In summary, a high-fructose diet significantly increased female rats' triglyceride concentrations. Both the combined high-fructose and stearic acid as well as combined fenofibrate and high-fructose intervention did not prevent this increase. Nonetheless, using stearic acid alone as a dietary supplement reduced triglyceride concentration.

In male rats, the plasma cholesterol ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.13$) and triglycerides ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.68$) concentrations were not significantly different across treatment groups (table 3.2 B). Therefore, treatment regimens had no effect on male rats' plasma concentrations of the two lipids (table 3.2 B).

Table 3.2 A. The effect of stearic acid on cholesterol and triglycerides concentrations in growing female Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Cholesterol (mg/dL)	4.6 ± 1.1 [b]	6.6 ± 1.6 [a]	5.7 ± 1.8 [ab]	3.7 ± 0.6 [b]	6.6 ± 1.1 [a]	(p = 0.0001)
Triglycerides (mmol./L)	0.4 ± 0.2 [b]	0.7 ± 0.2 [a]	0.5 ± 0.2 [ab]	0.2 ± 0.1 [a]	0.6 ± 0.2 [ab]	(p = 0.0003)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. [a,b] within row means with different superscript significantly different at $p < 0.05$;Control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 3.2 B. The effect of stearic acid on cholesterol and triglycerides concentrations in growing male Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Cholesterol (mg/dL)	3.9 ± 1.8[a]	3.8 ± 1.5[a]	3.7 ± 1.1[a]	4.0 ± 1.1[a]	4.0 ± 1.7[a]	ns (p = 0.98)
Triglycerides (mmol/L)	0.6 ± 0.3[a]	0.6 ± 0.2[a]	0.5 ± 0.1[a]	0.7 ± 0.2[a]	0.7 ± 0.3[a]	ns (p = 0.68)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$.Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

3.4.3.2 Changes in fasted blood glucose and plasma insulin, adiponectin concentrations and Homeostatic model of insulin resistance (HOMA-IR) index

The effects of stearic acid on fasted blood glucose, plasma insulin, adiponectin concentrations and HOMA-IR indexes in female and male rats fed a high-fructose diet following a 13-week dietary treatment period are shown in table 3.3 A and B respectively.

Table 3.3 (A) shows that, in female rats, overall fasted blood glucose concentrations across the treatment groups were not significant different ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.86$). The female rats' plasma insulin concentrations were not significantly different ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 2.53$) across the treatment groups. The overall female rats' adiponectin concentrations were significantly different ($p < 0.0001$, one-way ANOVA, $F_{(4, 34)} = 782.2$) across treatment groups. Female rats fed high-fructose alone had significantly lower ($p < 0.0001$) adiponectin concentrations than negative control rats fed a standard diet ($p = 0.0475$) and their counterparts who received a treatment of combined stearic acid and high-fructose diet ($p < 0.0001$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose diet ($p < 0.0001$). Female rats given a combined high-fructose and stearic acid diet ($p < 0.0001$), as well as the group given stearic acid alone ($p < 0.0001$), showed considerably greater adiponectin concentrations as compared to female rats fed high-fructose alone, respectively. Female rats on a combination of fenofibrate and high-fructose diet, on the other hand, had the highest adiponectin concentrations ($p < 0.0001$). The computed HOMA-IR indexes of the female rats across treatment regimens were similar ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 1.54$).

Table 3.3 (B) show that, in male rats, there were no significant differences in the glucose ($p = 0.51$, one-way ANOVA, $F_{(4, 34)} = 0.84$), insulin ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 1.75$) and the computed HOMA-IR indexes of the male rats across treatment regimens ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 1.21$), respectively. In male rats, the overall adiponectin concentrations were significantly different ($p < 0.0001$, one-way ANOVA, $F_{(4, 34)} = 1376$) across treatment groups. Male rats fed a high-fructose diet and negative control rats on a standard diet had similar adiponectin concentrations ($p = 0.9911$). However, as compared to the combination high-fructose and stearic acid ($p < 0.0001$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.0001$) treatment groups, these were extremely low.

Table 3.3 A. The effect of stearic acid on the glucose, insulin, adiponectin concentrations and HOMA-IR index in growing female Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Glucose (mmol/L)	4.05 ± 0.44 [a]	3.93 ± 0.67 [a]	4.23 ± 0.67 [a]	3.86 ± 0.75 [a]	3.70 ± 0.30 [a]	ns (p = 0.50)
Insulin (ng/mL)	1.04 ± 0.98 [a]	1.21 ± 0.65 [a]	1.19 ± 0.77 [a]	1.27 ± 0.56 [a]	2.11 ± 0.76 [a]	ns (p = 0.06)
Adiponectin (pg/mL)	64.88 ± 11.96 [a]	47.49 ± 3.18 [b]	141.80 ± 9.88 [c]	163.20 ± 6.78 [d]	235.00 ± 2.56 [e]	* (p < 0.0001)
HOMA-IR Index	0.19 ± 0.18 [a]	0.21 ± 0.12 [a]	0.23 ± 0.17 [a]	0.22 ± 0.10 [a]	0.35 ± 0.12 [a]	ns (p = 0.21)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. [a, b, c] within row means with different superscript significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8). [a] = Within rows means with similar superscripts are not significantly different at $p > 0.05$.

Table 3.3 B. The effect of stearic acid on the glucose, insulin, adiponectin concentrations and HOMA-IR index in growing male Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Glucose (mmol/L)	4.06 ± 0.82 [a]	3.96 ± 0.76 [a]	3.85 ± 0.44 [a]	3.59 ± 0.43 [a]	3.68 ± 0.39 [a]	ns (p = 0.51)
Insulin (ng/mL)	1.35 ± 0.81 [a]	1.50 ± 0.74 [a]	1.41 ± 0.96 [a]	1.51 ± 0.59 [a]	1.95 ± 0.47 [a]	ns (p = 0.16)
Adiponectin (pg/mL)	53.52 ± 1.54 [a]	52.49 ± 3.56 [a]	109.00 ± 5.81 [b]	153.2 ± 6.39 [c]	185.00 ± 2.56 [d]	* p < 0.0001
HOMA-IR Index	0.25 ± 0.18 [a]	0.27 ± 0.16 [a]	0.24 ± 0.17 [a]	0.24 ± 0.10 [a]	0.41 ± 0.26 [a]	ns (p = 0.33)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

3.5 Discussion

This discussion focuses on the effect of administration of a diet enriched with stearic acid on metabolic health as well as the potential protective effects of stearic acid against the development of ill-health risk factors associated with metabolic dysfunction induced by the consumption of a high-fructose diet. We assessed growth performance, fasting blood glucose, plasma lipids and hormones regulating metabolism and the visceral and epididymal (in male rats) fat mass as a marker of obesity.

3.5.1 A high-fructose and/or stearic acid diet fed to growing female and male Sprague-Dawley rats showed no adverse effects on growth performance

Body mass is frequently influenced by a variety of factors such as age, developmental determinants, diet, gender, genetic makeup (Bouchard, 1991), level of physical activity (Kohl III and Hobbs, 1998), social and some environmental factors. Sex variations in growth rates in rats are caused by hormonal changes that favour males and are frequently preceded by an increase in testosterone concentrations (Garcia-Mayor et al., 1997). Indeed, it was observed that the male rats gained greater mass across treatment regimens in this study than female rats. There were no differences in the terminal masses of the female rats across the groups. Similarly, apart from the group treated with fenofibrate having a lower body mass than the negative control group, there were no significant differences in the terminal mass of the rats across the different groups.

A combined fenofibrate and high-fructose diet reduced the terminal body masses of the male rats in the present study (figure 3.1 B). These data imply that the fenofibrate effects in reducing body mass in male rats can possibly be attributed to enhanced fat catabolism in liver mostly through the stimulation of target enzymes involved in hepatic lipid metabolism (Ferreira et al., 2006, Mancini et al., 2001), also owing to the action of fenofibrate, which works as an agonist for peroxisome proliferator-activated receptor - alpha as well as increased fatty acid beta-oxidation (Prakash, 2018). Our findings are consistent with other metabolic research that have found fenofibrate reduces body mass in rat models of diet-induced obesity through its effect on tissue lipid metabolism (Ferreira et al., 2006, Ji et al., 2005).

Despite the general lack of differences in body mass observed across the groups, body mass alone does not indicate the relative contributions of lean and fat mass to the animal's weight.

Therefore, other metrics are used to evaluate growth performance including empty carcass mass and long bone characteristics. The tibiae and femora, in particular, are more dependable long term growth indicators (Black and Scheuer, 1996). They have a dose-dependent relationship with growth hormone and insulin-like growth factor -1 activity, a fundamental bone growth hormone throughout life (Ernst, 1989). The bone parameters are also more stable long term measures of growth than body mass which can acutely change due to degree of gut fill and hydration status amongst several factors (Baum et al., 1996).

Eviscerating the carcass helps to reduce the impact of viscera on the body mass. This study assessed both empty carcass masses and long bone parameters and we found that there were no adverse effects caused by the interventions in both female and male rats. In addition, insignificant differences were observed in terms of tibial and femoral mass and bone indexes of both female and male rats across the treatment groups. Therefore, these insignificant differences in the empty carcass mass and long bone parameters measurements for female and male rats advocate those different treatments had no adverse effects on the growth performance of the rats.

3.5.2 Sexual dimorphism in the changes in the visceral and epididymal (in male rats) fat masses following administration of a high-fructose and/or stearic acid diet

Obesity indicators were assessed in the current study by directly measuring the masses of the rats' epididymal (in male rats) and visceral fat pads (in both sexes).

Consumption of high-fructose diets is often associated with excess body mass increase and visceral fat deposition (Henry et al., 1991, Tappy and Lê, 2010). Investigations have been undertaken in rat models employing varying fructose dosages as treatments (Mahmoudi et al., 2022, Sabir et al., 2022, Wu et al., 2023a). Fructose consumption accounts for 15-23% of total daily calorie intake; thus, this study employed a 20% fructose solution to imitate the quantity of fructose usually found in foods commonly taken daily, such as beverages (Gillespie et al., 2023).

Fructose is lipogenic by nature and promotes metabolic alterations such as hyperlipidaemia and hypertriglyceridemia, which are harmful and induce metabolic disruptions (Gillespie et al., 2023). Previous research has shown that high fructose consumption causes animals (rats) and human models to gain mass (Gunawan et al., 2021), as well as an increase in visceral adipose tissue and raised circulating concentrations of total cholesterol and triglycerides (Li et al., 2022c). Furthermore, there is an imbalance between energy intake and expenditure associated

with chronic over nutrition, resulting in a surplus energy supply that is stored in adipocytes as triglycerides (Coronati et al., 2022).

Obesity is on the rise, as are poor reproductive consequences, such as infertility (George et al., 2023). Male infertility has been related with a wide variety of factors including epididymal fat accumulation (Katib, 2015), endocrine factors such as those creating increases in oxidative stress thereby influencing the actions of intracellular enzymes (Alahmar, 2019) and factors producing epigenetic modifications impacting spermatogenesis and sperm function (Marzouni et al., 2022). While visceral fat is associated with metabolic diseases, the build-up of epididymal fat affects the quality of sperm and semen, as well as the ability to fertilize (George et al., 2023). As a result, dietary changes at a young age may affect epididymal fat accumulation and can negatively affect fertility.

As indicated in figures 3.4 A (I and II) and figure 3.4 B (I and II), administration of stearic acid alone and/or combined high-fructose and stearic acid diet showed significant changes in the visceral fat masses of female and male rats. Female rats fed a high-fructose diet or a combination of fenofibrate and high-fructose diet had significantly heavier absolute visceral fat masses than control rats fed a standard diet. Our findings are consistent with other metabolic research that have found an association between a high-fructose diet and visceral adiposity (Hernández-Díazcouder et al., 2019). Consumption of the diet enriched with stearic acid did not prevent the rats from increasing visceral fat build-up caused by the high-fructose diet.

In male rats, the high-fructose diet alone did not induce visceral obesity. However, the combined high-fructose and stearic acid diet significantly increased absolute visceral fat mass while male rats whose diet had high-fructose only, stearic acid alone, those fed combined fenofibrate and high-fructose resulted in similar visceral fat. Additionally, epididymal (in male rats) fat masses in male rats was similar across treatment groups. The similarity in visceral and epididymal fat pad masses of male rats on a high-fructose diet shows that it did not contribute to increased adiposity. Therefore, while it is suggestive that due to absence of differences in epidiymal fat accumulation sperm and semen quality might not have been affected by the interventions, there is need for more direct fertility assessments to be carried out in future.

The sexually dimorphic effects noticed in this study confirm previous reports that female rats are more susceptible to fat accumulation than male rats (Braga Tibaes et al., 2022, Velasco et al., 2020). These differences have been attributed to sex steroid hormone differences in female and male rats (Chang et al., 2018). Additionally, differences in metabolic rates could also have had an impact. In humans, females tend to have more adipose tissue than males, these depots

offer a mechanism for long-term lipid storage and hunger resistance (Chang et al., 2018). Furthermore, these visceral and subcutaneous adipose tissues produce distinct adipokines which may explain the observed sexual dimorphism (Mittal, 2019). Therefore, long-term fructose-fed female rats exhibit visceral obesity, possibly due to significant lipogenic turnover from the diet.

In the current study, in male rats, stearic acid alone administration as an intervention resulted in a reduction of visceral fat compared to normal. This is most likely due to stearic acid's capacity to bind to the PPARα, which increases the expression of genes involved in fatty acid transport and oxidation (Varga et al., 2011).

After discussing the findings that visceral obesity can have an impact on metabolic health, it is important to consider the effect of stearic acid on plasma or fasting metabolite and hormones that regulate metabolism in both female and male growing Sprague-Dawley rats.

3.5.3 The administration of combined high-fructose and stearic acid had no impact on high-fructose diet-induced hypercholesterolemia and hypertriglyceridemia, although it led to hyperadiponectinaemia. The stearic acid alone as a supplement lowered cholesterol and resulted in hypotriglyceridaemia and hyperadiponectinaemia

In this study, the effect of a high-fructose diet on the development of metabolic dysfunction in female rats for 13 weeks caused hypercholesterolemia, hypertriglyceridemia, hypoadiponectinaemia and had no effect on plasma glucose, insulin and HOMA-IR index, as shown in tables 3.1-3.3 and figures 3.1-3.3. On the other hand, in male rats, high-fructose diet had no effect in all the parameters as shown in tables 3.1-3.3 and figures 3.1-3.3. The study showed that the rats' responses to high-fructose consumption differed by gender. Females were more vulnerable to high-fructose diet-induced metabolic dysfunction than males.

Fructose consumption increases non-esterified free fatty acids in the blood, elevates blood triglycerides, low-density lipoprotein cholesterol and decreases high-density lipoprotein cholesterol and is linked to enhanced hepatic *de novo* lipogenesis (Lorenzatti and Toth, 2020). Multiple studies on rats and humans have indicated that high-fructose diet induces hypercholesterolemia and hypertriglyceridemia (Özkan et al., 2022). Metabolic studies discovered that feeding female and male rats a high-fructose diet (20%) for 6 - 8 weeks induced hypercholesterolemia and hypertriglyceridemia (Chan et al., 2021, Wong et al., 2018). The current study observed that in female rats, high-fructose consumption induced

hypercholesterolemia and hypertriglyceridemia. Our findings are consistent with previous research that have found an association between high-fructose consumption in that it increases plasma cholesterol and triglyceride concentrations.

Several studies have demonstrated that consumption of saturated fatty acids increase low-density lipoprotein cholesterol and apolipoprotein concentrations, which can lead to cell and tissue lipotoxicity and as a result, increase the risk of heart disease (Hodson et al., 2020, Roumans et al., 2020). The current study observed that in female rats, stearic acid alone as a dietary supplement resulted in lower cholesterol than the negative control. In addition, stearic acid intervention had no impact on high-fructose induced hypercholesterolaemia and hypertriglyceridemia.

Stearic acid has been reported to reduce blood cholesterol concentrations more effectively than its counterpart's lauric, myristic and palmitic acids in several studies (Bonanome and Grundy, 1988, Grundy, 1994, Hunter, 2001). Although evidence for stearic acid's therapeutic benefits is growing, the mechanisms by which it mitigates hypercholesterolaemia and hypertriglyceridemia have yet to be fully explained. It is proposed that the blood cholesterol lowering ability of stearic acid is attributed to the inhibitory activity of 5-alpha reductase (Gobalakrishnan et al., 2020), which blocks the β-hydroxy β-methylglutaryl-CoA reductase in the cholesterol biosynthetic pathway (Igwe et al., 2015, Vadivel and Gopalakrishnan, 2011).

Fenofibrate reduces obesity by activating peroxisome proliferator-activated receptor alpha lipolysis and fatty acid oxidation, lowering triglyceride concentrations, low-density lipoprotein cholesterol and increasing high-density lipoprotein cholesterol (Ferreira et al., 2006). In the current study, the fact that fenofibrate lowered the triglyceride concentration caused by a high-fructose diet in female rats confirming the known lipid lowering effects of fenofibrate. However, it did not lower cholesterol, possibly due to the dose used. In several studies, fenofibrate has been shown to reduce high-fructose induced triglyceride and cholesterol concentrations more efficiently in females than males (Abdelmoneim et al., 2021, Mancini et al., 2001, Miceli et al., 2021).

In male rats, the current study observed no significant differences in the plasma triglyceride and cholesterol concentrations across the treatment groups fed a high-fructose diet and/or stearic acid for 13 weeks. This suggests sex differences in how rats respond to high fructose diets with female rats being more prone to metabolic response than males (Roca et al., 1999, Salinero et al., 2018, Stubbins et al., 2012). The physiological effect of fructose is

predominantly dependant on its concentration (De Souza et al., 2021) and duration of consumption (Beisner et al., 2020). For example, Sprague Dawley rats administered 20% fructose solution induced hypercholesterolaemia and hypertriglyceridemia (Ibrahim et al., 2017). The findings of this study vary from those of the previous studies, which used Sprague Dawley rats of the same sex, fructose content and duration of consumption. In another related study, male Wistar rat pups fed a 20% fructose solution for 8 weeks demonstrated an increase in plasma triglyceride and cholesterol contents (Sandeva et al., 2015).

Adiponectin influences several metabolic processes, including glucose control and fatty acid breakdown (Nguyen, 2020). Hyperadiponectinemia is associated with a decreased risk of type II diabetes and other obesity-related diseases affected by insulin resistance (Gariballa et al., 2019, Nguyen, 2020). Adiponectin increases insulin sensitivity and pancreatic-β cell survival and functionality (Nguyen, 2020). In adipocytes, adiponectin secretion, stimulated by the peroxisome proliferator-activated receptor gamma antagonists is said to have profound positive health benefit effects (Nguyen, 2020, Parida et al., 2019). In this study, high-fructose consumption significantly reduced adiponectin concentrations in female rats than negative control rats. Meanwhile, in male rats, high-fructose consumption did not significantly affect adiponectin concentrations. The findings in this study are consistent with those of Sharabi et al. (2007) who found that high fructose (60%) feeding to Sprague-Dawley rats for 5 weeks did not significantly plasma adiponectin concentrations. Visceral adiposity triggers the development of pro-inflammatory adipokines, which block adiponectin (Stranahan, 2022). In this study, we therefore linked the observed visceral adiposity due to high-fructose diet to the decreased adiponectin concentrations.

In this study, stearic acid combined with a high-fructose diet resulted in hyperadiponectinaemia in both female and male rats. Increased adiponectin concentrations hinder gluconeogenesis and contribute to the control of glucose absorption and lipid metabolism, hence lowering triglyceride build-up (Zhou et al., 2005). In this study, it is likely that stearic acid benefits both female and male rats via increasing adiponectin concentrations (see tables 3.3 A and B). As a result, in the current study, the variations observed in visceral fat accumulation (described in section 3.5.2) may potentially explain the changes in adiponectin concentrations. Our findings are consistent with other metabolic research that have found an association between hyperadiponectinaemia being inversely proportional with obesity, hyperlipidaemia and type 2 diabetes (Nguyen, 2020).

Furthermore, stearic acid alone as a dietary supplement resulted in hyperadiponectinemia in both female and male rats. In our study, administering stearic acid as a supplement improved metabolic dysfunction indicators such as circulating lipids and visceral fat in female rats. Furthermore, the increase in adiponectin concentrations had no effect on fasting blood glucose, plasma insulin, or HOMA-IR indices in female and male rats (see section 3.5.4). The increase in adiponectin may have contributed to stearic acid's overall beneficial effect on metabolic dysfunction.

In this study, both female and male rats fed a combined fenofibrate and high-fructose diet resulted in hyperadiponectinaemia. The increase in plasma adiponectin concentrations may be linked to fenofibrate's ability to lower the visceral fat induced by a high-fructose diet. This result supports prior studies indicating that fenofibrate stimulates peroxisome proliferator-activated receptor-alpha in adipose tissue, increasing adiponectin concentrations (Fougerat et al., 2020, Jin et al., 2023). As a result, the current study's observed rise in adiponectin concentration might be attributable to a same mechanism.

3.5.4 In terms of fasting glucose concentrations, insulin and Homeostasis model assessment of insulin resistance, administration of a high-fructose and/or stearic acid diet revealed no significant changes.

Increased fasting concentrations of glucose in the blood are indicative of type II diabetes mellitus. Administration of a high-fructose diet to rats has been demonstrated in most studies to raise blood glucose concentrations (Lutsey et al., 2008, Zhao et al., 2022). On the contrary, there have been studies that show no effect on blood glucose after high-fructose consumption (Ibrahim et al., 2019), for example, showed that a 20% fructose diet administered to rat pups for 7 weeks had no effect on the rats' fasting blood glucose concentrations. In the current study, fasting blood glucose concentrations were determined following a 12-hour fast and we anticipated that long-term high-fructose consumption by the rats would cause hyperglycemia. Our results showed that female and male fasting blood glucose concentrations in this study were similar across treatment groups. This implies that the combined high-fructose and stearic acid, stearic acid alone as well as combined fenofibrate and high-fructose diets as interventions had neither hyperglycaemic nor hypoglycaemic effects. Furthermore, our findings suggest that the insignificant differences might be due to feeding duration, particularly in growing rats where calories may be diverted toward development. Although blood glucose concentrations in the treatment groups may be normal, this could be due to increased insulin production due

to early stages of insulin resistance, as a result of fructose consumption, which maintains normal blood concentrations (Kovačević et al., 2021). As a result, measuring blood concentrations of insulin is critical in metabolic studies.

The main risk factor for the development of insulin resistance and hyperinsulinemia is an excessive calorie intake in the form of a high fructose diet (Basciano et al., 2005, Softic et al., 2020). The insulin concentrations of female and male rats in the current study were similar across treatment groups. This suggests that supplementation with high-fructose and/or stearic acid diet as well as with fenofibrate diet had no effect on insulin concentration. We therefore had to further analyse if these findings were physiologically meaningful and estimate insulin resistance by computing the homeostatic model assessment index. Insulin resistance is indicated by an increase in HOMA-IR (Wang et al., 2024). The current study found no differences in HOMA-IR across female and male rats in different treatment groups. The findings imply that the treatment interventions did not result in insulin resistance.

3.6 Conclusion

Given the worldwide health risk of metabolic dysfunction, there is currently no viable pharmacologic intervention and/or therapy for this entity as a whole. Novel strategies are needed to address the obesity epidemic. The study hypothesized that administering stearic acid alone or in combination with high-fructose will prevent the development of metabolic dysfunction in growing Sprague Dawley rats. This study found that of the measured parameters following the interventions, the high-fructose diet affected plasma metabolic substrates, adiponectin concentrations and visceral obesity. The results of the current study revealed that there are some sex variations in responsiveness to high-fructose and stearic acid treatments. Stearic acid resulted in a significant increase in adiponectin concentrations, it is probable that stearic acid has its positive effects via increasing the concentrations of adiponectin thereby reversing the high-fructose diet-induced metabolic dysfunction. Stearic acid might be employed to counteract diet-induced metabolic dysfunction.

The current chapter studied the effects of dietary supplementation with stearic acid (standard rat chow enriched with 3% stearic acid) on high-fructose (20% fructose drinking solution) diet-induced metabolic dysfunction in growing female and male Sprague Dawley rat pups, specifically growth performance, fasting blood glucose, determined fasting lipids (plasma triglycerides and total-cholesterol) and visceral obesity. The influence of these treatments on the development of non-alcoholic fatty liver disease is evaluated in the next chapter.

CHAPTER 4

THE COMBINED EFFECTS OF A DIET ENRICHED WITH STEARIC ACID AND HIGH FRUCTOSE ON THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE IN GROWING FEMALE AND MALE SPRAGUE-DAWLEY RATS

4.1 Introduction

The previous chapter described the combined effect of a stearic acid and high-fructose on the general metabolic health profiles of growing female and male Sprague-Dawley rats. The liver is an essential organ in regulating metabolism in rats. Inappropriate diets may cause developmental disorders, which result from hepatic malfunction. Non-alcoholic fatty liver disease is the most common pathology associated with calorie excess, caused by ingesting high-energy foods such as fructose. Therefore, this current chapter assesses the impact of a diet enriched with stearic acid and high fructose concentrations on the development of Sprague-Dawley rats.

4.1.1 Mechanisms of non-alcoholic fatty liver disease caused by high-fructose intake

The most prevalent chronic liver disease is non-alcoholic fatty liver disease, which is considered the hepatic manifestation of metabolic syndrome without any excessive alcohol consumption (Decraecker et al., 2022, Rinaldi et al., 2021). Histopathological abnormalities include simple steatosis progressing through to non-alcoholic steatohepatitis (with or without fibrosis) and then to cirrhosis (Federico et al., 2021).

In the literature, multiple pathways have been described that link high-fructose intake to the development of non-alcoholic fatty liver disease, including reduced peroxisome proliferator-activated receptor alpha expression and activity (Montagner et al., 2016), insulin resistance (Basciano et al., 2005), inflammation and oxidative stress (Federico et al., 2021, Scorletti and Carr, 2022, Varga et al., 2011). Given that fructose is a highly lipogenic monosaccharide, it can promote insulin resistance (Basciano et al., 2005) and eventually hepatic steatosis (Yustisia et al., 2022). Hyperinsulinemia occurs in response to systemic insulin resistance, which promotes hepatic *de novo* lipogenesis while impairing free fatty acid β-oxidation, resulting in triglyceride build-up in the liver and development to fatty liver (Leslie et al., 2022, Pal and Méndez-Sánchez, 2023, Singh et al., 2023).

Furthermore, *de novo* lipogenesis generates over twenty-five percent of the intrahepatic lipid in obese and non-alcoholic fatty liver people (Barazesh et al., 2024). Fructose enters hepatocytes via GLUT5 **(**transporter that specifically transports fructose) and intracellular fructose is converted into fructose-1-phosphate by fructokinase (Dholariya and Orrick, 2022, Hernández-Díazcouder et al., 2019). Fructose-1-phosphate is then converted into pyruvate and acetyl-CoA for use in the tricarboxylic acid cycle. Fructose-1-phosphate, fructose and their intermediates can activate carbohydrate-responsive element-binding protein-β (Santos et al.,

2022, Yu et al., 2021), c-Jun N-terminal kinase and/or insulin receptor substrates (Arslanbulut and Çiftçi, 2023, Yu et al., 2021), peroxisome proliferator-activated receptor gamma co-activator-1 (Arslanbulut and Çiftçi, 2023, Singh et al., 2023) and/or sterol regulatory element-binding protein-1 (Elseweidy et al., 2022) and/or acetyl-CoA carboxylase 1 and downstream *de novo* lipogenesis (Geidl-Flueck and Gerber, 2023, Softic et al., 2016) pathways during this process. Genes encoding fatty acid oxidizing enzymes are downregulated in response to increased fructose intake (DiStefano, 2020), therefore worsening hepatic lipid build-up. Therefore, the consumption of diets rich in fructose can result in the manifestation non-alcoholic fatty liver disease.

The next section will elaborate how short-chain fatty and amino acids are also substrates for hepatic *de novo* lipogenesis, resulting in accumulation of lipids in hepatocytes and an increase in circulating fatty acids.

4.1.2 Fat deposition and hepatic pathology

In *in vivo* studies in rats, dietary consumption of rat chow enriched with high-fructose and saturated fatty acids causes' fat accumulation in the liver (Park et al., 2020, Roumans et al., 2020). This fat accumulation is visualised as lipid droplets on histological sections of hepatic tissue. The condition is characterised as hepatic steatosis, which eventually leads to cirrhosis on long-term exposure to this combined high-fructose and saturated fatty acid diet. Not only are there increased concentrations of triglycerides, but there is also increased synthesis of cholesterol in the hepatocytes (Heida et al., 2021, Horn et al., 2022).

A high intake of saturated fatty acids has also been observed in non-alcoholic steatohepatitis patients (Berná and Romero-Gomez, 2020). When one consumes fats in the diet, they are instantly broken down into free fatty acids, monoglycerides and cholesterol for ultimate packing and transportation (Malik et al., 2023). The liver secretes bile acids and bile salts, which enter the intestinal lumen via the duct and act on the fat globules further breaking them down (Torcello-Gómez and Foster, 2014). Pancreatic lipases additionally act on these fat globules, breaking them down further to produce surface-optimal free fatty acids, monoglycerides and cholesterol (Chen et al., 2023). These surface-optimal lipid components (free fatty acids, monoglycerides and cholesterol) are then bundled into micelles, which are passively absorbed by small intestine enterocytes. Once finally, in the enterocyte, free fatty acids and monoglycerides are resynthesized into triglycerides by the endoplasmic reticulum (Zhang et al., 2021b). These triglycerides are then packaged into chylomicrons (Borén et al., 2020, Rahmany and Jialal, 2019), which diffuse out of the basolateral membrane of the

enterocyte into the lacteal system. The lacteal system transports lipids into the circulation, where they are transported to numerous tissues throughout the body, including the liver, adipose tissue and muscle, where they are stored (Malik et al., 2023).

As a result, consuming lipid-rich diets might result in the development of non-alcoholic fatty liver disease. Saturated fatty acid metabolism in the liver generates substrates for *de novo* lipogenesis (Hodson et al., 2020, Roumans et al., 2020) and stimulates hepatic lipid synthesis and storage (Mashek, 2021), resulting in an increase in liver mass (Tamer et al., 2020).

When there is excess adiposity and insulin resistance, adipocytes generate more free fatty acids (Ahmed et al., 2021). Following release, free fatty acids enter the circulation and may overwhelm the liver's capacity to metabolize or export lipids, contributing to the development of steatosis (Yustisia et al., 2022). The liver's intake of circulating fatty acids is mostly dependent on fatty acid transporters, which include fatty acid transport proteins and the cluster of differentiation 36, which are situated in the hepatocyte plasma membrane (Ipsen et al., 2018, Keles et al., 2023). Fatty acid transport proteins 2 and 5 are largely located in the liver and function as a facilitator of hepatic steatosis through fatty acid transport protein-mediated lipid uptake (Ipsen et al., 2018). Additionally, the fatty acid translocase protein, cluster of differentiation 36, aids in the transport of long-chain fatty acids (Jia et al., 2023).

We have addressed the mechanism by which dietary lipids are converted to fatty acids, as well as how fatty acids are supplied to the liver from adipose. Dietary treatments to cure and/or prevent and/or ameliorate liver problems are being sought after in medicine.

4.1.3 Stearic acid may be useful

Stearic acid is a saturated fatty acid that can be found in the food chain (Sampath and Ntambi, 2005). Diets high in saturated fat are considered detrimental to metabolic health given that they have been associated with the development of obesity, hepatic steatosis, metabolic dysfunction and type II diabetes in humans (Nishitani et al., 2007, Zhou et al., 2020). *In vivo* studies show that feeding rats high-fat diets raises hepatic and plasmatic triglyceride concentrations (Lozano et al., 2016, Roumans et al., 2020). These findings are consistent with those seen in human investigations.

Lifestyle changes are the major therapy method for non-alcoholic fatty liver disease. Diet appears to be vital for the development of high-fructose induced hepatic steatosis. Due to the complicated pathophysiology of this disorder, there are currently no precise dietary suggestions

for the treatment of non-alcoholic fatty liver disease. It was predicted in the current study that a stearic acid diet would decrease hepatic steatosis caused by excessive caloric consumption.

Unlike most other saturated fats, the physiological effects of stearic acid on the human body are interesting. Stearic acid has hypolipidaemic action (Bonanome and Grundy, 1988, Mensink, 2005) to it possessing a 5-alpha reductase inhibitor activity (Vadivel and Gopalakrishnan, 2011), which hinders 3-hydroxy-3-methylglutaryl-CoA reductase, a critical enzyme in the cholesterol synthesis pathway. In fact, contrary to the expected increase in fat deposition that would occur when saturated fat is consumed in excess, it has been reported that stearic acid decreases cholesterol accumulation in the liver (Nie et al., 2022, Pan et al., 2010). Secondly, stearic acid may operate as a natural ligand of the peroxisome proliferator-activated receptor, protecting cells against oxidative damage (Balakumar et al., 2019, Montagner et al., 2016). Activation of the peroxisome proliferator-activated receptor protects by targeting hypolipidemic fibrates and increasing the uptake of fatty acids and activates their β-oxidation (Berger and Moller, 2002). Furthermore, stearic acid-treated hepatocytes generated higher interleukin-10, an anti-inflammatory cytokine (Nishitani et al., 2007, Pan et al., 2010). The anti-inflammatory cytokine interleukin-10 has hepatoprotective properties (Nishitani et al., 2007). Stearic acid has been shown to stimulate interleukin-10 production in hepatocytes. Stearic acid has been proven to protect the liver against cholestasis-induced damage (Pan et al., 2010).

The body of literature on stearic acid is not extensive. There has been very little research on the influence of dietary fatty acids on the development of non-alcoholic fatty liver disease. We opted to include stearic acid in the diet to test stearic acid's function in preventing the effects of a high-fructose diet based on studies that suggest a hepatic steatosis lowering activity of stearic acid, which may also be hepatoprotective. So we asked the question, ‘Does stearic acid-enriched diet intake from early in life protect against the development of non-alcoholic fatty liver disease in growing female and male Sprague-Dawley rats?’ This study's findings will provide the groundwork for future research into the use of stearic acid to prevent the development of high-fructose-induced non-alcoholic fatty liver disease in people.

4.2 Aim and objectives

This study aimed to investigate the effect of a stearic acid alone and/or combinatorial high-fructose and stearic acid diet in the physiopathology of high-fructose diet-induced non-alcoholic fatty liver disease in growing female and male Sprague-Dawley rats.

