

**TANNIC ACID AS A DIETARY SUPPLEMENT FOR
PROTECTION AGAINST METABOLIC DISORDERS
CAUSED BY SWEETENED ALCOHOL INTAKE IN RATS**



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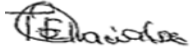
Toluwase Eniola Olanipekun

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

Johannesburg, 2025.

DECLARATION

I, Toluwase Eniola Olanipekun, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of Candidate)

25 day of August 2025

DEDICATION

I dedicate this PhD thesis to God Almighty, the Alpha and Omega, for His guidance from start to finish. To Jesus, whose limitless power makes all things possible, and to the Holy Spirit for His wisdom throughout my journey.

PRESENTATIONS ARISING FROM THIS STUDY

Grad-Flash Presentation

Olanipekun, T.E., Oladiran, O. and Erlwanger, K.H. Impact of Sweetened Alcohol and Tannic acid on body weight gain, fasting blood glucose, and visceral obesity in male and female rats. Presented at the 14th Wits Cross Faculty Postgraduate Symposium, 6- 8 September 2023.

Oral Presentations

Olanipekun, T.E., Oladiran O. and Erlwanger, K.H. Tannic acid and sweetened alcohol reduced feed intake without impacting triglyceride concentration but increased visceral adiposity in female compared to male rats. Presented at the School of Physiology Research Day, 13th September 2023.

Olanipekun, T.E., Oladiran O. and Erlwanger, K.H. Effect of tannic acid against metabolic disorders caused by sweetened alcohol consumption in Sprague Dawley rats and presented at the University of Witwatersrand Faculty of Health Sciences Research Day, 5th September 2024.

Poster Presentation

Olanipekun, T.E., Oladiran, O., Manilall, A. and Erlwanger K.H. Effect of voluntary consumption of tannic acid and sweetened alcohol on the liver of Sprague Dawley rats. Presented at the 50th Anniversary Congress of the Physiological Society of Southern Africa (PSSA), 25th –28th August 2024, hosted by the University of Limpopo, South Africa.

PUBLICATIONS ARISING FROM THIS STUDY

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ABSTRACT

Excessive sugar and alcohol intake contribute to the rising prevalence of metabolic disorders. Sweetened alcoholic beverages are increasingly popular among adolescents. While the individual effects are well studied, the combined impact remains unclear. Phytochemicals show promise in managing metabolic dysfunction, but their role in sugar- and alcohol-induced metabolic damage is not well understood. This study investigated tannic acid (TA) as a potential intervention for mitigating sweetened alcohol (SAC)-induced metabolic disorders, with a focus on the liver and kidneys of young adult rats. A voluntary gelatin-based model was used to mimic adolescent alcohol shot consumption.

Sixty-four male and female adolescent Sprague Dawley rats (42 days old) were treated for 10 weeks with SAC (10% ethanol + 20% fructose in gelatine) and/or TA (50 mg/kg in gelatine), or control (plain gelatine). Measurements included feed intake, body mass gain, fasting glucose, visceral fat, insulin levels, HOMA-IR, blood parameters (Hb, HCT, MCHC), and lipid profiles (TG, HDL-C, TG-HDL-C ratio). Liver absolute mass, hepatosomatic index, TBARS, and gene expression (CYP2E1, SREBP-1, IL-10, NF- κ B1, TNF- α), and histology (hepatocyte density, micro/macro steatosis, fibrosis) were assessed. Kidney evaluations included mass, index, function biomarkers (KIM-1, NGAL), TBARS, and histology (urinary space, glomerular and Bowman's capsule areas, fibrosis).

All rats grew significantly ($P < 0.001$), but body mass remained consistent within sexes. From week 3, feed intake declined in SAC and SAC+TA groups compared to PG and TA ($P < 0.05$). SAC increased visceral fat, especially in females, and raised HDL-C levels in males ($P < 0.05$), while other metabolic and blood markers remained unchanged ($P > 0.05$).

In females, SAC and TA each reduced hepatocyte density by ~38%, with a smaller reduction in the SAC+TA group ($P < 0.05$). In males, SAC+TA resulted in lower hepatocyte density compared to PG and TA ($P < 0.05$). Steatosis was more severe in females across most groups, except SAC, where males showed higher micro-vesicular steatosis ($P < 0.05$). Hepatic fibrosis was unchanged in females but reduced in SAC and TA males ($P < 0.05$).

Kidney mass and function markers (KIM-1, NGAL) were unaffected ($P > 0.05$), but TBARS were elevated in female kidneys ($P < 0.05$). Urinary space was enlarged in TA and SAC females but reduced in SAC males ($P < 0.05$). Bowman's capsule area increased in TA females ($P < 0.05$),

with no change in males. Glomerular tuft area was higher in TA and SAC+TA females, but lower in TA males, compared to other groups ($P<0.05$).

Moderate voluntary alcohol consumption in gelatine did not induce overt metabolic dysfunction in rats. Where abnormalities occurred, tannic acid (50 mg/kg) offered limited protection, improving only hepatic oxidative stress in males. SAC and TA had no significant impact on body mass, blood parameters, insulin resistance, triglycerides, or renal injury markers. However, SAC increased visceral fat, hepatic steatosis, lipid peroxidation, and glomerular hyperfiltration in females, while elevating HDL-C in males, suggesting greater hepatic and renal vulnerability in females. Caution is advised for females consuming sweetened alcohol.

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NOMENCLATURE/LIST OF ABBREVIATIONS AND SYMBOLS

AACE:	American Association of Clinical Endocrinology
AChE:	Acetylcholine esterase
ADH:	Alcohol dehydrogenase
AFP:	Alpha-fetoprotein
AGEs:	Advanced glycation end products
AKD:	Acute kidney disease
AKI:	Acute kidney injury
ALD:	Alcohol-related liver disease
ALT:	Alanine aminotransferase
ANOVA:	Analysis of variance
AREC:	Animal Research Ethics Committee
ARRIVE:	Animal Research: Reporting of In Vivo Experiments
AST:	Aspartate aminotransferase
AT1R:	Angiotensin type 1 receptor
ATP:	Adenosine triphosphate
BChE:	Butyrylcholinesterase
BHA:	Butylated hydroxyanisole
BHT:	Butylated hydroxytoluene
CAT NO:	Catalog No
CD14:	Cluster of differentiation 14
cDNA:	Complementary deoxyribonucleic acid

CIV:	Type IV collagen
CK18:	Cytokeratin 18
CK-18:	Cytokeratin-18
CKD:	Chronic kidney disease
CT:	Computed tomography
CYP2E1:	Cytochrome P450 2E1
db/db mice:	Leptin receptor-deficient mice
DILI:	Drug-induced liver injury
DKD:	Diabetic kidney disease
DS/obese rats:	DahlS.Z-Lepr fa/Lepr fa rats
eGFR:	Estimated glomerular filtration rate
EGIR:	European Group for the Study of Insulin Resistance
ELISA:	Enzyme-Linked Immunosorbent Assay
EMT:	Epithelial-mesenchymal transition
ER:	Endoplasmic reticulum
ESKD:	End-stage kidney disease
FDA:	The US Food and Drug Administration
GFR:	Glomerular filtration rate
GK rats:	Goto-Kakizaki rats
GLDH:	Glutamate dehydrogenase
GST-α:	Glutathione-S-transferase- α
H&E:	Haematoxylin and eosin
HA:	Hyaluronic acid

Hb:	Haemoglobin concentration
HBV:	Hepatitis B viruses
HCT:	Haematocrit
HCV:	Hepatitis C viruses
HDL-C:	High-density lipoprotein cholesterol
HE:	Hepatic encephalopathy
HHDP:	Hexahydroxy diphenic acid
HIV:	Human immunodeficiency virus
HMGB1:	High mobility group box 1
HOMA-IR:	Homeostatic Model Assessment of Insulin Resistance
HPV:	Human papillomavirus
HSCs:	Hepatic stellate cells
HSPs:	Heat shock proteins
HSV-1:	Herpes simplex virus type 1
HSV-2:	Herpes simplex virus type 2
IAV:	Influenza A virus
IDF:	International Diabetes Federation
IL-10:	Interleukin-10
KIM-1:	Kidney injury molecule-1
LD50:	Median lethal dose or lethal dose 50
LDLR:	Low-density lipoprotein receptor
LN:	Laminin
MCHC:	Mean corpuscular haemoglobin concentration

MDH:	Malate dehydrogenase
MEOS:	Microsomal ethanol-oxidizing system
MetS:	Metabolic syndrome
miRNAs:	microRNAs
MPO:	Myeloperoxidase
MRI:	Magnetic resonance imaging
mRNA:	messenger Ribonucleic acid
MT:	Masson Trichrome
NAFLD:	Non-alcoholic fatty liver disease
NASH:	Non-alcoholic steatohepatitis
NCEP ATP III:	National Cholesterol Education Program Adult Treatment Panel III
NESARC:	National Epidemiological Survey on Alcohol and Related Conditions
NF-κB1:	Nuclear factor kappa B1
NGAL:	Neutrophil gelatinase-associated lipocalin
ob/ob mice:	Leptin-deficient mice
OD value:	Optical density value
OLT:	Orthotopic liver transplantation
PCNA:	Proliferating cell nuclear antigen
PFA:	Paraformaldehyde
PG:	Plain gelatine
PIIINP:	Procollagen III N-terminal peptide
RAAS:	Renin-angiotensin-aldosterone system

RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RRT:	Renal replacement therapy
RT-qPCR:	Real-time polymerase chain reaction
SAC:	Sweetened Alcohol (20% fructose and 10% alcohol)
SAC+TA:	Sweetened Alcohol (20% fructose, 10% alcohol and Tannic acid- 50mg/kg)
SREBP-1:	Sterol regulatory element-binding protein-1
TA:	Tannic acid (50mg/kg)
TBA:	Thio-barbituric acid
TBARS:	Thio-barbituric acid-reactive substances
TG:	Triglycerides
TNF-α:	Tumour necrosis factor- α
UPR:	Unfolded protein response
US:	Ultrasound
USD:	United States dollar
VEGF:	Vascular endothelial growth factor
WHO:	World Health Organisation
WRAF:	Wits Research Animal Facility
ZDF rats:	Zucker diabetic fatty rats,
ZF rats:	Zucker fatty rats
β1/JNK signalling pathway:	Beta-1/Jun N-terminal kinase signalling pathway

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CHAPTER ONE

INTRODUCTION

1.1 Thesis structure

Following the Wits Faculty of Health Sciences ‘style guide for theses, dissertations and research reports’, this thesis adopted the conventional monograph (block) format, consisting of six chapters (Introduction, Literature Review, Methods, Results, Discussion and Conclusions), a references section, and appendices.

Chapter One introduces the study and outlines the thesis structure. It provides background on sweetened alcohol (SAC) consumption and its link to metabolic disorders. The potential protective effects of tannic acid (TA), especially on the liver and kidneys in adolescents, are briefly mentioned. The chapter also states the study’s justification, aims, objectives, and hypotheses.

Chapter Two provides a comprehensive review of relevant literature, covering the prevalence, definition, components, and pathophysiology of metabolic disorders. It describes the contribution of SAC consumption to metabolic, hepatic, and renal diseases. Additionally, it explores conventional and non-conventional approaches to managing metabolic disorders, with a focus on the possible health benefits of TA. This chapter establishes the theoretical foundation for the study.

Chapter Three details the experimental design, including dietary interventions, preparation procedures, and termination protocols. It outlines the methodology used to assess general health performance, metabolic profile, liver and kidney function analysis, ensuring a systematic approach to data collection and analysis.

Chapter Four presents the study’s findings, supported by figures, tables, and representative photomicrographs of liver and kidney histological sections. The results are systematically organised to address each research objective.

Chapter Five interprets and contextualises the findings, drawing comparisons with existing literature and expanding on it, considering the research findings. It explores the potential role of

TA in mitigating metabolic, hepatic, and renal impairments associated with SAC consumption, offering insights into the biological mechanisms underlying these effects.

Chapter Six concludes the thesis by summarising the key findings, emphasising their significance, and discussing study limitations. It also provides recommendations for future research directions.

This structured approach ensures a logical and coherent flow, guiding the reader through the research journey from background and methodology to results, discussion, and conclusions.

1.2 Overview and background of the study

The global rise in metabolic disorders represents a growing public health challenge (Papaioannou, Kadi and Nilsson, 2022). Among adults, the prevalence is estimated at approximately 25% (Papaioannou, Kadi and Nilsson, 2022). In children and adolescents, metabolic dysfunction affects about 3% and 5%, respectively, with an estimated 25.2 million children and 35.2 million adolescents diagnosed worldwide (Noubiap *et al.*, 2022). This upward trend is strongly linked to the increasing rates of obesity within these age groups (Wentzel *et al.*, 2025). In South Africa, 21.56% of adolescents are classified as obese, with females at a greater risk of developing obesity than males (Nwosu *et al.*, 2022). Excessive intake of sugar (Mai and Yan, 2019) or alcohol (Åberg *et al.*, 2023), individually, has also been strongly associated with the development of metabolic syndrome.

Metabolic syndrome can be described as a collection of risk factors (of metabolic origin) that contribute to the development of serious illnesses such as chronic diabetes and atherosclerotic heart disease (O'Neill and O'Driscoll, 2015). Insulin resistance, obesity, dyslipidaemia, hypertension, hyperinsulinemia, hyperuricaemia, and an altered blood lipid profile are all considered metabolic risk factors (Papaioannou, Kadi and Nilsson, 2022). Individuals with these medical conditions have a fivefold increased risk of developing Type II diabetes mellitus and a threefold higher likelihood of experiencing heart issues (O'Neill and O'Driscoll, 2015).

Chronic consumption of alcohol increasingly impairs metabolic profiles and promotes the development of metabolic syndrome components (Fujita and Takei, 2011). Globally, 136 million

females and 264 million males aged 15 years and above are predicted to have an alcohol use disorder, which is a significant risk factor for metabolic dysfunction (WHO, 2024). Also, alcohol use disorder is responsible for approximately 2.6 million deaths each year, resulting in significant social, economic, and medical burdens (WHO, 2024). Sugar-sweetened alcohol is particularly appealing to adolescents because of its pleasing flavour, and this usually leads to increased sugar and alcohol consumption (Hughes *et al.*, 1997).

The rising use of sweeteners like fructose in beverages has been linked to increasing rates of obesity, insulin resistance, liver damage, and the onset of diabetes mellitus, a leading global cause of chronic kidney disease (CKD) (Mai and Yan, 2019). Alcoholism also contributes significantly to CKD progression (Grant *et al.*, 2017), particularly in individuals with fatty liver and other metabolic disorders (Wang *et al.*, 2022). In recent years, both non-infectious (e.g., non-alcoholic fatty liver disease [NAFLD], alcohol-related liver disease [ALD], and drug-induced liver injury [DILI]) and infectious liver diseases (e.g., hepatitis B and C virus infections) have become more prevalent (Xiao *et al.*, 2019). Cirrhosis, hepatocellular carcinoma, and hepatic failure are also on the rise (Xiao *et al.*, 2019). Among these, NAFLD and ALD are the most common, with estimated global prevalence rates of 25% and 6%, respectively and are primarily driven by excessive sugar and alcohol consumption (Sharma, Suk and Kim, 2021).

Molecular markers such as Cytochrome P450 2E1 (*CYP2E1*), Sterol regulatory element-binding protein-1 (*SREBP-1*), Nuclear factor kappa B1 (*NF-κB1*), and inflammatory cytokines play critical roles in the development of ALD (Nowak and Relja, 2020). *CYP2E1*, a member of the cytochrome P450 enzyme family, is particularly implicated in the pathogenesis of ALD (Zhang *et al.*, 2024). *SREBP-1* regulates fatty acid synthesis, contributing to hepatic lipid accumulation (Ren *et al.*, 2023). Inflammatory cytokines also significantly influence the development of metabolic syndrome, with elevated levels of Interleukin-10 (*IL-10*) and Tumour necrosis factor- α (*TNF- α*) activating the NF- κ B signalling pathway (Chojnacka and Lewandowska, 2023).

Currently, the many components of metabolic diseases are managed and treated through lifestyle changes and pharmacological medications that modify specific metabolic biochemical pathways (Fang *et al.*, 2020). Conventional pharmacological agents are typically mono-therapeutic, targeting individual risk factors, necessitating the administration of multiple medications at the same time

(Makhoba *et al.*, 2020). Pharmaceuticals can be costly and are often associated with unpleasant side effects, which might impair patient compliance (Abdulghani and Al-Fayyadh, 2024). Non-conventional approaches for the management of metabolic problems include the use of therapeutic plants rich in phytochemicals (Fang *et al.*, 2020). These medicinal plants are thought to have a more integrative effect than conventional medications and are readily available and less harmful than conventional medications (Fang *et al.*, 2020; Tran, Pham and Le, 2020). The consumption of fruits and vegetables, which are high in phytochemicals such as polyphenolic compounds, has been shown to reduce the development of metabolic syndrome (Papaioannou, Kadi and Nilsson, 2022). One of these compounds is TA.

TA is a polyphenolic chemical obtained from plants (Nagesh *et al.*, 2020). Anti-inflammatory, anti-diabetic, anti-bacterial, anti-cancer, and antioxidant properties have been demonstrated as biological activities of TA (Türkan, Taslimi and Saltan, 2019). According to Liu *et al.* (2005), TA improves glucose absorption and inhibits adipocyte differentiation and lipid accumulation. TA has also been shown to help inhibit the development of non-insulin-dependent diabetes and inhibit fat storage (Liu *et al.*, 2005; Türkan *et al.*, 2019).

Furthermore, TA also prevents diet-induced non-alcoholic fatty liver disease (NAFLD) by inhibiting the activity of histone acetyltransferase, an enzyme that disrupts hepatic fat homeostasis (Chung *et al.*, 2019). It also reduced body weight gain and protected against the development of hepatic steatosis caused by long-term ethanol treatment (Jordao *et al.*, 2010). TA consumption reduced body mass, liver mass, and serum Thio-barbituric acid-reactive substance (TBARS) levels in another study on faecal steroid excretion in rats (Nakamura, Tsuji and Tonogai, 2003).

1.3 Justification of the study

Prevalence and risk

According to the World Health Organisation's 2024 Global Status Report on Alcohol and Health and Treatment of Substance Use Disorder, approximately 143 million adolescents aged 15 to 19, representing 22% of this age group, are current drinkers (WHO, 2024). This includes about 18.9 million adolescents from the African region. In addition, young people aged 15 to 24 years have a greater prevalence of binge drinking than the general population in all the W.H.O. regions (WHO,

2019). The prevalence of alcohol intake among teenagers with a higher risk of developing an alcohol use disorder range from 3.9% in those aged 12 to 13 to 51.6% in those aged 18 to 20 (Ben El Jilali *et al.*, 2020).

Research gaps in sweetened alcohol studies

Sugar-sweetened beverages are major contributors to the global rise in metabolic syndrome (Malik and Hu, 2022). Among adolescents, there is a growing trend of consuming sweetened alcoholic drinks. Diets high in sugar (Kobi *et al.*, 2023) and alcohol intake (Tsermpini, Plemenitaš Ilješ and Dolžan, 2022) promote the generation of reactive oxygen species, leading to oxidative stress, an important risk factor for the development of chronic metabolic conditions in adolescents (Samsonov and Urlacher, 2025). This may ultimately result in kidney damage and metabolic-associated fatty liver disease (MAFLD) (Dobrowolski *et al.*, 2022). Adolescents are specifically vulnerable to metabolic and organ damage, as external stressors like unhealthy diets can disrupt hormones, hinder growth, and impair organ development, raising the risk of long-term health issues (Mousikou, Kyriakou and Skordis, 2023). Therefore, the combined burden of excessive sugar and alcohol consumption presents a significant challenge, potentially increasing the incidence of cardiometabolic disorders among teenagers.

Furthermore, there is a lack of studies investigating the combined effects of sugar and alcohol, as well as the potential protective role of TA against SAC-induced metabolic dysfunction. As a result, more research is needed to better understand the impact of the diseases related to the practice of drinking SAC. Similarly, there is a need to investigate other therapies, such as phytochemicals, to prevent the development of metabolic illnesses.

Sex differences in alcohol consumption

While men drink alcohol more frequently and consistently than women, there has been an increase in the number of women consuming alcohol worldwide in recent years (WHO, 2024). Similarly, National Epidemiological Survey on Alcohol and Related Conditions (NESARC) data show that between 2001–2002 and 2012–2013, high-risk drinking and alcohol use disorders among women increased by 57.9% and 83.7%, respectively (Grant *et al.*, 2017). This pattern indicates a gender difference in the prevalence and consequences of alcohol consumption. Given the usual bias

towards males in research, there is a need to include females, as sex variations in illness susceptibility and metabolism have been well established.

Rationale for the use of TA

Although the impacts of TA on high sugar or high alcohol intake have been reported independently, there are no research findings regarding its effect on a combination of sugar and alcohol, such as in a sweetened alcoholic beverage. TA has well-established mechanisms, such as antioxidant, anti-inflammatory, and antifibrotic properties, that support its potential as a therapeutic candidate. In studies where rodents are used as experimental models for alcohol consumption, a forced drinking paradigm is often used. However, such models may not accurately represent the physiological patterns of alcohol consumption. To better mimic adolescent behaviour and enhance the real-world applicability of TA as a dietary supplement, this study employed a voluntary SAC consumption model in rats. Thus, the study aimed to examine the protective effects of TA, an FDA-approved supplement, against the development of metabolic disorders resulting from the combined voluntary intake of alcohol and sugar in adolescent male and female rats.

The specific aim and objectives of the study are presented in the following subsection.

1.4 Aim and objectives

The study aimed to investigate whether TA, when administered as a dietary supplement in adolescence, will prevent the development of metabolic disorders associated with combined alcohol and sugar intake in both sexes of Sprague Dawley rats.

To achieve this, three key objectives were set:

1. To assess the protective potential of TA against the adverse effects of SAC on overall metabolic health by measuring:
 - Feed intake
 - Body weight gain as an indicator of growth performance
 - Obesity-related markers: visceral and epididymal fat pad accumulation

- Haematological parameters, specifically haematocrit (HCT), haemoglobin (Hb) concentration, and mean corpuscular haemoglobin concentration (MCHC)
 - Circulating lipid metabolite levels: triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and the TG-to-HDL-C ratio
 - Circulating glucose concentration, insulin levels and insulin resistance using the homeostatic model assessment of insulin resistance (HOMA-IR) index
2. To evaluate the protective potential of TA against the adverse effects of SAC on liver function and integrity by analysing:
- Liver weight and hepatosomatic index
 - Oxidative stress markers in the liver (TBARS)
 - Expression of hepatic genes related to alcoholic liver disease, including metabolic markers– Cytochrome P450 2E1 (*CYP2E1*), Sterol regulatory element-binding protein-1 (*SREBP-1*) and pro-inflammatory markers– Interleukin-10 (*IL-10*), Nuclear factor kappa B1 (*NF-κB1*), and Tumour necrosis factor- α (*TNF- α*).
 - Liver steatosis and hepatic injury through histomorphometry scoring of steatosis, hepatocyte count and fibrosis analysis
3. To assess the protective potential of TA against the adverse effects of SAC on renal function and nephropathy by focusing on
- Kidney weight and kidney index
 - Serum levels of the renal function biomarkers, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL)
 - Oxidative stress markers (TBARS) in the kidney tissue
 - Renal microstructure, assessed via urinary space, glomerular tuft area, Bowman's capsule area measurements and renal fibrosis analysis

This study will provide insights into TA's potential role in preventing metabolic, hepatic, and renal impairments associated with SAC consumption.

1.5 Hypotheses

The null hypotheses (H_0) and the alternative hypotheses (H_1) for this study are stated below:

1.5.1 Hypothesis for objective one

H_0 : Supplementation with TA during adolescence does not modulate the metabolic outcomes linked to the voluntary intake of alcohol that has been sweetened with fructose in both sexes of Sprague Dawley rats.

H_1 : Supplementation with TA during adolescence modulates metabolic outcomes linked to the voluntary intake of alcohol that has been sweetened with fructose in both sexes of Sprague Dawley rats.

1.5.2 Hypothesis for objective two

H_0 : Adolescent supplementation with TA does not attenuate liver-related alterations induced by voluntary consumption of fructose-sweetened alcohol in female and male Sprague Dawley rats.

H_1 : Adolescent supplementation with TA attenuates liver-related alterations induced by voluntary consumption of fructose-sweetened alcohol in female and male Sprague Dawley rats.

1.5.3 Hypothesis for objective three

H_0 : TA supplementation during adolescence does not prevent renal dysfunction associated with voluntary intake of fructose-sweetened alcohol in female and male Sprague Dawley rats.

H_1 : TA supplementation during adolescence prevents renal dysfunction associated with voluntary intake of fructose-sweetened alcohol in female and male Sprague Dawley rats.

The next chapter presents a critical review of the literature relevant to this study.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Metabolic syndrome

Metabolic syndrome (MetS) is a cluster of interrelated conditions that co-occur in an individual, including obesity, hyperglycaemia, hypertension, dyslipidaemia, and other metabolic abnormalities (Gargari and Chatterjee, 2024). These components collectively impact energy metabolism and distribution (Alemany, 2024). MetS is primarily associated with chronic low-grade inflammation and insulin resistance, both of which are major risk factors for cardiovascular diseases and diabetes (Lin and Sun, 2024). Systemic inflammation contributes to MetS by activating innate immune cells, leading to persistent inflammation even in the absence of infection (van de Vyver, 2023). Similarly, insulin resistance disrupts lipid and protein metabolism, further compromising overall metabolic health (Banerji and Chaiken, 2010).

2.1.1 History of metabolic syndrome

The idea that metabolic disorders often co-occur dates as early as the 12th century, when a group of medical practitioners observed that diabetes and cardiovascular disease could manifest in the same individual (Shah and Vella, 2016). However, the term "metabolic syndrome" was first introduced in 1975 by Hermann Haller, who described it as a combination of obesity, diabetes, hyperlipidaemia, hyperproteinaemia, hyperuricaemia, and liver steatosis (Haller, 1977). In 1988, Gerald Reaven introduced the terminology "Syndrome X," emphasising the link between insulin resistance and various components of MetS (Szychlińska *et al.*, 2020). The following year, Kaplan Norman renamed it the "Deadly Quartet," highlighting its severe health implications (Kaplan, 1989). Kaplan established that MetS is primarily driven by insulin resistance, which, when accompanied by hyperinsulinemia, contributes to metabolic complications.

The definition and diagnostic criteria of MetS have evolved. The World Health Organisation (WHO) provided the first formal definition in 1998, identifying MetS as the presence of diabetes, impaired glucose tolerance, impaired fasting glucose, or insulin resistance, along with at least two of the following: high blood pressure ($\geq 160/90$ mmHg), hyperlipidaemia (triglycerides ≥ 150 mg/dL or low HDL cholesterol), central obesity, or microalbuminuria (Alberti and Zimmet, 1998).

This definition was later refined by the European Group for the Study of Insulin Resistance (EGIR), which emphasised insulin resistance as the central component of MetS (Shah and Vella, 2016). However, definitions provided by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), the International Diabetes Federation (IDF), and the American Association of Clinical Endocrinology (AACE) differ from the EGIR definition, as they do not require insulin resistance for diagnosis but instead prioritise visceral obesity alongside other risk factors (Akintunde *et al.*, 2011). Despite these variations, the definitions proposed by EGIR and NCEP ATP III remain widely accepted in clinical and research settings.

2.1.2 Prevalence of metabolic syndrome

The prevalence of MetS varies across different populations, sexes, and age groups, largely due to differences in diagnostic criteria (Huang *et al.*, 2022). Globally, MetS affects approximately 25% of the population, equating to over two billion individuals (Arantes *et al.*, 2023). It is responsible for 7% of total deaths and contributes to 17% of cardiovascular disease-related mortality (Arantes *et al.*, 2023). Recent studies indicate that MetS prevalence ranges between 12.5% and 31.4%, with women exhibiting higher rates than men in certain populations (Noubiap *et al.*, 2022). Additionally, the prevalence of MetS among adolescents has risen significantly, with estimates of approximately 4.2% based on IDF criteria and 7.7% according to NCEP definitions (Zong *et al.*, 2024). The early onset of MetS components in adolescents has serious health implications, increasing the likelihood of developing metabolic diseases in adulthood (Orsini *et al.*, 2023).