Hypotheses

H_0: Administration of a stearic acid alone and/or combinatorial high-fructose and stearic acid diet to growing Sprague-Dawley rats has no effect on the development of non- alcoholic fatty liver disease.

H_1: Administration of a stearic acid alone and/or combinatorial high-fructose and stearic acid diet to growing Sprague-Dawley rats has an effect on the development of non- alcoholic fatty liver disease.

The specific objectives of this part of the study focused on non-alcoholic fatty liver disease by assessing the effects of the dietary interventions on:

4.2.1 Liver masses, both absolute and relative to body mass.

4.2.2 Hepatic lipid content and profile of liver fatty acids.

4.2.3 Hepatic histomorphology by computing hepatic scores of steatosis as markers of non-alcoholic fatty liver disease and non-alcoholic steatosis.

4.3 Materials and methods

4.3.1 Ethical clearance for the use of animals and study site

Details of ethical clearance and study site are as described in Chapter 3, subheading 3.3.3.

4.3.2 Animals, housing and feeding

Housing, feeding and care of the experimental rats were as described in Chapter 3, subheading 3.3.4.

4.3.3 Experimental design and treatments

The detailed experimental design and treatments administered to the rats was as described in chapter 3, subheading 3.3.5.

4.3.4 Liver sample collection

The detailed liver sample collection was as described in chapter 3, subheading 3.3.7. The collected livers were weighed to get absolute masses while the mass relative to body mass (hepatosomatic index) was computed by dividing the mass of each liver by the respective terminal body mass of each rat and expressed as a percentage (%).

4.3.5 Determination of hepatic lipid content and fatty acid profiling

The previously stored, frozen at -20 ºC stored liver samples were allowed to stand at room temperature for 20 to 30 minutes to thaw out. A portion of each liver was cut and pooled. About 50 g of liver samples (per treatment group) was weighed into a pre-weighed plastic container and then freeze dried using a desktop preconditioned freeze dryer (Specht Scientific Engineering (SSE), Cologne, Germany) connected to a vacuum pump (Sogevac SV28, Leybold GmbH, Cologne, Germany) with a conditioner temperature of -50ºC and shelf temperature of 26ºC. The combined liver samples from each treatment regimen were then ground into powder using a laboratory mortar and pestle. These were then weighed and packaged into ziplock plastic bags and sent to the Agricultural Research Council Irene Analytical Services Laboratories for hepatic fatty acid profiling and the analysis of the total hepatic lipid content using the soxhlet method. The methods are listed by the South African National Accreditation System (SANAS) with ASM 044 for hepatic total lipid content using a Soxtec System HT 1043 Extraction Unit (Foss Analytical, HillerØd, Denmark) and ASM 023 for fatty acid profiling. See appendix for raw results.

4.3.6 Processing of liver for histology analysis

For morphological analysis of liver samples, the previously formalin-preserved tissues were used. The liver tissue samples were processed using an automatic tissue processor (Microm GmbH STP 120, Walldorf, Germany) which lasted for 16 hours. Following processing, each tissue sample was embedded in paraffin wax using a waxing machine (Thermolyne, Histo-Center II – N, Labotec, South Africa) and made into blocks and then sections of the liver were cut at 3µm thickness using a rotary microtome (Leica Biosystems, model RM2125 RTS, Nussloch, Heildelburger, Germany) and MB35 Premier microtome blade (34〞 80 mm). Thereafter the sections were mounted on glass slides with frosted ends (Lasec Laboratories) before staining. The slides were stained with haematoxylin and eosin (H & E) for tissue architecture and Masson's trichrome (MT) for connective tissue using methods as previously described by (Bancroft and Gamble, 2008). The slides were then placed in mounting xylene and mounted with DPX mounting media (Merck Chemicals, Pty Ltd) and cover slip (Deckglaser 24 x 40 mm).

4.3.7 Liver histological analyses

4.3.7.1 NAFLD Activity Score

A light microscope (Reichert®, Austria) with CCD camera was used to view the slides at 400x magnification. The H&E stained liver sections images were used to score for steatosis and inflammation as described by (Brunt et al., 1999, Kleiner et al., 2005) modified by (Liang et al., 2014). Based on the total area affected and by analysing hepatocellular vesicular, steatosis was determined. Both the macrovesicular steatosis (when vacuoles displaced the nucleus to the side) and microvesicular steatosis (vacuoles not displaced the nucleus to the side) were scored based on the percentage of the total area affected or field of the liver parenchyma. Both steatosis and hypertrophy were assessed at a 100X magnification. The following categories (table 4.1) were used to grade steatosis (Santiago-Rolón et al., 2015). By counting the number of inflammatory cell aggregates in the liver parenchyma, inflammation was scored (at 100X magnification) and graded using the criteria described by Liang et al., (2014) as shown in table 4.1 below.

Table 4.1. Grading system for rodent non-alcoholic fatty liver disease (adapted from Liang et al., 2014).

Histological feature	**Score**			
	0	**1**	**2**	**3**
Macrovascular steatosis	<5%	5–33%	33–66%	>66%
Microvascular steatosis	<5%	5–33%	33–66%	>66%
Hypertrophy	<5%	5–33%	33–66%	>66%
Inflammation: # of inflammatory foci per field	<0.5	0.5–1.0	1.0–2.0	>2.0

4.3.7.2 Quantitative analysis of hepatic fibrosis by stereological method

Masson trichrome stained slides were utilized to analyse the fibrosis in the liver based on images collected at 100x objective. The fibrosis was assessed using ImageJ software (ImageJ 1.47v/Java 1.6.0_045, Oracle America Inc.) (Schneider et al., 2012). The area fraction ($A_{fraction}$) was utilized to provide quantitative information on the area per point (A_p), length, numerical density, and volume using a simple point counting method. Thereafter, the point count technique was used to calculate the area per point (A_p) and area fraction ($A_{fraction}$) of connective tissue in each liver section, resulting in the total of points ($\sum_p$). The area per point was determined using the following formula (Ibrahim et al., 2019):

$A = A_p \times \sum p$,

A_p = area per point (17.39)

A = area

$\sum p$ = sum of points falling on the connective tissue within a camera field of each liver section (7339.52 μm²)

Distance in pixels was set at 162.

The area fraction of an image measuring 594501.7232 μm² was calculated using the following formula:

$A_{fraction} = [(A \div 594501.7232\ \mu m^2) \times 100]$.

$A_{fraction}$ = area fraction

A = area

4.3.8 Statistical analysis

Data analysis was done using GraphPad Prism 7 software (Graph-pad Software Inc., San Diego, USA). All normally distributed data were expressed as mean ± standard deviation and skewed data (non-alcoholic fatty liver disease score, NAS) are expressed as median and range (min, max). Group means for comparisons between different dietary treatment groups were analysed using one-way analysis of variances (ANOVA) and mean comparison using a Tukey post-hoc test with statistical significance of $p < 0.05$ considered. A Kruskal-Wallis test was used to analyse non-alcoholic fatty liver disease score (NAS) multiple group data followed by a Dunns post hoc test to compare the medians. The Dunn's test was selected instead of other post-hoc tests to determine exactly which groups are different. Statistical significance was set at $p < 0.05$. For the data presented in the table, the statistical level of significance (p-value) is that of the overall group differences before applying post hoc test.

4.4 Results

4.4.1 Liver masses

The liver is an essential organ for the processing and storage of metabolites, the quantity of metabolites stored can have an impact on liver mass. The absolute and relative (to body mass) masses of the livers obtained from female rats following the 13-week treatment intervention are presented in table 4.2 A.

In female rats, overall absolute liver masses of the rats across treatment groups were significantly different ($p = 0.0246$), one-way ANOVA, $F_{(4, 34)} = 3.204$. The female rats whose diet was supplemented with a high-fructose alone had similar absolute liver masses ($p = 0.72$) when compared to the negative control rats fed a standard diet. Similarly, female rats that received a treatment of combined stearic acid and high-fructose diet ($p = 0.87$) and their counterparts whose diet had stearic acid alone ($p = 0.99$) as well as those whose diet had combined fenofibrate and high-fructose ($p = 0.06$) had similar absolute liver masses in comparison to the negative control rats fed a standard diet ($p > 0.05$). However, stearic acid alone as a dietary supplement caused a lower absolute liver mass ($p = 0.02$) than female rats fed a combined fenofibrate and high-fructose diet (table 4.2 A).

In summary, a high-fructose diet had no impact on absolute liver masses in female rats. Furthermore, a combined stearic acid and high-fructose, stearic acid alone and a combined fenofibrate and high-fructose diets all resulted in similar absolute liver masses. Stearic acid alone fed rats, on the other hand, resulted in lower absolute liver masses than those fed a combined fenofibrate and high-fructose diet.

The overall differences observed in relative (to body mass) liver masses of the female rats across treatment groups were statistically significant ($p = 0.0068$), one-way ANOVA, $F_{(4, 34)} = 4.250$. As shown in table 4.2 A, there were insignificant differences ($p > 0.05$) in relative (to body mass) liver masses of the female rats whose diet was high-fructose ($p = 0.3014$), combined stearic acid and high-fructose ($p = 0.2704$) as well as those fed a stearic acid alone ($p = 0.9951$) when compared to the negative control rats fed a standard diet. Rats administered a combined fenofibrate and high-fructose diet had significantly ($p =0.0362$) heavier relative (to body mass) liver masses compared to the mass of the livers of rats administered a standard diet. Stearic acid alone as a dietary intervention resulted in significantly ($p = 0.0139$) lower relative (to body mass) liver masses than female rats fed a combined fenofibrate and high-fructose diet.

In summary, the relative (to body mass) liver masses of the female rats fed a high-fructose, combined stearic acid and high-fructose and those fed stearic acid alone diets were similar. We observed that a combined fenofibrate and high-fructose diet, on the other hand, resulted in significant higher relative (to body mass) liver masses compared to those fed a negative control standard diet. We also observed heavier relative (to body mass) liver masses in female rats as a result of administration of combined fenofibrate and high-fructose diet than those fed a stearic acid diet.

The absolute and relative (to body mass) masses of liver of the male rats following a 13-week dietary treatment period are presented in table 4.2 B. In males, overall absolute liver masses of the rats across treatment groups were not significantly different ($p = 0.5809$), one-way ANOVA, $F_{(4, 34)} = 0.7251$.

The overall, relative (to body mass) liver masses of the male rats across treatment groups were significantly different ($p = 0.0094$), one-way ANOVA, $F_{(4, 34)} = 3.98$. Male rats administered a combined fenofibrate and high-fructose diet had significantly ($p = 0.0092$) heavier relative (to body mass) liver masses compared to the masses of the livers of negative control male rats administered a standard diet. On the other hand, the relative (to body mass) liver masses of the male rats were similar ($p > 0.05$) in those whose diet was high-fructose ($p = 0.2096$), combined stearic acid and high-fructose ($p = 0.2320$) as well as those fed stearic acid alone ($p = 0.7354$) when compared to the negative control rats fed a standard diet. Male rats administered a combined fenofibrate and high-fructose diet had significantly ($p = 0.0184$) heavier relative (to body mass) liver masses than the mass of the livers of rats administered stearic acid alone as a dietary intervention.

In summary, the fenofibrate with high fructose dietary intervention induced heavier relative (to body mass) liver masses in male rats compared to negative control male rats.

Table 4.2 A. The effects of stearic acid on absolute and relative to body mass (rBM), liver masses in growing Sprague-Dawley female rats fed a high-fructose diet and/or stearic acid for 13 weeks.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Liver mass (g)	7.6 ± 0.5^{ab}	8.1 ± 0.8^{ab}	8.0 ± 0.9^{ab}	7.5 ± 0.6^{b}	8.6 ± 0.7^{a}	* (p = 0.0246)
Liver rBM (%)	2.8 ± 0.2^{a}	3.0 ± 0.3^{ab}	3.1 ± 0.2^{ab}	2.8 ± 0.1^{a}	3.2 ± 0.2^{b}	** (p = 0.0068)

Data presented as mean ± standard deviation. [a] Within rows, means with similar superscripts are not significantly different at $p < 0.05$. [a, b] Within row means with different superscripts differ significantly at ($P \leq 0.05$). The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 4.2 B. The effect of stearic acid on absolute and relative to body mass (rBM) liver masses in growing Sprague-Dawley male rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Liver mass (g)	12.5 ± 1.5^{a}	12.5 ± 1.6^{a}	12.8 ± 1.5^{a}	12.1 ± 1.2^{a}	13.3 ± 1.7^{a}	($p > 0.05$)
Liver rBM (%)	2.8 ± 0.3^{a}	3.0 ± 0.3^{ab}	3.0 ± 0.2^{ab}	2.8 ± 0.2^{a}	3.2 ± 0.2^{b}	** ($p = 0.0094$)

Data presented as mean ± standard deviation. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. [a, b] Within row means with different superscripts differ significantly at ($P \leq 0.05$). Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

4.4.2 Hepatic lipid content

Figures 4.1 A and B show the effect of combined diet enriched with stearic acid and high fructose on hepatic lipid content of growing female and male Sprague-Dawley rats following 13-week dietary treatments.

In female rats, the overall liver lipid content was statistically different ($p < 0.0001$, one-way ANOVA, $F_{(4, 10)} = 103.8$) in all rats across treatment groups.

Female rats fed a high-fructose diet had the highest liver lipid content ($p < 0.0001$) compared to negative control rats fed a standard diet, rats fed a combined stearic acid and high-fructose diet ($p < 0.0001$), rats given stearic acid alone diet ($p = 0.0002$) and rats given a combined fenofibrate and high-fructose diet ($p < 0.0001$). The female rats given a combined stearic acid and high-fructose diet as well as the group to which a combined fenofibrate and high-fructose diet was given had similar ($p > 0.999$) liver lipid content but significantly lower when compared to both the negative control rats ($p = 0.0003$) and the stearic acid only group ($p < 0.0001$). Female rats administered a high-fructose diet exhibited a 22.9% significant increase ($p < 0.0001$) in hepatic lipid content compared to negative control rats fed a standard diet, as shown in figure 4.1 A, whilst those given stearic acid alone had a 6.6% higher ($p = 0.0819$) hepatic lipid content. Female rats fed a combined stearic acid and high-fructose diet ($p = 0.0003$), as well as those provided a combined fenofibrate and high-fructose diet ($p = 0.0003$), exhibited a 15.5% decrease in hepatic lipid content when compared to negative control rats fed a standard diet. While a high-fructose diet increased ($p < 0.0001$) hepatic lipid content, female rats fed a combined high-fructose and stearic acid diet, as well as those fed a combined high-fructose and fenofibrate diet both as interventions, significantly decreased ($p < 0.0001$) high-fructose-induced hepatic lipid accumulation by approximately 31%. When compared to negative control rats on a standard diet, stearic acid alone resulted in a 15.5% reduction in hepatic lipid content in female rats.

In summary, a high-fructose diet increased hepatic lipid content. The administration of combined stearic acid and high-fructose diet prevented the high-fructose-induced hepatic lipid content accumulation. The hepatic lipid content of rats fed a stearic acid-only diet and those fed a standard diet was similar. Fenofibrate, as an intervention, prevented high-fructose-induced hepatic lipid content even below the hepatic lipid content of negative control rats. Additionally, fenofibrate, as an intervention, lowered hepatic lipid content compared to the

stearic acid alone diet fed rats. The hepatic lipid content of the combined stearic acid and high-fructose diet was similar to that of the combined fenofibrate and high-fructose diet.

In male rats, the overall hepatic lipid content of the rats across treatment groups were significantly different ($p < 0.0001$), one-way ANOVA, $F_{(4, 10)} = 20.39$). Male rats fed a high-fructose diet had the highest liver lipid content compared to the rest of the rats in different treatment groups. Male rats administered a high-fructose diet exhibited a 8.6% significant increase ($p = 0.0062$) in hepatic lipid content compared to negative control rats fed a standard diet, as shown in figure 4.1 A, whilst those administered a combined fenofibrate and high-fructose diets demonstrated a significant 6.6% decrease ($p = 0.0305$) in hepatic lipid content. Combined fenofibrate and high-fructose diet significantly decreased (14%, $p < 0.0001$) the hepatic lipid content induced by the high-fructose diet in male rats. On the other hand, figure 4.1 B shows that a combined stearic acid and high-fructose diet ($p > 0.05$), failed to prevent high-fructose diet-induced hepatic lipid content. Stearic acid alone diet showed a significant ($p = 0.0014$, 10%) more hepatic lipid content than the combined fenofibrate and high-fructose diet hepatic lipid content.

In summary, a high-fructose diet increased hepatic lipid content in male rats when compared to the control, but combined fenofibrate and high-fructose treatment significantly lowered hepatic lipid content. Diet treatments of combined stearic acid and high-fructose failed to attenuate high-fructose-induced hepatic lipid accumulation. Fenofibrate treatment reduced high-fructose-induced hepatic lipid content. In compared to the combined fenofibrate and high-fructose treatment, the combined stearic acid and high-fructose treatment showed a higher hepatic lipid content. Stearic acid alone dietary treatment resulted in increased hepatic lipid content than fenofibrate and high-fructose combined treatment.

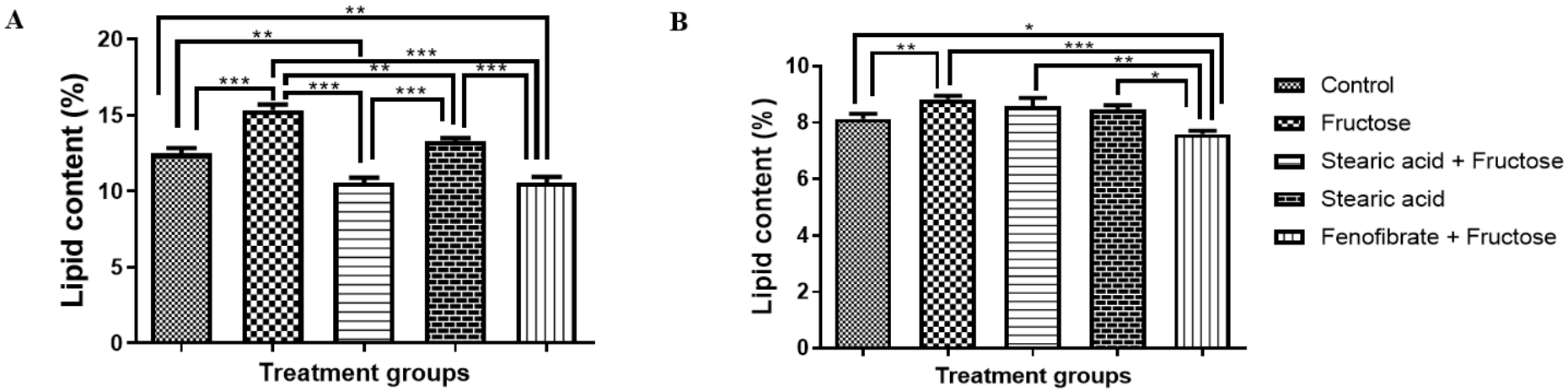


Figure 4.1. The effect of combined diet enriched with stearic acid and high fructose on hepatic lipid content of growing female (A) and male (B) rats. Data presented as mean ± standard deviation. A two-way ANOVA test was used to analyse induction and terminal body masses of the rats across treatment interventions. * = significantly different at $p < 0.05$; ** = significantly different at $p < 0.001$ and *** = significantly different at $p < 0.0001$. The control group is the negative control in which rats received standard rat chow and plain water to drink (n = 7 for males and n = 8 for females); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8 for males and n = 7 for females); the stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8) and the fenofibrate and fructose group rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

4.4.3 Fatty acid profile

The hepatic fatty acid profile of the female rats in each group after a 13-week dietary treatment is shown in Table 4.3 A.

Total saturated fatty acid concentration in female rats was similar across treatment groups ($p > 0.05$, $F_{(4, 5)} = 2.145$). Palmitic acid and stearic acid constituted the majority of the total saturated fatty acid composition. When compared to rats on a high-fructose diet, the combined stearic acid and high-fructose diet significantly ($p = 0.0001$) lowered palmitic acid content in the livers. Furthermore, the stearic acid alone diet resulted in a significant ($p = 0.0008$) drop in palmitic acid content in the livers when compared to rats on a high-fructose diet.

In comparison to the control, the high-fructose diet reduced stearic acid content significantly ($p = 0.0003$), but the combined stearic acid and high-fructose diet increased stearic acid content significantly ($p = 0.0068$). When compared to the high-fructose diet, the combined stearic acid and high-fructose ($p < 0.0001$), stearic acid alone ($p = 0.0002$) and combined fenofibrate and high-fructose ($p = 0.0001$) diets significantly raised the stearic acid content in the livers. The livers of female rats fed the combination stearic acid and high-fructose diet had a greater stearic acid content ($p = 0.0260$) than those fed the stearic acid alone diet. The combined stearic acid and high-fructose group, as well as the combined fenofibrate and high-fructose group, had different stearic acid content ($p = 0.0318$).

In the current study, capric, lauric, myristic, pentadecylic acid, margaric acid, arachidic acid, heneicosylic acid and behenic acid accounted for less than 0.5% of the total saturated fatty acid profile in the liver and while included in the data, they are not reported on in detail.

Total monounsaturated fatty acids content across treatment groups was different ($p = 0.0013$, $F_{(4, 5)} = 28.01$) with oleic acid comprising the greatest proportion of the monounsaturated fatty acid. When compared to the control, the high-fructose diet significantly ($p = 0.0023$) increased the total monounsaturated fatty acid content. The combined stearic acid and high-fructose ($p = 0.0019$), stearic acid alone ($p = 0.0018$) and combined fenofibrate and high-fructose ($p = 0.0192$) dietary groups significantly decreased the total monounsaturated fatty acid content resulting from the high-fructose diet.

The combined fenofibrate and high-fructose ($p = 0.0360$) and high-fructose ($p = 0.0001$) diets significantly increased the oleic acid content in the livers compared to the control, but the combined stearic acid and high-fructose diet decreased ($p = 0.0055$) the oleic acid content. The oleic acid content, on the other hand, was similar ($p > 0.05$) between the control and stearic

acid alone groups of rats. The combined stearic acid and high-fructose ($p < 0.000$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose ($p = 0.0008$) diets all considerably reduced the oleic acid content in the livers caused by the high-fructose diet. The oleic content in the livers of the stearic acid alone was lower ($p = 0.0004$) than that of the combined fenofibrate and high-fructose diet.

In the current study, myristoleic acid, pentadecenoic acid, heptadecanoic acid, elaidic acid and gadoleic acid accounted for less than 0.5% of the total monounsaturated fatty acid fatty acid profile in the liver, and hence are not reported on in detail.

The total polyunsaturated fatty acid content differed significantly across treatment groups (p 0.05, $F_{(4, 5)} = 10.29$). In compared to the control, the polyunsaturated fatty acid content of the livers of rats fed a high-fructose diet ($p = 0.0531$), the combination stearic acid and high-fructose diet ($p = 0.9934$), the stearic acid alone diet ($p = 0.7558$) and the combined fenofibrate and high-fructose ($p = 0.1296$) diets were similar. However, both combined stearic acid and high-fructose ($p =0.0371$) and stearic acid alone ($p = 0.0187$) dietary interventions significantly increased polyunsaturated fatty acid content compared to the high-fructose diet group. The stearic acid alone diet had more (p 0.0406) polyunsaturated fatty acid content than combined fenofibrate and high-fructose diet.

Linoleic acid (C18:2n6c) and arachidonic acid (C20:4n6) fatty acids made up the majority of the total polyunsaturated fatty acid content. Linoleic acid concentration differed significantly ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 1164$) across treatment groups. Linoleic acid content was significantly lower in the livers of rats fed high-fructose ($p < 0.0001$), combined stearic acid and high-fructose ($p < 0.0001$) and combined fenofibrate and high-fructose ($p = 0.0024$) diets in comparison with the control. When compared to high-fructose diet fed group, the stearic acid alone diet significantly reduced ($p < 0.0001$) and the combined fenofibrate and high-fructose diet significantly increased ($p = 0.0021$) the linoleic acid content, whereas the combined stearic acid and high-fructose diet resulted in similar ($p = 0.0921$) linoleic acid content. Linoleic acid content was significantly higher in the livers of rats fed stearic acid alone diet ($p = 0.0198$) than those fed a combined fenofibrate and high-fructose diet.

The arachidonic acid (C20:4n6) differed across treatment groups ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 98.68$). When compared to the control, a combined stearic acid and high-fructose diet lowered ($p < 0.0001$) arachidonic acid content, but a combined fenofibrate and

high-fructose diet raised (p= 0.0133) arachidonic acid content. When compared to the high-fructose fed group, both combined stearic acid and high-fructose diet ($p < 0.0001$) and combined fenofibrate and high-fructose diets (p= 0.0037) significantly increased arachidonic acid. The livers from rats fed a combination of stearic acid and high-fructose diet had higher arachidonic acid concentration ($p < 0.0001$) than the livers of a stearic acid alone diet as well as the livers of a combined fenofibrate and high-fructose diet (p =0.0324).

The current study results showed that trans-linolenic acid (C18:2n6t), α-Linolenic acid (C18:3n3), γ-linolenic acid (C18:3n6), Eicosadienoic acid (C20:2), Eicosatrienoic acid (C20:3n3), Eicosapentaenoic acid (EPA) (C20:5n3) and Docosadienoic acid (DHA) (C22:2) accounted for less than 0.5% of the total polyunsaturated fatty acid profile in the liver. They are therefore not expounded on further.

All treatment groups exhibited similar ($p > 0.05$) trans fatty acid content in the livers of female rats.

The combined stearic acid and high-fructose diet ($p < 0.0001$) and the combined fenofibrate and high-fructose diet ($p = 0.0013$) significantly reduced cis fatty acid content in the livers when compared to rats on a control diet. Cis fatty acid content was highest in stearic acid alone diet than in a combined stearic acid and high-fructose ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.0001$) diets.

Total omega 3 fatty acids content across treatment groups was similar ($p > 0.05$). The total omega 6 fatty acid content differed significantly across treatment groups ($p < 0.0001$, $F_{(4, 5)} = 10.29$). The high-fructose ($p = 0.0005$) and a combined fenofibrate and high-fructose ($p = 0.0024$) diets had lowest omega 6 fatty acid content compared to the control. Both combined stearic acid and high-fructose (p =0.0004) and stearic acid alone ($p = 0.0002$) dietary interventions significantly increased omega 6 fatty acid content compared to the high-fructose diet group.

The total omega 9 fatty acid content differed significantly across treatment groups ($p < 0.0001$, $F_{(4, 5)} = 128.8$). The combined fenofibrate and high-fructose ($p = 0.0153$) and high-fructose ($p = 0.0002$) diets greatly raised the omega 9 fatty acid content, but the combined stearic acid and high-fructose ($p = 0.0005$) significantly decreased it. The combined stearic acid and high-fructose diet ($p < 0.0001$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose ($p = 0.0021$) diets all reduced the increase in omega 9 fatty acid content caused by the high-fructose diet.

The lowest ratio of omega 6: omega 3 fatty acid was observed in the livers of rats fed a stearic acid alone diet, while rats receiving a combined fenofibrate and high-fructose had a highest omega 6: omega 3 ratio fatty acid.

The saturated to unsaturated fatty acid ratio was lowest in the livers of the rats receiving a high-fructose alone diet and the highest in the livers of rats receiving a combined fenofibrate and high-fructose diet.

In summary, the total saturated fatty acid content in livers of female rats was comparable between treatment groups. Female rats on a high-fructose diet indicated increased hepatic monounsaturated fatty acid contents compared to other treatment groups. Stearic acid alone as a dietary supplement resulted in the highest hepatic total polyunsaturated fatty acid content in female rats, followed by a standard diet fed female rats and those fed a stearic acid and high-fructose diet. All treatment groups exhibited similar trans fatty acid and omega-3 fatty acid levels in the livers of female rats.

Table 4.3 A. Fatty acid profile of the livers in growing Sprague-Dawley female rats fed a high-fructose diet and or stearic acid for 13 weeks.

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Saturated Fatty acids						
Capric acid (C10:0)	0.01 ± 0.01 [a]	0.01 ± 0.02 [a]	0.00 ± 0.00 [a]	0.10 ± 0.09 [a]	0.07 ± 0.10 [a]	ns (p = 0.4628)
Lauric acid (C12:0)	0.04 ± 0.00 [a]	0.05 ± 0.00 [a]	0.06 ± 0.03 [a]	0.06 ± 0.04 [a]	0.18 ± 0.17 [a]	ns (p = 0.4304)
Myristic acid (C14:0)	0.40 ± 0.00 [a]	0.43 ± 0.06 [a]	0.16 ± 0.20 [a]	0.39 ± 0.01 [a]	0.37 ± 0.05 [a]	ns (p = 0.1668)
Pentadecylic acid (C15:0)	0.10 ± 0.03 [a]	0.08 ± 0.02 [a]	0.06 ± 0.01 [a]	0.55 ± 0.71 [a]	0.07 ± 0.01 [a]	ns (p = 0.5330)
Palmitic acid (C16:0)	16.89 ± 0.03 [ab]	18.82 ± 0.84 [a]	15.21 ± 0.48 [b]	15.59 ± 0.31 [b]	17.07 ± 0.08 [ab]	* p = 0.0030
Margaric acid (C17:0)	0.38 ± 0.00 [a]	0.29 ± 0.01 [b]	0.23 ± 0.00 [b]	0.33 ± 0.01 [a]	0.25 ± 0.04 [a]	* p = 0.0027
Stearic acid (C18:0)	26.46 ± 0.12 [c]	22.12 ± 0.53 [b]	28.69 ± 0.22 [ac]	27.06 ± 0.41[ac]	27.14 ± 0.28 [ac]	* p < 0.0001
Arachidic acid (C20:0)	0.09 ± 0.01[a]	0.07 ± 0.01 [a]	0.12 ± 0.03 [a]	0.07 ± 0.02 [a]	0.04 ± 0.05 [a]	ns (p = 0.2082)
Heneicosylic acid (C21:0)	0.26 ± 0.02 [ab]	0.22 ± 0.03 [ab]	0.19 ± 0.15 [ab]	0.31 ± 0.01 [b]	0.00 ± 0.00 [a]	* p = 0.0395
Behenic acid (C22:0)	0.13 ± 0.02[a]	0.03 ± 0.05 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	* p = 0.0109
Total saturated fatty acid	**44.78 ± 0.26 [a]**	**42.11 ± 1.57 [a]**	**44.72 ± 1.13 [a]**	**44.47 ± 1.61 [a]**	**45.19 ± 0.78 [a]**	**ns (p = 0.2122)**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Mono-unsaturated fatty acids						
Myristoleic acid C14:1	0.01 ± 0.01 ^{ab}	0.07 ± 0.03 ^{a}	0.04 ± 0.01 ^{ab}	0.06 ± 0.02 ^{ab}	0.00 ± 0.00 ^{b}	* p = 0.0355
Pentadecenoic acid C15:1	0.01 ± 0.01 ^{a}	0.02 ± 0.02 ^{a}	0.01 ± 0.01 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	ns (p = 0.4246)
Palmitoleic acid (C16:1)	1.03 ± 0.02 ^{a}	1.99 ± 0.10 ^{b}	1.21 ± 0.03 ^{a}	1.02 ± 0.11 ^{a}	1.36 ± 0.06 ^{b}	* p = 0.0002
Heptadecanoic acid (C17:1)	0.08 ± 0.02 ^{a}	0.12 ± 0.03 ^{a}	0.05 ± 0.02 ^{a}	0.08 ± 0.01 ^{a}	0.08 ± 0.01 ^{a}	ns (p = 0.1133)
Oleic acid (C18:1n9c)	17.04 ± 0.77 ^{a}	22.59 ± 0.22 ^{b}	14.24 ± 0.29 ^{bc}	15.98 ± 0.11 ^{ac}	19.35 ± 0.34 ^{bc}	* p < 0.0001
Elaidic acid (C18:1n9t)	0.10 ± 0.01 ^{a}	0.15 ± 0.00 ^{a}	0.09 ± 0.12 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	ns (p = 0.1288)
Gadoleic acid (C20:1)	0.23 ± 0.03 ^{a}	0.23 ± 0.01 ^{a}	0.22 ± 0.03 ^{a}	0.23 ± 0.10 ^{a}	0.08 ± 0.01 ^{a}	ns (p = 0.9953)
Nervonic acid (C24:1)	6.37 ± 0.29 ^{a}	6.58 ± 0.00 ^{a}	8.71 ± 0.72 ^{b}	7.11 ± 0.10 ^{ab}	6.63 ± 0.09 ^{ab}	* p = 0.0058
Total monounsaturated fatty acids	**24.88 ± 1.16 ^{a}**	**31.78 ± 0.44 ^{b}**	**24.56 ± 1.23 ^{a}**	**24.47 ± 0.43 ^{a}**	**27.50 ± 0.52 ^{a}**	*** p = 0.0013**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Poly-unsaturated fatty acids						
Linoleic acid (C18:2n6c)	13.58 ± 0.12 ^{ac}	9.19 ± 0.09 ^{bc}	8.77 ± 0.21 ^{b}	14.60 ± 0.05 ^{a}	8.14 ± 0.09 ^{bc}	* p < 0.0001
trans-linolenic acid (C18:2n6t)	0.11 ± 0.02 ^{ab}	0.13 ± 0.02 ^{ab}	0.17 ± 0.08 ^{a}	0.07 ± 0.01 ^{ab}	0.00 ± 0.00 ^{b}	* p = 0.0415
α-Linolenic acid (C18:3n3)	0.26 ± 0.03 ^{a}	0.14 ± 0.01 ^{a}	0.07 ± 0.02 ^{a}	0.33 ± 0.02 ^{a}	0.19 ± 0.03 ^{a}	ns (p = 0.9996)
γ-linolenic acid (C18:3n6)	0.36 ± 0.03 ^{a}	0.47 ± 0.21 ^{a}	0.19 ± 0.03 ^{a}	0.30 ± 0.32 ^{a}	0.00 ± 0.00 ^{a}	ns (p = 0.2102)
Eicosadienoic acid (C20:2)	0.18 ± 0.07 ^{a}	0.51 ± 0.19 ^{a}	0.39 ± 0.03 ^{a}	0.21 ± 0.05 ^{a}	0.51 ± 0.30 ^{a}	ns (p = 0.2530)
Eicosatrienoic acid (C20:3n3)	0.04 ± 0.06 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.02 ± 0.03 ^{a}	0.00 ± 0.00 ^{a}	ns (p = 0.6838)
Dihomo-gamma-linolenic acid (C20:3n6)	0.57 ± 0.04 ^{ab}	0.65 ± 0.06 ^{b}	0.82 ± 0.03 ^{c}	0.49 ± 0.01 ^{ac}	0.73 ± 0.04 ^{c}	* p = 0.0024
Arachidonic acid (C20:4n6)	14.97 ± 0.36 ^{a}	14.66 ± 0.56 ^{ac}	19.89 ± 0.09 ^{b}	15.14 ± 0.23 ^{ac}	17.55 ± 0.08 ^{ab}	* p < 0.0001
Eicosapentaenoic acid (EPA) (C20:5n3)	0.28 ± 0.03 ^{a}	0.29 ± 0.06 ^{a}	0.43 ± 0.23 ^{a}	0.32 ± 0.09 ^{a}	0.09 ± 0.01 ^{a}	ns (p = 0.1931)
Docosadienoic acid (DHA) (C22:2)	0.00 ± 0.00 ^{a}	0.37 ± 0.41 ^{a}	0.00 ± 0.00 ^{a}	0.06 ± 0.09 ^{a}	0.10 ± 0.15 ^{a}	ns (p = 0.4755)
Total polyunsaturated fatty acids	**30.34 ±0.77 ^{ab}**	**26.41 ± 1.61 ^{b}**	**30.73 ± 0.72 ^{a}**	**31.53 ± 0.89 ^{c}**	**27.31 ± 0.68 ^{ab}**	*** p = 0.0125**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Trans fatty acids	0.21 ± 0.03 [a]	0.28 ± 0.02 [a]	0.26 ± 0.20 [a]	0.07 ± 0.01 [a]	0.00 ± 0.00 [a]	ns (p = 0.0939)
Cis fatty acids	30.62 ± 0.65 [a]	31.78 ± 0.13 [a]	23.00 ± 0.09 [b]	30.58 ± 0.16 [a]	27.49 ± 0.42[b]	* p < 0.0001
Omega 3	0.58 ± 0.06 [a]	0.43 ± 0.06 [a]	0.50 ± 0.21 [a]	0.67 ± 0.10 [a]	0.28 ± 0.01 [a]	ns (p = 0.9999)
Omega 6	29.58 ± 0.46 [a]	25.10 ± 0.36 [bc]	29.83 ± 0.44 [a]	30.60 ± 0.47 [ab]	26.41 ± 0.04 [c]	* p = 0.0001
Omega 9	17.15 ± 0.78 [a]	22.77 ± 0.26 [c]	14.32 ± 0.17[b]	15.98 ± 0.11 [ab]	19.35 ± 0.34 [b]	* p < 0.0001
SFA: UFA	0.81: 1	0.72: 1	0.81: 1	0.79: 1	0.82: 1	
Omega 6: Omega 3	51.0: 1	58.4: 1	59.7: 1	45.7: 1	94.3: 1	

[a, b, c] Means in the same row with different superscripts differ significantly at $p < 0.05$. ns = not significant with $p > 0.05$. * = significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink; fructose group rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. n = 2. SFA, saturated fatty acids; UFA, unsaturated fatty acids.