2.1.3 Components of metabolic syndrome

The key components of MetS include abdominal obesity, hypertriglyceridemia, low circulating high-density lipoprotein cholesterol (HDL-C), hypertension, and hyperglycaemia (Alberti and Zimmet, 1998). The presence of at least three of these factors in an individual doubles the risk of cardiovascular disease and increases the likelihood of developing type II diabetes by fivefold (Alberti and Zimmet, 1998). Obesity, a major contributor to MetS, results from excessive adipose tissue accumulation due to an energy imbalance (Rodrigues *et al.*, 2016). Genetic variations affecting lipid metabolism may further contribute to hyperlipidaemia. Low HDL-C levels are also strongly associated with obesity and metabolic disorders, as they impair cholesterol clearance (Zvintzou *et al.*, 2023). Elevated cholesterol levels, in turn, promote the accumulation of low-

density lipoprotein (LDL), contributing to atherosclerotic plaque formation and raising cardiovascular disease risk (Mankar, B. Pawar and Y. Pawar, 2024). This risk is exacerbated by the coexistence of diabetes and hypertension, which drive disease progression through mechanisms such as insulin resistance and endothelial dysfunction (Mwende, 2024). The interplay among these components is complex, involving numerous biological pathways that contribute to the syndrome's development and associated health risks (Shi *et al.*, 2023).

2.1.4 Pathophysiology of metabolic syndrome

The development of MetS is influenced by genetic, lifestyle, and environmental factors, making it a complex global health concern (Lin and Sun, 2024). Genetic predisposition plays a significant role, with approximately 50% of MetS cases attributed to hereditary factors, including alterations in DNA sequences and chromosomal variations (Rana *et al.*, 2022). Environmental exposure to endocrine-disrupting chemicals also contributes to MetS by interfering with metabolic pathways and causing hormonal imbalances (Ziki and Mani, 2016). Additionally, lifestyle choices such as poor dietary habits, physical inactivity, and substance abuse significantly impact MetS development (Haverinen *et al.*, 2021). Unhealthy diets are strongly associated with a higher prevalence of MetS, whereas increased physical activity has been shown to mitigate its effects by improving insulin sensitivity and reducing obesity (Shackelford, 2023). Moreover, substance abuse, particularly excessive alcohol consumption, is linked to a high incidence of MetS (Bathla *et al.*, 2017).

South Africa ranks among the highest alcohol-consuming countries globally, with recent estimates indicating that approximately 32.5% of the population consumes alcohol, comprising 43.4% of males and 22.6% of females (WHO, 2024). A particular concern is the early initiation of alcohol use, with evidence indicating that 12% of South African adolescents start consuming alcohol before the age of 13 years (Mafa *et al.*, 2019). Similarly, despite the introduction of the South African Health Promotion Levy in 2018, which imposed a 10% tax on sugar-sweetened beverages, sugar consumption has not significantly declined. Adolescent intake of added sugars remains persistently high (Wrottesley *et al.*, 2021).

2.1.5 Impact of alcohol on metabolic syndrome

The effects of alcohol consumption on metabolic health remain a topic of debate (Skultecka *et al.*, 2024). While some studies suggest that moderate to high alcohol consumption may lower the risk of MetS compared to low consumption, excessive alcohol intake is undeniably harmful (Musto, Brown and Lucey, 2025). Chronic alcohol consumption is associated with the development of metabolic conditions such as obesity, insulin resistance, and dyslipidaemia (Åberg *et al.*, 2023). Alcohol disrupts metabolic pathways that regulate glucose and lipid metabolism, leading to oxidative stress and cellular damage. Furthermore, alcohol consumption and abuse often begin in early adolescence, increasing the likelihood of long-term metabolic complications (Wakabayashi *et al.*, 2021).

Globally, the prevalence of alcohol consumption among adolescents aged 15 to 19 is estimated at 22% (WHO, 2024). In South Africa, the prevalence is slightly higher at 23.1% (Belete *et al.*, 2024). This trend places adolescents at increased risk of alcohol-related mortality and long-term metabolic disorders, including diabetes and cardiovascular diseases (WHO, 2024). Alcohol consumption trends also vary by gender, with males typically preferring beer and spirits, while females more often choose wine and flavoured drinks. Notably, adolescents of both sexes tend to favour sweetened alcoholic beverages (Mafa *et al.*, 2019).

2.1.6 Impact of sugar on metabolic syndrome

Sugar, a type of carbohydrate, includes monosaccharides (e.g., glucose, galactose, and fructose) and disaccharides (e.g., sucrose, lactose, and maltose) (Walton *et al.*, 2023). While sugars occur naturally in many foods, they are also frequently added as sweeteners (Walton *et al.*, 2023). The excessive intake of added sugars is a key contributor to the rising prevalence of MetS (Johnson *et al.*, 2007). High sugar consumption is strongly linked to core MetS components, including insulin resistance, obesity, and cardiovascular disease (Muñoz-Cabrejas *et al.*, 2023). A meta-analysis has demonstrated a dose-dependent relationship between the intake of sugar-sweetened beverages (SSBs) and the risk of MetS (Zhang *et al.*, 2021).

Despite some reductions in global sugar intake in recent years, consumption levels remain above the recommended guidelines (Walton *et al.*, 2023). Adolescents and children have the highest

sugar intake as a percentage of total energy consumption (12-14%), compared to 8% in the adult population (Walton *et al.*, 2023). Excessive sugar consumption in younger age groups significantly contributes to the increasing prevalence of MetS in adolescents and children (Shim, Lee and Hwang, 2024).

2.2 Sweetened alcohol consumption trends

The consumption of sugar-sweetened beverages (SSBs) has been consistently linked to an increased risk of metabolic syndrome (MetS) and its associated components across various populations (Muñoz-Cabrejas *et al.*, 2023). In recent times, there has been a rise in the consumption of jello shots (alcohol-infused flavoured gelatine) among adolescents (Grossman, Binakonsky and Jernigan, 2018). The appeal of sweetened alcoholic beverages lies in their enhanced palatability due to added flavours (Wakabayashi *et al.*, 2021). This trend contributes to increased alcohol intake, leading to a higher incidence of heavy episodic drinking and greater alcohol-related harm compared to the consumption of non-flavoured alcoholic beverages (Albers *et al.*, 2015).

Additionally, females consume jello shots more frequently than males (Siegel *et al.*, 2016), likely due to their preference for milder bitterness and richer, layered flavours (Muggah and McSweeney, 2017). Both alcohol and sugar activate similar neurobiological pathways, contributing to addictive behaviours and excessive consumption (Fortuna, 2010). Excessive intake of alcohol (Åberg *et al.*, 2023) and sugar (Muñoz-Cabrejas *et al.*, 2023) increases the risk of MetS in adolescents, highlighting the need to investigate the combined metabolic impact of both, as we have in sweetened alcoholic beverages.

2.3 Experimental animal models of alcohol consumption

Animal models play a crucial role in elucidating the relationship between alcohol consumption and metabolic syndrome (MetS) (Buyco *et al.*, 2021). Several models are utilised to study alcohol consumption, including operant models, non-operant models, and binge-drinking models (Favoretto *et al.*, 2024). These models of alcohol use and abuse involve precise adjustments to the timing, duration, and frequency of alcohol availability, along with designated periods of abstinence and prior exposure (Becker and Lopez, 2024). Various alcohol drinking patterns, including

intermittent consumption, binge drinking, and mixed intake, can be modified in both frequency and duration (Becker and Lopez, 2024).

The operant alcohol model involves modifying behaviour through reinforcement or inhibition based on consequences, such as self-administration, intermittent access, chronic alcohol dependence and withdrawal (Becker and Lopez, 2024), and the saccharin-fading procedure (Calleja-Conde *et al.*, 2023). The scheduled access alcohol model demonstrates that intermittent alcohol availability promotes binge-like drinking patterns, which intensify alcohol consumption behaviours (Becker and Lopez, 2024). The saccharin-fading procedure, which introduces alcohol after initial exposure to a sweetener, aids in understanding voluntary alcohol consumption (Calleja-Conde *et al.*, 2023).

Conversely, non-operant protocols involve investigator-controlled alcohol administration, enabling researchers to manipulate alcohol doses and observe the resulting physiological and behavioural responses without requiring the subject's active participation (Favoretto *et al.*, 2024). Non-operant animal models include the conditioned place preference model, which assesses the association between a specific environment and the rewarding effects of alcohol, as well as home-cage drinking paradigms, which evaluate voluntary alcohol consumption in the absence of operant conditioning (Tunstall, Vendruscolo and Allen–Worthington, 2020).

2.4 Experimental animal models of fructose consumption

The relationship between sugar consumption and metabolic syndrome is investigated using animal models that modify diet, genetics, or pharmacological interventions to provide critical insights into the pathophysiology of the condition (Wong *et al.*, 2016). Dietary models of sugar consumption in metabolic syndrome research include carbohydrate-enriched food, such as high-fructose diets and high-sucrose diets (Oron-Herman *et al.*, 2008). Some dietary models combine various carbohydrates with high-fat diets to better simulate real-world dietary patterns (Wong *et al.*, 2016). Similarly, the addition of fructose to drinking water is a well-established model for the induction of metabolic syndrome (MetS) in rodents (Mamikutty *et al.*, 2014).

Additionally, genetically modified experimental animal models examine the pathogenesis of MetS resulting from genetic mutations affecting leptin and leptin receptor function. These models

include leptin-deficient (ob/ob) mice, leptin receptor-deficient (db/db) mice, Zucker fatty (ZF) rats, Zucker diabetic fatty (ZDF) rats, DahlS.Z-Lepr fa/Lepr fa (DS/obese) rats, Goto-Kakizaki (GK) rats, obese spontaneous hypertensive rats (Koletsy rats), and POUND mice (Wong *et al.*, 2016). Pharmacologically induced models of MetS include those triggered by glucocorticoid administration and antipsychotic medications (Wu *et al.*, 2014). These models provide valuable data on the metabolic disruptions associated with excessive sugar consumption and their role in the development of MetS.

2.5 Justification of model used

Different animal models used to study alcohol consumption each have unique advantages and limitations (Becker and Lopez, 2024). For instance, the forced consumption model does not accurately reflect human drinking behaviour. Human alcohol consumption is typically social and often aimed at reducing stress (Franzen *et al.*, 2018). Conversely, oral gavage introduces physiological stress in animal models by damaging the oesophagus of rodents (Arantes-Rodrigues *et al.*, 2012), thus misrepresenting the human idea of voluntary alcohol consumption. A previous study reported that oral gavage in mice raises mean arterial blood pressure and heart rate for up to five hours post-dosing and increases faecal corticosterone metabolites, which suggests systemic stress induction (Walker *et al.*, 2012).

In contrast, administering interventions in gelatine reduces the stress of oral gavage (Kamerman, Modisa and Mphahlele, 2004) and better mimics voluntary consumption in humans. Gelatine's capacity to create a thermo-reversible gel makes it an excellent option for drug administration (Foux and Zilberman, 2015). Furthermore, adolescent consumption of Jello shots, a cocktail made by combining alcohol with sweetened gelatine, is on the rise (Siegel *et al.*, 2016). Gelatine "shots" offer a simpler way to administer alcohol in a laboratory setting, effectively concealing its bitter taste (Ralevski *et al.*, 2006). Therefore, this study used gelatine to deliver sugar and alcohol to Sprague Dawley rats, a widely used strain for MetS research (Wong *et al.*, 2016).

2.6 Synergistic effects of alcohol and sugar on metabolic disorders

Excessive consumption of fructose and alcohol has detrimental effects on body function, promotes insulin resistance and inflammation, and increases the risk of cardiometabolic disorders. Alcohol

negatively impacts all body systems, regardless of the amount consumed (Musto, Brown and Lucey, 2025). In preclinical studies, assessing organ structure and function is a crucial step in evaluating pharmaceutical interventions (Sellers et al., 2007). Key organs affected by excessive sugar and alcohol intake include the brain, heart, liver, kidney, bones, nerves, pancreas, and gastrointestinal tract (GIT) (Musto, Brown and Lucey, 2025). This study explores the combined effects of alcohol and sugar intake on overall metabolism, liver function, and kidney health.

2.7 The role of sugar and alcohol in the pathophysiology of liver diseases

High alcohol consumption (Subramaniyan *et al.*, 2021) and excessive sugar (DiStefano and Shaibi, 2021) intake are major contributors to liver disease. Liver disease ranks as the 11th leading cause of death globally (Devarbhavi *et al.*, 2023) primarily due to the liver's constant exposure to circulating toxins and pathogens, which can disrupt its functional architecture (Acharya *et al.*, 2021). As the primary site for alcohol and sugar metabolism (Nutfillovevich, 2024), the liver becomes overburdened when exposed to excessive intake of these substances, leading to the development of common liver diseases such as alcoholic liver disease (ALD) and non-alcoholic liver disease (NAFLD) (Sharma, Suk and Kim, 2021). The addition of sweeteners like fructose to alcoholic beverages further exacerbates the metabolic damage caused by alcohol consumption. Thus, the combined impact of excessive alcohol and sugar intake on liver health and metabolic disorders warrants further investigation.

Alcohol (Åberg *et al.*, 2023)-and sugar intake (Li *et al.*, 2024) significantly contribute to the onset and progression of liver diseases. Alcohol consumption is directly linked to ALD, which includes conditions ranging from fatty liver disease to cirrhosis (Åberg *et al.*, 2024). Similarly, high sugar intake, particularly from fructose and sucrose, is associated with metabolic dysfunction-associated steatotic liver disease (MASLD) (Li *et al.*, 2024). Research indicates that the excessive intake of both alcohol (Seitz, Moreira and Neuman, 2023) and fructose (Faenza *et al.*, 2024) leads to liver damage through multiple metabolic pathways. Chronic alcohol consumption disrupts lipid metabolism, alters amino acid pathways, and increases oxidative stress, all of which contribute to liver injury and disease progression (Seitz, Moreira and Neuman, 2023). Likewise, fructose metabolism promotes fat accumulation in the liver, exacerbating oxidative stress and inflammation, which further accelerates liver damage and disease (Baharuddin, 2024).