Table 4.3 B shows the hepatic fatty acid profile of the male rats in each group following a 13-week dietary treatment. Fatty acids are expressed as mean ± standard deviation of total fatty acids within composite liver tissue sample with n = 2.

Total saturated fatty acid concentration in livers of the male rats was different across treatment groups ($p = 0.0103$, $F_{(4, 5)} = 11.21$). The total saturated fatty acid content of the livers of rats fed a high-fructose diet and those fed a stearic acid alone diet were similar ($p > 0.05$), whereas the combined stearic acid and high-fructose ($p = 0.0164$) and combined fenofibrate and high-fructose ($p = 0.084$) diet interventions significantly decreased it.

Palmitic acid and stearic acid constituted the majority of the total saturated fatty acid composition. When compared to rats on a control diet, the high-fructose ($p = 0.0082$) and combined stearic acid and high-fructose ($p = 0.0205$) diets significantly decreased palmitic acid content in the livers. Furthermore, the hepatic palmitic acid content was similar ($p > 0.05$) in the combined stearic acid and high-fructose diet and combined fenofibrate and high-fructose diet when compared to rats on a high-fructose diet whilst stearic acid alone diets significantly ($p = 0.0002$) increase it. The stearic acid alone diet had the highest liver palmitic acid content than combined stearic acid and high-fructose ($p = 0.0039$) and combined fenofibrate and high-fructose ($p = 0.0104$) diets.

The high-fructose diet had similar ($p > 0.05$) hepatic stearic acid content to the control, but the combined stearic acid and high-fructose diet ($p = 0.0237$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose ($p = 0.0003$) diets significantly decreased hepatic stearic acid content. All the dietary interventions, including the combined stearic acid and high-fructose ($p = 0.0017$), the stearic acid alone ($p < 0.0001$) and the combined fenofibrate and high-fructose ($p < 0.0001$) significantly reduced the high-fructose induced increase in stearic acid content in the livers.

In the current study, capric acid, lauric acid, pentadecylic acid, arachidic acid, heneicosylic acid and behenic acid accounted for less than 0.5% of the total saturated fatty acid profile in the liver.

Total monounsaturated fatty acid content in the livers of male rats differed across treatment groups ($p = 0.0001$, $F_{(4, 5)} = 71.00$), with oleic acid accounting for the highest amount of the monounsaturated fatty acid. Total monounsaturated fatty acid content in male rat livers was similar in the high-fructose ($p = 0.3291$), combined stearic acid and high-fructose ($p =$

0.9038) as well as combined fenofibrate and high-fructose ($p = 0.2761$) dietary groups, however increased significantly ($p = 0.0003$) in the stearic acid alone diet when compared to control. When compared to the high-fructose diet, stearic acid alone ($p = 0.0001$) and the combined fenofibrate and high-fructose ($p = 0.0333$) diets significantly increased the total monounsaturated fatty acid content in male rat livers, whilst the combination stearic acid and high-fructose diet resulted in similar ($p > 0.05$) total monounsaturated fatty acid content. Among the dietary interventions, stearic acid alone had the greatest total monounsaturated fatty acid concentration (0.0004), followed by the combined fenofibrate and high-fructose group (0.0007) and then combined stearic acid and high-fructose group (0.0004).

With oleic acid accounting for the highest amount of the monounsaturated fatty acid, when compared to the control group, the high-fructose diet significantly ($p = 0.0162$) decreased oleic acid content in male rats' livers, but the stearic acid alone diet increased ($p < 0.0001$) oleic acid content. The oleic acid content in male rats' livers of the combined stearic acid and high-fructose and combined fenofibrate diet were similar ($p > 0.05$) when compared to control.

Myristoleic acid, pentadecenoic acid, heptadecanoic acid, gadoleic acid and erucic acid accounted for less than 0.5% of the total monounsaturated fatty acid fatty acid profile in the liver.

Total polyunsaturated fatty acid content in the livers of male rats differed across treatment groups ($p < 0.0001$, $F_{(4, 5)} = 323.3$), with linoleic acid (C18:2n6c) and arachidonic acid (C20:4n6) accounting for the highest amount of the polyunsaturated fatty acid. When compared to the control group, total polyunsaturated fatty acid content in male rat livers was significantly ($p = 0.05$) higher in the high-fructose ($p = 0.0005$), combined stearic acid and high-fructose ($p = 0.0032$) and combined fenofibrate and high-fructose ($p = 0.0071$) dietary groups. The stearic acid alone diet, on the other hand, significantly ($p < 0.0001$) decreased the total polyunsaturated fatty acid content in male rat livers. In male rat livers, the combined stearic acid and high-fructose diet group showed equivalent ($p > 0.05$) total polyunsaturated fatty acid content to the high-fructose diet group. When compared to the high-fructose diet group, both the stearic acid alone ($p < 0.0001$) and the combined fenofibrate and high-fructose ($p = 0.0216$) dietary treatments significantly decreased the total polyunsaturated fatty acid content in male rat livers. Stearic acid alone dietary treatment had the lowest total polyunsaturated fatty acid content in male rat livers than combined stearic acid and high-fructose ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.0001$) diets.

Linoleic acid concentration differed significantly ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 743.6$) across treatment groups. In comparison with the control, the linoleic acid content in the livers of male rats was fed a high-fructose diet ($p = 0.0031$), combined stearic acid and high-fructose diet ($p = 0.0038$) and those combined fenofibrate and high-fructose diet ($p < 0.0001$) was significantly increased. However, the stearic acid alone diets significantly reduced ($p < 0.0001$) the linoleic acid content in the livers of male rats in comparison with the control. When compared to high-fructose diet group, the combined stearic acid and high-fructose diet had similar ($p = 0.9968$) amount of linoleic acid, whilst the stearic acid alone diet significantly reduced ($p < 0.0001$) and the combined fenofibrate and high-fructose diet significantly increased ($p = 0.0090$) the linoleic acid content in the livers of male rats. The livers of male rats fed stearic acid alone diet had the lowest linoleic acid content ($p < 0.05$) than the livers of male rats fed a combined stearic acid and high-fructose diet ($p < 0.0001$) as well as those fed combined fenofibrate and high-fructose diet ($p < 0.0001$).

The arachidonic acid (C20:4n6) differed across treatment groups ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 1068$). When compared to the control, a high-fructose diet ($p < 0.0001$), a combined stearic acid and high-fructose diet ($p = 0.0001$) and a combined fenofibrate and high-fructose diet ($p = 0.0356$) significantly increased the arachidonic acid content in the male rats' livers whilst a stearic acid alone diet significantly ($p < 0.0001$) decreased the arachidonic acid content. The combined stearic acid and high-fructose ($p = 0.0164$), stearic acid alone ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.0001$) dietary interventions significantly decreased the high-fructose diet induced increase of arachidonic acid content in the livers of the male rats.

Trans-linolenic acid (C18:2n6t), α-Linolenic acid (C18:3n3), γ-linolenic acid (C18:3n6), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3n3) and eicosapentaenoic acid (EPA) (C20:5n3) accounted for less than 0.5% of the total polyunsaturated fatty acid profile in the liver.

The cis fatty acid content in the livers of male rats differed across treatment groups ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 100.4$). Compared to control diet, the high-fructose diet significantly reduced ($p = 0.0268$) the cis fatty acid content. However, the stearic acid alone ($p = 0.0002$) and the combined fenofibrate and high-fructose diet ($p = 0.0311$) dietary interventions significantly increased cis fatty acid content in the livers of male rats. All the dietary interventions, including the combined stearic acid and high-fructose ($p = 0.0337$), the stearic acid alone ($p < 0.0001$) and the combined fenofibrate and high-fructose ($p = 0.0013$)

significantly increased cis fatty acid content in the livers of male rats when compared to high-fructose diet group. The livers of male rats fed a stearic acid alone diet had the highest cis fatty acid content ($p < 0.05$) than the livers of male rats fed a combined stearic acid and high-fructose diet ($p = 0.0002$) as well as those fed combined fenofibrate and high-fructose diet ($p = 0.0012$).

The omega 6 fatty acid content in the livers of male rats differed across treatment groups ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 1293$). When compared to the control, a high-fructose diet ($p < 0.0001$), a combined stearic acid and high-fructose diet ($p = 0.0001$) and a combined fenofibrate and high-fructose diet ($p = 0.0010$) significantly increased the omega 6 fatty acid content in the male rats' livers whilst a stearic acid alone diet significantly ($p < 0.0001$) decreased the omega 6 fatty acid content. The combined stearic acid and high-fructose ($p = 0.0190$), stearic acid alone ($p = 0.0001$) and combined fenofibrate and high-fructose ($p = 0.0010$) dietary treatments all significantly reduced the high-fructose diet caused rise in omega 6 fatty acid content in male rats' livers. Stearic acid alone diet showed significantly lower omega 6 fatty acid concentration in male rats' livers than combined stearic acid and high-fructose ($p < 0.0001$) and combined fenofibrate and high-fructose ($p < 0.0001$).

The content of omega 9 fatty acids in male rat livers differed across treatment groups ($p < 0.0001$, one-way ANOVA, $F_{(4, 5)} = 184.9$). A high-fructose diet significantly decreased ($p = 0.0082$) the quantity of omega 9 fatty acids in male rat livers as compared to the control. A stearic acid alone diet, on the other hand, significantly increased ($p < 0.0001$) the content of omega 9 fatty acids in male rat livers, whereas a combined stearic acid and high-fructose ($p = 0.3600$) and combined fenofibrate and high-fructose ($p = 0.5923$) diet were similar ($p > 0.05$). When compared to the high-fructose diet group, all the dietary interventions, including the combined stearic acid and high-fructose ($p = 0.0428$), the stearic acid alone ($p < 0.0001$) and the combined fenofibrate and high-fructose ($p = 0.0031$) significantly increased omega 9 fatty acid content in the livers of male rats.

The lowest ratio of omega 6: omega 3 fatty acid was observed in the livers of rats fed a combined fenofibrate and high-fructose diet, while rats receiving a standard diet (control) had a highest omega 6: omega 3 ratio fatty acid.

The saturated to unsaturated fatty acid ratio was lowest in the livers of the rats receiving fed a combined fenofibrate and high-fructose diet and the highest in the livers of rats receiving a standard diet (control) diet.

In summary, the total saturated fatty acid content in livers of male rats was highest in the standard diet fed rats, followed by rats fed a high-fructose diet and those fed a stearic acid alone diet as a supplement. Male rats fed a stearic acid alone diet showed a significant increase in hepatic total monounsaturated fatty acid contents compared to other treatment groups. Meanwhile, the total monounsaturated fatty acid content of male rats' livers was comparable across the control, high-fructose, combined stearic acid and high-fructose and combined fenofibrate and high-fructose diets groups. In male rats, the high-fructose diet exhibited highest hepatic total polyunsaturated fatty acid level, followed by rats fed a combined stearic acid and high-fructose diet and those fed a combined fenofibrate and high-fructose diet. All treatment groups exhibited similar omega-3 fatty acid levels in the livers of male rats.

Table 4.3 B. Fatty acid profile of the livers in growing Sprague-Dawley male rats fed a high-fructose diet and or stearic acid for 13 weeks.

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Saturated Fatty acids						
Capric acid (C10:0)	0.02 ± 0.00 [a]	0.01 ± 0.02 [a]	0.02 ± 0.00 [a]	0.04 ± 0.03 [a]	0.03 ± 0.04 [a]	ns (p = 0.7715)
Lauric acid (C12:0)	0.06 ± 0.01 [a]	0.03 ± 0.01 [a]	0.05 ± 0.01 [a]	0.08 ± 0.01 [a]	0.05 ± 0.07 [a]	ns (p = 0.6666)
Myristic acid (C14:0)	1.40 ± 0.24 [a]	0.30 ± 0.01 [b]	0.47 ± 0.02 [bc]	2.42 ± 0.28 [b]	0.55 ± 0.04 [ab]	* p = 0.0004
Pentadecylic acid (C15:0)	0.39 ± 0.05 [a]	0.10 ± 0.01 [b]	0.09 ± 0.01 [b]	0.43 ± 0.03 [ac]	0.13 ± 0.01 [ab]	* p = 0.0001
Palmitic acid (C16:0)	24.25 ± 0.89 [a]	19.63 ± 0.57 [b]	20.52 ± 0.36 [b]	25.96 ± 1.11 [a]	21.58 ± 0.52 [b]	* p = 0.0016
Margaric acid (C17:0)	0.76 ± 0.04 [a]	0.32 ± 0.00 [b]	0.24 ± 0.01 [b]	0.93 ± 0.03 [ac]	0.28 ± 0.02 [ab]	* p < 0.0001
Stearic acid (C18:0)	22.29 ± 0.63 [b]	23.54 ± 0.25 [b]	20.75 ± 0.17 [d]	14.56 ± 0.12 [bc]	18.14 ± 0.01 [ac]	* p < 0.0001
Arachidic acid (C20:0)	0.15 ± 0.01 [a]	0.09 ± 0.05 [a]	0.06 ± 0.02 [a]	0.14 ± 0.03 [a]	0.09 ± 0.01 [a]	ns (p = 0.0974)
Heneicosylic acid (C21:0)	0.33 ± 0.01 [a]	0.42 ± 0.03 [a]	0.33 ± 0.01 [a]	0.22 ± 0.13 [a]	0.40 ± 0.02 [a]	ns (p = 0.1090)
Behenic acid (C22:0)	0.23 ± 0.01 [a]	0.20 ± 0.04 [ab]	0.06 ± 0.01 [ab]	0.05 ± 0.08 [b]	0.16 ± 0.04 [ab]	* p = 0.0297
Total saturated fatty acid	**49.90 ± 2.02 [a]**	**44.65 ± 1.01** [a]	**42.60 ± 0.61** [bc]	**44.85 ± 1.85** [ab]	**41.38 ± 0.76** [bc]	*** p = 0.0103**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Mono-unsaturated fatty acids						
Myristoleic acid C14:1	0.15 ± 0.04 [a]	0.04 ± 0.00 [a]	0.06 ± 0.00 [b]	0.83 ± 0.10 [ac]	0.07 ± 0.01 [ab]	* p < 0.0001
Pentadecenoic acid C15:1	0.18 ± 0.01 [a]	0.01 ± 0.02 [b]	0.01 ± 0.01 [b]	0.19 ± 0.00 [a]	0.00 ± 0.00 [b]	* p < 0.0001
Palmitoleic acid (C16:1)	1.30 ± 0.10 [b]	1.24 ± 0.04 [b]	2.17 ± 0.01 [ac]	3.57 ± 0.18 [a]	2.40 ± 0.01 [b]	* p < 0.0001
Heptadecanoic acid (C17:1)	0.18 ± 0.03 [b]	0.07 ± 0.01 [b]	0.07 ± 0.02 [b]	0.71 ± 0.05 [a]	0.07 ± 0.04 [b]	* p < 0.0001
Oleic acid (C18:1n9c)	19.43 ± 0.61 [bc]	13.21 ± 0.52 [a]	18.01 ± 0.35 [b]	39.61 ± 2.43 [ac]	22.14 ± 0.40 [b]	* p < 0.0001
Elaidic acid (C18:1n9t)	0.97 ± 0.02 [a]	0.21 ± 0.03 [b]	0.10 ± 0.06 [a]	1.96 ± 0.10 [b]	0.01 ± 0.02 [a]	* p < 0.0001
Gadoleic acid (C20:1)	0.21 ± 0.02 [a]	0.20 ± 0.04 [a]	0.34 ± 0.08 [ab]	0.24 ± 0.03 [ab]	0.40 ± 0.02 [b]	* p = 0.0215
Erucic acid C22:1 n9	0.03 ± 0.04 [a]	0.01 ± 0.02 [a]	0.01 ± 0.02 [a]	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	p = 0.6653
Nervonic acid (C24:1)	4.71 ± 0.59 [a]	8.79 ± 0.22 [b]	7.74 ± 0.21 [b]	0.67 ± 0.03 [a]	5.72 ± 0.17 [bc]	* p < 0.0001
Total monounsaturated fatty acid	**27.16 ± 1.47** [a]	**23.78 ± 0.90** [a]	**28.50 ± 0.75** [a]	**47.79 ± 2.91** [b]	**30.81 ± 0.66** [ac]	*** p = 0.0001**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Poly-unsaturated fatty acids						
Linolenic acid (C18:2n6c)	9.58 ± 0.17 [b]	10.77 ± 0.08 [c]	10.72 ± 0.07 [c]	4.27 ± 0.23 [a]	11.71 ± 0.16 [bc]	* p < 0.0001
trans-linolenic acid (C18:2n6t)	0.10 ± 0.04 [a]	0.10 ± 0.03 [a]	0.10 ± 0.04 [a]	0.36 ± 0.12 [b]	0.11 ± 0.04 [ab]	*p = 0.0313
α-Linolenic acid (C18:3n3)	0.15 ± 0.04 [b]	0.12 ± 0.01 [b]	0.13 ± 0.01 [b]	0.17 ± 0.02 [b]	0.26 ± 0.00 [a]	* p = 0.0061
γ-linolenic acid (C18:3n6)	0.07 ± 0.03 [a]	0.32 ± 0.09 [c]	0.05 ± 0.01 [a]	0.21 ± 0.05 [a]	0.00 ± 0.00 [b]	* p = 0.055
Eicosadienoic acid (C20:2)	0.14 ± 0.06 [b]	1.05 ± 0.03 [a]	0.56 ± 0.01 [c]	0.05 ± 0.08 [b]	0.67 ± 0.03 [ac]	* p < 0.0001
Eicosatrienoic acid (C20:3n3)	0.04 ± 0.02 [a]	0.01 ± 0.02 [a]	0.03 ± 0.01 [a]	0.00 ± 0.00 [a]	0.40 ± 0.49 [a]	p = 0.4108
Dihomo-gamma-linolenic acid (C20:3n6)	0.68 ± 0.08 [b]	1.09 ± 0.01 [a]	0.95 ± 0.01 [a]	0.18 ± 0.00 [b]	1.02 ± 0.05 [a]	* p < 0.0001
Arachidonic acid (C20:4n6)	12.17 ± 0.45 [a]	17.40 ± 0.10 [b]	16.02 ± 0.01 [c]	2.12 ± 0.10 [d]	13.31 ± 0.34 [e]	* p < 0.0001
Eicosapentaenoic acid (EPA) (C20:5n3)	0.00 ± 0.00 [a]	0.41 ± 0.05 [bc]	0.15 ± 0.17 [ab]	0.03 ± 0.04 [a]	0.24 ± 0.06 [ac]	*p = 0.0236
Total polyunsaturated fatty acid	**22.93 ± 0.89** [a]	**31.56 ± 0.42** [b]	**28.76 ± 0.35** [b]	**7.38 ± 0.64** [c]	**27.80 ± 1.17** [b]	*** p < 0.0001**

Fatty acid (% of total lipid)	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Trans fatty acids	1.06 ± 0.01 [a]	0.31 ± 0.01 [b]	0.20 ± 0.09 [b]	2.32 ± 0.22 [a]	0.12 ± 0.02 [b]	* $p < 0.0001$
Cis fatty acids	29.01 ± 0.78 [b]	23.99 ± 0.43 [a]	28.73 ± 0.28 [b]	43.88 ± 2.18 [a]	33.85 ± 0.25 [c]	* $p < 0.0001$
Omega 3	0.19 ± 0.06 [a]	0.55 ± 0.10 [a]	0.29 ± 0.18 [a]	0.20 ± 0.03 [a]	0.90 ± 0.45 [a]	ns ($p = 0.0974$)
Omega 6	22.60 ± 0.36 [a]	29.68 ± 0.10 [b]	27.85 ± 0.09 [c]	7.12 ± 0.50 [d]	26.15 ± 0.49 [e]	* $p < 0.0001$
Omega 9	20.44 ± 0.60 [a]	13.43 ± 0.57 [b]	18.13 ± 0.28 [a]	41.58 ± 2.32 [b]	22.15 ± 0.42 [c]	* $p < 0.0001$
SFA: UFA	0.99: 1	0.81: 1	0.74: 1	0.81: 1	0.71: 1	
Omega 6: Omega 3	118.9: 1	54.0: 1	96.0: 1	35.6: 1	29.1: 1	

[a, b, c, d., e] Means in the same row with different superscripts differ significantly at $p < 0.05$. ns = not significant with $p > 0.05$. * = significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink; fructose group rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. n = 2. SFA, saturated fatty acids; UFA, unsaturated fatty acids.

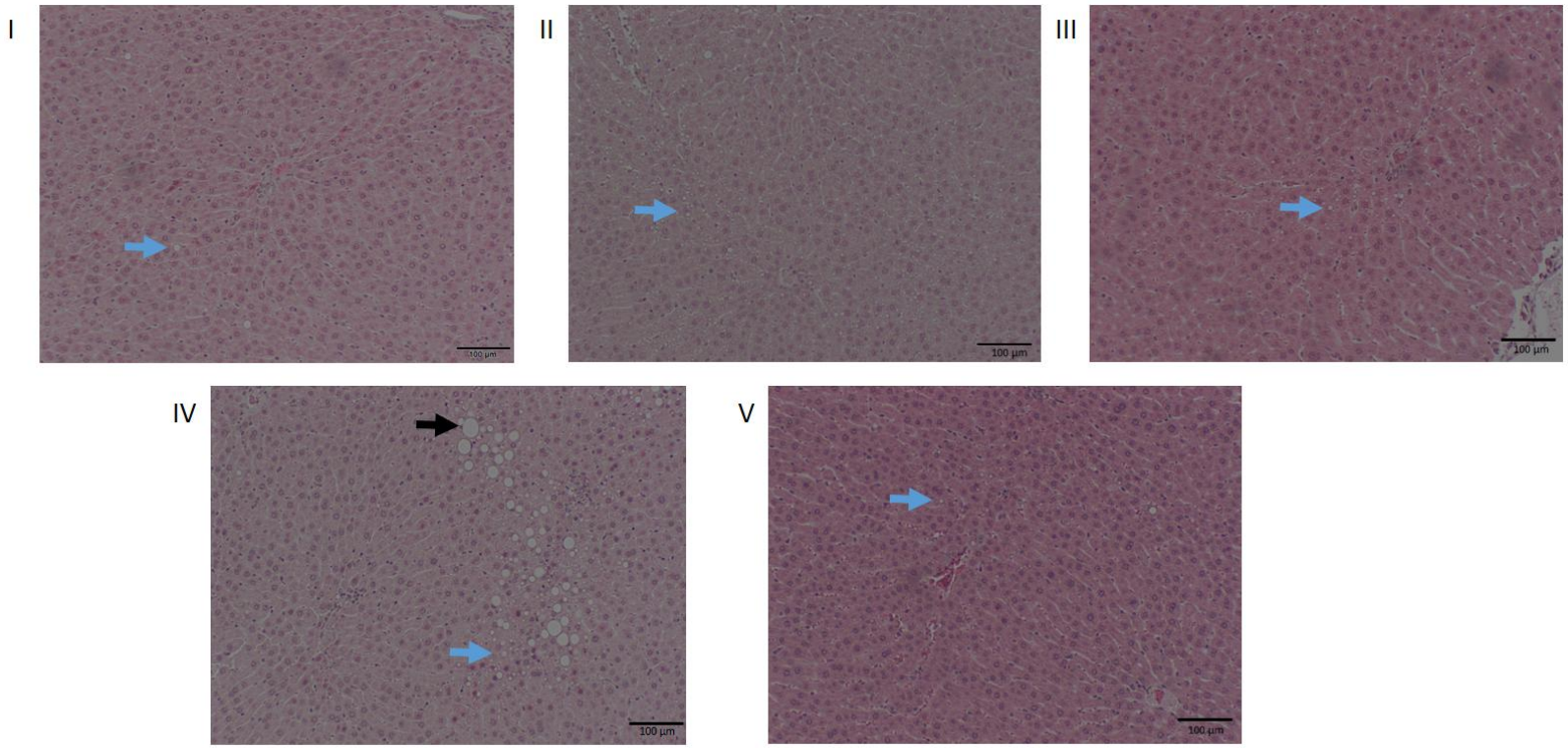
4.4.4 Hepatic histomorphology, fat accumulation and fibrosis

Having considered the fatty acid profile of the livers, it is important to characterise the microscopic distribution of the lipids in the liver (macrovesicular and microvesicular steatosis). Figures 4.2 show representative haematoxylin and eosin (H & E) stained liver photomicrographs (100x magnification) of female (A) and male (B) rats following 13-week treatment period. Each group had one photomicrograph selected at random for display in the figures below.

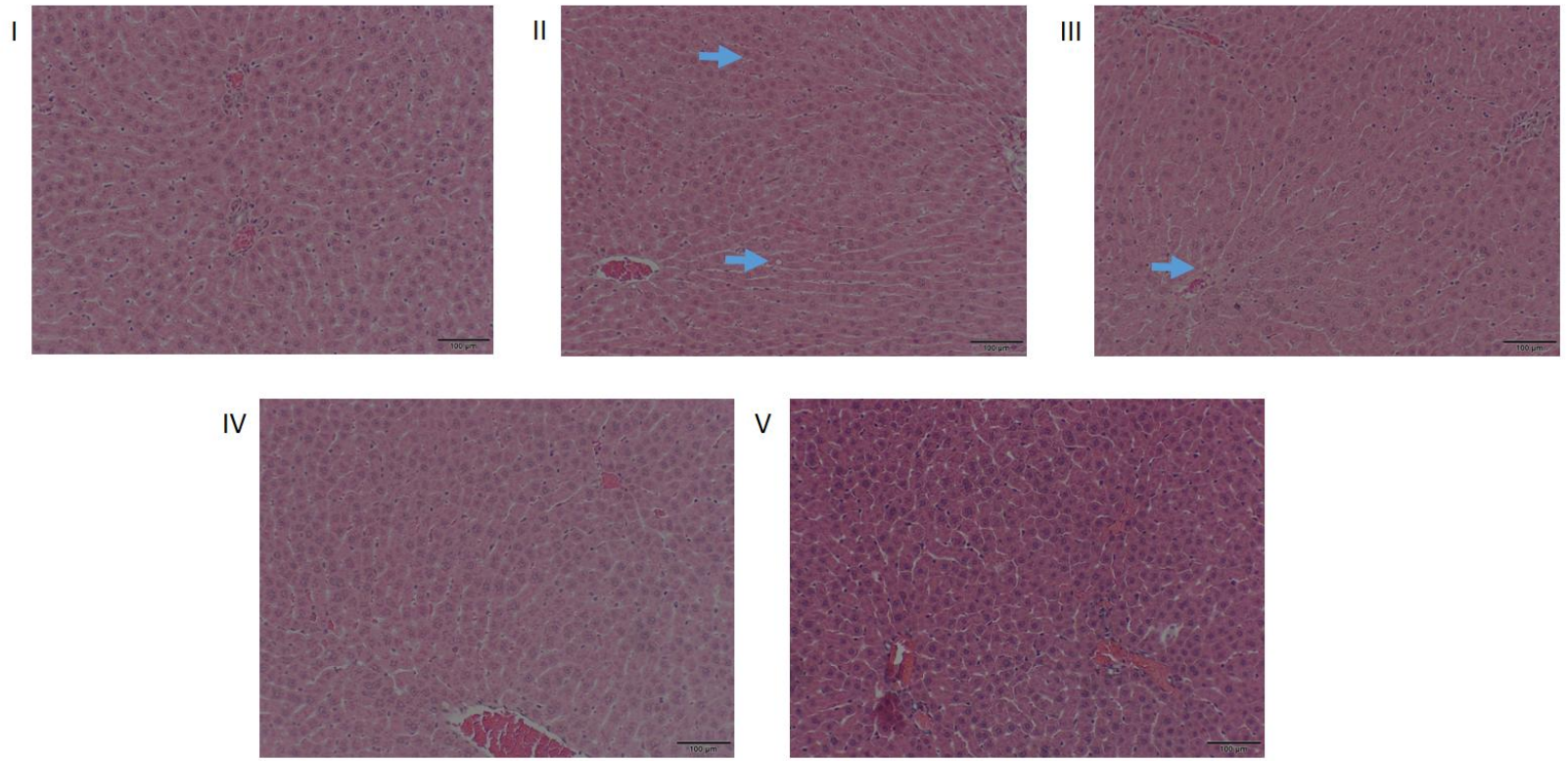
Female rats in the control group (I) fed conventional rat chow for the duration of the study had normal typical hepatocellular architecture with no evident histomorphological abnormalities. Notably, female rats fed high-fructose (II), a combination of stearic acid and high-fructose (III) and a combination of fenofibrate and high-fructose (V) diets showed more pronounced micro-vesicular hepatic steatosis, disordered arrangement of hepatocytes and inflammatory cell infiltration (figure 4.2 A). The rats fed a stearic acid-only (IV) diet developed both micro- and macro-vesicular hepatic steatosis (figure 4.2 A).

The hepatocellular architecture of the male rats was generally normal across the treatment groups. However, the high-fructose fed (II) and the combination of stearic acid and high-fructose (III) groups showed some evidence of micro-vesicular hepatic steatosis (figure 4.2 B).

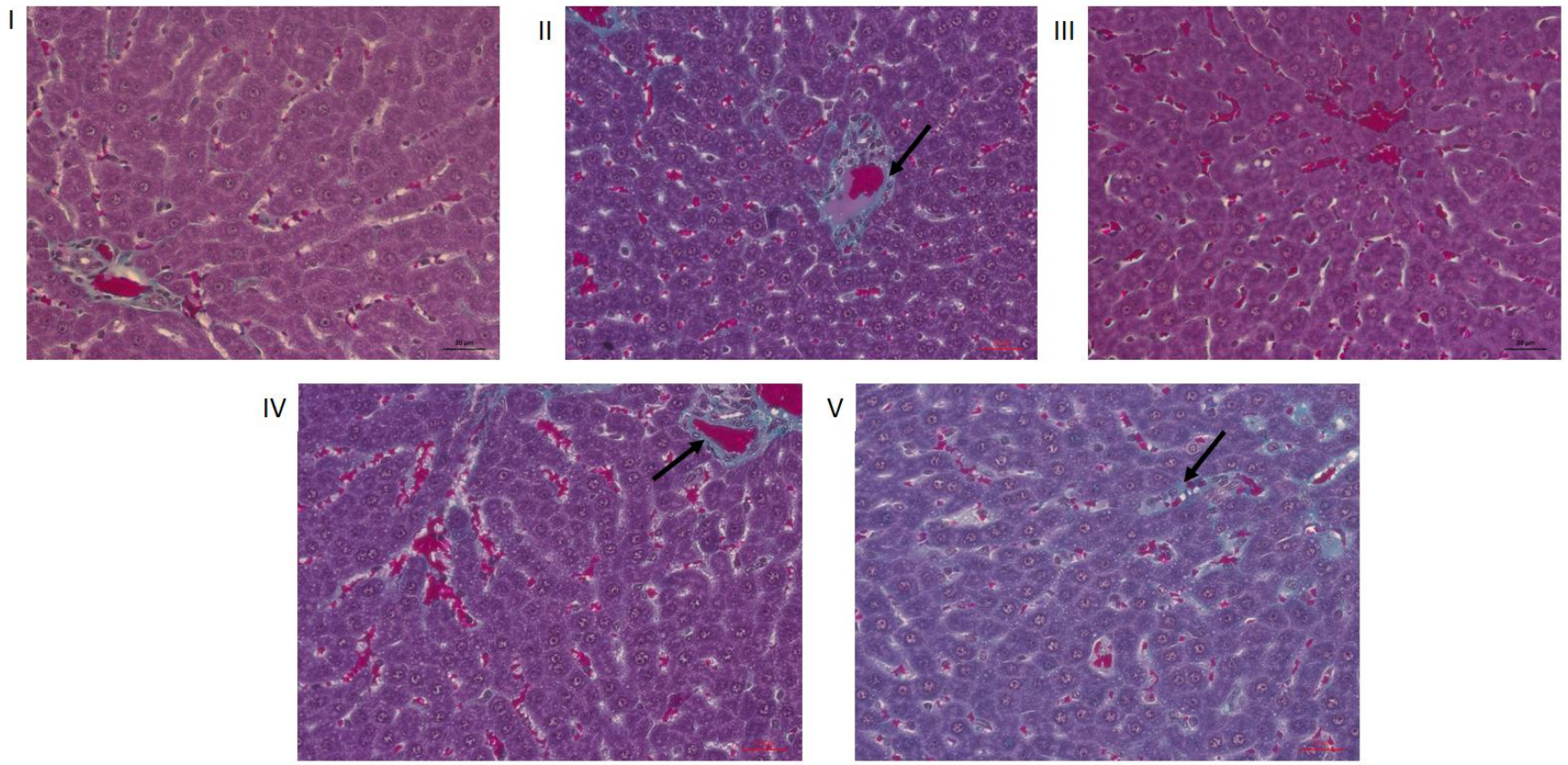
Figures 4.3 show representative Masson's trichrome (MT) stained liver photomicrographs (40x magnification) of female (A) and male (B) rats following 13-week treatment period. Each group had one photomicrograph selected at random. Notably, female rats fed high-fructose diet (II), stearic acid alone (IV), a fenofibrate plus high-fructose diet (V) had greater fibrosis in their livers. Meanwhile, female rats' livers in the control (I) and combined stearic acid and high-fructose (III) groups exhibited no fibrosis. Male rats fed combined stearic acid and high-fructose diet (III) and those fed a stearic acid alone diet (IV) developed similar pronounced fibrosis. Male rats on combined fenofibrate and high-fructose diets (V) showed minimal fibrosis. However, no fibrosis was seen in either the control group (I) or the high-fructose group (II) rats.