2.7.1 Liver diseases

Liver disease encompasses a range of conditions, diseases, and infections that affect hepatic cells, tissues, structures, or functions (Bhardwaj *et al.*, 2011). These conditions impact the liver's ability to detoxify substances, synthesise proteins, and produce compounds essential for digestion and other vital functions (Bhardwaj *et al.*, 2011). Approximately two million people die from liver disease each year (Devarbhavi *et al.*, 2023), with common conditions including hepatitis, hepatic steatosis, liver fibrosis, cirrhosis, hepatocellular carcinoma, and drug-induced liver toxicity (Akçakaya, 2024).

Hepatitis involves liver necrosis and inflammation caused by viral infections (Llerena *et al.*, 2016), alcohol consumption (Hosseini, Shor and Szabo, 2019), or exposure to toxins (Nithiyanandam and Evan Prince, 2021). Hepatic steatosis, characterised by excessive lipid accumulation in liver cells, occurs when fat content exceeds 5% of liver weight, encompassing both alcoholic and non-alcoholic fatty liver diseases (Israelsen *et al.*, 2024). Hepatic fibrosis results from excessive extracellular matrix (ECM) protein deposition, primarily cross-linked collagens type I and III, replacing damaged normal tissue (Friedman, 2003). Cirrhosis represents the final stage of progressive liver fibrosis, where extensive scarring distorts liver architecture, potentially progressing to liver failure or hepatocellular carcinoma (Sapanlou *et al.*, 2020).

2.7.2 Liver structure

The liver is a complex organ crucial for metabolic processes (Kanel, 2015). It is included among the largest organs in the human body and is in the right upper quadrant of the abdomen. Anatomically, it consists of two major lobes (right and left) and two accessory lobes (caudate and quadrate) (Kanel, 2015). The liver receives nutrient-rich blood from the portal vein and oxygenated blood from the hepatic artery. Venous drainage occurs through the hepatic veins (right, middle, and left), which empty into the inferior vena cava (Mahadevan, 2020).

Lymphatic drainage originates in the hepatic sinusoids within the space of Disse, connecting to deep portal and sub-lobular lymphatics (Ohtani and Ohtani, 2008). The deep portal lymphatics merge at the liver hilum and drain into peripancreatic and paraaortic lymph nodes, ultimately linking with the cisterna chyli and thoracic duct (Smith, Woodside and Abizaid, 2022). The

sublobular lymphatics follow the hepatic veins to the inferior vena cava, while capsular lymphatics drain into the mediastinum or the liver hilum (Smith, Woodside and Abizaid, 2022). Microscopically, the liver comprises functional units called hepatic lobules, consisting of hepatocytes arranged in plates. Small bile canaliculi transport bile from hepatocytes to the bile ducts. Other liver cells found in the sinusoid include Kupffer cells, which function as macrophages and play a vital role in the body's immune system (McCuskey, 2024).

2.7.3 Liver functions

The liver's structure directly supports its numerous functions, including bile production, drug metabolism, and nutrient processing (Sumadewi, 2023). It is also responsible for bilirubin metabolism, protein synthesis, blood storage, metabolic detoxification, thermogenesis and the storage of essential vitamins and minerals (Ozougwu, 2017).

Bile, a yellowish-green alkaline fluid containing bile salts, cholesterol, bilirubin, electrolytes, and water, is secreted by hepatocytes into bile canaliculi (Ozougwu, 2017). Bile salts facilitate fat emulsification and absorption in the intestine (Maldonado-Valderrama *et al.*, 2011). Bilirubin, derived from the breakdown of aged red blood cells, undergoes metabolism in the liver (Wang, Chowdhury and Chowdhury, 2006). When haemoglobin from old red blood cells is broken down into haem and globin, the globin component is further degraded into amino acids for protein synthesis, while the haem portion is converted into biliverdin through enzymatic removal of iron (Ozougwu, 2017). Biliverdin is then reduced to bilirubin by enzymes within the mononuclear phagocytic system and released into plasma (Ozougwu, 2017).

Furthermore, the liver plays a central role in metabolic regulation, including carbohydrate, protein, and lipid metabolism (Mitra and Metcalf, 2012). Carbohydrate metabolism encompasses glycogenesis, glycolysis, gluconeogenesis, and glycogenolysis, which collectively maintain glucose homeostasis (Nogueira-Ferreira, Oliveira and Ferreira, 2024). Protein metabolism involves synthesising essential proteins, regulating amino acid levels, and detoxifying harmful substances. The liver also conducts metabolic detoxification, transforming xenobiotics and endogenous metabolites into less toxic forms for excretion (Shah and Vella, 2016). Additionally,

it serves as a storage site for minerals such as iron and copper, as well as vitamins A, D, E, K, and B12. The liver's functional anatomy makes it particularly susceptible to disease.

2.7.4 Common causes of liver diseases

Liver diseases can result from various factors, including microbial infections, obesity, genetic predisposition, and alcohol consumption (Akçakaya, 2024). Viral hepatitis remains a leading cause, with hepatitis viruses A, B, C, D, and E capable of inducing both acute and chronic liver conditions (Quirino *et al.*, 2024). Obesity is a significant risk factor for non-alcoholic fatty liver disease (NAFLD), as it promotes fat accumulation in the liver, which can progress to steatohepatitis and cirrhosis (An *et al.*, 2024). Genetic factors also play a crucial role, with mutations in genes such as Patatin-like Phospholipase Domain-Containing Protein 3 (PNPLA3), Transmembrane 6 Superfamily Member 2 (TM6SF2), and Membrane Bound O-Acyltransferase Domain-Containing 7 (MBOAT7) being linked to the development of liver diseases (Tkachuk *et al.*, 2024). While alcohol consumption has long been recognised as a major contributor to liver steatosis, cases of liver steatosis have also been reported in individuals who do not consume alcohol (Israelsen *et al.*, 2024).

2.7.5 Pathophysiological mechanisms of liver diseases

The pathophysiology of liver diseases varies depending on their underlying cause. Liver fibrosis develops through chronic activation of wound-healing responses, oxidative stress-related molecular pathways, and disruptions in epithelial-mesenchymal interactions (Pinzani, 2015). In alcoholic liver disease (ALD), hepatic enzymes such as alcohol dehydrogenase (ADH), CYP2E1, and catalase metabolise alcohol into acetaldehyde, generating reactive oxygen species (ROS) and increasing oxidative stress in the liver (Gramenzi *et al.*, 2006). NAFLD, on the other hand, results from a complex interplay of genetic, metabolic, and environmental factors that contribute to insulin resistance and excessive fat accumulation in hepatocytes (Marchisello *et al.*, 2019). This accumulation triggers inflammation, immune cell infiltration, and cytokine secretion, leading to cell damage and fibrosis (Heyens *et al.*, 2021). At the cellular level, hepatocyte injury and inflammation activate quiescent hepatic stellate cells (HSCs), which differentiate into collagen-producing myofibroblasts. This transformation promotes fibrosis by stimulating the proliferation and activation of HSC progenitors (Heyens *et al.*, 2021).

2.7.6 Clinical manifestations of liver diseases

Liver diseases present with a wide range of clinical symptoms, which can be classified into hepatic (Meza *et al.*, 2022), dermatological (Jiménez, 2024), infectious, and haematological categories (Shan, Zhao and Jia, 2022). Hepatic manifestations often include hepatomegaly, splenomegaly (Meza *et al.*, 2022), and hepatic encephalopathy (HE) (Dellatore *et al.*, 2020). Haematological complications may involve hyperbilirubinemia and portal hypertension (Shan, Zhao and Jia, 2022). Dermatological signs of liver disease include Terry's nails, characterised by leukonychia with a distal pink band, which is commonly associated with chronic liver disease (Jiménez, 2024). Pruritus, frequently linked to cholestatic liver conditions, is often managed with bile acid chelators (Thébaud, Debray and Gonzales, 2018). Additional dermatological features, such as palmar erythema and spider angiomas (arachnoid nevi) are vascular markers commonly observed in chronic liver disease, particularly in cases related to alcohol consumption (Jiménez, 2024). While these clinical indicators are valuable for diagnosis, some patients may present with atypical or no symptoms, complicating early detection and delaying treatment (Ghulam *et al.*, 2022).

2.7.7 Diagnosis of liver diseases

Diagnostic markers for liver diseases play a crucial role in early detection, monitoring, and treatment evaluation (Heyens *et al.*, 2021). While liver biopsy is invasive, costly, and time-consuming, it remains the gold standard for diagnosis (Heyens *et al.*, 2021). Pathological diagnoses are based on the assessment of steatosis severity, inflammation, and cellular ballooning (Yip *et al.*, 2023).

Non-invasive diagnostic methods rely on serological and biochemical markers, each serving specific functions in identifying conditions such as liver fibrosis, non-alcoholic fatty liver disease (NAFLD), and other liver-related disorders. Commonly used serological markers, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are biochemical indicators of hepatocyte inflammatory damage (Piazzolla and Mangia, 2020). However, ALT and AST levels alone are not reliable for determining active inflammation in NAFLD (Piazzolla and Mangia, 2020). Diagnostic biomarkers, such as proinflammatory cytokines, show potential in identifying patients with non-alcoholic steatohepatitis (NASH) (Mounika *et al.*, 2024).

Furthermore, total bilirubin serves as a key indicator of liver health, with elevated levels often pointing to liver dysfunction or bile duct obstruction (Guerra Ruiz *et al.*, 2021). Alpha-fetoprotein (AFP) is widely recognised as a marker for hepatocellular carcinoma, a common form of liver cancer (Force *et al.*, 2022). Additionally, hepatic markers such as hyaluronic acid (HA), laminin (LN), type IV collagen (CIV), and procollagen III N-terminal peptide (PIIINP) are linked to liver fibrosis and inflammation (Ma *et al.*, 2021). Meanwhile, emerging biomarkers like high mobility group box 1 (HMGB1), cytokeratin 18 (K18), glutathione-S-transferase- α (GST- α), glutamate dehydrogenase (GLDH), malate dehydrogenase (MDH), and various microRNAs (miRNAs) are being explored for their potential in diagnosing and monitoring liver diseases (Tamber *et al.*, 2023). Newly introduced diagnostic and monitoring tools for liver diseases include ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI), particularly for evaluating hepatic steatosis and fibrosis (Vernuccio *et al.*, 2021).

2.7.8 Management and treatment of liver diseases

The management of liver diseases involves various therapeutic approaches with differing levels of efficacy (Zahmatkesh *et al.*, 2023). Management of liver diseases through lifestyle modifications involves adopting a healthier diet and increasing physical activity (Zeng, Fan and Francque, 2024). Dietary changes include consuming a low-calorie diet while avoiding unhealthy foods high in sugar and alcohol. Regular exercise also plays a crucial role in improving liver health (Zeng, Fan and Francque, 2024).

In addition to lifestyle adjustments, pharmacological interventions may be necessary, including anti-obesity, anti-diabetic, antilipidemic, and antihypertensive medications (Negi *et al.*, 2022). For NAFLD, treatment options include vitamin E supplementation and medications commonly used to manage type 2 diabetes (T2DM) and obesity, which may help improve liver health (Heyens *et al.*, 2021). The most effective treatment for end-stage liver disease remains orthotopic liver transplantation (OLT) (Zahmatkesh *et al.*, 2023). However, its application is limited by donor availability and the subjection to lifelong immunosuppression (Zahmatkesh *et al.*, 2023).

2.8 The role of sugar and alcohol in the pathophysiology of kidney diseases

2.8.1 Definition of kidney diseases

Globally, there is an increase in the number of persons living with kidney diseases, including acute kidney injury (AKI), acute kidney disease (AKD), chronic kidney disease (CKD) and renal replacement therapy (RRT), with an estimate of over 850 million individuals affected (Jager *et al.*, 2019). “Kidney disease is defined as a heterogeneous group of disorders that affects kidney structure and function” (Levey, Levin and Kellum, 2013) and is the 12th leading cause of death worldwide, contributing significantly to the global burden of non-communicable diseases (Cockwell and Fisher, 2020). It is predicted to become the 5th leading cause of years of life lost by the year 2040 (Foreman *et al.*, 2018). Abnormalities in renal structure or function that have health implications are classified as follows: AKI when present for less than 7 days, AKD when lasting less than 3 months, and CKD when present for more than 3 months (Lameire *et al.*, 2021). Severe complications from these kidney diseases can lead to the initiation of RRT (X. Li *et al.*, 2021) which includes dialysis therapies and renal transplantation (Fidan and Ağırbaş, 2023).

2.8.2 Prevalence of kidney diseases

Kidney disease is strongly associated with metabolic disorders such as obesity, hypertension, and diabetes (Zhang and Lerman, 2017). According to the Global Burden of Disease Study, over 336 million men and 417 million women worldwide have some form of renal dysfunction (Tomlinson and Clase, 2019). However, the number of individuals requiring dialysis or RRT differs between sexes, with approximately 1.3 million women and 0.3 million men undergoing RRT compared to 1.7 million men and 0.4 million women undergoing dialysis (Tomlinson and Clase, 2019). This disparity highlights that while the prevalence of CKD is higher in women, the progression to end-stage kidney disease (ESKD), as evidenced by the need for dialysis, is more common in men (García *et al.*, 2022). The higher prevalence of kidney disease in women may be attributed to their greater susceptibility to risk factors such as obesity (WHO, 2021). Conversely, men are at greater risk of CKD progression to ESKD (García *et al.*, 2022) due to their increased exposure to kidney injuries and other risk factors like smoking and alcohol consumption (Kazancioğlu, 2013). This underscores the importance of considering both sexes in preclinical and clinical research (Steiger *et al.*, 2024). Focusing research solely on one sex and then applying those findings to the other sex

can lead to negative outcomes, such as diminished effectiveness or harmful side effects (Loeffler and Ziller, 2023).