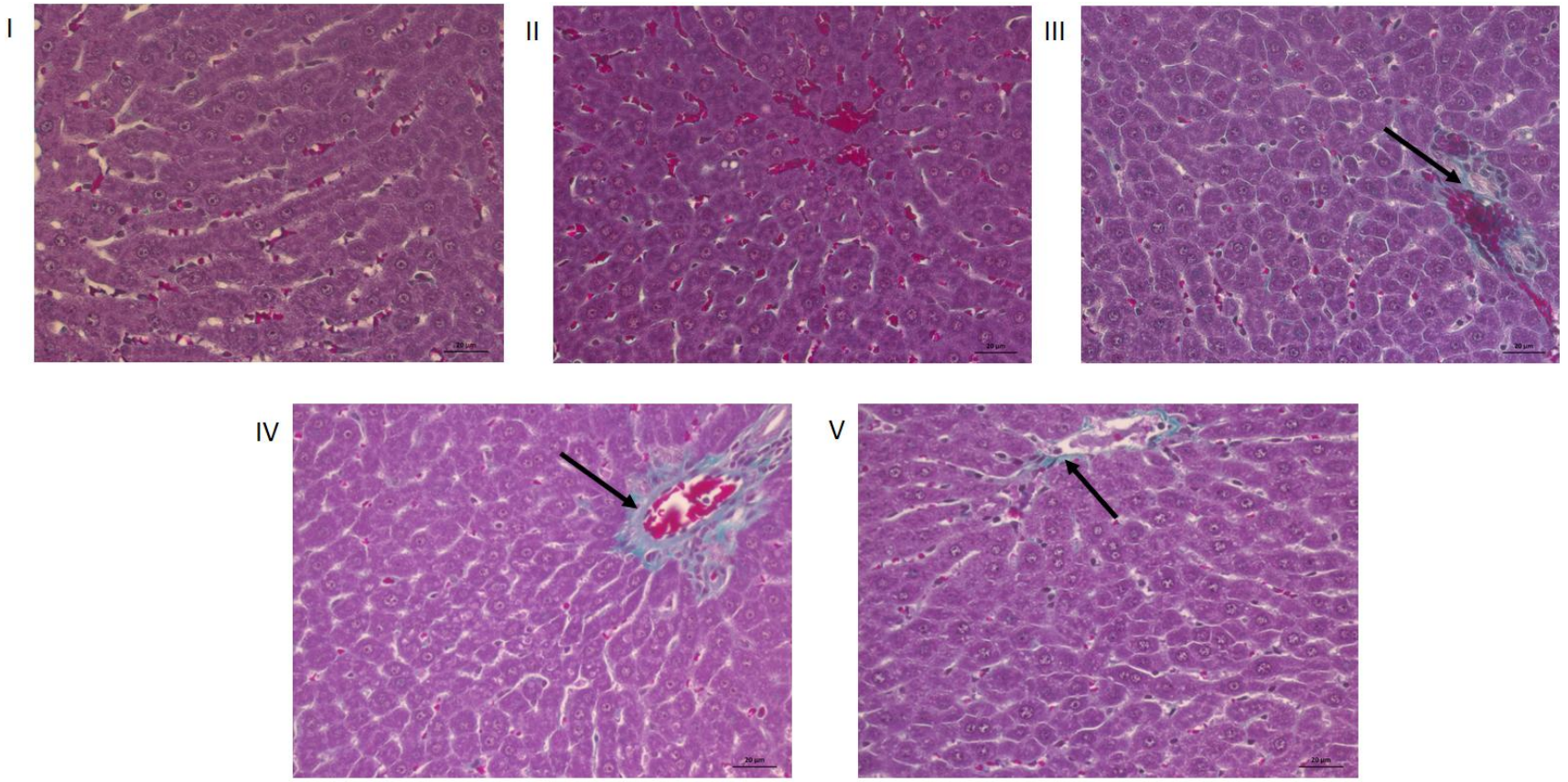
Figures 4.2 A. The representative haematoxylin and eosin (H & E) stained liver histology photomicrographs (100x magnification) of female rats from different treatment groups. Blue arrows indicate hepatic micro-vesicular steatosis and black arrow point to macro-vesicular steatosis. The control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Blue arrow = lipid droplets. Scale bar = 100 µm.



Figures 4.2 B. The representative haematoxylin and eosin (H & E) stained liver histology photomicrographs (100x magnification) of male rats from different treatment groups. Blue arrows indicate hepatic micro-vesicular steatosis. Control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Blue arrow = lipid droplets. Scale bar = 100 μm.



Figures 4.3 A. The representative Masson's trichrome (MT) stained liver histology photomicrographs (40x magnification) of female rats from different treatment groups. Black arrows indicate fibrosis. The control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Scale bar = 20 μm.



Figures 4.3 B. The representative Masson's trichrome (MT) stained liver histology photomicrographs (40x magnification) of male rats from different treatment groups. Black arrows indicate fibrosis. The control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Scale bar = 20 μm

4.4.5 Non-alcoholic fatty liver disease activity score (NAS)

The non-alcoholic fatty liver disease activity scores of the female rats for the different dietary groups are illustrated in table 4.4 A.

In female rats, the overall microvesicular steatosis of the rats across treatment groups were significantly different ($p = 0.0029$), one-way ANOVA, $F_{(4, 34)} = 4.972$). Rats fed a high-fructose diet had significantly increased ($p = 0.0082$) microvesicular steatosis when compared their counterparts control rats fed a standard diet. However, the female rats fed a combined stearic acid and high-fructose ($p = 0.9999$), counterpart fed stearic acid alone ($p = 0.8145$) as well as counterpart fed a combined fenofibrate and fructose diet ($p > 0.9999$) had similar microvesicular steatosis compared to the control rats fed a standard diet. Both the female rat groups fed a combined stearic acid and high-fructose ($p = 0.0076$) as well as those administered combined fenofibrate and high-fructose ($p = 0.0082$) had significantly prevented reduced high-fructose diet induced microvesicular steatosis. Whilst the stearic acid alone did not induce microvesicular steatosis ($p = 0.1088$).

Overall, there were no significant changes in female rat macrovesicular steatosis scores across treatment groups ($p = 0.3542$, one-way ANOVA, $F_{(4, 34)} = 1.14$). In female rats, there was no significant difference in hepatocyte hypertrophy scores ($p > 0.05$) across treatment groups. In the female rats, there was a significant difference ($p = 0.0001$, one-way ANOVA, $F_{(4, 34)} = 7.979$) in the inflammation scores of the hepatocytes across the treatment groups. When compared to control rats fed a standard diet, female rats fed a high-fructose diet ($p < 0.0001$), rats fed a combined stearic acid and high-fructose diet ($p = 0.0030$), rats fed stearic acid alone diet ($p = 0.0313$) and rats fed a combined fenofibrate and high-fructose diet ($p = 0.0459$) had significantly induced inflammation. The overall differences observed in the hepatic fibrosis scores across treatment groups was statistically significant ($p < 0.001$, one- way ANOVA, $F_{(4, 34)} = 8.09$). As shown in table 4.4 A, the female rats fed a high-fructose diet significantly ($p = 0.003$) induced fibrosis when compared to control rats fed a standard diet. Female rats fed a combined stearic acid and high-fructose diet in their diet had significantly ($p = 0.001$) mitigated the high-fructose induced fibrosis. Female rats whose diet was combined stearic acid and high-fructose and their counterparts whose diet had combined fenofibrate and high-fructose had different ($p < 0.05$) fibrosis. Stearic acid alone as a supplement had higher fibrosis ($p < 0.05$) compared to their counterparts fed a standard diet but the fibrosis was similar ($p > 0.05$) to the high-fructose fed rats.

In summary, a high-fructose diet caused microvesicular steatosis whereas a combination of stearic acid and high-fructose, and a combination of fenofibrate and high-fructose diets all failed to prevent this high-fructose induced microvesicular steatosis. There were no differences in macrovesicular steatosis across the treatment groups. Hepatic inflammation developed in all dietary treatment groups. A high-fructose diet induced fibrosis that was mitigated by stearic acid. Fenofibrate failed to prevent the high-fructose induced fibrosis. Stearic acid alone as a supplement induced fibrosis.

The non-alcoholic fatty liver disease activity scores in the male rats for the different dietary groups are illustrated in Table 4.4 B.

In male rats, the overall microvesicular steatosis of the rats across treatment groups were significantly different ($p = 0.0001$), one-way ANOVA, $F_{(4, 34)} = 7.774$). The microvesicular steatosis for high-fructose ($p = 0.4316$), combined stearic acid and high-fructose ($p = 0.8945$) as well as stearic acid alone ($p = 0.1197$) diets were similar. Only combined of fenofibrate and high-fructose diet significantly ($p =0.0374$) did not show microvesicular steatosis when compared to control male rats fed a standard diet. Stearic acid alone diet fed male rats microvesicular steatosis was lower ($p = 0.0010$) than microvesicular steatosis of the high-fructose diet fed male rats. Combined of fenofibrate and high-fructose as a dietary intervention resulted in significantly lower ($p = 0.0002$) microvesicular steatosis when compared to the control.

Male rats' macrovesicular steatosis scores did not change significantly ($p > 0.05$) across treatment groups. In male rats, there was no significant difference in hepatocyte hypertrophy scores ($p > 0.05$) across treatment groups. In the male rats, there was a significant difference ($p = 0.0029$, one-way ANOVA, $F_{(4, 34)} = 4.979$) in the inflammation scores of the hepatocytes across the treatment groups. The inflammation of the hepatocytes for high-fructose ($p = 0.5447$), combined stearic acid and high-fructose ($p = 0.6511$), stearic acid alone ($p > 0.9999$) as well as combined of fenofibrate and high-fructose ($p = 0.0997$) diets were similar when compared to inflammation of the hepatocytes for control male rats fed a standard diet. A combined stearic acid and high-fructose diet significantly decreased (0.0368) the high-fructose diet induced inflammation of the hepatocytes. Additionally, combined stearic acid and high-fructose diet resulted in lower ($p = 0.0023$) inflammation of the hepatocytes than the combined of fenofibrate and high-fructose diet. The overall differences observed in the hepatic fibrosis across treatment groups was statistically significant ($p < 0.001$, one- way ANOVA, $F_{(4, 34)} = 4.12$). Male rats fed a high-fructose diet had similar ($p > 0.05$) fibrosis when compared to the

control rats fed a standard diet. A combined stearic acid and high-fructose induced fibrosis ($p < 0.05$) when compared to the male rats fed a high-fructose diet. A combined fenofibrate and high-fructose diet had similar ($p = 0.07$) fibrosis when compared to the male rats fed a high-fructose diet. However, when compared to the combined stearic acid and high-fructose fed rats, the combined fenofibrate and high-fructose had significantly lower ($p < 0.05$) fibrosis. Stearic acid alone fed rats induced ($p < 0.05$) fibrosis in comparison with the control rats fed a standard diet.

In summary, a high-fructose, combined stearic acid and high-fructose, as well as stearic acid alone diet treatment had similar microvesicular steatosis when compared to control diet. Whereas, combined of fenofibrate and high-fructose diet induced microvesicular steatosis when compared to control diet. There were no differences in macrovesicular steatosis across the treatment groups. A high-fructose diet induced inflammation of the hepatocytes. The high-fructose diet did not lead to fibrosis. Instead, the combination stearic acid and high-fructose diet induced fibrosis. Fenofibrate similar fibrosis as the male rats fed high-fructose. Stearic acid as a supplement caused fibrosis.

Table 4.4 A. Liver micro-steatosis, macro-steatosis, hypertrophy and inflammation scores of female Sprague Dawley rats after 13 weeks of receiving either normal rat chow or a high-fructose diet and/or stearic acid or fenofibrate

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Microvesicular steatosis	0.5 (0, 2) [a]	2 (1, 3) [b]	1 (0, 1) [a]	1 (0, 2) [ab]	1 (0, 1) [a]	***
Macrovesicular steatosis	0 (0, 0) [a]	0 (0, 1) [a]	0 (0, 2) [a]	0 (0, 2) [a]	0 (0, 0) [a]	ns
Hypertrophy	0.00 (0;0) [a]	1.00 (1;1) [a]	0.00 (0;0) [a]	0.50 (0;1) [a]	1.00 (0;1) [a]	ns
Inflammation	0 (0, 2) [a]	1 (0, 2) [b]	1 (0, 2) [b]	1 (0, 2) [b]	1 (0, 2) [b]	***
Fibrosis (%)	3.49 ± 1.04 [a]	7.43 ± 1.05 [b]	3.07 ± 0.93 [a]	7.59 ± 1.44 [b]	6.75 ± 1.08 [b]	**

Data are presented as median (interquartile range). [a,b] Within row medians with different superscripts are significantly different at $p < 0.05$. ** $p < 0.001$ and *** $p < 0.0001$. The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 4.4 B. Liver micro-steatosis, macro-steatosis, hypertrophy and inflammation scores of male Sprague Dawley rats after 13 weeks of receiving either normal rat chow or a high-fructose diet and /or stearic acid or fenofibrate.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Microvesicular steatosis	1 (0, 1) [ab]	1 (0, 2) [b]	0.5 (0, 1) [ab]	0 (0, 1) [a]	0 (0, 0) [a]	***
Macrovesicular steatosis	0 (0, 0) [a]	0 (0, 0) [a]	0 (0, 0) [a]	0 (0, 0) [a]	0 (0, 0) [a]	ns
Hypertrophy	0.00 (0;0) [a]	1.00 (0;1) [a]	0.00 (0;1) [a]	0.00 (0;0) [a]	0.50 (0;1) [a]	ns
Inflammation	0 (0, 1) [ab]	1 (0, 2) [b]	0 (0, 1) [a]	0 (0, 1) [ab]	1 (0, 2) [b]	**
Fibrosis (%)	2.69 ± 1.55 [a]	2.58 ± 1.49 [a]	6.68 ± 1.78 [b]	6.55 ± 1.94 [b]	2.31 ± 1.32 [a]	**

Data are presented as median (interquartile range). [a,b,c] Within row medians with different superscripts are significantly different at $p < 0.05$. ** $p < 0.001$ and *** $p < 0.0001$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

4.5 Discussion

In a normal liver, less than 5-10% of the fresh liver mass should be made up of lipid droplets/stores (Younossi et al., 2019, Chalasani et al., 2018). Any more lipid stored in the liver is classified as steatosis. Non-alcoholic fatty liver disease develops when the lipids accumulate in the hepatocytes without a history of alcohol excessive drinking or other underlying pathological disorders that might induce steatosis (Coronati et al., 2022). The histological range of non-alcoholic fatty liver disease is broad, comprising from simple steatosis to non-alcoholic steatohepatitis, non-alcoholic steatohepatitis-related cirrhosis and hepatocellular carcinoma (Hliwa et al., 2021). Non-alcoholic steatohepatitis is distinguished from simple steatosis by ballooning hepatocyte degeneration with or without fibrosis. Consumption of high-fructose diets raise liver triglycerides, this causes the development of hepatic steatosis (Yustisia et al., 2022). Additionally, high-fructose consumption induces hepatic steatosis, due to increased *de novo* lipogenesis (Geidl-Flueck and Gerber, 2023), reduced hepatic fatty acid oxidation and increased triglyceride inflow into the liver from extrahepatic mobilization of fatty acid from adipose tissues (Softic et al., 2016, Yu et al., 2021).

In the present study, consumption of a high-fructose diet over a long term had no effect on absolute and relative (to body mass) liver masses but, increased hepatic lipid content in both female and male rats. In both female and male rats, a high-fructose diet induced micro-vesicular hepatic steatosis, inconsistent hepatocyte organization and inflammatory cell infiltration. Furthermore, a high-fructose diet caused fibrosis in female rats but not in male rats. In contrast to our findings, research suggests that men are more likely than women to develop hepatic fibrosis (Sabir et al., 2022). This sex bias was also detected in previous experiments on rats (Tacke and Weiskirchen, 2021), indicating that men's higher vulnerability to hepatocellular carcinoma is unlikely to be related to increased exposure to one or more metabolic risk factors.

The findings of the current study revealed that a combined stearic acid and high-fructose diet had no effect on absolute and relative (to body mass) liver masses but prevented high-fructose-induced hepatic lipid content in female rats, micro-vesicular hepatic steatosis and induced inflammation. This study also observed that a combined stearic acid and high-fructose diet mitigated high-fructose-induced fibrosis in female rats while inducing fibrosis in male rats.

We also found that combined fenofibrate and high-fructose caused heavier relative (to body mass) liver masses, inhibited high-fructose-induced increase in hepatic lipid content, inhibited high-fructose-induced micro-vesicular hepatic steatosis and induced inflammation. Reported

in the current study, dietary supplementation with stearic acid alone had no effect on hepatic lipid concentrations. However, it resulted in inflammation.

4.5.1 On the bright side, a high-fructose diet and/or stearic acid had no effect on the liver masses, suggesting its unlikeliness to cause hepatomegaly.

Evidence from studies on both humans and animals demonstrate that high-fructose diets are associated with increased lipogenesis, visceral obesity, non-alcoholic fatty liver disease in growing children, adolescents and adults (Ipsen et al., 2018, Jensen et al., 2018, Le et al., 2022).

An enlarged liver, also known as hepatomegaly, is a symptom of an underlying disease caused by accumulation of lipid and/or metabolic substrates such as glycogen, hepatic inflammation (hepatitis), or a result of cell damage, cancer and hepatocellular hypertrophy, all of which induce higher liver masses (Jensen et al., 2020, Kubitz et al., 2009) . The results of the present study show that the female and male rats after 13 weeks of receiving either a high-fructose diet and /or stearic acid did not have an increase in their liver masses. My results demonstrate that following a high-fructose diet for 13 weeks has no effect on liver mass. Similarly, administering stearic acid to both female and male rats had no effect on liver masses. On the other hand, after 13 weeks of receiving fenofibrate, the rats showed an increased the liver masses (table 4.2 A and B). Previous research has revealed that fenofibrate increases liver masses (Abdelmoneim et al., 2021). It is hypothesized that PPARα activation by fenofibrate promotes hepatic tissue growth resulting in hepatomegaly (Berger and Moller, 2002, Zhang et al., 2022a). Also, Hong et al. (2007) found that fenofibrate exacerbated hepatomegaly caused by a high-fat diet, which supports the current study's finding of increased liver mass. Due to unfavourable results, fenofibrate should be used with caution as an intervention therapy, since it may have trophic effects on organs including the liver.

On the bright side, a combinatorial diet of high-fructose diet and /or stearic acid had no effect on their liver masses, suggesting the unlikeliness of stearic acid to cause hepatomegaly.
Liver mass alone does not give full insights into the actual composition and structure of the liver. In order to assess the treatments' possible lipotoxicity effects, this study looked at their impact on hepatic lipid levels.

4.5.2 Stearic acid reduced the hepatic lipid content caused by a high-fructose diet

The results of the present investigation demonstrated that female rats fed a high-fructose diet had hepatic lipid contents that were significantly greater (22.94%) than those of the negative

control group. According to published research, consumption of high-fructose diet causes hepatic *de novo* lipid synthesis and storage to rise as well as β-oxidation to decrease (Roumans et al., 2020, Softic et al., 2016). Therefore, in this study, female rats' hepatic lipid content significantly increased after consuming a 20% high-fructose solution for 13 weeks. The findings from the current investigation are consistent with (Stanhope et al., 2015, Vos and Lavine, 2013) who reported that the consumption of a 20% high-fructose diet resulted in increased hepatic lipid. Fructolysis in the liver proceeds much more quickly than glycolysis does because the route skips the rate-limiting step phosphofructokinase-catalyses (Coronati et al., 2022), leading to an increase in triglycerides generation and *de novo* lipogenesis, both of which prolong unregulated production lead in the progression of non-alcoholic fatty liver disease (Attia et al., 2022, Ipsen et al., 2018), which substantiates the increased hepatic lipid findings of the present study.

In addition, in female rats, we found that after stearic acid was added in high-fructose diet (combinatorial effects); stearic acid managed to decrease the hepatic lipid content induced by high-fructose diet by 31.25%. In contrast to our findings, earlier research has found that the synergistic effects of fructose and added fat result in metabolic alterations that lead to the development of non-alcoholic fatty liver disease (Im et al., 2021). We were unable to find literature directly explaining the mechanisms by which stearic acid could reduce lipid content in non-alcoholic fatty liver disease. However, potentially the gastro-intestinal tract microbiome could play a role in altering the hepatic lipid content. In a study by Nie et al. (2022) indicated that stearic acid prevented alcohol-induced liver damage by controlling the gut microbiota to limit lipid production and increase liver lipid oxidation output.

In a research by Pan et al. (2010), it indicated that stearic acid could act as a hepatoprotective agent through decreasing cholestasis-induced hepatic damage. Meaning that, prolonged stearic acid intake was observed to prevent liver damage by raising serum biochemicals, ductular response, fibrosis and inflammatory pathophysiologic changes (Pan et al., 2010). This might explain why the combined stearic acid and high-fructose diet in female rats reduced high-fructose diet-induced fibrosis, as seen in this study (figure 4.3 A). Previous study found that stearic acid's anti-fibrotic effects were accompanied by alterations in α-smooth muscle actin-positive matrix-producing cells and production of the key fibrogenic cytokine transforming growth factor beta-1 (Pan et al., 2010). Unfortunately, we did not extend this study to evaluate circulating transforming growth factor-beta 1 as a marker of hepatic function impairment in the liver (Chen et al., 2012, Flisiak et al., 2000).

While saturated fatty acids are typically thought as unhealthy, some saturated fatty acids, in the opinion of nutritionists, may be advantageous. In our study, stearic acid stands out since its steatosis inducing effect in the liver was minimal in both female and male rats (table 4.4 A and B). According to Athira et al. (2022). Stearic acid has a hypolipidemic impact because it contains a 5-alpha reductase inhibitor, which may have hindered 3-hydroxy-3-methylglutaryl-CoA reductase, a critical enzyme in the cholesterol synthesis pathway (Igwe et al., 2015, Vadivel and Gopalakrishnan, 2011).

Similar effects were observed in rats fed a combined fenofibrate and high-fructose diet in which fenofibrate ameliorated hepatic lipid content induced by high-fructose diet by 31.25%. Multiple studies have found that fenofibrate reduces diet-induced liver lipid accumulation by increasing hepatic fatty oxidation (Abdelmoneim et al., 2021, Mahmoudi et al., 2022, Zhang et al., 2022a). Thus, it is likely that fenofibrate reduced the liver lipid high-fructose high-fat diet-induced liver lipid accretion in female rats in the current investigation through increasing hepatic fatty oxidation. Despite the rise in liver mass in rats administered fenofibrate, lipids seem unlikely to be the cause.

The lipid threshold in fresh liver is less than 5-10% of its mass. Beyond is associated with an increased risk of developing steatosis. In the current study, the hepatic lipids content were measured in dried hepatic tissue. The current study observed that across dietary interventions, the male rats' hepatic lipid content was within the 5-10% threshold for clinically diagnosing steatosis. Therefore, one can infer that this amount of fat is likely not to cause steatosis since male rats were less than 10% even on a dry matter basis. In female rats the hepatic lipid content ranged from 9-15% on a dry matter basis which although higher than males, on a wet basis would be less than the 105 threshold.

In the current study, the general summary of the hepatic lipid content is that female rats are prone to the detrimental effects of supplied high-fructose diets, whereas male rats are unaffected by the adverse repercussions. Therefore, our next task was to look at fatty acid profiles of the livers.

4.5.3 Fatty acid profile

Having considered the hepatic lipid content, it was important to look and analyse the hepatic fatty acid profile. Saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids are the three primary types of fatty acid species defined by their degree of saturation.

According to hepatic lipid profiling of female rats, the high-fructose diet had no effect on total saturated fatty acid content, increased total monounsaturated fatty acid, decreased polyunsaturated fatty acid, had a significant cis fatty acid content, increased omega 9 fatty acid and decreased omega 6 fatty acid content. Furthermore, hepatic lipid profile of female rats revealed a lower amount of stearic acid and a higher concentration of oleic acid.

Total saturated fatty acid: Capric acid has antiviral characteristics (Waehler, 2021), whereas myristic and lauric acids are linked to higher blood cholesterol levels in humans and has been shown in several studies to increase high-density lipoprotein cholesterol by at least as much as low-density lipoprotein cholesterol (Shramko et al., 2020).

Consumption of a combined stearic acid and high-fructose diet resulted in no increase in saturated fatty acid content, a decrease in total monounsaturated fatty acid content and an increase in polyunsaturated fatty acid content in female rat livers. Furthermore, omega 6 content increased in female rat livers fed a stearic acid and high-fructose diet.

A diet high in stearic acid only fed to female rats had no effect on their hepatic saturated fatty acid content. However, we observed that stearic acid-only female rat livers had identical palmitic acid and stearic acid concentration to those fed a standard diet. We additionally noticed that a stearic acid-only diet had the same total monounsaturated fatty acid content as the control group. This was demonstrated by a comparable oleic acid fatty acid concentration in female rat livers.

The total polyunsaturated fatty acids in the livers of female rats fed a high stearic acid diet significantly increased as compared to the control group rats. However, the linoleic acid as well as arachidonic concentrations were comparable.

According to hepatic lipid profiling of male rats, the high-fructose diet had no effect on total saturated fatty acid level but caused a lower concentration of palmitic fatty acid and a higher content of stearic acid. In addition, the male rats' livers showed no effect on total monounsaturated fatty acid content, with increased stearic acid content and decreased oleic acid content. The high-fructose diet raised total polyunsaturated fatty acid levels, as seen by increases in linoleic acid and arachidonic acid in male rats' livers. The hepatic lipid profile of male rats fed a high-fructose diet revealed a decrease in cis and omega 9 fatty acids, as well as an increase in omega 6 fatty acids.

A combination stearic acid and high-fructose diet reduced total saturated fatty acids significantly, with stearic and palmitic acids being lower than their control group counterparts in male rats' livers. Total monounsaturated fatty acids in the livers of male rats' fed combined

stearic acid and high-fructose diet were unaffected when compared to the control group; oleic acid content was similar to controls but significantly greater than the male rats livers in high-fructose fed rats. The combined stearic acid and high-fructose diet raised total polyunsaturated fatty acid levels when compared to total polyunsaturated fatty acid levels of the control group rats, as seen by significant increases in linoleic acid and arachidonic acid.

The cis fatty acid content in the livers of the male rats fed a combined stearic acid and high-fructose diet were raised when compared with the high-fructose diet fed male rats' livers. The omega 6 fatty acid content in the livers of the male rats fed a combined stearic acid and high-fructose diet were raised when compared with the standard diet fed male rats' livers. The omega 9 fatty acid content in the livers of male rats fed a combined stearic acid and high-fructose diet was similar to that of male rats fed a standard diet, but higher than that of male rats fed a high-fructose diet.

We also found that the group fed a stearic acid-only diet had the same total saturated fatty acid content as those fed a high-fructose diet only and a combination stearic acid and high-fructose diet, as well as similar palmitic fatty acid content than its counterparts fed a standard diet. Despite being fed a stearic acid diet, male rats' livers had the lowest stearic acid content in all treatment groups.

As indicated by an increase in oleic acid fatty acid content, the overall total monounsaturated fatty acid content of the male rats fed a stearic acid only diet increased compared to its counterparts across treatment groups. Total polyunsaturated and omega 6 fatty acids were drastically lower in the livers of male rats fed a stearic acid-only diet than in any other dietary treatment groups. Male rats fed only stearic acid exhibited considerably greater cis fatty acid and omega 9 fatty acid content in their livers than the other dietary treatment groups.

The fatty acid concentration, length and degree of saturation of dietary lipids influence their physico-chemical properties (Fave et al., 2004). Diet has a profound impact on the kind of lipids that exist in animal beings (Papotti et al., 2021). The fatty acid profile of the liver is regulated by the dietary fat consumed by the rats during development phases, which influences metabolism and eventual lipid deposition (Lian et al., 2020). Saturated fatty acids in the diet are detrimental to metabolic health because they contribute to the development of metabolic dysfunction and non-alcoholic fatty liver disease, obesity and chronic inflammation. Actually, the presence of excessive amounts of saturated fatty acids in the diet triggers the increase lipid storage within adipose tissues. Several studies have discovered clear correlations between high caloric and saturated fatty acid diets and the prevalence of obesity, hepatic steatosis and type

II diabetes in human beings (Lian et al., 2020, Melanson et al., 2009, Siri-Tarino et al., 2010, Zhou et al., 2020).

It is well known that a high fructose and saturated diet alters the distribution of fatty acids in the liver (Lian et al., 2020). However, there has been little evidence on the impact of a long-term diet on liver fatty acid distribution (da Silva-Santi et al., 2016). In the current study, we showed changes in liver fatty acid composition after 13 weeks of the consumption of stearic acid alone and/or combined high-fructose and stearic acid diet. We discovered that after 13 weeks of feeding, a combined high-fructose diet and a high-stearic acid diet had no effect on the saturated fat level of female rats' livers while decreasing the total saturated fat content of male rats' livers.

Despite an increase in the combined stearic acid and high-fructose diet (female rats' livers), as well as high-fructose alone and combined stearic acid and high-fructose diet (both female rats' livers and female rats' livers), poly-unsaturated fatty acids are healthy. Polyunsaturated fats comprise omega-3 and omega-6 fatty acids, both of which are required for brain function, blood sugar regulation and the prevention of type II diabetes (Fauzy et al., 2023, Kapoor et al., 2021). Omega 3, are essential fatty acids that provide a number of health benefits. Diets high in omega-3 fatty acids have favourable metabolic effects by lowering blood lipid levels while increasing levels of heart-protective high-density lipoprotein cholesterol, inflammation and blood pressure, therefore maintaining cardiovascular health (Covington, 2004). Omega-6 are also necessary and have metabolic characteristics that differ from omega-3 fatty acids (Djuricic and Calder, 2021). While the human body is unable to synthesize omega-3 and omega-6 fatty acids, it may metabolize them through elongation and desaturation processes (Saini and Keum, 2018).

In the current study, compared to the control, a high-fructose diet only and a combined stearic acid and high-fructose diet increased the omega 6 fatty acid content in the male rats' livers whilst a stearic acid alone diet decreased the omega 6 fatty acid content. On the other hand, the combined stearic acid and high-fructose dietary intervention and stearic acid alone dietary intervention reduced the high-fructose diet caused rise in omega 6 fatty acid content in male rats' livers. Nevertheless, of noteworthy is that the stearic acid alone diet showed significantly lower omega 6 fatty acid concentration in both female and male rats' livers. These results imply that dietary stearic acid supplementation reduced omega-6 fatty acid levels. These decreases in omega-6 fatty acid content corresponds directly to lowering the omega-6 to omega 3 ratio, which may be a useful technique for lowering inflammation and autoimmune responses (Fu et al., 2021, Simopoulos, 2002). Indeed, in the current study, dietary supplementation with stearic

acid alone significantly lowered the omega 6 to omega 3 ratios in both the female and male rats' livers. Previous research has demonstrated that a high dietary omega-6 to omega-3 ratio induces supraphysiologic inflammatory responses and maintains chronic inflammation and that a low omega 6 to omega 3 ratio reduces the risk of autoimmune disorders, asthma and allergies (Araya et al., 2004, DiNicolantonio and O'Keefe, 2021, Promrat et al., 2010). Furthermore, the concentration of omega 6 polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, in the livers of rats fed a stearic acid-only diet were significantly lower in the current study than in the control group.

α-linoleic acid, an omega 3 polyunsaturated fatty acid and linolenic acid, an omega 6 polyunsaturated fatty acid, are competitors for three enzymes that convert them, i.e., delta-5-desaturase, delta-6-desaturase and elongase (Rezapour-Firouzi, 2017, Vagner and Santigosa, 2011). These enzymes are a bottleneck because they have limited conversion capacity (Lee et al., 2016, Vagner and Santigosa, 2011). The important point is that the pro-inflammatory molecules are derived from omega 6 fatty acids and anti-inflammatory molecules from omega-3 fatty acids (Balić et al., 2020, Kang and Weylandt, 2008).