While kidney disease is more prevalent in the adult population than in the paediatric population (Kovesdy, 2022), there has been an increase in the age-specific incidence rate of early-onset kidney diseases (Sun *et al.*, 2024). The occurrence of kidney disease in children and adolescents significantly impacts their health-related quality of life (Ruidiaz-Gómez and Higuaita-Gutiérrez, 2021). Risk factors for renal disorders in children include hypertension, anaemia (Masalskienė *et al.*, 2021), genetic predispositions, perinatal complications, childhood kidney diseases, chronic conditions, lifestyle factors (e.g. diet) (Stern-Zimmer *et al.*, 2021), chemicals and environmental pollutants (Tsai *et al.*, 2021). In recent years, environmental pollutant microplastics have emerged as a growing health concern (Tsai *et al.*, 2021). Exposure to these contaminants during childhood and adolescence may increase the risk of developing kidney disease in adulthood.

2.8.3 Kidney structure

In humans and rats, the kidneys are bean-shaped organs located on the posterior abdominal wall, on either side of the lumbar spine (O’g’li *et al.*, 2023). When sectioned vertically, they reveal two distinct regions: the renal cortex and the renal medulla (Tanner, 2009). The kidneys consist primarily of nephrons—the functional units responsible for filtration—along with an extensive vascular network that supports their function (El-Sabbahy and Fikry, 2024).

A nephron comprises a glomerulus and a series of tubules, including the proximal tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct (Pollak *et al.*, 2014). The glomerulus, a network of capillaries, serves as the primary filtration site, while the tubules facilitate the reabsorption of water and solutes, leading to urine formation (Pollak *et al.*, 2014). The kidneys receive blood through the renal arteries, which branch into smaller arterioles that supply the glomeruli. This vital vascular network is essential for renal function and blood pressure regulation (Tanner, 2009).

2.8.4 Kidney functions

The kidneys' intricate structure supports their vital physiological functions, including filtration, fluid balance, and hormonal regulation (O'g'li *et al.*, 2023). Their primary role is urine formation, which occurs through three key processes: glomerular filtration, tubular reabsorption, and tubular secretion (Tanner, 2009).

Glomerular filtration is facilitated by the glomerulus, the glomerular basement membrane, and specialised visceral epithelial cells, which form slit diaphragms between interdigitating foot processes (Pollak *et al.*, 2014). This process enables the continuous filtration of plasma (Pollak *et al.*, 2014). Tubular reabsorption allows essential substances such as ions, water, and metabolites to be transported from the tubular fluid back into the bloodstream (Tanner, 2009). On the other hand, tubular secretion involves the active transport of substances like hydrogen ions and ammonia from tubular cells into the urine (Tanner, 2009).

Beyond urine production, the kidneys play a crucial role in homeostasis by regulating water and mineral balance, controlling arterial pressure, and maintaining electrolyte and acid-base equilibrium (John and Pasha, 2024). Additionally, they have essential endocrine functions, including the production of erythropoietin, renin, and prostaglandins, which contribute to red blood cell production, blood pressure regulation, and inflammatory response modulation (O'g'li *et al.*, 2023).

The kidneys also play a role in metabolism, particularly in gluconeogenesis. Renal gluconeogenesis accounts for about 40% of systemic glucose production, synthesising glucose from non-carbohydrate precursors like lactate, pyruvate, glycerol, and glutamine (Gerich *et al.*, 2001). This process is regulated by four key enzymes: pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBP1), and glucose-6-phosphatase (G6Pase) (Dalga, Verissimo and De Seigneux, 2023). Pyruvate is first converted to oxaloacetate by PC, then PEPCK decarboxylates and phosphorylates it into phosphoenolpyruvate (Dalga, Verissimo and De Seigneux, 2023). FBP1 hydrolyses fructose-1,6-bisphosphate to fructose-6-phosphate, and G6Pase dephosphorylates glucose-6-phosphate to release free glucose (Dalga, Verissimo and De Seigneux, 2023). Energy for these processes is derived from glycolysis

and fatty acid oxidation (FAO), with proximal tubule cells actively contributing to glucose homeostasis (Mather and Pollock, 2011).

2.8.5 Causes of kidney diseases

The pathogenesis of kidney diseases can stem from either infectious or non-infectious aetiologies. Infectious kidney diseases are commonly triggered by the invasion of viral, bacterial, fungal, or other microbial pathogens, as well as by the activation of immune responses (Prasad and Patel, 2018). Non-infectious kidney diseases may have hereditary origins, such as inborn errors of metabolism (Edvardsson *et al.*, 2013), or non-hereditary causes, including exposure to toxins (Chi *et al.*, 2021), pharmacological agents (Loh, Hons and Cohen, 2009), metabolic disorders like diabetes (Pecoits-Filho *et al.*, 2016), and dietary factors (Kramer, 2019).

2.8.6 Pathophysiological mechanisms of kidney diseases

The pathophysiology of kidney diseases involves a complex interplay of factors, including the progression from acute kidney injury (AKI) to chronic kidney disease (CKD), the development of diabetic kidney disease (DKD), and the impact of inflammation, oxidative stress, and hormonal imbalances (Yeh *et al.*, 2024).

AKI can progress to CKD due to mechanisms such as renal hypoperfusion, nephrotoxicity, and immune responses, which lead to maladaptive repair processes and fibrosis (Yeh *et al.*, 2024). DKD arises from chronic hyperglycaemia, triggering metabolic, hemodynamic, inflammatory, and fibrotic pathways that contribute to renal damage (Sinha and Nicholas, 2023). Inflammatory cytokines and reactive oxygen species (ROS) play a significant role in DKD progression (Sinha and Nicholas, 2023). Additionally, the activation of the renin-angiotensin-aldosterone system (RAAS) exacerbates oxidative stress and fibrosis, further accelerating kidney deterioration (Sinha and Nicholas, 2023).

2.8.7 Clinical manifestations of kidney diseases

The clinical presentation of infection-related kidney diseases varies depending on the nature of the infection, with manifestations ranging from acute to chronic (Prasad and Patel, 2018). Kidney injury may result from direct pathogen invasion or indirect immune-mediated mechanisms, leading

to conditions such as post-infectious or infection-related glomerulonephritis (Prasad and Patel, 2018).

AKI can impact multiple body systems, with renal, respiratory and cardiovascular failure being among the most common clinical manifestations (Kellum *et al.*, 2021). Key indicators of kidney disease include albuminuria and a reduced glomerular filtration rate (GFR) (Afkarian *et al.*, 2016). Other common manifestations include nephromegaly, glomerular hyperfiltration, and, in advanced cases, kidney failure (Adeva-Andany *et al.*, 2023). Chronic kidney disease (CKD) is also associated with dermatological symptoms such as xerosis (dry skin), pruritus (itching), and eczema (Dwivedi *et al.*, 2021).

Furthermore, kidney disease disrupts essential physiological processes, leading to various complications. One major consequence is azotaemia, characterised by the accumulation of nitrogenous waste products such as creatinine and urea in the blood due to impaired filtration (Kushnirenko *et al.*, 2023). Additionally, reduced erythropoietin production by the kidneys contributes to anaemia, resulting in fatigue and decreased oxygen delivery to tissues (Portolés *et al.*, 2021). The loss of calcium homeostasis further leads to bone defects, as impaired renal function affects vitamin D activation and calcium-phosphorus balance, increasing the risk of bone demineralisation and fractures (Rodrigues *et al.*, 2021).

2.8.8 Diagnosis of kidney diseases

Clinical diagnostic tests for kidney disease include estimating the glomerular filtration rate (GFR) using serum markers of filtration, such as creatinine and cystatin C, or urine biomarkers like albumin and protein (Kovesdy, 2022). Creatinine levels often become detectable only after significant kidney damage has occurred, complicating the early diagnosis of AKI (Shang & Wang, 2017). Elevated urea levels are associated with kidney disease (Laville *et al.*, 2023), and a high urea-to-creatinine ratio indicates a poorer outcome in CKD patients (Brookes and Power, 2022).

Consequently, biomarkers such as kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) are increasingly valuable for the early detection of AKI and predicting recovery or progression to CKD (Kashani and Kellum, 2015). Advancements in renal

imaging have significantly improved the assessment of kidney structure, morphology, perfusion, function, metabolism, and oxygenation (Caroli, Remuzzi and Lerman, 2021). Key imaging modalities include ultrasonography, computed tomography (CT), positron emission tomography (PET), renal scintigraphy, and multiparametric magnetic resonance imaging (MRI) (Caroli, Remuzzi and Lerman, 2021). Additional insights into the severity of kidney pathology, particularly regarding interstitial, tubular, and glomerular alterations, can be obtained through renal biopsy (Quinn *et al.*, 2021), which remains the gold standard for diagnosing most renal diseases (Feng and Liu, 2023).

2.8.9 Effect of high sugar intake on the kidney

An unhealthy diet is a significant risk factor for the development of kidney disease (Fotheringham *et al.*, 2022). Unhealthy diets include high sugar (Karalius and Shoham, 2013) and alcohol consumption (Das *et al.*, 2008). Individuals with metabolic syndrome face an elevated risk of kidney disease, which is further amplified by high sugar intake (Andres-Hernando *et al.*, 2020).

Adolescents and children tend to consume more added sugars than the older population (Welsh *et al.*, 2011), increasing their risk of developing kidney-related conditions. A commonly consumed sugar is fructose. Fructose-induced renal alterations occur due to reduced mitochondrial function and adenosine triphosphate (ATP) activity in the kidneys (Johnson *et al.*, 2020). This contributes to the development of insulin resistance, elevated blood pressure, and other components of metabolic syndrome, increasing the likelihood of renal abnormalities (Andres-Hernando *et al.*, 2020). Diets rich in fructose exert harmful effects, often characterised by local inflammatory responses in the renal tubules (Flisiński *et al.*, 2022) and increased renal histopathological scores (Elsisy, El-Magd and Abdelkarim, 2021). These fructose-induced kidney alterations are caused by the increased burden on renal clearance of advanced glycation end products (AGEs), which form when sugar interacts with lipids and proteins (Fotheringham *et al.*, 2022).

2.8.10 Alcohol impact on the kidneys

The impact of alcohol on renal function remains controversial, with studies reporting conflicting outcomes. Some show detrimental effects, others suggest beneficial effects, and others report no significant impact. For example, one study highlighted the potential benefits of alcohol

consumption, finding that moderate alcohol intake was associated with a slower decline in the estimated glomerular filtration rate (eGFR) among men, non-smokers, and individuals under 60 years of age (Lee, Cho and Kim, 2021). Conversely, another study identified a detrimental relationship between alcohol use disorder and CKD in individuals under 50 years old (Pan *et al.*, 2018). In contrast, a separate study reported that alcohol consumption was not a significant risk factor for CKD in the older population, particularly in males (Yuan *et al.*, 2021). These contradictory findings may be attributed to undefined drinking patterns and the presence of bioactive compounds, such as nephroprotective polyphenols, in certain types of alcoholic beverages, like wine (Fan *et al.*, 2019).

2.8.11 Impact of SAC on the kidneys

Although there are conflicting reports on the effects of alcohol on kidney damage (Pan *et al.*, 2018; Yuan *et al.*, 2021), the detrimental effects of high sugar consumption on renal structure and function are well established in literature (Andres-Hernando *et al.*, 2023; Flisiński *et al.*, 2022). Adolescents worldwide are consuming large quantities of sugar-sweetened beverages (Ooi *et al.*, 2021), including alcoholic drinks. This high intake of alcohol and sugar simultaneously, can potentially predispose them to the development of kidney diseases. The combined burden of high sugar and high alcohol on kidney function and structure as we have in SAC is yet to be fully understood, therefore this study intends to fill that gap.

2.8.12 Management of renal disease (acute, chronic, conventional, non-conventional)

Kidney diseases are currently managed with conventional medications, which are costly (Essue *et al.*, 2013) and have diverse side effects (Breyer and Susztak, 2016). Pharmacological interventions, such as renin–angiotensin–aldosterone system (RAAS) modulators and sodium-glucose transporter 2 (SGLT2) inhibitors, help preserve kidney function by lowering intraglomerular pressure while also aiding in blood pressure and glucose regulation (Kalantar-Zadeh *et al.*, 2021). Additionally, non-steroidal mineralocorticoid receptor antagonists protect the kidneys through anti-inflammatory and antifibrotic effects (Kalantar-Zadeh *et al.*, 2021). End-stage kidney disease may require renal replacement therapy, such as dialysis or a kidney transplant (Fidan & Ağırbaş, 2023).

There is a growing call for effective strategies to lower the development of renal disorders and slow their progression (Kovesdy, 2022). Nonpharmacological interventions, such as diet and lifestyle modifications, are also proposed for managing kidney diseases (Kalantar-Zadeh et al., 2021). Dietary modifications include consuming a diet low in protein and salt, while lifestyle changes involve regular physical exercise and weight loss (Kalantar-Zadeh *et al.*, 2021). These non-conventional strategies include the use of phytochemicals, such as polyphenolic compounds (Ashkar, Bhullar and Wu, 2022). Polyphenols have antioxidant properties that can protect the kidney from damage due to oxidative stress (Das and Vasudevan, 2008) and an example is TA.

2.9 Prevention of metabolic syndrome

Metabolic syndrome (MetS) has become a growing public health concern in both paediatric and adult populations (Assadi, Mazaheri and Kelishadi, 2022); due to its increasing prevalence and the substantial healthcare costs associated with its management (Shukla *et al.*, 2018). The risk of developing metabolic disorders can be significantly minimised by adopting healthy lifestyle habits from an early age, including a balanced diet, regular physical activity, and effective weight management (Assadi, Mazaheri and Kelishadi, 2022). Additionally, preventive strategies should focus on increasing public awareness of the various risk factors linked to MetS and its components (Musa, Elyamani and Dergaa, 2022).

2.10 Management and treatment of metabolic syndrome

Current pharmacological treatments for MetS primarily target individual components such as hypertension, hyperglycaemia, and hypertriglyceridemia (Jha *et al.*, 2023). However, the high cost and potential side effects of these medications have led to a growing interest in alternative management strategies (Abdulghani and Al-Fayyadh, 2024).

Dietary interventions have emerged as a promising approach, with researchers exploring the role of gut microbiota in metabolic regulation using probiotics, prebiotics, and synbiotics (Qureshi, Dar and Rather, 2024). Natural products, including phytochemicals, medicinal herbs, and dietary supplements, are increasingly recommended due to their affordability and reduced side effects (Tabatabaei-Malazy, Lavari and Abdollahi, 2024). Certain medicinal plants, particularly those rich in tannins, contain bioactive compounds that influence metabolic pathways, improve insulin

sensitivity, and mitigate oxidative stress, making them viable alternatives for managing MetS (Ahmed *et al.*, 2022).