Our bodies need both and the right mix of omega 6 and omega 3 is important because an imbalance could promote a development of a lot of metabolic related diseases particularly cardiovascular disease. The omega 6 to omega 3 ratio is a measure that has been used as an indicator to assess if a person is consuming the proper types of fat in the right amounts. In a diet, generally, the recommended omega 6 to omega 3 fatty acid ratio is 4: 1for the cardio-protective purposes (Huber et al., 2018, Krebs, 2006). This implies that an individual should strive to consume 1g of omega 3s for every 4g of omega 6. Furthermore, it is suggested that adults consume between 1.1g to 1.6g of omega-3s each day. However, westernized diets have a significantly greater ratio.

In the current study, stearic acid alone as a dietary intervention decreased the omega 6 to omega 3 fatty acid ratio in both female (45.7: 1) and male (35: 1) rats' livers, though not to the recommended ratio. Whereas a combined stearic acid and high-fructose diet increased the omega 6 to omega 3 fatty acid ratio in both female (59.7: 1) and male (96: 1) rats' livers. Perhaps, the extremely low fatty acid content of eicosapentaenoic acid and docosahexaenoic acid in both female and male rats' livers is the contributing factor for this increased omegas 6 to omega 3 fatty acids ratios. Furthermore, omega 3 polyunsaturated fatty acids, such as docosahexaenoic acid and eicosapentaenoic acid, provide health professionals an alternative therapeutic option for non-alcoholic fatty liver disease (Cicero et al., 2021, Rizzo et al., 2023b).

Our study results showed extremely low fatty acid content of eicosapentaenoic acid and docosahexaenoic acid in both female and male rats' livers. These are often received from food, which includes fatty fish or through marine and synthetic-based supplements.

Palmitic and stearic acids were the most prevalent among total saturated fatty acids in the current investigation. In the current study, although the rats were fed a high stearic acid diet, liver fatty acid profiling revealed that the combination of stearic acid and high-fructose diet, as well as the stearic acid alone diet, dramatically reduced stearic acid concentration while the high fructose diet increased it. It has been reported that stearic acid has a considerable cholesterol-lowering impact (Bonanome and Grundy, 1988, Mensink, 2005), but other saturated fatty acids, such as myristic acid and palmitic acid, are positively related with hepatic inflammation (Saraswathi et al., 2022, Saraswathi et al., 2020). Stearic acid is involved in many different liver activities, including cholesterol metabolism and lipoprotein biogenesis (Goradel et al., 2016). Unlike oleic and linoleic acids, eating stearic acid has been shown to lower obesity (Sampath and Ntambi, 2005). Indeed, as shown earlier in figure 3.4 A (I), stearic acid alone diet decreased visceral fat in female rats. In agreement with other previous researchers, dietary stearic acid raises oleic acid levels as shown in table 4.4 B, most likely by activating hepatic enzymes.

In the current study, as demonstrated in table 4.3 A and B, oleic acid content (in monounsaturated fatty acids) is comparable to omega 9 content. Omega 9 fatty acid is another name for oleic acid. Oleic acid is more oxidatively stable than its polyunsaturated counterparts are and has been related to a number of health advantages including positive influence on cholesterol levels and promotes proper inflammatory responses in the body. It also provides favorable health effects such as reducing atherosclerosis, decreasing insulin resistance, improving immunological function and protecting against some types of cancer (Wang et al., 2022). In the current study, the omega 9's was quite high in the profiles of both female and males. Though not considered essential fatty acids, they can be produced in small amounts if omega 3 and omega 6 are available. The findings of this study support the idea that if the body lacks omega 3 and/or omega 6, it will be unable to produce enough omega 9. This study reported the highest omega 9's because the body is unable to generate omega-9, it converts it into an important fatty acid.

Unsaturated fats reduce the amount of low-density lipoprotein in the blood, which lowers the risk of cardiovascular disease (Shaya et al., 2022, Tindall et al., 2020). However, not all saturated fats are equal, with trans fats being the most detrimental since they increase low-

density lipoprotein cholesterol "bad cholesterol" while reducing high-density lipoprotein cholesterol "good cholesterol." As a result, a high-trans-fat diet increases the risk of heart disease (Perry et al., 2023). In the current investigation, it is of the utmost importance to highlight that a combined stearic acid and fructose dietary intervention, as well as stearic acid alone, reduces the trans fatty acid level of the female rats' livers, while cis fatty acids were dramatically increased in the livers of both female and male rats.

After analysing the rats' hepatic fatty acid profile, this study assessed histological alterations in liver lipid accumulation.

4.5.4 Histology

Histopathological examination of rat livers was performed to determine the impact of stearic acid in high-fructose induced pathological changes in growing Sprague-Dawley female and male rats fed a high-fructose diet and or stearic acid for 13 weeks (figures 4.2 A and B; figure 4.3 A and B). It was also important to characterise the nature of the fat deposition in the liver. Non-alcoholic fatty liver disease is a typical characteristic of metabolic dysfunction, characterized by hepatic steatosis, hypertrophy, inflammation and fibrosis (Caturano et al., 2021). With the liver being the primary site of fructose metabolism, an expanding body of research suggests that a high-fructose diet affects hepatic lipid metabolism (Brkljačić et al., 2019). Studies on rats have revealed a variety of histological changes in liver tissue following high-fructose diet consumption (Demirci et al., 2021, Lee et al., 2015, Zaki et al., 2019). Huang et al. (2004) observed increases in hepatic fat content in rats after six weeks of high fructose diet. The current study observed more evidence of hepatic pathology in females than males (figure 4.2 A and B).

Histomorphological examination of the liver revealed the existence of sex-specific steatosis in rats from treatment groups (Ballestri et al., 2017, Lonardo and Suzuki, 2020).

Both male and female rats on high-fructose diets developed microvesicular steatosis, with female rats also developing macrovesicular steatosis. As a result, our findings suggest that long-term high-fructose consumption may have distinct effects on metabolic function in male and female rats. Vilà et al. (2011) found that female rats responded more negatively to hepatic metabolic changes than male rats, which is consistent with our findings. The disparities could possibly be attributed to variances in male and female reactivity to high-fructose caused by sex hormone levels. In a sense that the male rats have more microsomal oxidative enzymes,

whereas female's rats have more microsomal reductive enzymes (Van Thiel and Gavaler, 1992).

The combination of stearic acid and a high-fructose diet resulted in a reduction in diet-induced hepatic lipid content (figure 4.1 A), as demonstrated by a substantial reduction in hepatic steatosis scores in female rats (table 4.3 A). Suggesting that stearic acid intake reduced fat caused by the subsequent high-fructose overload. Female rats administered stearic acid alone as a supplement developed microvesicular and macrovesicular steatosis, yet male rats had general normal hepatocellular architecture. The current study's findings imply that stearic acid alone had no effect on liver dysfunction in male rats. However, its usage in females should be cautiously monitored. The fenofibrate diet effectively prevented high-fructose diet-induced microvesicular steatosis in both female and male rats. This implies that fenofibrate tends to increase fatty acid oxidation rather than adipogenesis, which contributes to its lipid-lowering properties. Our findings suggest that stearic acid could serve as a hepatoprotective agent, its inclusion could attenuate non-alcoholic fatty liver induced by high-fructose diet especially in females.

4.6 Conclusion

This study found that a high-fructose diet could cause fatty liver disease. Sex-specific variations have been noted in response to high fructose diets. Non-alcoholic fatty liver disease in female rats was characterized by increased hepatic lipid accumulation, micro- and macro-vesicular hepatic steatosis, inflammation and fibrosis. In male rats, it was characterized by microvesicular steatosis and hepatic inflammation. This study also showed, for the first time, that stearic acid treatment reduced the development of fructose-induced non-alcoholic fatty liver disease by lowering hepatic lipid content. It is worth noting that stearic acid was effective in substantially lowering high-fructose diet-induced hepatic steatosis. These results suggests that stearic acid may protect against the development of non-alcoholic fatty liver disease. As a result, stearic acid supplementation has the potential preventive effect against on fructose diet-induced non-alcoholic fatty liver disease.

High-fructose consumption can also lead to development of nephropathy. The next chapter will explore the potential protective effects of stearic acid against renal impairment caused by a high-fructose diet in both male and female rats.

CHAPTER 5

THE COMBINED EFFECTS OF A DIET ENRICHED WITH STEARIC ACID AND HIGH-FRUCTOSE ON KIDNEY FUNCTION OF GROWING FEMALE AND MALE SPRAGUE-DAWLEY RATS

5.1 Introduction

The previous chapter focused on the combined effects of a diet enriched with stearic acid and high-fructose on the development of non-alcoholic fatty liver disease of growing female and male Sprague-Dawley rats. Long term and excessive consumption of high-fructose and/or high fat diets can potentially damage essential organs such as kidneys (Elsisy et al., 2021). In addition to filtering blood, kidneys produce and regulate essential hormones in the body that serve to manage blood pressure, maintain body fluid levels necessary for the body to operate and govern body chemistry (Lee and Mather, 2022). These regulatory actions controlled by the kidney are accomplished by managing the quantity of salt, water and other chemicals circulating around the body (Lee and Mather, 2022). Therefore, this current chapter will focus on the impact of a diet enriched with stearic acid and high-fructose concentrations on the possible development of nephropathy on Sprague-Dawley rats.

According to the International Society of Nephrology-Global Kidney Health Atlas, chronic kidney disease affects roughly 850 million individuals globally (Oguejiofor et al., 2021). Due to high treatment costs and widespread effects on the health and well-being of persons living with kidney disease, the worldwide burden of kidney failure remains enormous. According to the Pan American Health Organization, kidney disorders caused 254,028 fatalities in the region in 2019, with men accounting for 131,008 deaths and women accounting for 123,020 deaths (PAHO, 2021). Furthermore, in most nations, men outnumber women in terms of kidney disease mortality (van de Luijtgaarden et al., 2019). Using a population-based study, (George et al., 2019) evaluated the prevalence of chronic kidney disease in four sub-Saharan African countries: Burkina Faso, Ghana, Kenya and South Africa. Chronic kidney disease was found in 10.7% of the population (George et al., 2019). In comparison to East and West African countries, South Africa had the highest frequency of 12.9% (Mousa et al., 2021).

Diabetic nephropathy is one of the chronic consequences of diabetes mellitus that occurs more frequently in patients who have had diabetes for a long time (Sagoo and Gnudi, 2020) and has become the main cause of chronic kidney disease (Vodošek Hojs et al., 2020). Diabetic nephropathy affects 20% to 40% of diabetic people and is a significant impediment to the patient's quality of life (Hamza and Burton, 2023). Research suggests that the burden caused by excessive plasma glucose, which then has a deleterious impact on kidney function (Joshi et al., 2021), leads to the development of diabetic nephropathy (Clemente-Suárez et al., 2022).

5.1.1 The mechanisms of renal damage from hyperglycaemia.

Hyperglycaemia inhibits the polyol and hexosamine pathways from being activated, resulting in irregular glucose metabolism (Ohiagu et al., 2021). Renal structural changes and dysfunction are caused by abnormal glucose metabolism. The polyol pathway converts glucose to fructose in two steps. Glucose is reduced to sorbitol, which is then oxidized to fructose in this process (Ohiagu et al., 2021). Excessive activation of the polyol pathway leads to excessive accumulation of sorbitol in cells, which disrupts the antioxidant capability of the cells (Yagihashi et al., 2010, Zhang et al., 2021a). As a result, the activation of many cellular damage processes is accelerated, leading to the development of a variety of ill-metabolic complications including diabetic nephropathy (Nellaiappan et al., 2022).

Whereas, the hexosamine pathway is an important anabolic pathway whose product, uridine, is required for glycosylation activities (Paneque et al., 2023). The latter, hexamine pathway is a mediator of the effect of excessive hyperglycaemia on the build-up of the mesangial and renal tubular matrix (Ohiagu et al., 2021, Paneque et al., 2023). Previous research has revealed that hexamine pathway is implicated in hyperglycaemia-induced synthesis of transforming growth factor-1 (Robson et al., 2018, Sun et al., 2021), which facilitates hyperglycaemia-induced mesangial and tubular matrix development, hence speeding up the nephrosclerosis process in diabetic nephropathy (Kökény et al., 2021). Glycosylation is the process by which glucose undergoes a series of non-enzymatic reactions that result in advanced glycation end products (Lee and Mather, 2022). The increased development of advanced glycation end products in patients with diabetic nephropathy may be connected to the exposure of kidneys to a high glucose environment over a lengthy period of time, which enhances the likelihood of glycosylation and accumulation of advanced glycation end products (Parwani and Mandal, 2023, Thomas et al., 2005).

5.1.2 Fructose metabolism in the kidney

In the kidney, fructose is primarily metabolized at the proximal tubule (Björkman and Felig, 1982, Gonzalez-Vicente et al., 2019) and uses glucose as a substrate of gluconeogenesis during normal physiologic conditions; contributes to maintaining systemic glucose concentrations under starvation (Cirillo et al., 2009a). Long-term and excessive high-fructose consumption disrupts normal metabolism and becomes deleterious, eventually leading to the start and development of chronic kidney disease. Fructose metabolism in the liver produces glucose, lactate and elevated uric acid. Excessive uric acid production, which is a by-product of high-

fructose consumption causes oxidative stress and kidney disease (Asghar et al., 2016). This uric acid can cause tubule-interstitial scarring and kidney fibrosis, increasing pressures within the kidneys and causing kidney damage (Johnson et al., 2013, Kang and Nakagawa, 2005).

Furthermore, high saturated fat and high cholesterol diets are linked to the development of chronic kidney disease (Kochan et al., 2021, Odermatt, 2011). A significant number of animal studies have shown that eating a diet rich in saturated fat and cholesterol can lead to kidney damage (García-Arroyo et al., 2021, Thongnak et al., 2020). High-fructose diets have been demonstrated to induce as monocyte chemotactic protein-1 in proximal tubular cells (Chan and Lin, 2023, Wang et al., 2023). Monocyte chemotactic protein-1 recruits inflammatory mediators that eventually lead to tubule damage and contribute to kidney injury development (Cirillo et al., 2021).

5.1.3 Kidney injury molecule-1

Kidney injury molecule-1 is a type 1 transmembrane protein that is increased in the renal proximal tubule following acute kidney damage in rats. Several additional studies have shown that KIM-1 might be a useful biomarker for detecting and diagnosing acute kidney injury caused by nephrotoxin exposure. Kidney injury molecule-1 is involved in kidney tissue development and regeneration. Thus, during tubular injury, kidney cells produce kidney injury molecule-1, which is recognized as a distinct biomarker for tubular injury evaluation (Tanase et al., 2019).

5.1.4 The function of stearic acid in the prevention of high-fructose-induced kidney damage.

The fast growth in the prevalence of chronic kidney disease is cause for great concern and thus there is a need for disease protection. Stearic acid in the current study has shown hypolipidaemic and hepatoprotective effects and thus might suggest the potential role in the protection against high-fructose induced kidney injury. Furthermore, stearic acid is of relevance in this study since no studies have investigated its protective effects against kidney injury in high-fructose diet-fed rats as an experimental model for translation to humans.

5.2 Aim and objectives

This study aimed to investigate the effects of a stearic acid alone and/or combined high-fructose and stearic acid diet in the pathophysiology of high-fructose diet-induced kidney dysfunction in growing female and male Sprague-Dawley rats.

Hypotheses

H_0: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has no effect on nephropathy.
H_1: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has an effect on nephropathy.

The specific objective of this part of the study focused on:

5.2.1 Nephropathy by assessing:

5.2.1.1 kidney (absolute, relative to body mass and relative to tibial) masses.

5.2.1.2 kidney histomorphometry.

5.2.1.3 the circulating surrogate marker of renal tubular damage (plasma kidney injury molecule -1).

5.3 Materials and methods

5.3.1 Ethical clearance for the use of animals and study site

Details of ethical clearance and study site are as described in Chapter 3, subheading 3.3.3.

5.3.2 Animals, housing and feeding

Housing, feeding and care of the experimental rats were as described in Chapter 3, subheading 3.3.4.

5.3.3 Experimental design and treatments

The detailed experimental design and treatments administered to the rats are as described in chapter 3, subheading 3.3.5.

5.3.4 Kidney sample collection

The detailed kidney sample collection was as described in chapter 3, subheading 3.3.7. The collected kidneys were weighed to get absolute masses and mass relative to body mass was computed by dividing the mass of kidneys by the respective terminal body mass of each rat and expressed as a percentage (%). Relative to tibial length (rTL) masses of the kidneys was computed by dividing the mass of kidneys (g) by the respective tibia length (mm) of each rat and expressed as g/mm.

5.3.5 Processing of kidney samples for histology analysis

For morphological analysis of kidney samples, the previously formalin-preserved tissues were used. The kidney tissue samples were processed using an automatic tissue processor (Microm GmbH STP 120, Walldorf, Germany) over a duration of 16 hours. Following processing, each tissue sample was embedded in paraffin wax using a waxing machine (Thermolyne, Histo-Center II – N, Labotec, South Africa) and made into blocks and then sections of the kidney were cut at 5µm thickness using a rotary microtome (Leica Biosystems, model RM2125 RTS, Nussloch, Heildelburger, Germany) and MB35 Premier microtome blade (34⁰⁄ 80 mm). Thereafter the sections were mounted on glass slides with frosted ends (Lasec Laboratories) before staining. The slides were stained with haematoxylin and eosin (H&E) and Masson's trichrome (MT) for general tissue architecture and fibrosis examination respectively (Reyes-Gordillo et al., 2007). The slides were then placed in mounting xylene and mounted with DPX mounting media (Merck Chemicals, Pty Ltd) and cover slipped.

5.3.6 Kidney histological analyses and fibrosis

A light microscope (Reichert®, Austria) with CCD camera (AxioCam ERc 5s Rev.2; ZEISS, Germany) was used to view the slides at 400x magnification. Triplicate digital images of the kidney sections were obtained from different fields. Changes to kidney morphology were analysed by measuring renal corpuscle area and glomerulus area using ImageJ software (ImageJ 1.47v, Java 1.8.0_191; LOCI, University of Wisconsin). The area of Bowman's space also known as urinary space was determined using these measurements:

Bowman's space = renal corpuscle area (μm^2) - glomerulus area (μm^2). (Demirci et al., 2020)

Glomerular density was calculated as follows:

$$\text{Glomerular density} = \frac{\text{number of glomeruli in a section (N)}}{\text{total area of cortex (}\mu\text{m2)}}$$

To avoid bias, in the histopathological examination, the scorer was blinded. Two independent histologists scored; one from the School of Anatomical Sciences, at the University of Witwatersrand and the other from the Department of Human and Animal Physiology at the University of Johannesburg. The histologists were blinded to both the sex and the rats' treatment regimens (identity) and scoring was done independently. I also scored blindly, using rats' numbers instead of rats' treatment regimens. The supervisors did not score however, they cross-checked the final scores.

ImageJ software (ImageJ 1.47v/Java 1.6.0_045, Oracle America Inc.) was used to quantify the development of fibrosis in the kidneys using MT-stained slides at a 40x objective (Schneider et al., 2012). To determine the area per point (A_p) and area fraction ($A_{fraction}$) of each kidney section with connective tissue at 10x objective, the point count technique was used to calculate the sum of points ($\sum_p$). The formula used to calculate the area per point was:

$A = Ap \times \sum p$,

Ap = area per point (17.39)

A = area

$\sum p$ = sum of points falling on the connective tissue within a camera field of each liver section (7339.52 μm^2)

Distance in pixels was set at 162.

The area fraction of an image measuring 594501.7232 μm^2 was calculated using the following formula:

Afraction = [(A ÷ 594501.7232 μm²) × 100].

Afraction = area fraction

A = area

5.3.7 Determination of surrogate marker of kidney health

The plasma kidney injury molecule-1 (pg/mL) concentration was measured using a kidney injury molecule-1 (ELISA) kit for rats (Elabscience Biotechnology Co, Wuhan, Hubei Province, China). A detailed description of the protocol used is attached in appendix VI. A standard curve was constructed using known concentrations. The concentrations of kidney injury molecule-1 in the samples were then determined from the constructed standard curve. The control group rats had plasma kidney injury molecule-1 concentrations below the lower reportable limit for a rat kidney injury molecule-1 assay, hence they were assigned the lowest detectable ELISA KIM-1 concentration of 11.72 (pg/mL) of a healthy population in order to perform the statistical comparisons as recommended by the kit manufacturer.

5.3.8 Statistical analysis

Data analysis was done using GraphPad Prism 7 software (Graph-pad Software Inc., San Diego, USA). All normally distributed data are expressed as mean ± standard deviation. Group means for comparisons between different dietary treatment groups were analyzed using one-way analysis of variances (ANOVA) and mean comparison using a Tukey post-hoc test with statistical significance of $p < 0.05$ considered. For the data presented in the table, the statistical level of significance (p-value) is that of the overall group differences before applying post hoc test.

5.4 Results

5.4.1 The effect of oral administration of stearic acid on absolute, relative to body mass and relative to tibial length masses of the kidneys in growing female and male Sprague - Dawley rats fed a high-fructose diet.

The absolute, relative to body mass (rBM) and relative to tibial length (rTL) masses of the kidneys obtained from female rats following the 13-week treatment intervention are presented in table 5.1 A.

In female rats, the overall absolute kidney masses of the rats across treatment groups were significantly different ($p = 0.0372$), one-way ANOVA, $F_{(4, 34)} = 2.88$). The female rats whose diet was supplemented with a high-fructose alone had no significant difference in absolute kidney masses ($p = 0.9996$) when compared to the negative control rats fed a standard diet. Similarly, female rats that received a treatment of combined stearic acid and high-fructose diet ($p = 0.2397$) and their counterparts whose diet had stearic acid alone ($p = 0.9970$) as well as those whose diet had combined fenofibrate and high-fructose ($p = 0.7389$) had similar absolute kidney masses in comparison to the negative control rats fed a standard diet ($p > 0.05$). However, female rats fed a combined fenofibrate and high-fructose diet had significantly higher absolute kidney mass ($p = 0.0186$) than female rats fed a combined stearic acid and high-fructose diet (table 5.1 A).

The overall differences observed in relative to body mass kidney masses of the female rats across treatment groups were statistically significant ($p = 0.0464$), one-way ANOVA, $F_{(4, 34)} = 2.71$). As shown in table 5.1 A. The high-fructose diet had no effect ($p > 0.05$) on relative (to body mass) kidney masses in female rats when compared to the negative control rats fed a standard diet ($p = 0.9710$). Similarly, female rats that received a treatment of combined stearic acid and high-fructose diet ($p = 0.5525$) and their counterparts whose diet had stearic acid alone ($p = 0.8838$) as well as those whose diet had combined fenofibrate and high-fructose ($p = 0.4923$) had similar absolute kidney masses in comparison to the negative control rats fed a standard diet ($p > 0.05$). The female rats that were fed a combined fenofibrate and high-fructose diet had significantly higher ($p = 0.0299$) relative (to body mass) kidney masses when compared to the female rats fed a combined stearic acid and high-fructose diet (table 5.1 A).

In female rats, the overall relative to tibial length masses of the kidneys across treatment groups were not significantly different ($p > 0.05$), one-way ANOVA, $F_{(4, 34)} = 0.976$). In summary, in female rats, fenofibrate as an intervention resulted in a significantly heavier absolute and relative (to body mass) masses of the kidneys when compared to stearic acid intervention.

The absolute and relative (to body mass) masses of kidney of the male rats following a 13-week dietary treatment period are presented in table 5.1 B. In males, the overall absolute kidney masses of the rats between the five treatment groups were not significantly different ($p > 0.05$), one-way ANOVA, $F_{(4, 34)} = 0.476$). Similarly, the overall, relative (to body mass) kidney masses of the male rats between the five treatment groups were not significantly different ($p = 0.2522$), one-way ANOVA, $F_{(4, 34)} = 1.408$). In male rats, overall relative to tibial length masses of the kidneys across treatment groups were not significantly different ($p > 0.05$), one-way ANOVA, $F_{(4, 34)} = 1.76$).

In summary, in male rats, a high-fructose diet, stearic acid and fenofibrate interventions as well as stearic acid alone as a dietary supplement resulted in similar absolute, relative (to body mass) and relative to tibial length masses of kidney.

Table 5.1 A. The effects of stearic acid on absolute, relative to body mass (rBM) and relative to tibial length (rTL) kidney masses in growing female Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Kidney mass (g)	1.83 ± 0.11 [ab]	1.81 ± 0.16 [ab]	1.69 ± 0.11 [a]	1.85 ± 0.09 [ab]	1.90 ± 0.15 [b]	* (p = 0.0372)
Kidney rBM (%)	0.67 ± 0.04 [ab]	0.68 ± 0.03 [ab]	0.65 ± 0.03 [a]	0.69 ± 0.03 [ab]	0.70 ± 0.04 [b]	* (p = 0.0464)
Kidney rTL (g/mm)	5.20 ± 0.32 [a]	5.40 ± 0.42 [a]	5.28 ± 0.62 [a]	5.54 ± 0.49 [a]	5.60 ± 0.51 [a]	ns (p = 0.4333)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. [a, b] within row means with different superscript were significantly different at $p < 0.05$. The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 5.1 B. The effects of stearic acid on absolute, relative to body mass (rBM) and relative to tibial length (rTL) kidney masses in growing male Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Kidney mass (g)	2.87 ± 0.15 [a]	2.73 ± 0.21 [a]	2.73 ± 0.32 [a]	2.74 ± 0.21 [a]	2.74 ± 0.21 [a]	ns (p = 0.7531)
Kidney rBM (%)	0.64 ± 0.03 [a]	0.64 ± 0.03 [a]	0.62 ± 0.04 [a]	0.64 ± 0.03 [a]	0.67 ± 0.04 [a]	ns (p = 0.2522)
Kidney rTL (g/mm)	8.03 ± 0.77 [a]	7.43 ± 0.87 [a]	7.15 ± 0.85 [a]	7.24 ± 0.51 [a]	7.52 ± 0.56 [a]	ns (p = 0.1591)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

5.4.2 The effect of oral administration of stearic acid on kidney histomorphometry in growing female and male Sprague - Dawley rats fed a high-fructose diet.

Now that we have investigated renal mass as part of determining some of the negative consequences of diets, it is time to look at kidney histology. Table 5.2 A shows the quantification of the renal corpuscle area, glomerular area and area of the Bowman's/urinary space in female rats. Figures 5.1 A shows representative haematoxylin and eosin (H & E) stained kidney photomicrographs (400x magnification) of female rats after 13 weeks of treatment. One photomicrograph was randomly chosen for each group for representative inclusion in the images below.

The renal corpuscle area of female rats did not differ significantly among treatment groups ($p > 0.05$) when compared to female rats fed a standard rat diet. There was no statistically significant difference in the area of the glomeruli in female rats across treatment groups ($p > 0.05$). Female rats' Bowman's/urinary space was also not significant ($p > 0.05$). The glomeruli density of rat kidneys was similar among treatment regimens ($p > 0.05$).

Table 5.2 B shows the renal corpuscle area, glomerulus area and area of the Bowman's/ urinary space in male rats. Figure 5.1 B shows representative haematoxylin and eosin (H & E) stained kidney photomicrographs (400x magnification) of male rats after 13 weeks of treatment. One photomicrograph was chosen at random representative for each group. Overall, across treatment regimens, male rats' renal corpuscle area, glomerulus area, Bowman's/ urinary space and glomeruli density were similar ($p > 0.05$).

Figure 5.2 shows representative Masson's trichrome (MT) stained kidney photomicrographs (100x magnification) of female (A) and male (B) rats following 13-week treatment periods. Each group had one photomicrograph selected at random. There was no significant difference in liver fibrosis scores between female ($p > 0.09$, one-way ANOVA $F_{(4, 34)} = 0.63$) and male ($p > 0.05$, one-way ANOVA, $F_{(4, 34)} = 0.78$) rats following the different dietary treatments.

Table 5.2 A. The effects of stearic acid on the kidney histomorphometry of the female Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

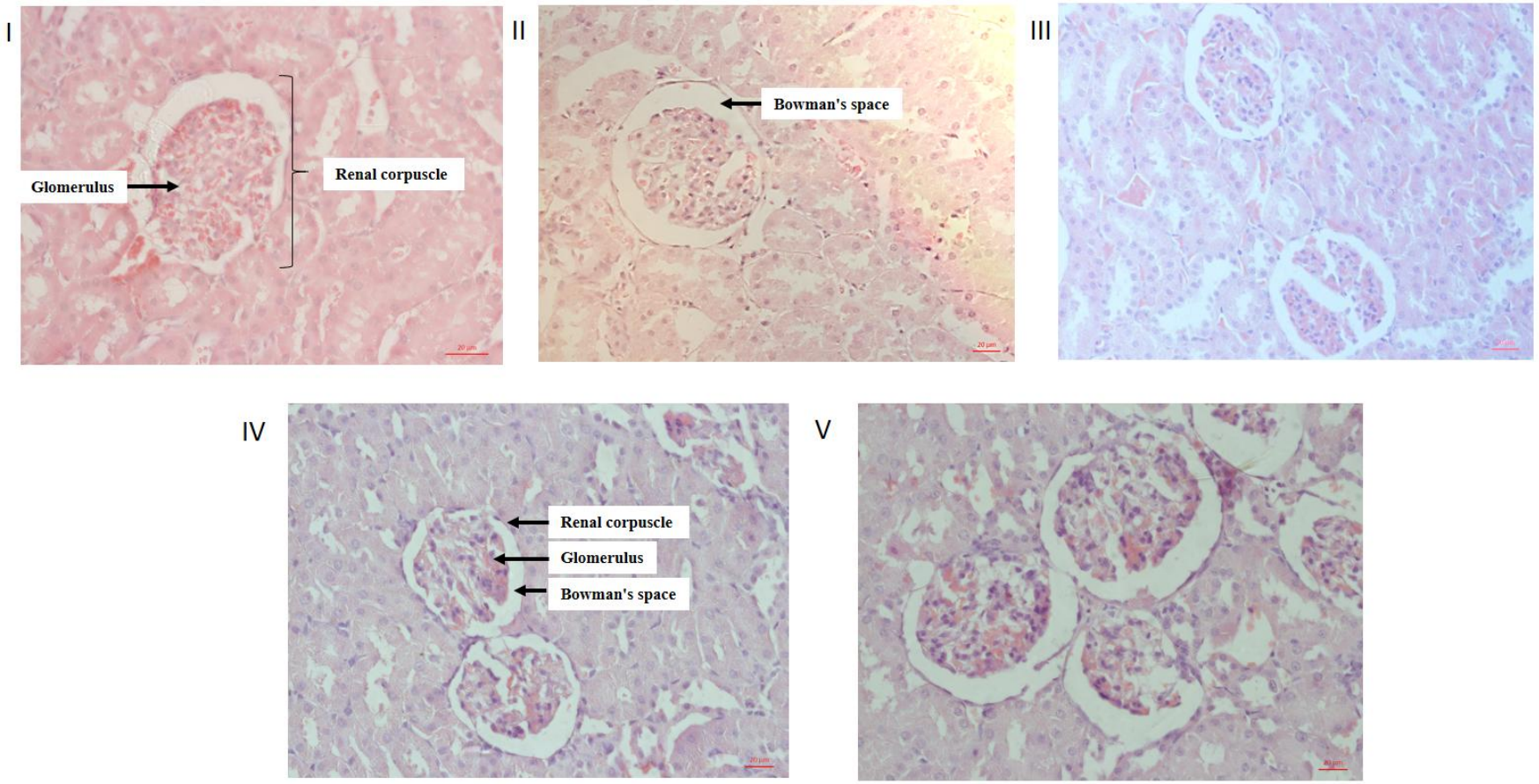
Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Renal corpuscle area (μm²)	8626 ± 2372 [a]	8836 ± 2252 [a]	8471 ± 2407 [a]	8533 ± 2108 [a]	8912 ± 2381 [a]	ns (p = 0.985)
Glomerulus area (μm²)	5540 ± 2176 [a]	5826 ± 2188 [a]	5436 ± 2176 [a]	5257 ± 2001 [a]	5686 ± 2023[a]	ns (p = 0.660)
Bowman's/urinary space (μm²)	3086 ± 1988 [a]	3010 ± 1680 [a]	3035 ± 1534 [a]	3276 ± 1785 [a]	3226 ± 1809 [a]	ns (p = 0.434)
Glomeruli density ($N/\mu m^2.10^{-5}$)	7.20 ± 1.32 [a]	6.40 ± 1.42 [a]	6.28 ± 1.62 [a]	5.54 ± 1.49 [a]	7.20 ± 1.51 [a]	ns (p = 0.175)
Fibrosis (%)	3.64 ± 1.50 [a]	3.83 ± 2.38 [a]	3.28 ± 1.52 [a]	3.56 ± 0.89 [a]	3.03 ± 1.45 [a]	ns (p = 0.092)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