2.11 Tannic acid

2.11.1 Definition of tannins

Tannins are naturally occurring polyphenolic compounds derived from plants, known for their diverse biological activities and potential health benefits (Kadigi, Muzzo and Schreiber, 2024). Based on their chemical structure and properties, tannins are categorised into two main types: hydrolysable and condensed tannins, each influencing their biomedical applications differently (Ahmed *et al.*, 2022).

Condensed tannins, also known as proanthocyanidins, are polymerised flavonoid chains (Schofield, Mbugua and Pell, 2001), while hydrolysable tannins consist of glucose-based polymers with various catechin derivatives (Sarwar and Ahmed, 2022). Hydrolysable tannins are further classified into two subgroups: gallotannins (GTs), which contain galloyl groups attached to a glycosyl unit, and ellagitannins (ETs), which also include hexahydroxy diphenic acid (HHDP) groups (Sarwar and Ahmed, 2022).

TA, a well-known hydrolysable gallotannin, is a natural polyphenol that has gained attention for its extensive applications in medicine and the biomedical industry due to its antimicrobial, anti-inflammatory, and other therapeutic properties (Baldwin and Booth, 2022; Jafari *et al.*, 2022).

2.11.2 Structure of tannic acid

TA is a complex polyphenolic molecule composed of a central glucose core with multiple galloyl groups derived from gallic acid (Baldwin and Booth, 2022). It is considered a mixture of poly-galloyl glucose molecules and is commonly represented by the empirical formula $C_{76}H_{52}O_{46}$ (Yan *et al.*, 2020). The number of galloyl units in TA ranges from 2 to 12, contributing to its average molecular mass of 1701.19 g/mol (Watrelet *et al.*, 2020). The biomedical significance of TA stems from the phenolic hydroxyl groups present on its galloyl units, which can form hydrogen bonds with amino acids and covalent bonds with metal ions, enhancing its bioactivity and potential therapeutic applications (Yan *et al.*, 2020; Baldwin and Booth, 2022).

2.11.3 Sources of tannic acid

The term "tannic acid" was first introduced by Seguin in the 17th century, derived from the French word *tan*, meaning oak bark, to describe a substance extracted from oak bark (Arapitsas, 2012). TA is widely distributed in various plants and food products and is primarily purified from the gallnuts of specific oak and sumac tree species (Baldwin and Booth, 2022).

In addition to gallnuts, TA can be found in vegetables, grains, fruits, tea, and legumes, as well as in several herbaceous and woody plants (Lu *et al.*, 2020). Recognised for its safety, TA is an FDA-approved food additive (Guo *et al.*, 2021). Beyond its culinary applications, it exhibits significant biological potential in preventing metabolic disorders (Chung *et al.*, 2019). Its therapeutic properties include anti-obesity, anti-diabetic, anti-inflammatory, and antioxidant effects (Türkan, Taslimi and Saltan, 2019). The next section reviews the literature on the biological potential of TA.

2.12 Biological and pharmacological potentials of tannic acid

2.12.1 Anti-obesity property

TA has demonstrated significant anti-obesity potential through various mechanisms. It exhibits a dose-dependent anti-lipogenic effect on 3T3-L1 adipocytes by promoting lipolysis in rats fed a high-fat diet (Kim *et al.*, 2008). Additionally, TA inhibits key digestive enzymes, including α -amylase, α -glucosidase, and pancreatic lipase, which are essential for carbohydrate and lipid metabolism (Ejiofor *et al.*, 2024). TA's interference with these enzymes slows carbohydrate absorption and reduces lipid accumulation in the body (Ejiofor *et al.*, 2024).

Notably, TA has shown greater efficacy in inhibiting α -amylase *in vitro* compared to commercial starch blockers and other polyphenols such as quercetin and rutin (Zhao *et al.*, 2013). Furthermore, it modulates gut microbiota by decreasing the abundance of obesity-associated bacteria like *Firmicutes* and *Bacteroidetes* while promoting the growth of beneficial bacteria such as *Bacteroides* and *Odoribacter* (J. Fang *et al.*, 2022).

2.12.2 Anti-diabetic potential

TA is recognised as a potent antidiabetic compound due to its ability to inhibit metabolic enzymes, including acetylcholine esterase (AChE), α -glucosidase, α -amylase, and butyrylcholinesterase (BChE) (Türkan, Taslimi and Saltan, 2019). Studies have shown that TA exhibits antihyperglycemic effects in streptozotocin-induced diabetic rats (Babby *et al.*, 2014; Esmail, Abo-Youssef and Tohamy, 2019). Moreover, it is utilised in hydrogel formulations to aid in wound healing, particularly in diabetic patients (K. Fang *et al.*, 2022).

2.12.3 Cholesterol-reducing and cardioprotective properties

TA plays a protective role against hypertension and hyperlipidaemia by inhibiting the angiotensin type 1 receptor (AT1R) and low-density lipoprotein receptor (LDLR) (Iyer, Zhang and Thekkumkara, 2017). It mitigates myocardial ischemia injury by downregulating the expression of apoptosis-related proteins and inflammatory cytokines while enhancing antioxidant activity (Hu *et al.*, 2015).

Additionally, TA protects against acute doxorubicin-induced cardiotoxicity by lowering the activity of lactate dehydrogenase, creatine kinase, and creatine kinase isoenzyme, as well as reducing the expression of proinflammatory cytokines (Zhang *et al.*, 2017). These properties make TA a promising natural compound for cardiovascular protection and metabolic health.

2.12.4 Anti-inflammatory potential

TA exhibits significant anti-inflammatory properties, making it a promising candidate for various therapeutic applications (Jing *et al.*, 2022). By crosslinking with proteins, TA suppresses inflammatory pathways associated with metabolic disorders (Jing *et al.*, 2022). Studies have shown that TA treatment enhances the production of anti-inflammatory cytokines such as IL-4 and IL-10, while simultaneously reducing the expression of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α , across different experimental models (Abolarin and Owoyele, 2024). Additionally, TA has been explored as a treatment for rheumatoid arthritis, demonstrating its potential in inflammation management (Wu *et al.*, 2023). Advances in TA-based hydrogels (Ninan *et al.*, 2016) and nanogels (Yeo *et al.*, 2020) have further enhanced its effectiveness as an anti-inflammatory agent.

2.12.5 Antioxidant potential

Beyond its anti-inflammatory effects, TA also possesses strong antioxidant capabilities, primarily through its ability to mitigate reactive oxygen species (ROS) production (Azimullah *et al.*, 2023). Research indicates that TA plays a crucial role in regulating oxidative stress and aiding tissue repair through its free radical scavenging activity (de Veras, da Silva and Cabral Ribeiro, 2021). Moreover, TA reduces oxidative damage by inhibiting myeloperoxidase (MPO), an enzyme responsible for generating reactive oxygen species (Soyocak *et al.*, 2019; de Veras, da Silva and Cabral Ribeiro, 2021). Its antioxidant effectiveness is comparable to standard antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α -tocopherol, and Trolox (Gülçin *et al.*, 2010).

2.12.6 Hepatoprotective property of tannic acid

TA has been proposed as a phytochemical intervention for treating hepatic disorders and metabolic derangements (Das *et al.*, 2022). It can crosslink with proteins in the body and suppress the activation of inflammatory pathways that cause hepatic disorders (Jing *et al.*, 2022). Furthermore, the administration of nanoparticles containing TA and vitamin E prevented liver damage caused by chronic alcohol consumption in mice (Nag *et al.*, 2020). Similarly, TA prevented the development of non-alcoholic fatty liver disease in mice consuming a Western diet by inhibiting the histone acetyltransferase pathway (Chung *et al.*, 2019).

2.12.7 Molecular modulation by tannic acid

TA modulates key metabolic enzymes, such as *CYP2E1* and *SREBP1*, and regulates the expression of inflammatory cytokines, including *IL-10*, *NF- κ B1*, and *TNF- α* . Studies have shown that TA inhibits *CYP2E1* expression in the liver of mice (Krajka-Kuźniak and Baer-Dubowska, 2003), a finding further supported by in vitro experiments (Yao *et al.*, 2008). Additionally, tannins are known to suppress *SREBP1*, a gene involved in fatty acid synthesis (Ren *et al.*, 2023). TA-rich extracts have also been shown to modulate the secretion of inflammatory cytokines, such as *IL-10* and *TNF- α* , in Necrotic Enteritis-infected broilers by inhibiting the NF- κ B signalling pathway (Xu *et al.*, 2023).

2.12.8 Nephroprotective potential

TA has demonstrated protective effects against kidney damage caused by various nephrotoxic agents in experimental studies (Akomolafe, Akinyemi and Anadozie, 2014; Jin *et al.*, 2020; Yesilkent and Ceylan, 2022). For example, research on male Wistar rats with unilateral kidney obstruction has shown that TA prevents renal injury by modulating the renin-angiotensin signalling pathway (Kianfar *et al.*, 2023). Furthermore, TA has been applied in medical technologies, such as coating polyether sulfone haemodialysis membranes, which has proven effective in reducing oxidative stress in patients undergoing haemodialysis (Chen *et al.*, 2022; Wei *et al.*, 2022; Zhang *et al.*, 2022).

At a dose of 50 mg/kg, TA promoted kidney function recovery following renal warm ischemia-reperfusion injury in a rat model (Alechinsky *et al.*, 2020) and protected proximal tubule cells from vascular calcification through paracrine signalling mechanisms in an *in vitro* assay (Boadi, Shin and Bandyopadhyay, 2021). Furthermore, treatment with TA nanospheres has shown promising results in curing acute kidney injury by inhibiting ferroptosis (Gao *et al.*, 2022), a type of programmed cell death driven by iron-dependent lipid peroxidation.

2.12.9 Antiviral activity

TA has demonstrated broad-spectrum antiviral properties, effectively inhibiting various viruses, including influenza A virus (IAV) (Theisen *et al.*, 2014), human papillomavirus (HPV) (Theisen *et al.*, 2014), herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (Szymańska *et al.*, 2018), and norovirus (Zhang *et al.*, 2012). Additionally, studies indicate that TA plays a role in reactivating latent human immunodeficiency virus (HIV) (Chen *et al.*, 2025). During latency, the virus remains dormant, avoiding immune detection due to the absence of active replication.

2.12.10 Antibacterial property

TA has exhibited antibacterial effects against *Clostridioides difficile*, a Gram-positive bacterium known for its recurrence following antibiotic treatment that disrupts gut microbiota (W. Wang *et al.*, 2023). Additionally, TA-based gold nanoparticles have been developed to target bacteria such as *Streptococcus mutans* (Selvaraj *et al.*, 2024), while TA-loaded chitosan nanoparticles have shown effectiveness against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*,

Klebsiella pneumoniae, and *Candida albicans* (Rashidipour *et al.*, 2024). Furthermore, TA-copper nanoparticles have been identified as potent bactericidal agents against *Escherichia coli* and *Staphylococcus aureus* (Liu *et al.*, 2024). The antibacterial action of TA is primarily attributed to its ability to disrupt bacterial cell membrane integrity, leading to microbial cell death (W. Wang *et al.*, 2023).

2.12.11 Antifungal property and antiparasitic effects

In vitro studies have confirmed the antifungal activity of TA against various fungal pathogens, including *Alternaria alternata* (H. Wang *et al.*, 2023), *Penicillium digitatum* (Zhu *et al.*, 2019), *Candida species* (Moreira *et al.*, 2024), *Aspergillus fumigatus* and *Fusarium solani* (Ríos-López *et al.*, 2024).

TA has also demonstrated antiparasitic properties. It has been shown to reduce the viability of *Haemonchus contortus* (Zhong *et al.*, 2014) and *Ichthyophthirius multifiliis* (Alavinia *et al.*, 2019) in *in vitro* experiments. Additionally, in combination with albendazole therapy, TA exhibited a nematocidal effect on *Trichinella spiralis* in mice (Nasreldin *et al.*, 2022). Diets supplemented with TA were found to reduce oocyst shedding, enhance nutrient absorption, and improve intestinal health in broilers infected with *Eimeria maxima* (Choi *et al.*, 2022). Furthermore, TA demonstrated both *in vitro* and *in vivo* antiparasitic activity against *Ichthyophthirius multifiliis* in zebrafish (Alavinia *et al.*, 2018).

2.12.12 Anticancer and antiproliferative properties

TA has demonstrated anticancer and antiproliferative effects across various cancer types. In prostate cancer cells, TA induces apoptosis by downregulating pro-survival genes and upregulating pro-apoptotic genes, leading to increased cancer cell death (Kandir *et al.*, 2024). In colorectal cancer, it triggers endoplasmic reticulum (ER) stress, activating the unfolded protein response (UPR), which promotes pro-apoptotic protein expression while reducing anti-apoptotic factors (Nejad, Karami and Darvish, 2023). Additionally, TA inhibits epithelial-mesenchymal transition (EMT) in colorectal cancer, reversing mesenchymal markers and suppressing cell migration and invasion (Barboura *et al.*, 2022). In non-small cell lung cancer, TA has been shown to reduce cell proliferation and angiogenesis by inhibiting the release of vascular endothelial growth factor (VEGF) (Hatami *et al.*, 2022).

2.12.13 Toxicity and safety of tannic acid

TA is regarded as safe for consumption, as approved by the Food and Drug Administration (FDA) and the European Union (Guo *et al.*, 2021). A daily intake of 1.5 to 2.5 g of tannins, including TA, is considered safe and does not pose significant health risks. However, excessive consumption beyond this range may interfere with dietary iron absorption (Sharma *et al.*, 2021). The lethal dose (LD50) of TA in rats is approximately 2.26 ± 0.083 g/kg of body weight (Boyd, Bereczky and Godi, 1965), further supporting its safety as a food additive.