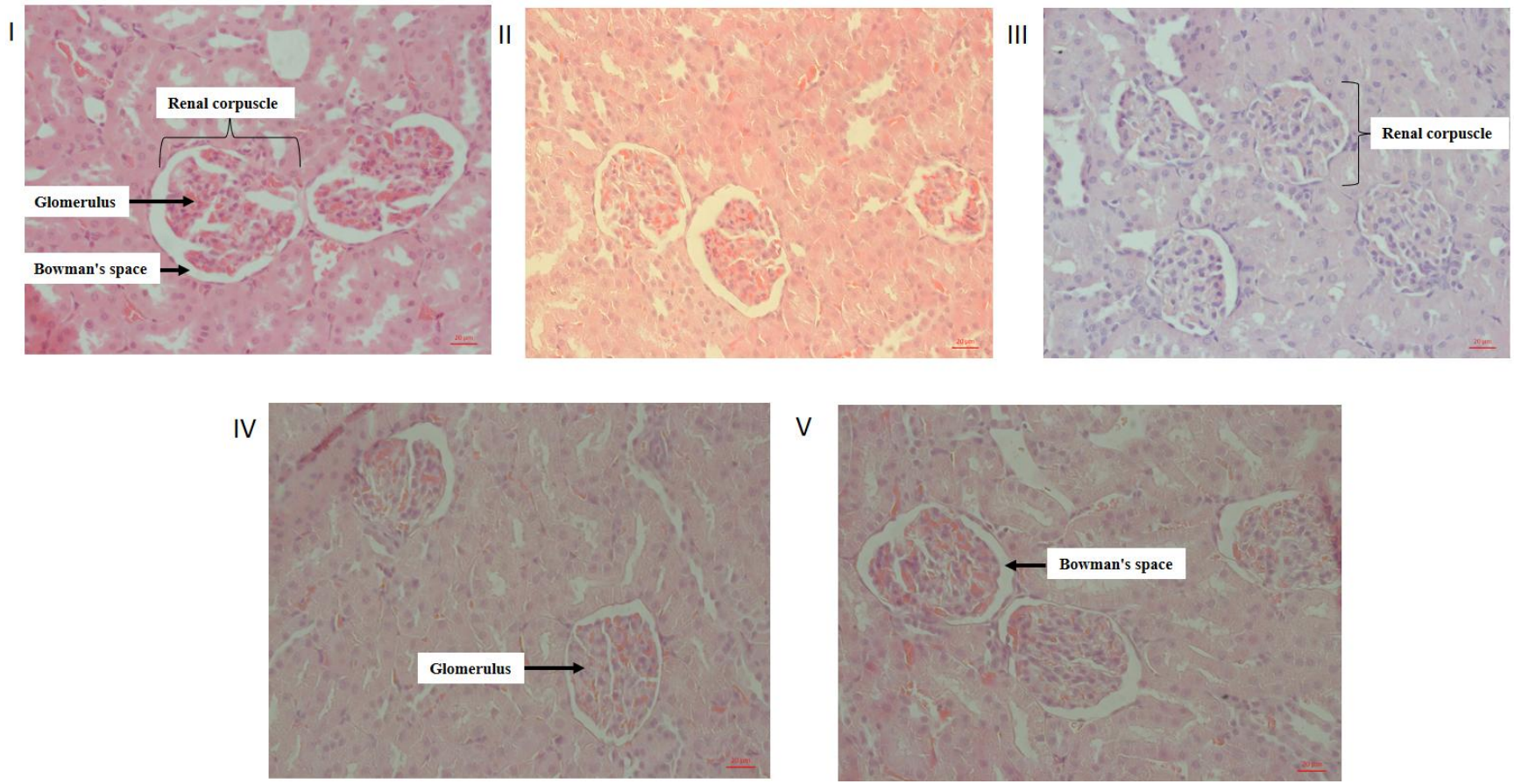
Table 5.2 B. The effects of stearic acid on the kidney histomorphometry of the male Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	**Treatment groups**					**Overall level of significance**
	Control	**Fructose**	**Stearic acid + Fructose**	**Stearic acid**	**Fenofibrate + Fructose**	
Renal corpuscle area (μm²)	10007 ± 3351 [a]	10469 ± 3217 [a]	10757 ± 3407 [a]	10469 ± 3642 [a]	11757± 4355 [a]	ns (p = 0.347)
Glomerulus area (μm²)	7816 ± 3142 [a]	8826 ± 2900 [a]	7636 ± 2176 [a]	8257 ± 2130 [a]	9902 ± 3622 [a]	ns (p = 0.250)
Bowman's/urinary space (μm²)	2191 ± 1898 [a]	1643 ± 1380 [a]	3121 ± 1231 [a]	2212 ± 1551 [a]	1855 ± 1809 [a]	ns (p = 0.243)
Glomeruli density ($N/\mu m^2.10^{-5}$)	5.65 ± 1.22 [a]	7.12 ± 2.88 [a]	5.24 ± 1.50 [a]	5.70 ± 0.99 [a]	6.86 ± 1.97 [a]	ns (p = 0.103)
Fibrosis (%)	5.76 ± 0.93 [a]	5.46 ± 1.44 [a]	5.86 ± 3.07 [a]	5.58 ± 2.775 [a]	5.74 ± 3.38 [a]	ns (p = 0.054)

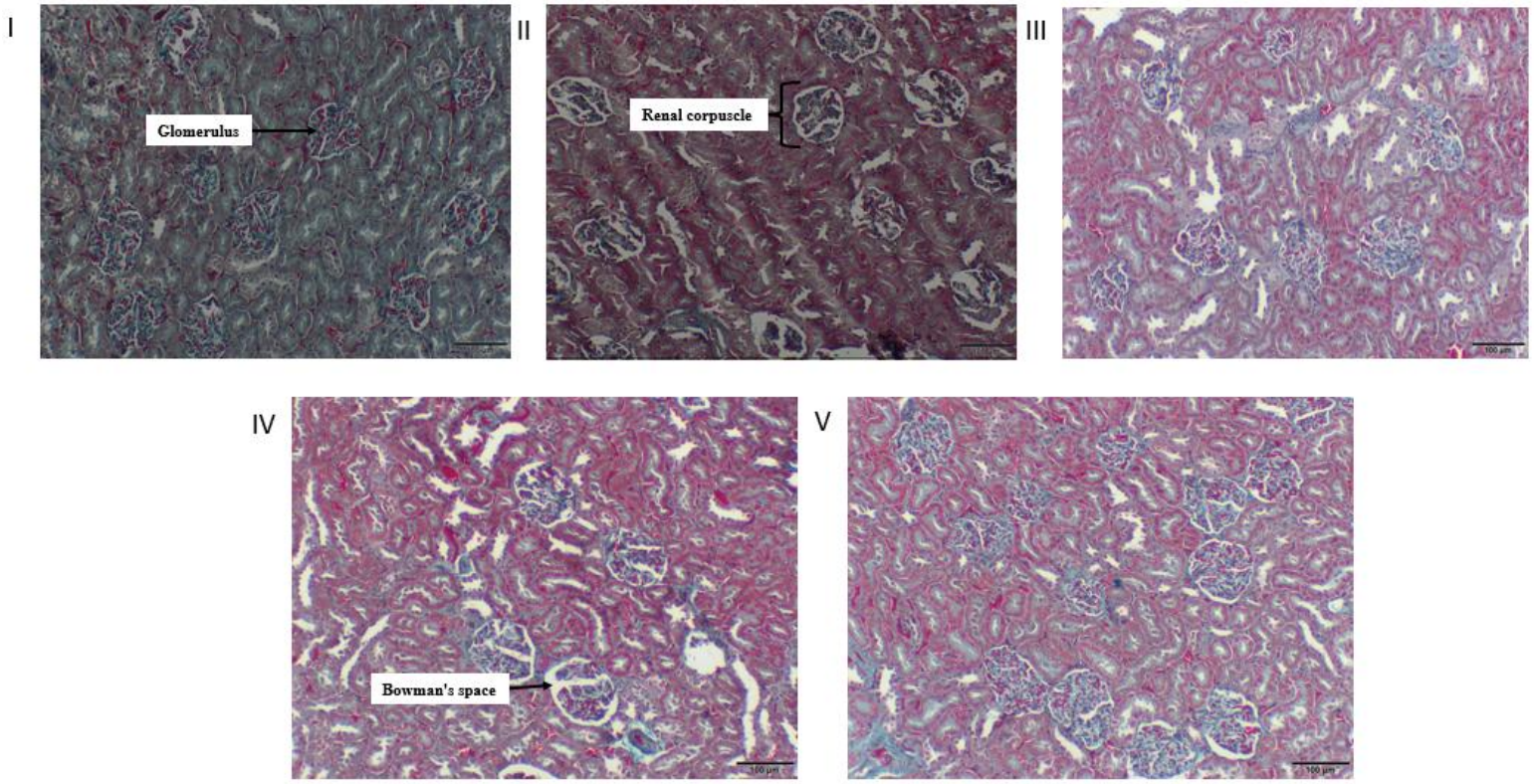
Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).



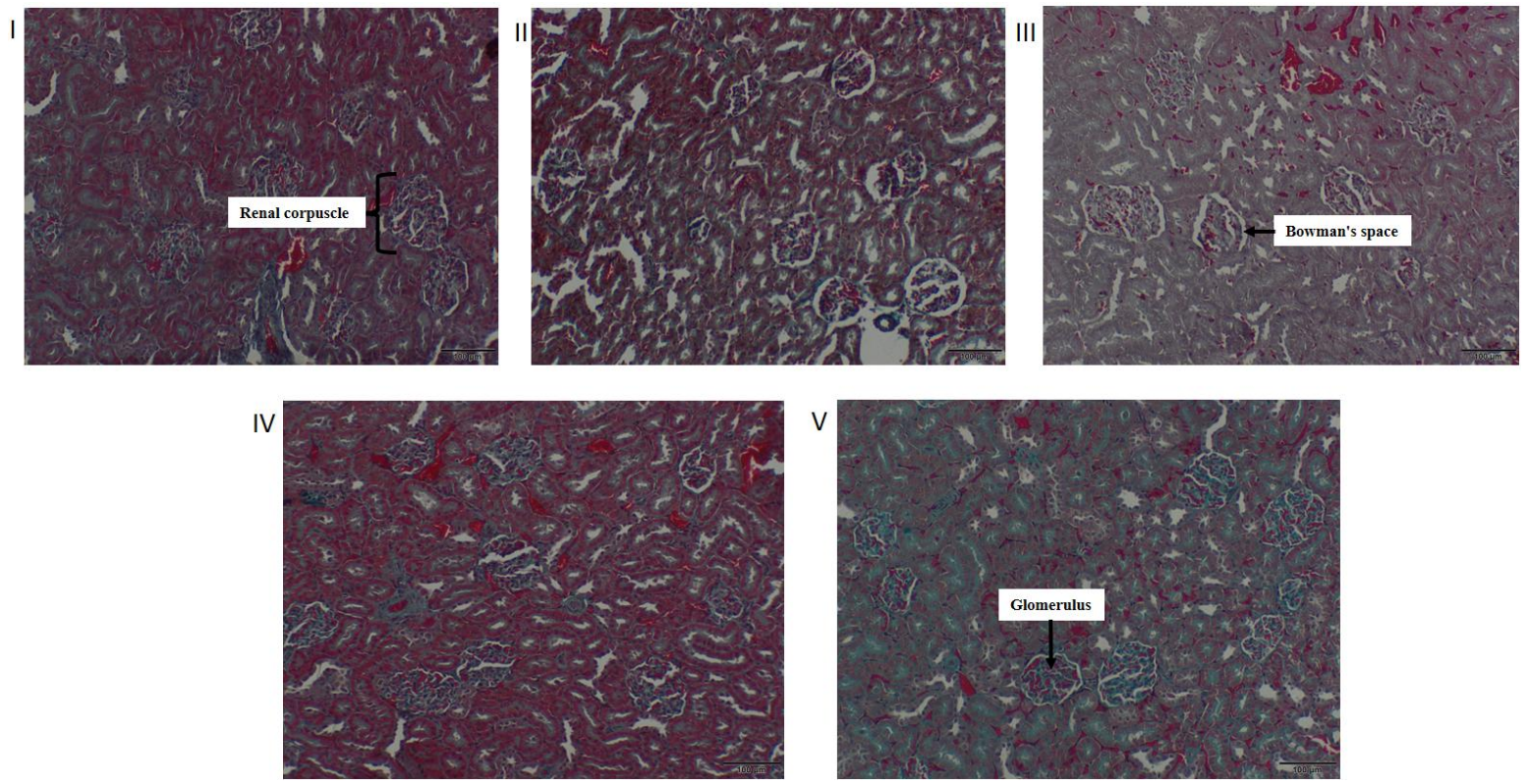
Figures 5.1 A. Photomicrographs of the kidney histological sections, stained with H & E, from representative female Sprague - Dawley rats (one from each dietary group) after 13 weeks of receiving standard rat chow and plain water to drink (I) or a standard rat chow and 20% fructose solution to drink (II) or a standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (III) or a standard rat chow enriched with 3% stearic acid and plain water to drink (IV) or a standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (V). The images were obtained at 400x magnification. Scale bar = 20 μm



Figures 5.1 B. Photomicrographs of the kidney histological sections, stained with H & E, from representative male Sprague Dawley rats (one from each dietary group) after 13 weeks of receiving standard rat chow and plain water to drink (I) or a standard rat chow and 20% fructose solution to drink (II) or a standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (III) or a standard rat chow enriched with 3% stearic acid and plain water to drink (IV) or a standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (V). The images were obtained at 400x magnification. Scale bar = 20 μm.



Figures 5.2 A. The representative Masson's trichrome (MT) stained kidney histology photomicrographs (40x magnification) of female rats from different treatment groups. The blue-green stain shows the extent of fibrosis in the cortical region of the kidney. The control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Scale bar = 100 μm.



Figures 5.2 B. The representative Masson's trichrome (MT) stained kidney histology photomicrographs (40x magnification) of male rats from different treatment groups. The blue-green stain shows the extent of fibrosis in the cortical region of the kidney. The control group (I) is the negative control in which rats received standard rat chow and plain water to drink; fructose group (II) rats were fed standard rat chow and 20% fructose solution to drink; stearic acid and fructose group (III) rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink; the stearic acid group (IV) rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink; the fenofibrate and fructose group (V) rats served as the positive control, rats were fed standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink. Scale bar = 100 μm.

5.4.3 The effect of oral administration of stearic acid on kidney injury molecule-1 in growing female and male Sprague - Dawley rats fed a high-fructose diet.

After assessing the kidney histology, it was critical to monitor the animals' health by evaluating any toxicity associated with the treatments. As a result, the concentration of kidney injury molecule-1, a surrogate marker of kidney function, was measured in this investigation. The plasma concentrations of kidney injury molecule-1 of female and male rats are shown in Tables 5.3 A and B, respectively.

In female rats, overall kidney injury molecule-1 concentrations across the treatment groups showed significant differences ($p < 0.0001$, one-way ANOVA, $F_{(4, 34)} = 3.77$). The KIM-1 concentration in the female rats whose diet was standard rat chow and plain water to drink was not detected. The female rats whose diet was high-fructose and their counterparts whose diet had fructose but supplemented with stearic acid and/or fenofibrate as well as the rats fed a stearic acid alone diet had significantly increased ($p < 0.0001$) plasma concentration of KIM-1 compared to the female rats fed the standard diet. However, female rats that had high-fructose and their counterparts whose diet had fructose but supplemented with stearic acid and/or fenofibrate as well as the rats fed a stearic acid alone diet had similar ($p > 0.05$) KIM-1 concentrations.

In summary, the high-fructose diet increased the plasma KMI-1 concentration. However, the stearic acid and fenofibrate intervention failed to protect against the high-fructose induced increase in KIM-1 concentration.

In male rats, overall kidney injury molecule-1 concentrations across the treatment groups showed significant differences ($p < 0.0001$, one-way ANOVA, $F_{(4, 34)} = 2.44$). The standard diet fed male rats' KIM-1 concentration is significantly different ($p < 0.0001$) compared to high-fructose fed and their counterparts whose diet had fructose but supplemented with stearic acid and/or fenofibrate. Although, high-fructose diet significantly ($p < 0.0001$) increased KIM-1 concentration when compared to the control rats, stearic acid and fenofibrate interventions failed to decrease the KIM-1 concentration ($p > 0.05$). Stearic acid alone as a dietary intervention significantly increased KIM-1 concentration compared to the male rats fed the standard diet ($p < 0.0001$).

Table 5.3 A. The effects of stearic acid on kidney injury molecule-1 in growing female Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
KIM-1 (pg/mL)	- [a]	201.10 ± 0.59 [b]	200.30 ± 0.0.71 [b]	201.50 ± 0.38 [b]	201.80 ± 0.63 [b]	* ($p < 0.0001$)

Data presented as mean ± standard deviation. - = below the assay detection limit, therefore lowest detectable concentration was used for statistical analyses. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. [a, b] within row means with different superscript significantly different at $p < 0.05$. The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 5.3 B. The effects of stearic acid on kidney injury molecule-1 in growing male Sprague-Dawley rats fed a high-fructose diet and or stearic acid for 13 weeks.

Parameter	Treatment groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
KIM-1 (pg/mL)	- [a]	181.08 ± 0.05 [b]	181.09 ± 0.04 [b]	181.10 ± 0.10 [b]	181.13 ± 0.06 [b]	* ($p < 0.0001$)

Data presented as mean ± standard deviation. - = below the assay detection limit, therefore lowest detectable concentration was used for statistical analyses. ns = not significant with $p > 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

5.5 Discussion

The study aimed to investigate the effects of a stearic acid alone and/or combined high-fructose and stearic acid diet in the pathophysiology of high-fructose diet-induced kidney dysfunction in growing female and male Sprague-Dawley rats. Previous research has revealed that prolonged high-fructose consumption predisposes rats to kidney disease. We found that the high-fructose diet had no influence on kidney mass, renal corpuscle area, glomerulus area, Bowman's/urinary space, glomeruli density in both female and male rats. Furthermore, the stearic acid intervention had no effect on absolute kidney mass and did not protect the rats from the high-fructose diet-induced elevation in kidney injury molecule-1. In female and male rats, fenofibrate intervention increased kidney mass but did not impair renal function compared to the other intervention groups. Stearic acid alone had no impact on kidney mass, renal corpuscle area, glomerulus area, Bowman's/ urinary space or glomeruli density in rats, but it did elevate kidney injury molecule-1 compared to the negative control in female and male rats.

5.5.1 Morphometry of the kidneys in stearic acid alone and/or combined high-fructose and stearic acid diet fed growing female and male Sprague-Dawley rats

Increased kidney mass is associated with chronic renal disease and implies abnormal kidney growth (Hsu et al., 2006). Given that, high-fructose intake alone can alter kidney function via glomerulonephritis (Sánchez-Lozada et al., 2007, Wu et al., 2022), there is a considerable chance of combined high-fructose and/or fat diet consumption, which has been linked to kidney damage via systemic release of pro-inflammatory triggers such as uric acid and oxidative stress (Lubawy and Formanowicz, 2023, Russo et al., 2020). The high-fructose diet had no effect on kidney masses in female or male rats across the treatment groups, according to this study. According to (table 5.1 A), while the kidney masses of the female rats fed a combined fenofibrate and high-fructose diet were significantly higher ($p < 0.05$) compared to the kidney masses of the female rats fed a combined stearic and high-fructose diet, the kidney masses of the female rats from other treatment interventions were similar ($p > 0.05$). These finding suggests that treatment with stearic acid alone or as an intervention did not cause damage to the kidney. The current investigation found that kidney masses when expressed relative to tibial length, fenofibrate had no significant differences across treatment groups in both female and male rats (Table 5.1 A and B). Previous studies have indicated that fenofibrate is renoprotective in rat metabolic syndrome models. (Yaribeygi et al., 2018). In addition, fibrates have been associated with decreased cardiovascular risk and are commonly used

alongside statins to treat hypertriglyceridemia (Hadjivasilis et al., 2022). Their use is typically limited due to a reduction in glomerular filtration rate at the start of treatment.
Kidney mass alone is an unreliable diagnosis for kidney disease. As a result, we next decided to evaluate general renal architecture assessment using histomorphometry.

5.5.2 Changes in kidney histomorphometry of the rats fed stearic acid alone and/or combined high-fructose and stearic acid diet.

The current study's kidney histological analysis revealed that the renal corpuscle area, glomerulus area, Bowman's/urinary space and glomeruli density were similar in female and male rats across the different treatment groups. As a result, a high-fructose diet did not cause kidney impairment. This study's findings contradict those of (Kretowicz et al., 2011), who found that high-fructose consumption promotes kidney harm, manifested as glomerular fibrosis. Although there was a tendency to slightly increased fibrosis in the current study, especially in male rats, there was no significant difference in the degree of fibrosis in the cortical area of the kidneys between treatment groups in both male and female rats. Therefore, our data suggest that treatment regimens did not result in the deposition of connective tissue in the glomerular tuft because of glomerular sclerosis. Perhaps a longer-term study could have produced different results in line with previous studies that used longer intervention durations.

Furthermore, fenofibrate has been linked to nephrotoxicity in investigations (Attridge et al., 2013, Hernandez-Arroyo et al., 2022). Nephrotoxicity can be caused by an imbalance in drug influx and efflux into and out of renal cells, resulting in an increase in intracellular drug concentration (Izzedine et al., 2005). The consistent size of the renal corpuscles across treatment groups could imply that Bowman's capsule did not expand due to glomerular hyperfiltration (Kotyk et al., 2016, Sutherland et al., 2021). Furthermore, the size of the glomerular region in female and male rats was similar across treatment groups. These findings may imply that no inflammation occurred in the kidneys because of podocyte hypertrophy and aberrant mesangial enlargement because of glomerular hypercellularity (Akhtar et al., 2020, Gusev et al., 2021).

KIM-1 is a sensitive marker of renal damage which might not be discernible histologically. It was therefore important to assess renal damage in the rats by determining plasma kidney injury molecule-1 concentrations.

5.5.3 A high-fructose diet, stearic acid alone and/or combined high-fructose and stearic acid diet increases plasma kidney injury molecule-1 concentrations.

Research has confirmed that KIM-1 as a biomarker is adequate for assessing nephrotoxicity in a variety of chronic renal disorders (Hassan et al., 2023, Vaidya et al., 2006). Therefore, increased plasma levels of kidney injury molecule-1 have shown to be indications of kidney damage (Vaidya et al., 2006). Increased plasma KIM-1 levels in rats correlate with severe kidney damage, both histological and functional. KIM-1 levels in healthy renal tissue are extremely low, if not undetectable (Khonsha et al., 2024).

In the current study, female and male rats were fed a high-fructose diet for 13 weeks, which resulted in an increase in the concentration of kidney damage molecule-1. Furthermore, stearic acid alone or in combination with a high-fructose and stearic acid diet, as well as fenofibrate as a dietary intervention, failed to reduce kidney damage molecule-1 levels. These data imply that the high-fructose diet was nephrotoxic or induced tubular damage, while intervention treatment with stearic acid alone or in combination with a high-fructose and stearic acid diet as well as fenofibrate failed to protect the kidneys. Our findings correspond with those of Liu et al. (2023), Yustisia et al. (2022), who found that feeding saturated fats and/or cholesterol to adult male rats for eight weeks predisposed to kidney damage, as seen by higher than normal kidney injury molecule-1 levels. Furthermore, the results from the current study are consistent with Cirillo et al. (2009b) who reported that high-fructose diet causes functional renal tubule damage.

Although the kidney histomorphometry data indicated that there was no inflammation in the kidneys, we cannot infer that stearic acid, as a dietary intervention in this study is safe for the kidneys. The present study demonstrated that stearic acid did not prevent high-fructose-induced proximal tubular damage, as evaluated by plasma renal injury molecule-1 concentrations in female and male rats fed a combined stearic acid and high-fructose diet. My investigation found that supplementing with stearic acid alone raised plasma kidney damage molecule-1 levels. However, it must be noted that increased renal proximal tubular epithelial cell regeneration has been linked to an increase in kidney injury molecule-1 (Lim et al., 2013, Stamellou et al., 2021). It is uncertain if stearic acid increased kidney injury molecule-1 via nephrotoxicity or proximal tubular epithelial cell regeneration, as we were unable to probe for specific markers. As a result, further investigation is necessary.

Kidney damage molecule-1 is a more specific, sensitive, as well as accurate measure of kidney injury in rats, outperforming creatinine and urea as predictors of histopathological alterations

in response to toxicants (Chaturvedi et al., 2009, Obert et al., 2021). Therefore, in the present study, only plasma kidney damage molecule-1 was determined as a surrogate marker of kidney health.

5.6 Conclusion

The study findings confirm the previous reports that long term consumption of a high-fructose diet can produce renal dysfunction in both female and male rats as demonstrated by elevated kidney injury molecule-1 concentrations. Kidney injury molecule-1 concentrations were expressed at low levels below detection in the normal kidney. However, there were no discernible variations in renal corpuscle area, glomerulus area, Bowman's/urinary space or glomeruli density were seen between treatment groups in female and male rats. The study findings also imply that stearic acid supplementation like fenofibrate may induce kidney abnormalities and should hence be administered with caution.

Having shown that long-term consumption of stearic acid in growing female and male Sprague-Dawley rats may induce kidney dysfunction, the next supplementary chapter presents the effect of stearic acid on the gastrointestinal tract and visceral organ morphometry in growing female and male rats.

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CHAPTER 6

THE COMBINED EFFECTS OF A DIET ENRICHED WITH STEARIC ACID AND HIGH FRUCTOSE ON GASTROINTESTINAL AND VISCERAL ORGAN MORPHOMETRY IN GROWING FEMALE AND MALE SPRAGUE-DAWLEY RATS

6.1 Introduction

The preceding chapter examined the combined effects of a stearic acid and high-fructose diet on the development of nephropathy in growing female and male Sprague-Dawley rats. Oral delivery of dietary interventions is known to have the ability to change the function and structure of organs of the gastrointestinal tract (Gawat et al., 2023). Common changes include altered (reduced/increased) growth and development (e.g. impaired transport processes) (Hargreaves et al., 2022, Hoelscher et al., 2002). As the initial point of contact for digestion and absorption of any orally ingested dietary food (Gawat et al., 2023) it is therefore prone to toxic insults which can damage the gastrointestinal tract (Shahsavari and Parkman, 2022). Ingested substances can also alter the microbiome causing short- and long-term unfavorable metabolic effects including excessive lipogenesis, obesity, cardiovascular disease, type II diabetes and hypercholesterolaemia (Hoelscher et al., 2002, Stefan, 2020). Examining gastrointestinal tract and visceral organ morphometry, provides a simple tool to explore the possible negative/positive impact of diets on the structure of the gastrointestinal system.

Therefore, the present chapter will focus on the effect of a diet rich in stearic acid and high fructose concentrations on the potential change of gastrointestinal tract morphometry on Sprague-Dawley rats.

The impact of oral administration of the stearic dietary intervention is not known in the present study. The reason this study is important, is to determine whether or not, the dietary intervention will be harmful or protective towards organs of the gastrointestinal tract. Evidence on the impact of dietary changes and nutrition made early in childhood on neonatal growth and physiology has been shown to be significant over the life of the rats (Guilloteau et al., 2009, Langley-Evans, 2006). During this critical stage of developmental plasticity, changes that occur during the neonatal phase may have a positive health impact by reducing the development of metabolic and developmental disorders later in life (Dearden et al., 2018, Zhu et al., 2019).

6.1.1 How dietary fatty acids affects the gastrointestinal tract

Dietary fatty acids are essential for the normal functioning of the gastrointestinal system (Xu et al., 2021). They influences a variety of factors, including absorption and digestion (Xu et al., 2021), nutritional bioavailability (Fave et al., 2004), microbiota and gut health (Machate et al., 2020). Given that the intestine is the nutritional supply organ, dietary fatty acids can have an

impact on impairing nutrient absorption in the intestines (Xie et al., 2022). Dietary fatty acids regulates the rate at which nutrients enter the circulatory system, which can be beneficial to health (Machate et al., 2020). Previous research has revealed that fat can slow stomach emptying, disrupting the overall absorption process (Abumrad and Davidson, 2012). In addition, fat intake might affect the availability of digestive enzymes in the colon, hence influencing nutritional digestion and absorption (Ravindran et al., 2016, Xu et al., 2021). The digestibility of fat-containing diets impacts the absorption and use of nutrients by tissues, affecting their bioavailability (Fave et al., 2004). Furthermore, the physical structure of meals, especially fat content, influences their functional impact and the availability of chemicals in the gastrointestinal tract (Maxton et al., 1989).

The caecum, small and large intestines and stomach (and pancreas as an accessory organ) are the organs that were measured. The fat composition of the diet influences the growth and development of the various organs of the gastrointestinal system in rats and other animals. In animal studies, Gill et al. (2021) showed that diets containing 3-4% fibre stimulate gastrointestinal tract development and the synthesis of digestive enzymes.

6.1.2 Gastrointestinal tract macromorphometery is an important toxicological measure

In summary, dietary fat influences the gastrointestinal tract's functioning, nutrient absorption, and overall health. Understanding these mechanisms helps us appreciate the complex interplay between diet, digestion and well-being. Therefore, gastrointestinal tract macromorphometery is an essential toxicological assessment, especially when determining the effects of drugs as well as compounds on the digestive system (Maxton et al., 1989). By examining the macroscopic aspects of the gastrointestinal tract in the current study, we may get insight into the possible protective and/or harmful effects of stearic acid, as well as completely examine its safety and influence on gastrointestinal health. As a result, the dietary implications of the combined consumption of stearic acid and high-fructose, as well as stearic acid alone, are unknown and this is the reason for this chapter.

6.2 Aim and Objectives

This study aimed:

To investigate the long-term effects of a stearic acid alone and/or combinatorial high-fructose and stearic acid diet fed to growing female and male Sprague-Dawley rat's gastrointestinal visceral organ morphometry.

Hypotheses

H_0: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has no effect on gastrointestinal and visceral organ morphometry.

H_1: Administration of a stearic acid alone and/or combined high-fructose and stearic acid diet to growing Sprague-Dawley rats has an effect on gastrointestinal and visceral organ morphometry

The specific objectives of this part of the study focused on:

6.2.1 Gastrointestinal and visceral organ morphometry

6.2.1.1 To assess the structure of the gastrointestinal tract which is the first point of contact of orally administered substances by assessing gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestine.

6.3 Materials and methods

6.3.1 Ethical clearance for the use of animals and study site

Details of ethical clearance and study site are as described in Chapter 3, subheading 3.3.3.

6.3.2 Animals, housing and feeding

Housing, feeding and care of the experimental rats were as described in Chapter 3, subheading 3.3.4.

6.3.3 Experimental design and treatments

The detailed experimental design and treatments administered to the rats was as described in chapter 3, subheading 3.3.5.

6.3.4 Determination of gastrointestinal tract and viscera organ morphometry

The abdomen was cut open by a midline incision after blood was collected. The stomach, small and large intestines, caecum and pancreas were carefully dissected out. Thereafter the stomach, small and large intestines and caecum were all gently emptied of content and weighed using an electronic balance (Presica 310M, Presica Instruments AG, Switzerland). The small and large intestine lengths were measured using a ruler attached to the dissection board. Organ mass relative to body mass (rBM) was computed by dividing the mass of each organ by the respective terminal body mass of each rat and expressed as a percentage (%).

6.3.5 Statistical analysis

Data analysis was done using GraphPad Prism 7 software (Graph-pad Software Inc., San Diego, USA). All normally distributed data are expressed as mean ± standard deviation. Group means for comparisons between different dietary treatment groups were analyzed using one-way analysis of variances (ANOVA) and mean comparison using a Tukey post-hoc test with statistical significance of $p < 0.05$ considered. For the data presented in the table, the statistical level of significance (p-value) is that of the overall group differences before applying post hoc test.

6.4 Results

Table 6.1 shows the effects of stearic acid on gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestine in growing female Sprague-Dawley rats fed a high-fructose diet and/or stearic acid for 13 weeks.

There was no significant difference ($p > 0.05$) in caecum absolute (one-way ANOVA, $F_{(4, 34)} = 1.149$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 1.102$) masses across treatment groups, indicating that the diet treatments had no effect on the caecum.

The large intestine absolute (one-way ANOVA, $F_{(4, 34)} = 2.665$) and relative body mass (one-way ANOVA, $F_{(4, 34)} = 2.374$) masses and large intestine lengths (one-way ANOVA, $F_{(4, 34)} = 0.2584$) were similar ($p > 0.05$) across treatment groups.

The pancreas absolute (one-way ANOVA, $F_{(4, 34)} = 0.8323$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 2.265$) masses were similar ($p > 0.05$) across treatment groups.

Across the treatment groups for female rats, there were no significant difference ($p > 0.05$) in small intestine absolute (one-way ANOVA, $F_{(4, 34)} = 2.387$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 1.603$) masses across treatment groups.

Additionally, the stomach absolute (one-way ANOVA, $F_{(4, 34)} = 1.115$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 1.340$) masses were not significantly different ($p > 0.05$) across treatment groups.

In summary, in female rats, a high-fructose diet, stearic acid and fenofibrate interventions as well as stearic acid alone as a dietary supplement did not affect the gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestines.

Table 6.1. The effects of stearic acid on gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestine in growing female Sprague-Dawley rats fed a high-fructose diet and/or stearic acid for 13 weeks.

GIT Parameters	Treatment Groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Caecum mass (g)	1.22± 0.47 [a]	1.04 ± 0.18 [a]	0.99 ± 0.11 [a]	1.02 ± 0.07 [a]	1.02 ± 0.18 [a]	ns (p = 0.3504)
Caecum rBM (%)	0.45 ± 0.16 [a]	0.39 ± 0.04 [a]	0.38 ± 0.04 [a]	0.38 ± 0.03 [a]	0.38 ± 0.05 [a]	ns (p = 0.3713)
LI length (mm)	212.50 ± 25.63 [a]	217.50 ± 19.27 [a]	217.10 ± 12.86 [a]	209.00 ± 14.30 [a]	215.00 ± 18.13 [a]	ns (p = 0.9025)
LI mass (g)	1.49 ± 0.14 [a]	1.34 ± 0.14 [a]	1.36 ± 0.21 [a]	1.54 ± 0.09 [a]	1.39 ± 0.16 [a]	ns (p = 0.0500)
LI rBM (%)	0.55 ± 0.05 [a]	0.50 ± 0.05 [a]	0.52 ± 0.07 [a]	0.57 ± 0.03 [a]	0.51 ± 0.05 [a]	ns (p = 0.0716)
Pancreas mass (g)	1.04 ± 0.31 [a]	1.15 ± 0.16 [a]	1.08 ± 0.20 [a]	1.02 ± 0.18 [a]	1.17 ± 0.13[a]	ns (p = 0.5141)
Pancreas rBM (%)	0.38 ± 0.10 [a]	0.43 ± 0.05 [a]	0.41 ± 0.08 [a]	0.37 ± 0.07 [a]	0.43 ± 0.05 [a]	ns (p = 0. 405)
SI length (mm)	1263 ± 39.18 [a]	1268 ± 73.38 [a]	1248 ± 81.34 [a]	1266.00 ± 77.20 [a]	1298 ± 68.66 [a]	ns (p = 0.6015)
SI mass (g)	7.32 ± 0.50 [a]	7.45 ± 0.56 [a]	6.90 ± 0.96 [a]	7.03 ± 0.73 [a]	7.70 ± 0.61 [a]	ns (p = 0.0697)
SI rBM (%)	2.69 ± 0.11 [a]	2.81 ± 0.32 [a]	2.63 ± 0.29 [a]	2.60 ± 0.17 [a]	2.83 ± 0.18 [a]	ns (p = 0. 196)
Stomach mass (g)	1.44 ± 0.14 [a]	1.48 ± 0.17 [a]	1.43 ± 0.19 [a]	1.48 ± 0.21 [a]	1.59 ± 0.10 [a]	ns (p = 0.3656)
Stomach rBM (%)	0.53 ± 0.05 [a]	0.56 ± 0.03 [a]	0.54 ± 0.05 [a]	0.55 ± 0.07 [a]	0.58 ± 0.04 [a]	ns (p = 0.2751)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$. * $p < 0.05$. [a] Within rows means with similar superscripts are not significantly different at $p < 0.05$. The control group is the negative control which received standard rat chow and plain water to drink (n = 8); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 7); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats in this group received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

Table 6.2 shows the effects of stearic acid on gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestine in growing male Sprague-Dawley rats fed a high-fructose diet and/or stearic acid for 13 weeks.