Considering the abovementioned multitude of biological activities, TA presents potential as a good candidate for use as a prophylactic agent against the development of sweetened alcohol induced metabolic dysfunction. The next chapter presents the materials and methodology used to evaluate the general health performance, metabolic profile, liver, and kidney function of the study's experimental animals.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Ethical approval and study location

The study received ethical clearance (Reference: AREC/2022/11/06C) from the Animal Research Ethics Committee of the University of the Witwatersrand, Johannesburg. All animal experiments were conducted at the Wits Research Animal Facility (WRAF) following the South African National Standards for the care and use of animals. Additionally, the study adhered to the ARRIVE 2.0 guidelines for animal research (Percie du Sert *et al.*, 2020).

3.2 Animal husbandry and housing

The Wits Research Animal Facility (WRAF) provided 64 adolescent Sprague Dawley rats (42 days old). Each rat was housed individually in a Perspex cage with a stainless-steel mesh lid (365 × 207 × 140 mm). The bedding, consisting of wood shavings, was changed twice a week. A 12-hour light-dark cycle was maintained, with lights on from 07:00 to 19:00. The ambient temperature was kept between 24 – 26 °C, and proper room ventilation was ensured. All rats were given commercially sourced rat chow (LabChef, Rodent Breeder, Nutritionalhub (Pty) Ltd, Stellenbosch 7602, South Africa) as feed and water *ad libitum*.

3.3 Study design

All rats in this prospective, intervention animal-based randomised trial were given four days to acclimatise to handling, housing conditions, and the consumption of flavoured gelatine. The rats were randomly assigned to one of four treatment groups (n = 8 males and 8 females per group). Simple randomisation was carried out by an independent researcher who was not involved in the study. Each rat was assigned a unique number, and group allocation was determined through a ballot draw. To ensure blinding, treatment groups were coded, and the researcher remained unaware of group identities throughout the randomisation process.

The sample size was calculated using the formula below (Erdoğan, 2024).

$$n = DF/k + 1$$

where n = number of rats per group, k = no of groups, DF = degree of freedom, N = Total no of animals

$$n = DF/k + 1 = 20/4 + 1 = 5 + 1 = 6 \text{ rats}$$

To adjust for attrition (Serdar *et al.*, 2021),

$$n = 6 + 1.8 = 7.8 \approx 8 \text{ rats per group}$$

$$N = n \times k = 8 \times 4 = 32 \text{ rats per sex (i.e. 64 rats)}$$

The experimental rats were divided into four groups:

- **Group 1 (PG):** Received gelatine (6% w/v and 0.5% w/v fructose), serving as the vehicle for intervention administration.
- **Group 2 (TA):** Received gelatine containing TA (50 mg/kg and 0.5% w/v fructose) to assess the effects of TA alone.
- **Group 3 (SAC):** Received SAC in gelatine (20% w/v fructose-sweetened gelatine + 10% v/v ethanol) to investigate the metabolic effects of SAC.
- **Group 4 (SAC+TA):** Received SAC and TA (20% w/v fructose-sweetened gelatine + 10% v/v ethanol + 50 mg/kg TA) to evaluate whether TA could mitigate metabolic dysfunction induced by SAC.

Fresh preparations of all dietary interventions were administered daily between 11:00 AM and 12:00 PM throughout the 10-week study. Figure 3.1 illustrates the experimental timeline and group allocation.

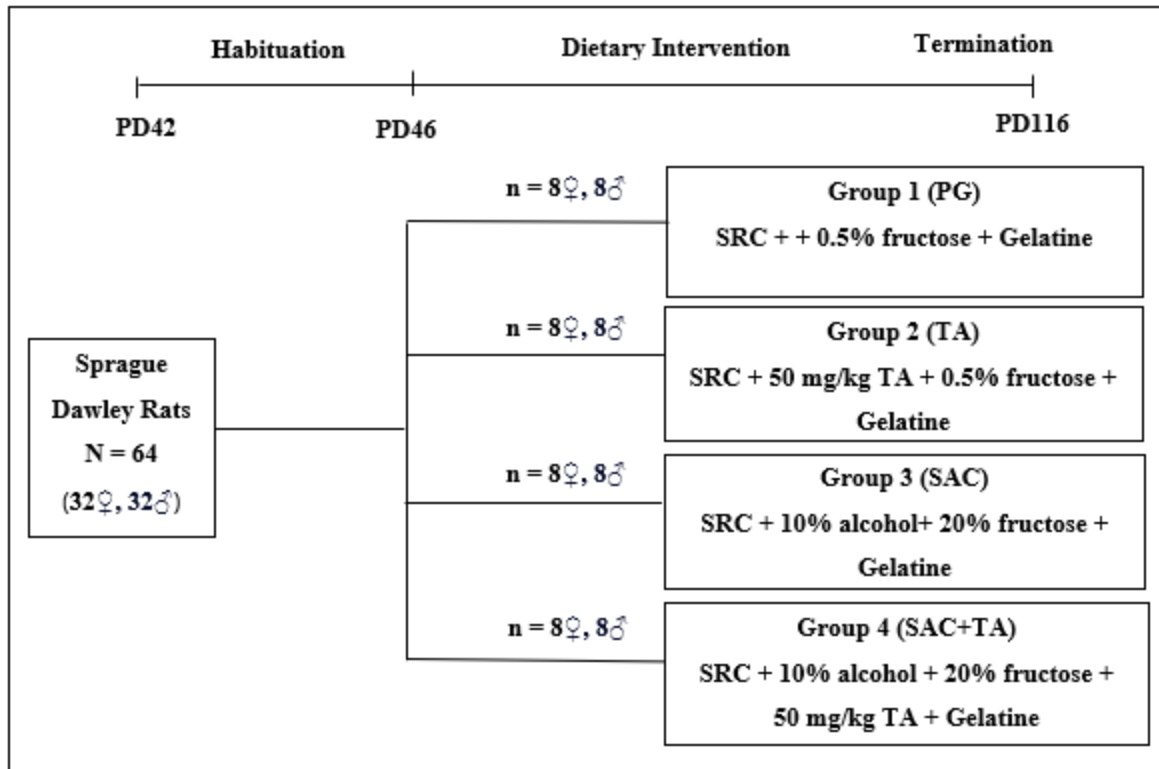


Figure 3.1: An illustration of the description of each experimental group and study timeline.

PD = Postnatal day, ♀ = Female, ♂ = Male, SRC = Standard rat chow, PG = Plain gelatine, TA = Tannic acid, SAC = Sweetened Alcohol

3.4 Dietary interventions

All treatments in the study were administered orally in glass jars using gelatine-based mixtures (Sheridan's Gelatine, Libstar Operations (Pty) Ltd, Dunkeld, South Africa). TA (*CAS No.: 1401-55-4; Sigma Aldrich, Missouri, USA*) was administered at a dose of 50 mg/kg, as per Zhang *et al.* (2017). Fructose (*Nature's Choice, Randvaal, South Africa*) was given at either 0.5% or 20% w/v, based on Wong *et al.* (2016). Ethanol (*99.9% ethanol; CAS No.: 64-17-5; Batch No. 37500; ACE Chemicals, South Africa*) was provided at a concentration of 10% v/v, following Liu *et al.* (2020).

3.4.1 Preparation of gelatine mixtures

- For Groups 1 and 2 (plain gelatine): 0.5 g fructose and 6 g gelatine powder were mixed in 100 ml of warm distilled water. A low concentration of fructose (0.5%) was added to the plain gelatine mixture solely to mask its taste and was not expected to exert any metabolic effects.
- For Groups 3 and 4 (SAC gelatine): 20 g fructose and 6 g gelatine powder were mixed with 10 ml of 99.9% ethanol and 90 ml of distilled water.
- TA at 0.05 mg per 100 ml was added to the respective mixtures for Groups 2 and 4, achieving a final dose of 50 mg/kg body mass.

The gelatine mixtures were refrigerated at 4° C and sealed overnight in glass jars to preserve the ethanol content. Although ethanol retention was not quantified in this study, it was assumed, based on previous findings, that ethanol content in gelatine as a vehicle remains stable, resulting in brain ethanol levels comparable to those achieved with other self-administration methods (Peris *et al.*, 2006).

During the first week of the study, the rats consumed more than 50% of the provided volume, increasing to about 75% by the second week. From the third week until the end of the study, they consistently consumed the entire gelatine. Each rat received a specific volume of gelatine mixture based on its body mass, as shown in Table 3.1:

Table 3.1: The volume of gelatine mixture provided per body mass

Body mass (g)	Volume of gelatine mixture (ml)
50 – 100	10 ml
101 – 150	15 ml
151 – 200	20 ml
201 – 250	25 ml
251 – 300	30 ml
301 – 350	35 ml
351 – 400	40 ml

3.5 Weekly feed intake and body mass measurements

Feed intake for each experimental group was measured weekly by subtracting the remaining feed at the end of the week from the total feed provided at the beginning. The initial body mass of each rat was recorded at the start of the study and then measured twice weekly throughout the experiment. This allowed for the assessment of growth performance for animal welfare monitoring and ensured accurate adjustments to the administered doses of gelatine, TA, and SAC, maintaining consistent dosage levels. At the end of the 10-week treatment period, the terminal body mass of each rat was recorded. All body mass and feed measurements were conducted using a digital weighing balance (*Presica 310M, Precision Instruments, Johannesburg, South Africa*).

3.6 Termination procedure

At the end of the 10-week dietary intervention, the rats were fasted overnight with access to water only. A small blood sample (0.05 ml) was collected from a tail pinprick to measure fasting blood glucose levels using a calibrated glucometer (*Accu-Chek Glucometer*). Additionally, haematocrit and haemoglobin levels were assessed using an HCT meter (*InSight HCT Meter, Woodley Equipment Company Ltd, Bolton, England*). The mean corpuscular haemoglobin concentration (MCHC), a red blood cell index, was determined using the following equation (Zhanyi Zhang *et al.*, 2022).

$$MCHC \text{ (g/dL)} = \text{Haemoglobin (g/dL)} / \text{Haematocrit (\%)} \times 100$$

Following these measurements, the rats were euthanised via an intraperitoneal overdose of sodium pentobarbital (200 mg/kg body mass).

3.6.1 Collection of cardiac blood samples

Blood samples were collected from the heart using 21G needles and 10 ml syringes and transferred into serum separator tubes (coagulant-coated). The samples were then centrifuged (*DLAB, DM0412, USA*) at 3000 rpm for 15 minutes. To analyse the clinical biochemical profile and determine hormone concentrations, the serum was separated and stored in microtubes at -80°C until biochemical analyses were performed.

3.6.2 Visceral and epididymal fat mass determination

A midline incision was made in the abdominal region of each rat to collect visceral adipose tissues and epididymal fat (for male rats). The fat was weighed using an electronic balance to assess adiposity. The relative fat mass was then calculated as the ratio of visceral or epididymal fat to final body weight.

3.7 Metabolic profile

3.7.1 Determination of TG, HDL-C and TG/HDL-C ratio

Serum triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) levels were measured using rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (TG CAT No: E-BC-K261-M; HDL CAT No: E-EL-R0504, *Elabscience Biotechnology Co., Ltd, Wuhan, China*).

The triglyceride (TG) assay employs a colorimetric enzymatic ELISA-based detection principle. TGs are hydrolysed to glycerol, which is enzymatically converted to hydrogen peroxide. In the presence of phenol and 4-aminoantipyrine, peroxidase catalyses the formation of a coloured quinone compound. The assay, performed according to the manufacturer's instructions, has a detection range of 0.14 to 10 mmol/L. Absorbance was measured at 510 nm using a microplate reader (*Labsystems Multiskan Ascent 354, Thermo Fisher Scientific, Waltham, MA, USA*), with the colour intensity directly proportional to TG concentration.

In contrast, the HDL assay utilises a sandwich ELISA method to detect rat HDL, involving a biotinylated detection antibody and an avidin–horseradish peroxidase (HRP) conjugate. The standard curve spans 3.31 to 200 ng/mL, with concentration points at 0, 3.31, 6.25, 12.5, 25, 50, 100, and 200 ng/mL. The assay was conducted according to the kit manual and has a sensitivity of 1.88 ng/mL. Absorbance was measured at 450 nm using a microplate reader.

Additionally, the TG/HDL-C ratio was calculated by dividing the TG value by the HDL-C concentration for each rat (Miralles *et al.*, 2015).

3.7.2 Quantification of insulin concentration and insulin resistance

The concentration of fasting blood insulin was measured using rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (CAT NO: E-EL-R3034, *Elabscience Biotechnology Co., Ltd, Wuhan, China*), following the manufacturer’s instructions. The insulin (INS) kit is based on a sandwich ELISA technique designed to detect Rat INS, using a biotinylated detection antibody in combination with an avidin–horseradish peroxidase (HRP) conjugate. The standard curve spans concentrations from 6.25 to 400 pg/mL, with specific points at 0, 6.25, 12.5, 25, 50, 100, 200, and 400 pg/mL. The assay offers a sensitivity of 3.75 pg/mL, and absorbance was read at 450 nm using a microplate reader (*Labsystems Multiskan Ascent 354, Thermo Fisher Scientific, Waltham, MA, USA*).

The fasting blood glucose concentration determined in section 3.4 and the insulin concentration were then used in the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) formula stated below to evaluate whole-body insulin sensitivity (Aref *et al.*, 2013).

$$HOMA - IR = \frac{\text{Fasting blood insulin } (\mu\text{U/ml}) \times \text{Fasting blood glucose (mmol/l)}}{22.5}$$

3.8 Liver analysis

3.8.1 Absolute and relative liver mass (hepatosomatic index)

At termination, the liver lobes were collected, and the absolute liver mass was measured using a digital analytical balance (*Presica 310M, Precision Instruments, Johannesburg, South Africa*). The hepatosomatic index was then calculated using the following formula (Ifthikhar *et al.*, 2022).

$$\text{Hepatosomatic index (\%)} = \frac{\text{Absolute liver mass}}{\text{Body mass}} \times 100$$

3.8.2 Storage of liver tissue

The left lobe of the liver was stored in 4% paraformaldehyde (PFA) in a 0.1 M phosphate solution at 4 °C for histological studies. The right lobe was preserved in RNAlater at –80 °C for molecular analyses. The remaining liver lobes were immediately frozen in liquid nitrogen and transferred to the –80 °C freezer for storage, pending oxidative stress marker analysis.