There were no significant differences in absolute and relative caecum masses ($p > 0.05$). However, the rats given a combination of stearic acid and fructose, had a lower caecum absolute ($p = 0.0497$) and relative to body mass ($p = 0.0486$) masses than male rats given stearic acid alone.

Male rats had similar large intestine lengths (one-way ANOVA, $F_{(4, 34)} = 1.450$) and large intestine masses relative to body mass (one-way ANOVA, $F_{(4, 34)} = 1.985$) across treatment groups ($p > 0.05$). However, rats given a combination of fenofibrate and fructose had significantly decreased absolute large intestine mass ($p = 0.0250$) than male rats receiving a standard diet.

The pancreas relative to body mass across all treatment groups was similar ($p > 0.05$). However, rats administered combined stearic acid and fructose had significantly ($p = 0.0366$) heavier pancreas absolute masses than the pancreas masses from rats administered stearic acid alone.

Across the treatment groups for male rats, there were no significant differences ($p > 0.05$) in small intestine absolute (one-way ANOVA, $F_{(4, 34)} = 1.574$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 1.340$) masses across treatment groups. There were no significant differences in the lengths (mm) of the small intestines ($p = 0.2770$, one-way ANOVA, $F_{(4, 34)} = 2.263$) of the male rats across treatment groups.

There was no significant difference ($p > 0.05$) in stomach absolute (one-way ANOVA, $F_{(4, 34)} = 0.5165$) and relative to body mass (one-way ANOVA, $F_{(4, 34)} = 2.441$) masses across treatment groups for male rats.

Table 6.2. The effects of stearic acid on gastrointestinal tract and visceral organ absolute and relative (to body mass) masses and lengths of the small intestine and large intestine in growing male Sprague-Dawley rats fed a high-fructose diet and/or stearic acid for 13 weeks.

GIT Parameters	Treatment Groups					Overall level of significance
	Control	Fructose	Stearic acid + Fructose	Stearic acid	Fenofibrate + Fructose	
Caecum mass (g)	1.31 ± 0.24 ^{ab}	1.50 ± 0.21 ^{ab}	1.25 ± 0.17^{a}	1.54 ± 0.27 ^{b}	1.28 ± 0.10 ^{ab}	* (p = 0. 0166)
Caecum rBM (%)	0.29 ± 0.05^{ab}	0.36 ± 0.06^{ab}	0.29 ± 0.05^{a}	0.36 ± 0.06 ^{b}	0.31 ± 0.02^{ab}	* (p = 0.0088)
LI length (mm)	242.90 ± 15.77^{a}	238.10 ± 13.61^{a}	231.30 ± 18.85^{a}	245.60 ± 8.21 ^{a}	234.40 ± 9.80^{a}	ns (p = 0.450)
LI mass (g)	1.99 ± 0.18^{a}	1.84 ± 0.21^{ab}	1.81 ± 0.21^{ab}	1.98 ± 0.28 ^{a}	1.66 ± 0.08^{b}	* (p = 0.0169)
LI rBM (%)	0.44 ± 0.05^{a}	0.43 ± 0.05^{a}	0.42 ± 0.02^{a}	0.46 ± 0.06 ^{a}	0.40 ± 0.03^{a}	ns (p = 0.1189)
Pancreas mass (g)	1.51 ± 0.25 ^{ab}	1.58 ± 0.18 ^{ab}	1.65 ± 0.19^{a}	1.36 ± 0.15 ^{b}	1.42 ± 0.22 ^{ab}	* (p = 0.0323)
Pancreas rBM (%)	0.33 ± 0.04^{a}	0.37 ± 0.05^{a}	0.38 ± 0.03^{a}	0.32 ± 0.05 ^{a}	0.35 ± 0.06^{a}	ns (p = 0.0825)
SI length (mm)	1478 ± 104.60^{a}	1463 ± 97.94^{a}	1394 ± 68.44^{a}	1448.00 ± 110.60 ^{a}	1383 ± 119.10^{a}	ns (p = 0.2770)
SI mass (g)	9.79 ± 1.17^{a}	9.89 ± 1.51^{a}	9.23 ± 0.76^{a}	9.24 ± 0.64 ^{a}	8.74 ± 0.95^{a}	ns (p = 0.2035)
SI rBM (%)	2.17 ± 0.22^{a}	2.32 ± 0.31^{a}	2.13 ± 0.18^{a}	2.15 ± 0.18 ^{a}	2.11 ± 0.10^{a}	ns (p = 0.2754)
Stomach mass (g)	1.96 ± 0.19^{a}	2.03 ± 0.21^{a}	2.11 ± 0.22^{a}	2.02 ± 0.21 ^{a}	1.97 ± 0.27^{a}	ns (p = 0.7241)
Stomach rBM (%)	0.43 ± 0.03^{a}	0.48 ± 0.04^{a}	0.49 ± 0.03^{a}	0.47 ± 0.04 ^{a}	0.48 ± 0.05^{a}	ns (p = 0.0655)

Data presented as mean ± standard deviation. ns = not significant with $p > 0.05$; * $p < 0.05$. ^{a} Within rows means with similar superscripts are not significantly different at $p < 0.05$. ^{a,b} Within rows means with different superscripts are significantly different at $p < 0.05$. Control group is the negative control which received standard rat chow and plain water to drink (n = 7); fructose group rats were fed standard rat chow and 20% fructose solution to drink (n = 8); stearic acid and fructose group rats were fed standard rat chow enriched with 3% stearic acid and 20% fructose solution to drink (n = 8); stearic acid group rats were fed standard rat chow enriched with 3% stearic acid and plain water to drink (n = 8); fenofibrate and fructose group is the positive control and the rats received standard rat chow and fenofibrate (100 mg/kg body mass per day) and 20% fructose solution to drink (n = 8).

6.5 Discussion

It is critical to quantify the organ masses because of the possibility of toxicological consequences when using or testing new dietary interventions. We used masses of the gastrointestinal tract and visceral organs adjusted by body mass for normalizing gastrointestinal tract and visceral organ weight (%BM). In the current study, interventions include consuming a high fructose diet and/or combining it with fenofibrate and/or stearic acid had no effect on caecum (female rats), large intestine (female rats), pancreas (female rats), stomach (both female and male rats), small intestines (both female and male rats) absolute and relative to body masses of the rats across treatment groups.

In male rats, a combined high fructose with stearic acid as an intervention, decreased caecum mass (relative to body mass) compared to high-fructose fed caecum mass. Caecum is an essential site for fermentation and the creation of short chain fatty acids, which creates more energy for the body (Zacchetti et al., 2007). Consumption of a fiber-rich diet can the mass of the caecum and increases the volume of faeces passing through the cecum. Also, chronic inflammation and/or infections in the cecum can induce edema (Hassan et al., 2021). Given the caecum's major purpose which is to absorb fluids and salts that remain after intestinal digestion and absorption (Svihus et al., 2013), the existence of osmotically active chemicals that may impact the caecum mass is possible. However, the potential negative effects of fructose and stearic acid on growth factors in cell proliferation and migration (Alison and Sarraf, 1994, Zhang et al., 2022b) should not be overlooked as a possible explanation for the decreased caecum mass observed in the combined stearic acid and high-fructose diet fed male rats. The mechanism behind our study's findings is unknown.

In this study, we observed that the large intestine mass (absolute) of male rats fed a combined fenofibrate and high fructose diet as an intervention was lighter than that of counterpart fed the control diet (table 6.2). The observed reduction in large intestine mass might be attributable to altered gut microbiota and consequent reduced short chain fatty acids which normal have a trophic effect on the large intestine (Machate et al., 2020, Agus et al., 2020). Although fluid consumption was not precisely assessed, this study noted that the rats fed fructose drank significantly more than those fed plain water, thus providing a substrate (fructose) which is known to have a negative impact on the gut microbiome (Agus et al., 2020).

Interventions including consuming a high fructose diet and/or a high fructose diet combined with fenofibrate and/or stearic acid as interventions had no effect on the pancreas when

compared to the male rats fed a standard diet, combined stearic acid and high fructose fed to male rats resulted in much heavier pancreas than male rats fed stearic acid alone. However, when expressed relative to relative body mass, there was no difference compared across treatment groups. Therefore, the observed difference in absolute mass, which is not normalised to body mass, may not be biologically significant. This finding suggests that stearic acid, as an intervention did not grossly impact the pancreas.

The small intestines and stomach masses were not significantly difference across treatment groups suggesting a lack of toxicity to these two organs.

The lack of differences in absolute and relative visceral macro-morphometry organ masses between the negative controls and stearic acid alone as supplement in both female and male rat's fructose suggests that oral administration of stearic acid alone, as a supplement did not cause visceral organ atrophy or hypertrophy.

6.6 Conclusion

This study looked into the long-term effects of a stearic acid alone and/or combinatorial high-fructose and stearic acid diet fed to growing female and male Sprague-Dawley rat's gastrointestinal visceral organ morphometry.

An unrestricted high-fructose diet (for drinking) was utilized, which has been shown to induce metabolic impairment in diet-induced rat models. A dietary saturated fatty acid was also fed to the rats as an intervention. An exciting finding was that there were no adverse effects observed with the administration of combinatorial high-fructose and stearic acid diet in both female and male rat's gastrointestinal visceral organ morphometry except for caecum (rTL).

The following chapter begins by summarizing the primary findings of this current research. Following that, the limits of the current research are noted and lastly, recommendations for further research are made.

CHAPTER 7

OVERALL CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

7.1 Overall conclusions

The overall aim of this study was to investigate the long-term effects of a stearic acid alone and/or combined high-fructose and stearic acid diet fed to growing female and male Sprague-Dawley rats against high-fructose diet-induced metabolic dysfunction.

In this study, both female and male rats were fed a high-fructose diet for 13 weeks, which has been demonstrated to be a successful experimental intervention in diet induced models for the development of metabolic abnormalities, non-alcoholic fatty liver disease and produced renal dysfunction. However, in the current study, the interventions did not result in development of all the characteristics of metabolic syndrome. There were notable sexually dimorphic differences in the outcomes to the interventions. Therefore, highlighting the need for research to experimental models to not be confined to a single sex (often males) (Beery and Zucker, 2011, Shansky and Murphy, 2021) as this leaves out key sex related information and reiterates the fact that findings in males cannot be extrapolated to females and vice versa.

Metabolic dysfunction in female rats was characterized by hypercholesterolemia, hypertriglyceridemia and increased visceral adiposity, whereas hypoadiponectinaemia was identified in males. The high-fructose diet-induced non-alcoholic fatty liver disease in female rats was characterized by increased hepatic lipid content, microvesicular steatosis and hepatic inflammation. Meanwhile, male rats with a high-fructose diet developed non-alcoholic fatty liver disease characterized by increased hepatic lipid content. Although there was not a noticeable kidney morphometry characterized by no variations in renal corpuscle area, glomerulus area, Bowman's/urinary space or glomeruli density across treatment groups. A high-fructose diet-induced nephropathy was confirmed by higher kidney injury molecule-1 concentrations in both female and male rats.

As a dietary intervention, the combined stearic acid and high-fructose diet protected female and male rats from high-fructose diet-induced metabolic dysfunction, non-alcoholic fatty liver disease and renal dysfunction. When rats were fed a combined stearic acid and high-fructose diet, it caused hyperadiponectinaemia but had no effect on visceral fat (female rats), fasting glucose concentrations, insulin, Homeostasis model assessment of insulin resistance. Furthermore, this study found that combining stearic acid and high-fructose protected against micro-steatosis and lowered hepatic lipid content therefore having no negative effects on liver and kidney function. Overall, this study found that stearic acid protects against the development of some clinically important elements of metabolic dysfunction including non-alcoholic fatty

liver disease and renal dysfunction caused by a long term and excessive consumption of a high-fructose diet.

Stearic acid, as a dietary supplement, had no effect on rats' growth performance, visceral and epididymal (male) obesity and gastrointestinal visceral organ morphometry. It is worth noting that when rats were administered stearic acid, total cholesterol (both sexes) did not change but total triglyceride concentrations decreased (females). In female and male rats, this study found that a diet that included stearic acid increased plasma adiponectin concentrations while having no negative effects on glucose, insulin and HOMA-IR.

Furthermore, this study discovered that stearic acid had no negative effects on hepatic and kidney morphology and function in males. However, in female rats, it caused hepatic micro and macrovesicular steatosis. Differences observed could be due to different steroid hormone profile, adiposity and metabolic rates, which have an impact on tissue/organ fat accretion. The hepatic fatty acid profiles have shown that the female rats administered stearic acid had the highest amount of total polyunsaturated fatty acids and the lowest omega 6 to omega 3 ratio, whereas male counterparts had increased total monounsaturated fatty acids but lower total polyunsaturated fatty acids and omega 6 to omega 3 ratios. Overall, this study found that stearic acid alone, as a dietary supplement, may have a significant benefit in mitigating metabolic dysfunction. In the fight against metabolic dysfunction, this study suggests using stearic acid instead of more adversely, metabolically active saturated fats found in food additives.

This is, to the best of my knowledge, the first study to report on the preventive benefits of a stearic acid enriched diet against high fructose-induced metabolic dysfunction and non-alcoholic fatty liver disease as well as hepatic fatty acid profiling.

7.2 Limitations and recommendations

It is a limitation that the study was descriptive in nature. Therefore, future research should examine employing cellular and molecular approaches to clarify the various pathways by which stearic acid exhibited its remarkable overall health positive effects. For example, experimental studies involving stearic acid at various doses and assessing renal tissue a) nuclear factor kappa B expression, a transcription factor that activates mesangial cells leading to kidney injury, b) neutrophil gelatinase-associated lipocalin (NGAL) and c) interleukin-18, a potent inflammatory cytokine constitutively expressed in kidney tubular epithelia, are required to back up the current study findings. Additionally, there is a need for molecular studies to investigate the underlying mechanisms of these pathologies, such as mechanisms involving activation of

adenosine monophosphate-activated protein kinase and sterol regulatory binding element protein 1, which are involved in the development of hepatic steatosis. It is also proposed that future studies look at urine composition, as it may contain extra protein, toxins, and blood after renal failure. It is also suggested that future research should consider analysis of the gut microbiome since it has been intricately associated with metabolic dysfunction.

Stearic acid dietary treatments protected the rats in a sexually dimorphic manner in several of the study targets, emphasizing the significance of future investigations assessing possible preventive agents in males and females. There is need for future studies to also consider investigating potential epigenetic changes including assessment of DNA methylation, microRNAs, histone modification and chromatin remodelling so as to investigate whether the protective effects of stearic acid can be intergenerational.

It is also recommended that future research should consider Micro-CT for a more detailed investigation of the impact of stearic acid on bone structure as well as fat distribution.

The take-home message from this study's findings is that saturated fatty acids in oils should be partially substituted with stearic acid to create modified oils rich in stearic acid. Stearic acid-rich modified oils can be used in a range of culinary applications to combat metabolic dysfunction and the non-alcoholic fatty liver disease pandemic. However, it is recommended that stearic acid be used as a dietary supplement with caution and monitoring of their health, especially in females where it could predispose to the development of steatosis considering that other health risks associated with metabolic dysfunction were not alleviated.

CHAPTER 8

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APPENDICES

Appendix 1: Animal Ethics Screening Committee Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/01/3/B

APPLICANT: Ms T Dladla

SCHOOL: Physiology
DEPARTMENT:
LOCATION:

PROJECT TITLE: The potential protective effects of stearic acid against diet-induced metobolic dysfunction in growing fructose-fed Sprague-Dawley rats

Number and Species

100X 21 day old rat pups male and female SD rats, 60X 6 day old rat pup male SD rats, 15X 8-12 pup/dam female SD rats (nursing dams) and 60X 6 day old rat pup female SD rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/01/30. This approval remains valid until 2020/03/25.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

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

Signed: _____ (Chairperson AESC) Date: 27/3/2018

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: 26 March 2018

cc: Supervisor: Professor K Erlwanger
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix 2: Enzyme Linked Immuno-Sorbent Assay (ELISA) Rat INS (Insulin) and Rat ADP/Acrp30 (Adiponectin) kits assay procedure

Elabscience
www.elabscience.com

6th Edition, revised in June, 2015

（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Rat INS (Insulin) ELISA Kit
Catalog No: E-EL-R2466
96T

This manual must be read attentively and completely before using this product.

May you have any problems, please contact our Technical Service Center for help.

Phone: 86-27-87805095
Email: techsupport@elabscience.com
techelabscience@gmail.com
Website: www.elabscience.com

Please kindly provide us the lot number(on the outside of the box) of the kit for more efficient service.

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Intended use
This ELISA kit applies to the invitro quantitative determination of RatINSconcentrations in serum, plasma and other biological fluids.

Sensitivity
The minimum detectable dose of RatINSis 1.875ng/mL (The sensitivity of this assay, orlowest detectable limit(LDL) was defined as the lowest protein concentration that could be differentiated from zero).

Detection Range
3.125-200ng/mL

Specificity
This kit recognizes natural and recombinantRatINS. No significant cross-reactivity or interference between RatINS and analogues was observed.
Note:
Limited by existing techniques, cross reaction may still exist, as it is impossible for us to complete the cross-reactivity detection between RatINS and all the analogues.

Repeatability
Coefficient of variation were<10%.

Statement: Thank you for choosing our products. This product is produced by using raw materialfrom world-renowned manufacturer and professional manufacturing technology of ELISA kits. Please read the instructions carefully before use and check all the reagent compositions! If in doubt, please contact Elabscience Biotechnology Co., Ltd.

Storage: All the reagents in the kit should be stored according to the labels on vials.Unused wellsshould bereturned to the foil pouch with the desiccant pack and resealed along entire edge of zip-seal.Substrate Reagent shouldn't be kept at -20℃(Check!). Exposure of reagents to strong light should beavoided in the process of incubation and storage. All the taps of reagents should be tightened to preventevaporation and microbial contamination. If not to store reagents according to above suggestions, erroneous results may occur.

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Kit Components:

Item	Specifications	Storage
Micro ELISA Plate	8 wells ×12 strips	4℃/-20℃#
Reference Standard	2 vials	4℃/-20℃#
Reference Standard & Sample Diluent	1vial 20mL	4℃
Concentrated Biotinylated Detection Ab	1vial 120μL	4℃/-20℃#
Biotinylated Detection AbDiluent	1vial 10mL	4℃
Concentrated HRP Conjugate	1vial 120μL	4℃(shading light)
HRP Conjugate Diluent	1vial 10mL	4℃
Concentrated Wash Buffer (25×)	1vial 30mL	4℃
Substrate Reagent	1vial 10mL	4℃(shading light)
Stop Solution	1vial 10mL	4℃
Plate Sealer	5pieces	
Manual	1 copy	
Certificate of Analysis	1 copy	

#: keep the kit at 4℃ if it's used within 30 days, keep at -20℃ for longer storage.

Test principle

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to INS. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for INSand Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain INS, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of INS.You can calculate the concentration of INSin the samples by comparing the OD of the samples to the standard curve.

Sample collection and storage

Samples should be clear and transparent and be centrifuged to remove suspended solids.

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4℃before centrifugation for 15 minutes at 1000×g. Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2 - 8℃within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Hemolysissamples are not suitable for ELISA assay!

Cell culture supernate: Centrifuge supernate for 20 minutes to remove insoluble impurity and cell debris at 1000×g at 2 - 8℃. Collect the clear supernate and carry out the assay immediately.

Tissue homogenates: You'd better get detailed references from other literatures before assay aiming at different tissue types. For general information, hemolysis blood may affect the result, so you should mince the tissues to small pieces and rinse them in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (the volume depends on the weight of the tissue) with a glass homogenizer on ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifugated for 5minutes at 5000×g to get the supernate.

Other biological fluids: Centrifuge samples for 20 minutes at 1000×g at 2 - 8℃. Collect the supernatant and carry out the assay immediately.

Note:

1. Samples should be used within 7 days when stored at 2-8℃, otherwise samples must be divided and stored at -20℃ (≤1month) or -80℃ (≤6months) to avoid the loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. Please take the samples to room temperature (18-25℃) without extra heating before performing the assay.
3. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

Sample preparation

1. Elabscience is only responsible for the kit itself, but not for the samples consumed during the experiment. The user should calculate the possible amount of the samples needed in the whole test. Reserving sufficient samples in advance is recommended.
2. If the samples are not mentioned in this manual, a pre-experiment to determine the validity of the kit is necessary.
3. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected Elisa results due to the impacts of certain chemicals.
4. Due to the possibility of mismatching between antigen from other origins and antibodies used in

our kits, some native or recombinant proteins from other manufacturers may not be detected by our kits.

5. Influenced by factors including cell viability, cell number or sampling time, molecular from cells culture supernatant may not be detected by the kit.
6. Grossly hemolyzed samples are not suitable for use in the assay.
7. Fresh samples without long time storage are recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

Other supplies required

Microplate reader with 450nm wavelength filter
High-precision transferpettor, EP tubes and disposable pipette tips
37℃ Incubator
Deionized or distilled water
Absorbent paper
Loading slot for Wash Buffer

Reagent preparation

Bring all reagents to room temperature(18-25℃) before use.

Wash Buffer - Dilute 30 mL of Concentrated Wash Buffer into 750 mL of Wash Buffer with deionized or distilled water. Put unused solution back at 4℃. If crystals have formed in the concentrate, you can warm it with 40℃water bath (Heating temperature should not exceed 50℃) and mix it gently until the crystals have completely dissolved. The solution should be cooled to room temperature before use.

Standard – Prepare standard within 15 minutes before use. Centrifuge at 10,000×g for 1 minute, and reconstitute the Standard with **1.0mL** of Reference Standard &Sample Diluent. Tighten the lid, let it stand for 10minutes and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 200ng/mL. Then make serial dilutions as needed (making serial dilution in the wells directly is not permitted). The recommended concentrations are as follows:**200、100、50、25、12.5、6.25、3.125、0 ng/mL**. If you want to make standard solution at the concentration of 100ng/mL, you should take0.5mL standard at 200ng/mL, add it to an EP tube with 0.5mL Reference Standard &Sample Diluent, and mix it.Procedures to prepare the remained concentrations are all the same. The undiluted standard serves as the highest standard (200ng/mL). The Reference Standard &Sample Diluent serves as the zero (0 ng/mL).

(Standards can also be diluted according to the actual amount，such as 200μL/tube)

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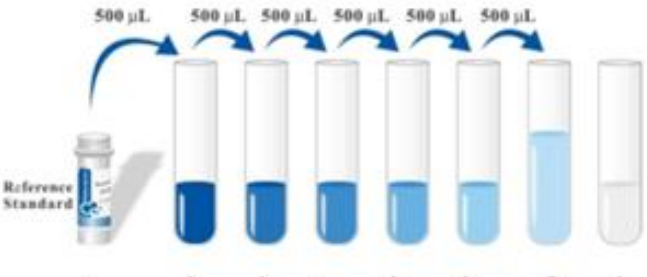


Biotinylated Detection Ab – Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Centrifuge the stock tube before use, dilute the concentrated BiotinylatedDetection Ab to the working concentration usingBiotinylated Detection AbDiluent(1:100).

Concentrated HRP Conjugate – Calculate the required amount before experiment (100μL/well). In actual preparation, you should prepare 100~200μL more. Dilute the Concentrated HRP Conjugate to the working concentration using Concentrated HRP ConjugateDiluent(1:100).

Substrate Reagent: As it is sensitive to light and contaminants, so you shouldn't open the vial until you need it! The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent shouldn't be dumped back into the vial again.

Note: Please don't prepare the reagent directly in the Diluent vials provided in the kit. Contaminated water or container for reagent preparation will influence the result.

Washing Procedure:

1. **Automated Washer:** Add 350μL wash buffer into each well, the interval between injection and suction should be set about 60s.
2. **Manual wash:** Add 350μLWash Buffer into each well, soak it for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean and toweling absorbent paper on a hard surface.

Assay procedure

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

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

Important Note:

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

(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSTICS !)

Rat ADP/Acrp30(Adiponectin) ELISA Kit

Synonyms: Acrp30, GBP28, ACDC, APM1, ADPN, AdipoQ, ADIPQTL1

Catalog No : E-EL-R0329
96T

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help (info in the header of each page).

Phone: 240-252-7368(USA) 240-252-7376(USA)
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

7th Edition, revised in April, 2017

Intended use

This ELISA kit applies to the in vitro quantitative determination of Rat ADP/Acrp30 concentrations in serum, plasma and other biological fluids.

Specification

●Sensitivity: 28.13 pg/mL
●Detection Range: 46.88-3000 pg/mL

●Specificity: This kit recognizes Rat ADP/Acrp30 in samples. No Significant cross-reactivity or interference between Rat ADP/Acrp30 and analogues was observed.

●Repeatability: Coefficient of variation is < 10%.

Test principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Rat ADP/Acrp30. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat ADP/Acrp30 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Rat ADP/Acrp30, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat ADP/Acrp30. You can calculate the concentration of Rat ADP/Acrp30 in the samples by comparing the OD of the samples to the standard curve.

6th Edition, revised in June, 2015

Calculation of results

Average the duplicate readings for each standardand samples, then subtract the average zero standard optical density. Create a standard curve by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. It is recommended to use some professional software to do this calculation, such as curve expert 1.3 or 1.4. In the software interface, a best fitting equation of standard curve will be calculated using OD values and concentrations of standard sample. The software will calculate the concentration of samples after entering the OD value of samples. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it after appropriate dilution. The actual concentration is the calculated concentration multiplied dilution factor.

SUMMARY

1. Add 100μL standard or sample to each well. Incubate 90mintues at 37℃
2. Remove the liquid. Add 100μLBiotinylated Detection Ab. Incubate 1 hour at 37℃
3. Aspirate and wash 3 times
4. Add 100μL HRP Conjugate. Incubate 30 minutes at 37℃
5. Aspirate and wash 5 times
6. Add 90μL Substrate Reagent. Incubate 15 minutes at 37℃
7. Add 50μL Stop Solution. Read at 450nm immediately
8. Calculation of results

7th Edition, revised in April, 2017

Kit components & Storage

An unopened kit can be stored at 4℃ for 1 month. If the kit is not used within 1 month, store the items separately according to the following conditions once the kit is received.

Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	8 wells ×12 strips	-20℃, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 μL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20°C(shading light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4°C, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	4°C(shading light)
Stop Solution	1 vial, 10 mL	4°C
Plate Sealer	5 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.
The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).

Other supplies required

Microplate reader with 450 nm wavelength filter
High-precision transfer pipette, EP tubes and disposable pipette tips
Incubator capable of maintaining 37℃
Deionized or distilled water
Absorbent paper
Loading slot for Wash Buffer

Note

1. Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
2. A freshly opened ELISA Plate may appear to have a water-like substance, which is normal and will not have any impact on the experimental results.
3. Do not reuse the diluted standard, biotinylated detection Ab working solution, concentrated HRP conjugate working solution. The unspent undiluted concentrated biotinylated detection Ab (100×) and other stock solutions should be stored according to the storage conditions in the above table.
4. The microplate reader should have a 450(±10 nm) filter installed and a detector that can detect the wavelength. The optical density should be within 0~3.5.
5. Do not mix or use components from other lots.
6. Change pipette tips in between adding standards, in between sample additions, and in between reagent additions. Also, use separate reservoirs for each reagent.

Sample collection

Serum: Allow samples to clot for 2 hours at room temperature or overnight at 4℃ before centrifugation for 15 min at 1000×g at 2~8℃. Collect the supernatant to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin.

Plasma: Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 min at 1000×g at 2~8℃ within 30 min of collection. Collect the supernatant to carry out the assay. Hemolysed samples are not suitable for ELISA assay!

Cell lysates: For adherent cells, gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin. Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g. Discard the medium and wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS to keep the cells suspended. Repeat the freeze-thaw process several times until the cells are fully lysed. Centrifuge for 10min at 1500×g at 4℃. Remove the cell fragments, collect the supernatant to carry out the assay. Avoid repeated freeze-thaw cycles.

Tissue homogenates: It is recommended to get detailed references from the literature before analyzing different tissue types. For general information, hemolysed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH≈7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then homogenized in PBS (tissue weight (g): PBS (mL) volume=1:9) with a glass homogeniner on ice. To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×g to get the supernatant.

Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g at 2~ 8℃. Collect the supernatant to carry out the assay.

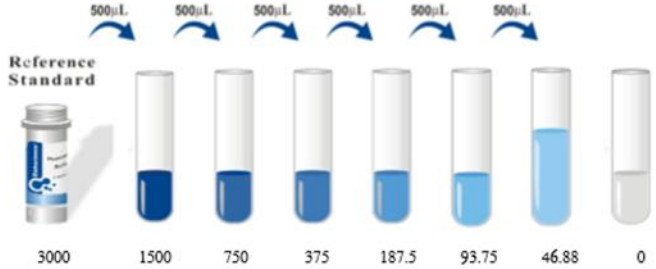
7th Edition, revised in April, 2017

Note for sample:

1. Samples should be assayed within 7 days when stored at 4℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze-thaw cycles.
2. Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.
4. If a lysis buffer is used to prepare tissue homogenates or cell culture supernatant, there is a possibility of causing a deviation due to the introduced chemical substance.
5. Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

Reagent preparation

1. Bring all reagents to room temperature (18~25℃) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved.
3. **Standard working solution:** Centrifuge the standard at 10,000×*g* for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 3000 pg/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 3000, 1500, 750, 375, 187.5, 93.75, 46.88, 0 pg/mL.
   Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 3000 pg/mL working solution to the first tube and mix up to produce a 1500 pg/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



4. **Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the stock tube before use, dilute the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.
5. **Concentrated HRP Conjugate working solution:** Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Dilute the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

7th Edition, revised in April, 2017

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

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

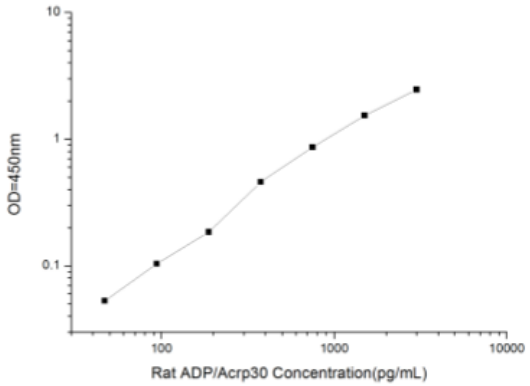
Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density.
Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.
If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

Concentration(pg/mL)	3000	1500	750	375	187.5	93.75	46.88	0
OD	2.528	1.599	0.927	0.521	0.248	0.167	0.116	0.063
Corrected OD	2.465	1.536	0.864	0.458	0.185	0.104	0.053	-



7th Edition, revised in April, 2017

SUMMARY

1. Add 100 µL standard or sample to each well. Incubate for 90 min at 37°C.
2. Remove the liquid. Add 100 µL Biotinylated Detection Ab. Incubate for 1 hour at 37°C.
3. Aspirate and wash 3 times.
4. Add 100 µL HRP Conjugate. Incubate for 30 min at 37°C.
5. Aspirate and wash 5 times.
6. Add 90 µL Substrate Reagent. Incubate for 15 min at 37°C.
7. Add 50 µL Stop Solution. Read at 450 nm immediately.
8. Calculation of results.

Declaration

1. Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.
2. The final experimental results will be closely related to the validity of products, operational skills of the operators and the experimental environments. Please make sure that sufficient samples are available.
3. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions!
4. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
5. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
6. Every kit has strictly passed QC test. However, results from end users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipments, and so on. Intra-assay variance among kits from different batches might arise from the above reasons, too.
7. Valid period: 6 months.

Appendix 3: Hepatic Lipid profiling Report

ARC • LNR
Excellence in Research and Development

ARC-Irene Analytical Services
LNR-Irene Analitiese Dienste

Sanas
Testing Laboratory
T0063

Private Bag/Privaatsak X2, Irene, 0062 Tel: (012) 672 9294 Fax: 086 607 7102

Enquiries: Penny Barnes
Tel: 012-672 9292/94

02/11/2018

The Manager
ARC-Vegetable and Ornamental Plant
Private Bag X293
Pretoria
0001

Attention: Dr A Ndhlala (VOP)

Tel: 012 808 8420
E-mail: NdhlalaA@arc.agric.za

TEST REPORT

Date received: **09/10/2018**
Date accepted: **16/10/2018**
Date completed: **02/11/2018**
Test report no: **2018-F-270**

RESULTS OF LIVER

Please take note that:

1. Test results relate only to the samples tested.
2. This report may not be reproduced without the written consent of the Quality Manager.
3. The samples received were thoroughly mixed before analysis.
4. Chromatogrammes, if applicable, are available on request.
5. Opinions and interpretations expressed herein are outside the scope of SANAS accreditation.
6. If the condition of the sample could affect the accuracy of analysis, a comment to the effect will be made.

Yours sincerely

PMB

P Barnes
Technical Signatory: Chemistry

ARC-IRENE ANALYTICAL SERVICES

Physical Address: ARC-AP, Old Olifantsfontein Rd, Irene

TEST REPORT 2018-F-270

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (1)
			Group 1 Male
Fat (ether extraction)	ASM 044	%	8.83
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.005
C14:0	ASM 023	% of product	0.109
C14:1	ASM 023	% of product	0.011
C15:0	ASM 023	% of product	0.031
C15:1	ASM 023	% of product	0.016
C16:0	ASM 023	% of product	2.086
C16:1	ASM 023	% of product	0.121
C17:0	ASM 023	% of product	0.065
C17:1	ASM 023	% of product	0.014
C18:0	ASM 023	% of product	2.008
C18:1n9c	ASM 023	% of product	1.678
C18:1n9t	ASM 023	% of product	0.084
C18:2n6c	ASM 023	% of product	0.835
C18:2n6t	ASM 023	% of product	0.011
C18:3n3	ASM 023	% of product	0.016
C18:3n6	ASM 023	% of product	0.004
C20:0	ASM 023	% of product	0.014
C20:1	ASM 023	% of product	0.020
C20:2	ASM 023	% of product	0.009
C20:3n3	ASM 023	% of product	0.005
C20:3n6	ASM 023	% of product	0.065
C20:4n6	ASM 023	% of product	1.103
C21:0	ASM 023	% of product	0.030
C22:0	ASM 023	% of product	0.029
C22:1n9	ASM 023	% of product	0.005
C24:1	ASM 023	% of product	0.453
Saturated fatty acids	ASM 023	% of product	4.380
Mono-unsaturated fatty acids	ASM 023	% of product	2.319
Poly-unsaturated fatty acids	ASM 023	% of product	2.037
Trans fatty acids	ASM 023	% of product	0.094
Cis fatty acids	ASM 023	% of product	2.513
Omega 3	ASM 023	% of product	0.021
Omega 6	ASM 023	% of product	2.018
Omega 9	ASM 023	% of product	1.767

TEST REPORT 2018-F-270

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (2)
			Group 1 Female
Fat (ether extraction)	ASM 044	%	15.33
C10:0	ASM 023	% of product	0.003
C12:0	ASM 023	% of product	0.007
C13:0	ASM 023	% of product	0.003
C14:0	ASM 023	% of product	0.061
C15:0	ASM 023	% of product	0.019
C16:0	ASM 023	% of product	2.593
C16:1	ASM 023	% of product	0.160
C17:0	ASM 023	% of product	0.058
C17:1	ASM 023	% of product	0.015
C18:0	ASM 023	% of product	4.044
C18:1n9c	ASM 023	% of product	2.696
C18:1n9t	ASM 023	% of product	0.017
C18:2n6c	ASM 023	% of product	2.068
C18:2n6t	ASM 023	% of product	0.019
C18:3n3	ASM 023	% of product	0.043
C18:3n6	ASM 023	% of product	0.051
C20:0	ASM 023	% of product	0.015
C20:1	ASM 023	% of product	0.033
C20:2	ASM 023	% of product	0.019
C20:3n3	ASM 023	% of product	0.013
C20:3n6	ASM 023	% of product	0.091
C20:4n6	ASM 023	% of product	2.256
C20:5n3	ASM 023	% of product	0.039
C21:0	ASM 023	% of product	0.038
C22:0	ASM 023	% of product	0.022
C24:1	ASM 023	% of product	0.945
Saturated fatty acids	ASM 023	% of product	6.865
Mono-unsaturated fatty acids	ASM 023	% of product	3.850
Poly-unsaturated fatty acids	ASM 023	% of product	4.579
Trans fatty acids	ASM 023	% of product	0.036
Cis fatty acids	ASM 023	% of product	4.764
Omega 3	ASM 023	% of product	0.095
Omega 6	ASM 023	% of product	4.485
Omega 9	ASM 023	% of product	2.713
EPA	ASM 023	% of product	0.039

TEST REPORT 2018-F-270

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (3)
			Group 2 Male
Fat (ether extraction)	ASM 044	%	8.13
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.003
C14:0	ASM 023	% of product	0.025
C14:1	ASM 023	% of product	0.003
C15:0	ASM 023	% of product	0.009
C15:1	ASM 023	% of product	0.002
C16:0	ASM 023	% of product	1.629
C16:1	ASM 023	% of product	0.103
C17:0	ASM 023	% of product	0.026
C17:1	ASM 023	% of product	0.006
C18:0	ASM 023	% of product	1.928
C18:1n9c	ASM 023	% of product	1.044
C18:1n9t	ASM 023	% of product	0.015
C18:2n6c	ASM 023	% of product	0.880
C18:2n6t	ASM 023	% of product	0.010
C18:3n3	ASM 023	% of product	0.009
C18:3n6	ASM 023	% of product	0.031
C20:0	ASM 023	% of product	0.004
C20:1	ASM 023	% of product	0.014
C20:2	ASM 023	% of product	0.084
C20:3n6	ASM 023	% of product	0.089
C20:4n6	ASM 023	% of product	1.409
C20:5n3	ASM 023	% of product	0.030
C21:0	ASM 023	% of product	0.036
C22:0	ASM 023	% of product	0.014
C22:2	ASM 023	% of product	0.024
C24:1	ASM 023	% of product	0.702
Saturated fatty acids	ASM 023	% of product	3.675
Mono-unsaturated fatty acids	ASM 023	% of product	1.873
Poly-unsaturated fatty acids	ASM 023	% of product	2.556
Trans fatty acids	ASM 023	% of product	0.026
Cis fatty acids	ASM 023	% of product	1.925
Omega 3	ASM 023	% of product	0.039
Omega 6	ASM 023	% of product	2.419
Omega 9	ASM 023	% of product	1.059
EPA	ASM 023	% of product	0.030

TEST REPORT 2018-F-270

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (4)
			Group 2 Female
Fat (ether extraction)	ASM 044	%	12.47
C12:0	ASM 023	% of product	0.006
C14:0	ASM 023	% of product	0.048
C14:1	ASM 023	% of product	0.011
C15:0	ASM 023	% of product	0.012
C16:0	ASM 023	% of product	2.272
C16:1	ASM 023	% of product	0.240
C17:0	ASM 023	% of product	0.037
C17:1	ASM 023	% of product	0.018
C18:0	ASM 023	% of product	2.805
C18:1n9c	ASM 023	% of product	2.836
C18:1n9t	ASM 023	% of product	0.019
C18:2n6c	ASM 023	% of product	1.138
C18:2n6t	ASM 023	% of product	0.018
C18:3n3	ASM 023	% of product	0.018
C18:3n6	ASM 023	% of product	0.040
C20:0	ASM 023	% of product	0.008
C20:1	ASM 023	% of product	0.030
C20:2	ASM 023	% of product	0.047
C20:3n6	ASM 023	% of product	0.086
C20:4n6	ASM 023	% of product	1.878
C20:5n3	ASM 023	% of product	0.031
C21:0	ASM 023	% of product	0.025
C22:0	ASM 023	% of product	0.008
C22:1n9	ASM 023	% of product	0.006
C22:2	ASM 023	% of product	0.011
C24:1	ASM 023	% of product	0.821
Saturated fatty acids	ASM 023	% of product	5.221
Mono-unsaturated fatty acids	ASM 023	% of product	3.953
Poly-unsaturated fatty acids	ASM 023	% of product	3.250
Trans fatty acids	ASM 023	% of product	0.037
Cis fatty acids	ASM 023	% of product	3.975
Omega 3	ASM 023	% of product	0.049
Omega 6	ASM 023	% of product	3.161
Omega 9	ASM 023	% of product	2.862
EPA	ASM 023	% of product	0.031

TEST REPORT 2018-F-270

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (5)
			Group 3 Male
Fat (ether extraction)	ASM 044	%	8.59
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.005
C14:0	ASM 023	% of product	0.041
C14:1	ASM 023	% of product	0.005
C15:0	ASM 023	% of product	0.007
C15:1	ASM 023	% of product	0.001
C16:0	ASM 023	% of product	1.785
C16:1	ASM 023	% of product	0.187
C17:0	ASM 023	% of product	0.020
C17:1	ASM 023	% of product	0.005
C18:0	ASM 023	% of product	1.772
C18:1n9c	ASM 023	% of product	1.526
C18:1n9t	ASM 023	% of product	0.012
C18:2n6c	ASM 023	% of product	0.925
C18:2n6t	ASM 023	% of product	0.011
C18:3n3	ASM 023	% of product	0.011
C18:3n6	ASM 023	% of product	0.004
C20:0	ASM 023	% of product	0.006
C20:1	ASM 023	% of product	0.024
C20:2	ASM 023	% of product	0.048
C20:3n3	ASM 023	% of product	0.002
C20:3n6	ASM 023	% of product	0.081
C20:4n6	ASM 023	% of product	1.377
C20:5n3	ASM 023	% of product	0.002
C21:0	ASM 023	% of product	0.029
C22:0	ASM 023	% of product	0.005
C22:1n9	ASM 023	% of product	0.002
C22:2	ASM 023	% of product	0.002
C23:0	ASM 023	% of product	0.014
C24:0	ASM 023	% of product	0.002
C24:1	ASM 023	% of product	0.678
Saturated fatty acids	ASM 023	% of product	3.688
Mono-unsaturated fatty acids	ASM 023	% of product	2.428
Poly-unsaturated fatty acids	ASM 023	% of product	2.450
Trans fatty acids	ASM 023	% of product	0.023
Cis fatty acids	ASM 023	% of product	2.451
Omega 3	ASM 023	% of product	0.014
Omega 6	ASM 023	% of product	2.398
Omega 9	ASM 023	% of product	1.540
EPA	ASM 023	% of product	0.002

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Analysis	Method Number	Unit	Sample (6)
			Group 3 Female
Fat (ether extraction)	ASM 044	%	10.54
C12:0	ASM 023	% of product	0.008
C14:0	ASM 023	% of product	0.032
C14:1	ASM 023	% of product	0.005
C15:0	ASM 023	% of product	0.005
C16:0	ASM 023	% of product	1.639
C16:1	ASM 023	% of product	0.130
C17:0	ASM 023	% of product	0.024
C17:1	ASM 023	% of product	0.007
C18:0	ASM 023	% of product	3.040
C18:1n9c	ASM 023	% of product	1.522
C18:2n6c	ASM 023	% of product	0.908
C18:2n6t	ASM 023	% of product	0.012
C18:3n3	ASM 023	% of product	0.006
C18:3n6	ASM 023	% of product	0.018
C20:0	ASM 023	% of product	0.015
C20:1	ASM 023	% of product	0.021
C20:2	ASM 023	% of product	0.039
C20:3n6	ASM 023	% of product	0.084
C20:4n6	ASM 023	% of product	2.090
C20:5n3	ASM 023	% of product	0.062
C21:0	ASM 023	% of product	0.009
C24:1	ASM 023	% of product	0.864
Saturated fatty acids	ASM 023	% of product	4.771
Mono-unsaturated fatty acids	ASM 023	% of product	2.550
Poly-unsaturated fatty acids	ASM 023	% of product	3.208
Trans fatty acids	ASM 023	% of product	0.012
Cis fatty acids	ASM 023	% of product	2.431
Omega 3	ASM 023	% of product	0.068
Omega 6	ASM 023	% of product	3.112
Omega 9	ASM 023	% of product	1.522
EPA	ASM 023	% of product	0.062

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Analysis	Method Number	Unit	Sample (7)
			Group 4 Male
Fat (ether extraction)	ASM 044	%	8.45
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.007
C13:0	ASM 023	% of product	0.002
C14:0	ASM 023	% of product	0.221
C14:1	ASM 023	% of product	0.076
C15:0	ASM 023	% of product	0.038
C15:1	ASM 023	% of product	0.016
C16:0	ASM 023	% of product	2.260
C16:1	ASM 023	% of product	0.312
C17:0	ASM 023	% of product	0.080
C17:1	ASM 023	% of product	0.063
C18:0	ASM 023	% of product	1.237
C18:1n9c	ASM 023	% of product	3.202
C18:1n9t	ASM 023	% of product	0.172
C18:2n6c	ASM 023	% of product	0.375
C18:2n6t	ASM 023	% of product	0.037
C18:3n3	ASM 023	% of product	0.015
C18:3n6	ASM 023	% of product	0.021
C20:0	ASM 023	% of product	0.010
C20:1	ASM 023	% of product	0.022
C20:3n6	ASM 023	% of product	0.015
C20:4n6	ASM 023	% of product	0.185
C21:0	ASM 023	% of product	0.027
C24:1	ASM 023	% of product	0.055
Saturated fatty acids	ASM 023	% of product	3.884
Mono-unsaturated fatty acids	ASM 023	% of product	3.746
Poly-unsaturated fatty acids	ASM 023	% of product	0.610
Trans fatty acids	ASM 023	% of product	0.209
Cis fatty acids	ASM 023	% of product	3.577
Omega 3	ASM 023	% of product	0.015
Omega 6	ASM 023	% of product	0.632
Omega 9	ASM 023	% of product	3.375

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Analysis	Method Number	Unit	Sample (8)
			Group 4 Female
Fat (ether extraction)	ASM 044	%	13.30
C10:0	ASM 023	% of product	0.005
C12:0	ASM 023	% of product	0.004
C14:0	ASM 023	% of product	0.051
C14:1	ASM 023	% of product	0.006
C15:0	ASM 023	% of product	0.006
C16:0	ASM 023	% of product	2.103
C16:1	ASM 023	% of product	0.126
C17:0	ASM 023	% of product	0.044
C17:1	ASM 023	% of product	0.009
C18:0	ASM 023	% of product	3.638
C18:1n9c	ASM 023	% of product	2.135
C18:2n6c	ASM 023	% of product	1.947
C18:2n6t	ASM 023	% of product	0.010
C18:3n3	ASM 023	% of product	0.046
C18:3n6	ASM 023	% of product	0.010
C20:0	ASM 023	% of product	0.011
C20:1	ASM 023	% of product	0.039
C20:2	ASM 023	% of product	0.024
C20:3n6	ASM 023	% of product	0.066
C20:4n6	ASM 023	% of product	1.991
C20:5n3	ASM 023	% of product	0.034
C21:0	ASM 023	% of product	0.040
C24:1	ASM 023	% of product	0.955
Saturated fatty acids	ASM 023	% of product	5.902
Mono-unsaturated fatty acids	ASM 023	% of product	3.270
Poly-unsaturated fatty acids	ASM 023	% of product	4.118
Trans fatty acids	ASM 023	% of product	0.010
Cis fatty acids	ASM 023	% of product	4.082
Omega 3	ASM 023	% of product	0.079
Omega 6	ASM 023	% of product	4.025
Omega 9	ASM 023	% of product	2.135
EPA	ASM 023	% of product	0.034

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Analysis	Method Number	Unit	Sample (9)
			Group 5 Male
Fat (ether extraction)	ASM 044	%	7.59
C12:0	ASM 023	% of product	0.007
C14:0	ASM 023	% of product	0.044
C14:1	ASM 023	% of product	0.006
C15:0	ASM 023	% of product	0.010
C16:0	ASM 023	% of product	1.666
C16:1	ASM 023	% of product	0.182
C17:0	ASM 023	% of product	0.022
C17:1	ASM 023	% of product	0.007
C18:0	ASM 023	% of product	1.377
C18:1n9c	ASM 023	% of product	1.702
C18:1n9t	ASM 023	% of product	0.002
C18:2n6c	ASM 023	% of product	0.880
C18:2n6t	ASM 023	% of product	0.006
C18:3n3	ASM 023	% of product	0.020
C20:0	ASM 023	% of product	0.007
C20:1	ASM 023	% of product	0.029
C20:2	ASM 023	% of product	0.049
C20:3n3	ASM 023	% of product	0.004
C20:3n6	ASM 023	% of product	0.080
C20:4n6	ASM 023	% of product	0.992
C20:5n3	ASM 023	% of product	0.021
C21:0	ASM 023	% of product	0.031
C22:0	ASM 023	% of product	0.014
C22:2	ASM 023	% of product	0.006
C24:1	ASM 023	% of product	0.425
Saturated fatty acids	ASM 023	% of product	3.178
Mono-unsaturated fatty acids	ASM 023	% of product	2.352
Poly-unsaturated fatty acids	ASM 023	% of product	2.052
Trans fatty acids	ASM 023	% of product	0.008
Cis fatty acids	ASM 023	% of product	2.583
Omega 3	ASM 023	% of product	0.044
Omega 6	ASM 023	% of product	1.958
Omega 9	ASM 023	% of product	1.704
EPA	ASM 023	% of product	0.021

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Analysis	Method Number	Unit	Sample (10)
			Group 5 Female
Fat (ether extraction)	ASM 044	%	10.54
C10:0	ASM 023	% of product	0.015
C12:0	ASM 023	% of product	0.006
C14:0	ASM 023	% of product	0.043
C15:0	ASM 023	% of product	0.008
C16:0	ASM 023	% of product	1.805
C16:1	ASM 023	% of product	0.139
C17:0	ASM 023	% of product	0.023
C17:1	ASM 023	% of product	0.008
C18:0	ASM 023	% of product	2.882
C18:1n9c	ASM 023	% of product	2.065
C18:2n6c	ASM 023	% of product	0.864
C18:3n3	ASM 023	% of product	0.018
C20:1	ASM 023	% of product	0.007
C20:2	ASM 023	% of product	0.032
C20:3n6	ASM 023	% of product	0.080
C20:4n6	ASM 023	% of product	1.844
C20:5n3	ASM 023	% of product	0.010
C24:1	ASM 023	% of product	0.692
Saturated fatty acids	ASM 023	% of product	4.781
Mono-unsaturated fatty acids	ASM 023	% of product	2.911
Poly-unsaturated fatty acids	ASM 023	% of product	2.848
Trans fatty acids	ASM 023	% of product	0.000
Cis fatty acids	ASM 023	% of product	2.929
Omega 3	ASM 023	% of product	0.029
Omega 6	ASM 023	% of product	2.787
Omega 9	ASM 023	% of product	2.065
EPA	ASM 023	% of product	0.010

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Analysis	Method Number	Unit	Sample (11)
			Group 1 Male
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.006
C14:0	ASM 023	% of product	0.139
C14:1	ASM 023	% of product	0.016
C15:0	ASM 023	% of product	0.037
C15:1	ASM 023	% of product	0.015
C16:0	ASM 023	% of product	2.197
C16:1	ASM 023	% of product	0.109
C17:0	ASM 023	% of product	0.070
C17:1	ASM 023	% of product	0.018
C18:0	ASM 023	% of product	1.929
C18:1n9c	ASM 023	% of product	1.754
C18:1n9t	ASM 023	% of product	0.087
C18:2n6c	ASM 023	% of product	0.856
C18:2n6t	ASM 023	% of product	0.006
C18:3n3	ASM 023	% of product	0.011
C18:3n6	ASM 023	% of product	0.008
C20:0	ASM 023	% of product	0.013
C20:1	ASM 023	% of product	0.017
C20:2	ASM 023	% of product	0.016
C20:3n3	ASM 023	% of product	0.002
C20:3n6	ASM 023	% of product	0.055
C20:4n6	ASM 023	% of product	1.047
C21:0	ASM 023	% of product	0.029
C22:0	ASM 023	% of product	0.011
C24:1	ASM 023	% of product	0.379
Saturated fatty acids	ASM 023	% of product	4.433
Mono-unsaturated fatty acids	ASM 023	% of product	2.307
Poly-unsaturated fatty acids	ASM 023	% of product	1.996
Trans fatty acids	ASM 023	% of product	0.093
Cis fatty acids	ASM 023	% of product	2.611
Omega 3	ASM 023	% of product	0.013
Omega 6	ASM 023	% of product	1.973
Omega 9	ASM 023	% of product	1.842

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Analysis	Method Number	Unit	Sample (12)
			Group 1 Female
C12:0	ASM 023	% of product	0.006
C14:0	ASM 023	% of product	0.061
C14:1	ASM 023	% of product	0.003
C15:0	ASM 023	% of product	0.013
C15:1	ASM 023	% of product	0.002
C16:0	ASM 023	% of product	2.587
C16:1	ASM 023	% of product	0.155
C17:0	ASM 023	% of product	0.058
C17:1	ASM 023	% of product	0.011
C18:0	ASM 023	% of product	4.069
C18:1n9c	ASM 023	% of product	2.529
C18:1n9t	ASM 023	% of product	0.015
C18:2n6c	ASM 023	% of product	2.095
C18:2n6t	ASM 023	% of product	0.015
C18:3n3	ASM 023	% of product	0.037
C18:3n6	ASM 023	% of product	0.058
C20:0	ASM 023	% of product	0.012
C20:1	ASM 023	% of product	0.039
C20:2	ASM 023	% of product	0.035
C20:3n6	ASM 023	% of product	0.083
C20:4n6	ASM 023	% of product	2.334
C20:5n3	ASM 023	% of product	0.046
C21:0	ASM 023	% of product	0.043
C22:0	ASM 023	% of product	0.017
C24:1	ASM 023	% of product	1.007
Saturated fatty acids	ASM 023	% of product	6.867
Mono-unsaturated fatty acids	ASM 023	% of product	3.746
Poly-unsaturated fatty acids	ASM 023	% of product	4.688
Trans fatty acids	ASM 023	% of product	0.029
Cis fatty acids	ASM 023	% of product	4.624
Omega 3	ASM 023	% of product	0.083
Omega 6	ASM 023	% of product	4.585
Omega 9	ASM 023	% of product	2.544
EPA	ASM 023	% of product	0.046

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Analysis	Method Number	Unit	Sample (13)
			Group 2 Male
C12:0	ASM 023	% of product	0.002
C14:0	ASM 023	% of product	0.024
C14:1	ASM 023	% of product	0.003
C15:0	ASM 023	% of product	0.008
C16:0	ASM 023	% of product	1.563
C16:1	ASM 023	% of product	0.098
C17:0	ASM 023	% of product	0.026
C17:1	ASM 023	% of product	0.005
C18:0	ASM 023	% of product	1.899
C18:1n9c	ASM 023	% of product	1.104
C18:1n9t	ASM 023	% of product	0.019
C18:2n6c	ASM 023	% of product	0.871
C18:2n6t	ASM 023	% of product	0.006
C18:3n3	ASM 023	% of product	0.010
C18:3n6	ASM 023	% of product	0.021
C20:0	ASM 023	% of product	0.010
C20:1	ASM 023	% of product	0.019
C20:2	ASM 023	% of product	0.087
C20:3n3	ASM 023	% of product	0.002
C20:3n6	ASM 023	% of product	0.088
C20:4n6	ASM 023	% of product	1.421
C20:5n3	ASM 023	% of product	0.036
C21:0	ASM 023	% of product	0.032
C22:0	ASM 023	% of product	0.019
C22:1n9	ASM 023	% of product	0.002
C22:2	ASM 023	% of product	0.024
C22:6n3	ASM 023	% of product	0.002
C24:0	ASM 023	% of product	0.001
C24:1	ASM 023	% of product	0.727
Saturated fatty acids	ASM 023	% of product	3.584
Mono-unsaturated fatty acids	ASM 023	% of product	1.959
Poly-unsaturated fatty acids	ASM 023	% of product	2.563
Trans fatty acids	ASM 023	% of product	0.025
Cis fatty acids	ASM 023	% of product	1.975
Omega 3	ASM 023	% of product	0.050
Omega 6	ASM 023	% of product	2.407
Omega 9	ASM 023	% of product	1.125
EPA	ASM 023	% of product	0.036
DHA	ASM 023	% of product	0.002

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Analysis	Method Number	Unit	Sample (14)
			Group 2 Female
C10:0	ASM 023	% of product	0.003
C12:0	ASM 023	% of product	0.006
C14:0	ASM 023	% of product	0.058
C14:1	ASM 023	% of product	0.006
C15:0	ASM 023	% of product	0.008
C15:1	ASM 023	% of product	0.004
C16:0	ASM 023	% of product	2.421
C16:1	ASM 023	% of product	0.257
C17:0	ASM 023	% of product	0.035
C17:1	ASM 023	% of product	0.013
C18:0	ASM 023	% of product	2.711
C18:1n9c	ASM 023	% of product	2.798
C18:1n9t	ASM 023	% of product	0.019
C18:2n6c	ASM 023	% of product	1.153
C18:2n6t	ASM 023	% of product	0.014
C18:3n3	ASM 023	% of product	0.017
C18:3n6	ASM 023	% of product	0.077
C20:0	ASM 023	% of product	0.009
C20:1	ASM 023	% of product	0.028
C20:2	ASM 023	% of product	0.081
C20:3n6	ASM 023	% of product	0.075
C20:4n6	ASM 023	% of product	1.779
C20:5n3	ASM 023	% of product	0.042
C21:0	ASM 023	% of product	0.030
C24:1	ASM 023	% of product	0.825
Saturated fatty acids	ASM 023	% of product	5.282
Mono-unsaturated fatty acids	ASM 023	% of product	3.932
Poly-unsaturated fatty acids	ASM 023	% of product	3.223
Trans fatty acids	ASM 023	% of product	0.033
Cis fatty acids	ASM 023	% of product	3.952
Omega 3	ASM 023	% of product	0.059
Omega 6	ASM 023	% of product	3.098
Omega 9	ASM 023	% of product	2.817
EPA	ASM 023	% of product	0.042

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Analysis	Method Number	Unit	Sample (15)
			Group 3 Male
C10:0	ASM 023	% of product	0.002
C12:0	ASM 023	% of product	0.004
C14:0	ASM 023	% of product	0.039
C14:1	ASM 023	% of product	0.005
C15:0	ASM 023	% of product	0.008
C16:0	ASM 023	% of product	1.741
C16:1	ASM 023	% of product	0.186
C17:0	ASM 023	% of product	0.021
C17:1	ASM 023	% of product	0.007
C18:0	ASM 023	% of product	1.793
C18:1n9c	ASM 023	% of product	1.568
C18:1n9t	ASM 023	% of product	0.005
C18:2n6c	ASM 023	% of product	0.917
C18:2n6t	ASM 023	% of product	0.006
C18:3n3	ASM 023	% of product	0.012
C18:3n6	ASM 023	% of product	0.005
C20:0	ASM 023	% of product	0.004
C20:1	ASM 023	% of product	0.034
C20:2	ASM 023	% of product	0.049
C20:3n3	ASM 023	% of product	0.002
C20:3n6	ASM 023	% of product	0.082
C20:4n6	ASM 023	% of product	1.376
C20:5n3	ASM 023	% of product	0.023
C21:0	ASM 023	% of product	0.028
C22:0	ASM 023	% of product	0.006
C22:2	ASM 023	% of product	0.005
C23:0	ASM 023	% of product	0.010
C24:1	ASM 023	% of product	0.652
Saturated fatty acids	ASM 023	% of product	3.656
Mono-unsaturated fatty acids	ASM 023	% of product	2.452
Poly-unsaturated fatty acids	ASM 023	% of product	2.471
Trans fatty acids	ASM 023	% of product	0.012
Cis fatty acids	ASM 023	% of product	2.485
Omega 3	ASM 023	% of product	0.036
Omega 6	ASM 023	% of product	2.387
Omega 9	ASM 023	% of product	1.574
EPA	ASM 023	% of product	0.023

This laboratory holds SANAS accreditation for analyses with an ASM number.
Results are expressed on a wet basis, therefore as samples were received.

Analysis	Method Number	Unit	Sample (16)
			Group 3 Female
C12:0	ASM 023	% of product	0.004
C14:0	ASM 023	% of product	0.002
C14:1	ASM 023	% of product	0.003
C15:0	ASM 023	% of product	0.007
C15:1	ASM 023	% of product	0.002
C16:0	ASM 023	% of product	1.568
C16:1	ASM 023	% of product	0.126
C17:0	ASM 023	% of product	0.024
C17:1	ASM 023	% of product	0.004
C18:0	ASM 023	% of product	3.007
C18:1n9c	ASM 023	% of product	1.479
C18:1n9t	ASM 023	% of product	0.018
C18:2n6c	ASM 023	% of product	0.940
C18:2n6t	ASM 023	% of product	0.024
C18:3n3	ASM 023	% of product	0.009
C18:3n6	ASM 023	% of product	0.022
C20:0	ASM 023	% of product	0.010
C20:1	ASM 023	% of product	0.025
C20:2	ASM 023	% of product	0.043
C20:3n6	ASM 023	% of product	0.089
C20:4n6	ASM 023	% of product	2.103
C20:5n3	ASM 023	% of product	0.028
C21:0	ASM 023	% of product	0.032
C24:1	ASM 023	% of product	0.972
Saturated fatty acids	ASM 023	% of product	4.654
Mono-unsaturated fatty acids	ASM 023	% of product	2.611
Poly-unsaturated fatty acids	ASM 023	% of product	3.233
Trans fatty acids	ASM 023	% of product	0.042
Cis fatty acids	ASM 023	% of product	2.418
Omega 3	ASM 023	% of product	0.037
Omega 6	ASM 023	% of product	3.177
Omega 9	ASM 023	% of product	1.496
EPA	ASM 023	% of product	0.028

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Analysis	Method Number	Unit	Sample (17)
			Group 4 Male
C10:0	ASM 023	% of product	0.005
C12:0	ASM 023	% of product	0.006
C13:0	ASM 023	% of product	0.001
C14:0	ASM 023	% of product	0.188
C14:1	ASM 023	% of product	0.064
C15:0	ASM 023	% of product	0.034
C15:1	ASM 023	% of product	0.016
C16:0	ASM 023	% of product	2.127
C16:1	ASM 023	% of product	0.291
C17:0	ASM 023	% of product	0.077
C17:1	ASM 023	% of product	0.057
C18:0	ASM 023	% of product	1.223
C18:1n9c	ASM 023	% of product	3.492
C18:1n9t	ASM 023	% of product	0.160
C18:2n6c	ASM 023	% of product	0.347
C18:2n6t	ASM 023	% of product	0.023
C18:3n3	ASM 023	% of product	0.013
C18:3n6	ASM 023	% of product	0.015
C20:0	ASM 023	% of product	0.014
C20:1	ASM 023	% of product	0.019
C20:2	ASM 023	% of product	0.009
C20:3n6	ASM 023	% of product	0.015
C20:4n6	ASM 023	% of product	0.173
C20:5n3	ASM 023	% of product	0.005
C21:0	ASM 023	% of product	0.011
C22:0	ASM 023	% of product	0.009
C24:1	ASM 023	% of product	0.059
Saturated fatty acids	ASM 023	% of product	3.694
Mono-unsaturated fatty acids	ASM 023	% of product	3.997
Poly-unsaturated fatty acids	ASM 023	% of product	0.576
Trans fatty acids	ASM 023	% of product	0.183
Cis fatty acids	ASM 023	% of product	3.838
Omega 3	ASM 023	% of product	0.018
Omega 6	ASM 023	% of product	0.572
Omega 9	ASM 023	% of product	3.652
EPA	ASM 023	% of product	0.005

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Analysis	Method Number	Unit	Sample (18)
			Group 4 Female
C10:0	ASM 023	% of product	0.022
C12:0	ASM 023	% of product	0.012
C14:0	ASM 023	% of product	0.053
C14:1	ASM 023	% of product	0.009
C15:0	ASM 023	% of product	0.140
C16:0	ASM 023	% of product	2.045
C16:1	ASM 023	% of product	0.146
C17:0	ASM 023	% of product	0.043
C17:1	ASM 023	% of product	0.011
C18:0	ASM 023	% of product	3.561
C18:1n9c	ASM 023	% of product	2.115
C18:2n6c	ASM 023	% of product	1.937
C18:2n6t	ASM 023	% of product	0.008
C18:3n3	ASM 023	% of product	0.042
C18:3n6	ASM 023	% of product	0.070
C20:0	ASM 023	% of product	0.008
C20:1	ASM 023	% of product	0.021
C20:2	ASM 023	% of product	0.033
C20:3n3	ASM 023	% of product	0.005
C20:3n6	ASM 023	% of product	0.064
C20:4n6	ASM 023	% of product	2.035
C20:5n3	ASM 023	% of product	0.050
C21:0	ASM 023	% of product	0.042
C22:2	ASM 023	% of product	0.016
C24:1	ASM 023	% of product	0.937
Saturated fatty acids	ASM 023	% of product	5.800
Mono-unsaturated fatty acids	ASM 023	% of product	3.239
Poly-unsaturated fatty acids	ASM 023	% of product	4.252
Trans fatty acids	ASM 023	% of product	0.008
Cis fatty acids	ASM 023	% of product	4.052
Omega 3	ASM 023	% of product	0.098
Omega 6	ASM 023	% of product	4.114
Omega 9	ASM 023	% of product	2.115
EPA	ASM 023	% of product	0.050

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Analysis	Method Number	Unit	Sample (19)
			Group 5 Male
C12:0	ASM 023	% of product	0.004
C14:0	ASM 023	% of product	0.040
C14:1	ASM 023	% of product	0.005
C15:0	ASM 023	% of product	0.009
C16:0	ASM 023	% of product	1.610
C16:1	ASM 023	% of product	0.183
C17:0	ASM 023	% of product	0.020
C17:1	ASM 023	% of product	0.003
C18:0	ASM 023	% of product	1.376
C18:1n9c	ASM 023	% of product	1.659
C18:2n6c	ASM 023	% of product	0.897
C18:2n6t	ASM 023	% of product	0.010
C18:3n3	ASM 023	% of product	0.020
C20:0	ASM 023	% of product	0.006
C20:1	ASM 023	% of product	0.031
C20:2	ASM 023	% of product	0.052
C20:3n3	ASM 023	% of product	0.057
C20:3n6	ASM 023	% of product	0.075
C20:4n6	ASM 023	% of product	1.029
C20:5n3	ASM 023	% of product	0.015
C21:0	ASM 023	% of product	0.029
C22:0	ASM 023	% of product	0.010
C22:2	ASM 023	% of product	0.007
C24:1	ASM 023	% of product	0.443
Saturated fatty acids	ASM 023	% of product	3.104
Mono-unsaturated fatty acids	ASM 023	% of product	2.324
Poly-unsaturated fatty acids	ASM 023	% of product	2.151
Trans fatty acids	ASM 023	% of product	0.010
Cis fatty acids	ASM 023	% of product	2.556
Omega 3	ASM 023	% of product	0.092
Omega 6	ASM 023	% of product	2.011
Omega 9	ASM 023	% of product	1.659
EPA	ASM 023	% of product	0.015

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Analysis	Method Number	Unit	Sample (20)
			Group 5 Female
C12:0	ASM 023	% of product	0.031
C14:0	ASM 023	% of product	0.036
C15:0	ASM 023	% of product	0.007
C16:0	ASM 023	% of product	1.793
C16:1	ASM 023	% of product	0.148
C17:0	ASM 023	% of product	0.029
C17:1	ASM 023	% of product	0.009
C18:0	ASM 023	% of product	2.840
C18:1n9c	ASM 023	% of product	2.014
C18:2n6c	ASM 023	% of product	0.851
C18:3n3	ASM 023	% of product	0.022
C20:0	ASM 023	% of product	0.008
C20:1	ASM 023	% of product	0.009
C20:2	ASM 023	% of product	0.076
C20:3n6	ASM 023	% of product	0.074
C20:4n6	ASM 023	% of product	1.856
C20:5n3	ASM 023	% of product	0.009
C22:2	ASM 023	% of product	0.022
C24:1	ASM 023	% of product	0.706
Saturated fatty acids	ASM 023	% of product	4.743
Mono-unsaturated fatty acids	ASM 023	% of product	2.886
Poly-unsaturated fatty acids	ASM 023	% of product	2.911
Trans fatty acids	ASM 023	% of product	0.000
Cis fatty acids	ASM 023	% of product	2.866
Omega 3	ASM 023	% of product	0.031
Omega 6	ASM 023	% of product	2.781
Omega 9	ASM 023	% of product	2.014
EPA	ASM 023	% of product	0.009

Sample	Sample type	Date analysis commenced
1 - 20	Liver	16/10/2018