3.8.3 Quantifying the concentration of oxidative damage indicators in the liver tissue

Oxidative damage in liver tissue was assessed by measuring the concentration of TBARS using rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (CAT NO: E-BC-K298-M, *Elabscience Biotechnology Co., Ltd, Wuhan, China*).

The TBARS assay was conducted based on a colorimetric, ELISA-like enzymatic detection principle. Under high-temperature conditions, TBARS react with thiobarbituric acid (TBA) to form a pink chromogen. The assay's standard curve ranged from 5 to 1000 ng/mL, with concentration points at 0, 5, 10, 20, 40, 60, 80, and 100 µmol/L. The detection sensitivity was 0.85 µmol/L. The intensity of the colour produced was directly proportional to the TBARS concentration.

a. Sample preparation

Frozen liver samples were thawed to room temperature (18 – 25 °C) and washed in cold PBS (0.01 M, pH 7.4). To each 0.1 g of liver tissue, 1 ml of cold PBS was added in a microtube (*Eppendorf, Abdos Labtech, P10138*), and the tissue was sonicated. The mixture was then centrifuged for 5 minutes at 10,000 rpm and 4 °C to remove insoluble materials. The supernatant was collected and kept on ice.

b. TBARS assay procedure

Standard solutions of known concentrations were prepared according to the manufacturer's instructions. Each standard solution or sample (0.1 ml) was transferred to a 10-ml glass tube. To each tube, 0.1 ml of clarificant and 4 ml of chromogenic agent (a mixture of 3 ml acid application

solution and 1 ml Thio-barbituric acid (TBA) application solution) were added. The tubes were sealed and incubated in a water bath at 95 – 100 °C for 60 minutes. After cooling to room temperature, the tubes were centrifuged at 1600 rpm for 10 minutes.

The supernatant (0.25 ml) was then transferred to a microplate, and the optical density (OD) was measured at 532 nm using a microplate reader ((*Labsystems Multiskan Ascent 354, Thermo Fisher Scientific, Waltham, MA, USA*)).

c. TBARS data analysis

The average of the duplicate readings for the standards was calculated. The mean OD value of the blank was subtracted from the standard values to obtain the absolute OD value. A standard curve was plotted with the absolute OD values on the y-axis and the concentrations on the x-axis using GraphPad software; the equation obtained was ($y = 0.003304x + 0.009712$). Finally, to quantify the TBARS concentration, the protein concentration in the supernatant of each sample was determined using the Bradford method (Bradford, 1976).

d. Bradford protein assay procedure

The Bradford reagent (*Sigma Aldrich, B6916*) was gently mixed and allowed to reach room temperature. A stock solution was prepared by dissolving 1 mg of Bovine Serum Albumin (BSA) in 1 ml of distilled water, resulting in a concentration of 1000 µg/ml. From this stock solution, a series of standard concentrations was prepared: 20 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, and 1000 µg/ml.

The samples were diluted to 1:100. Both the protein standards and diluted samples were added to a well plate in volumes of 5 µl each. Then, 250 µl of Bradford reagent was added to each well. The plate was gently shaken for 30 seconds and incubated at room temperature for 10 minutes. The absorbance was measured at 595 nm using a microplate reader ((*Labsystems Multiskan Ascent 354, Thermo Fisher Scientific, Waltham, MA, USA*)). The results were plotted against the protein concentration of each standard to generate the standard curve from which the protein concentration in the samples was interpolated.

e. Calculation of sample TBARS concentration

The formula below from the ELISA kit manual was used to determine the concentration of TBARS in each liver tissue sample.

$$TBARS (\mu mol/g protein) = \frac{\Delta A - b/a \times f}{Cpr}$$

f = Dilution factor of the sample before the test

Cpr = Concentration of protein in the sample (g protein/L)

ΔA = Absolute OD value of sample ($OD_{sample} - OD_{blank}$)

3.8.4 Molecular analyses of the liver

The RNAlater stored liver samples were thawed at room temperature, and 350 μ l of lysis solution and 3.5 μ l of β -mercaptoethanol were added to the tissues. The liver samples were then homogenised using a sonicator until no visible tissue fragments remained. RNA purification was performed using an RNeasy spin kit. The homogenised samples were transferred to an RNeasy spin mini filter inside a collecting tube and centrifuged for 1 minute at 11,000 rpm. cDNA was synthesised from the RNA via a reverse transcription process.

For the real-time polymerase chain reaction (RT-qPCR) analysis, TaqMan probes were used to determine the mRNA expression of markers associated with the development of alcoholic liver disease. The markers analysed included metabolic markers (*CYP2E1* and *SREBP-1*) and inflammatory markers (*IL-10*, *NF- κ B1*, and *TNF- α*). The TaqMan® assay ID of the primers used for quantitative RT-qPCR (ThermoFisher®, Life Science Technologies, Carlsbad, USA) is shown in Table 3.2. The fold change for each transcript was determined using the cycle threshold ($\Delta\Delta Ct$) method and normalised against β -actin.

Table 3.2: The TaqMan® assay ID of the primers used for RT-qPCR.

Genes	TaqMan® assay ID
<i>CYP2E1</i>	Rn00580624_m1
<i>SREBP-1</i>	Rn01495769_m1
<i>IL-10</i>	Rn01483988_g1
<i>NF-κB1</i>	Rn01399572_m1
<i>TNF-α</i>	Rn01525859_g1

3.8.5 Histomorphometry analysis of the liver

After fixation in 4% paraformaldehyde (PFA) in a 0.1 M phosphate solution and storage at 4 °C, the left lobes of the liver were processed for histology using a tissue processor (*Microm STP 120, Thermo Scientific, MA, USA*). The processed liver tissues were then embedded in paraffin wax and sectioned at 5 µm using a microtome (*Leica Biosystems Instruments (Pty) Ltd, model RM 2125, Wetzlar, Germany*). The tissue sections were stained with haematoxylin and eosin (H&E) for general morphology and Masson Trichrome (MT) to quantify the collagen-stained areas, an indicator for fibrosis. All histological slides were scanned using the VS200 scanner from Evident (*United States*). Histomorphometry was performed on the digitised images using QuPath software (*version 0.5.1*) and ImageJ software (*NIH, USA, version 1.47v*).

3.8.6 Determination of hepatocyte density

For the estimation of hepatocyte density, the scanned slides were analysed using the QuPath software (Pohl *et al.*, 2024). Counting grids (size = 250 µm × 250 µm) were inserted into the scanned sections to prevent repetitive counting of the same tissue area. Cell counting was conducted at every two counting grid intervals. In all, cell counting was conducted on 20 counting grids per section. Cell counting within each counting grid was performed using the fast cell count tool of the QuPath software. Therefore, the cell (i.e. hepatocyte) density was calculated using the formula below:

$$\text{Cell density (count}/\mu\text{m}^2) = \frac{\text{Number of detections}}{\text{Area } (\mu\text{m}^2)}$$

3.8.7 Micro and macro vesicular steatosis scores

For scoring micro and macro vesicular steatosis, digitised images from the scanned tissue sections were captured using the ImageJ plugin tool of QuPath software. The images were stored in .tiff format and reviewed by experienced and independent observers. Steatosis was scored based on the scoring chart provided in Table 3.3. The observers evaluated the presence and extent of micro and macro vesicular steatosis in the liver tissue sections according to the standardised scoring system (Yip and Burt, 2006). The observers were blinded to the identities and groups to which the rats whose tissues were being scored belonged.

Table 3.3: Micro and macro vesicular steatosis score cut-off standards (Yip and Burt, 2006)

Score	Percentage steatosis
0	<5%
1	5 - 33%
2	34 - 66%
3	>66%

3.8.8 Estimation of collagen distribution

For collagen distribution, digitised images from the scanned sections were captured using the ImageJ plugin tool of the QuPath software. The colour deconvolution plugin tool of ImageJ software was used to separate the image into colours 1, 2, and 3 under the Masson Trichrome vector selection tool of the ImageJ software using the method described by Chen *et al.* (2017). Colour 1 was analysed by adjusting the colour threshold (the minimum threshold was set at 90 and the maximum threshold was at 125; these thresholds were kept constant throughout the analyses) to obtain the area fraction of collagen distribution. The area of the image analysed was obtained and this area was kept constant for all the analyses. The percentage of the area of collagen distribution to the total area of the image was then calculated.

3.9 Kidney analysis

3.9.1 Absolute kidney mass and kidney index

The right and left kidneys were collected and weighed using a digital weighing balance (Presica 310M, Precision Instruments, Johannesburg, South Africa), and the absolute mass was determined. The kidney index was calculated using the formula below (Iftikhar *et al.*, 2022):

$$\text{Kidney index (\%)} = \frac{\text{Absolute kidney mass}}{\text{Terminal body mass}} \times 100$$

3.9.2 Storage of kidney tissue

The left kidney from each rat was fixed in 4% paraformaldehyde (PFA) in a 0.1 M phosphate solution and stored at 4 °C for histology studies. The right kidney was placed in a sealable zip lock bag, immediately frozen in liquid nitrogen, and transferred to a –80 °C freezer for future oxidative stress marker analysis.

3.9.3 Quantifying the serum concentration of surrogate indicators of renal functions

Stored serum was used to measure the levels of kidney injury molecule-1 (KIM-1) and Neutrophil gelatinase-associated lipocalin (NGAL) using rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kits (KIM-1 CAT No: E-EL-R3019; NGAL CAT No: E-EL-R0662 *Elabscience Biotechnology Co., Ltd, Wuhan, China*). The assay procedures outlined in the kit user manuals were followed.

Both the KIM-1 and NGAL kits utilise a sandwich ELISA principle for the detection of Rat KIM-1 and NGAL, respectively. Each assay employs a biotinylated detection antibody and an avidin–horseradish peroxidase (HRP) conjugate. The KIM-1 assay features a standard curve ranging from 0.31 to 20 ng/mL, with concentration points at 0, 0.31, 0.63, 1.25, 2.5, 5, 10, and 20 ng/mL, and has a sensitivity of 0.19 ng/mL. The NGAL assay includes a standard curve ranging from 62.5 to 4000 pg/mL, with points at 0, 62.5, 125, 250, 500, 1000, 2000, and 4000 pg/mL, and a sensitivity of 37.5 pg/mL. For both assays, absorbance was measured at an optical density of 450 nm using a microplate reader (*Labsystems Multiskan Ascent 354, Thermo Fisher Scientific, Waltham, MA, USA*).

The average of the duplicate readings of the standards was calculated. The mean OD value of the blank was subtracted from the standards to obtain the absolute OD value. A four-parameter logistic curve was then plotted with the standard concentrations on the x-axis and the absolute OD values of the standards and samples on the y-axis. The sample concentrations were interpolated using GraphPad Prism software.

3.9.4 Quantifying the concentration of indicators of oxidative damage in the kidney tissue: TBARS

Frozen kidney samples were thawed to room temperature (18–25 °C) and washed in cold PBS (0.01 M, pH 7.4). The concentrations of TBARS in the kidney tissues were measured using the method described in section 3.6.3.

3.9.5 Determining the renal micro morphometry

After being fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate solution and stored at 4 °C, the left kidney was histologically processed using the digital tissue processor, Thermo Scientific's Microm STP 120 (MA, USA). The kidney tissues were sectioned at 5 µm with a microtome (Leica Biosystems, model RM 2125, Wetzlar, Germany). Subsequently, the sections were stained with hematoxylin and eosin (H&E) for general morphology and Masson Trichrome for collagen quantification, indicating fibrosis. All histological slides were scanned using the VS200 Slide Scanner from Evident (USA). The area of the Bowman's capsule and glomerular tuft were measured on digitised images using QuPath software. The urinary space was calculated by subtracting the measured area of the glomerular tuft from the measured area of the Bowman's capsule.

3.9.6 Estimation of collagen distribution

A similar method described in section 3.8.8 was used to determine the collagen distribution of the kidney tissue.

3.10 Statistical analysis

GraphPad Prism version 10 software (*GraphPad Software Inc., San Diego, USA*) was used for data analysis. The normality of each data set was assessed using the Shapiro-Wilk test. Normally

distributed data were analysed using one-way analysis of variance (ANOVA) and expressed as mean $\pm$ standard deviation. If the test indicated significance, a Tukey post hoc test was performed.

Skewed data were analysed using the Kruskal-Wallis's test and expressed as median with range or interquartile range. When the test revealed significance, a Dunn post hoc analysis was performed. Two-way ANOVA was used to evaluate the effects of sex, treatment, and the interaction between female and male rats. Statistical differences of less than 5% were considered significant.

The next chapter presents the study's findings, supported by figures, tables, and representative photomicrographs of liver and kidney histological sections.

CHAPTER FOUR

4 RESULTS

4.1 Morbidity and mortality

In compliance with the AREC guidelines for terminal endpoints for welfare, one male rat was euthanised during the seventh week of the study due to a loss of 10% body mass and a stereotypical scratching behaviour. The post-mortem report revealed an underlying compromised cardiovascular system. The report is shown in Appendix VI. All other rats did not show signs of ill thrift during the study. The data from the terminated rat are not included in the results of this study.

4.2 Effect of TA and SAC on weekly feed intake

The weekly feed intake of the female (a) and the male rats (b) is shown in Figure 4.1. The feed intake was similar across the treatment groups in weeks 1 and 2 ($p>0.05$) in both sexes. In the female rats (Figure 4.1a), feed intake in the SAC and SAC+TA groups was consistently lower than the PG and TA groups throughout the 10-week treatment period, with significant reductions ($p<0.05$) in the SAC than in the TA (approx. 25%) from week 3 and the PG or the TA from week 4. Also at week 5, the food intake for the SAC or the SAC+TA was significantly lower (approx. 25%) than the PG or the TA. This trend persisted in the subsequent weeks of the study ($p<0.0001$). However, there was no significant difference in feed intake between the PG and the TA, as well as between the SAC and the SAC+TA, throughout the 10-week treatment period ($p>0.05$).

In the male rats (Figure 4.1b), a similar trend as in the female rats was observed, except that the SAC and SAC+TA groups had significantly lower (approximately 25%) feed intake than the control or the TA group at week 3. This trend persisted in the subsequent weeks of the study ($p<0.0001$). However, there was no significant difference in feed intake between the PG and the TA as well as between the SAC and the SAC+TA, throughout the 10-week treatment period ($p>0.05$).

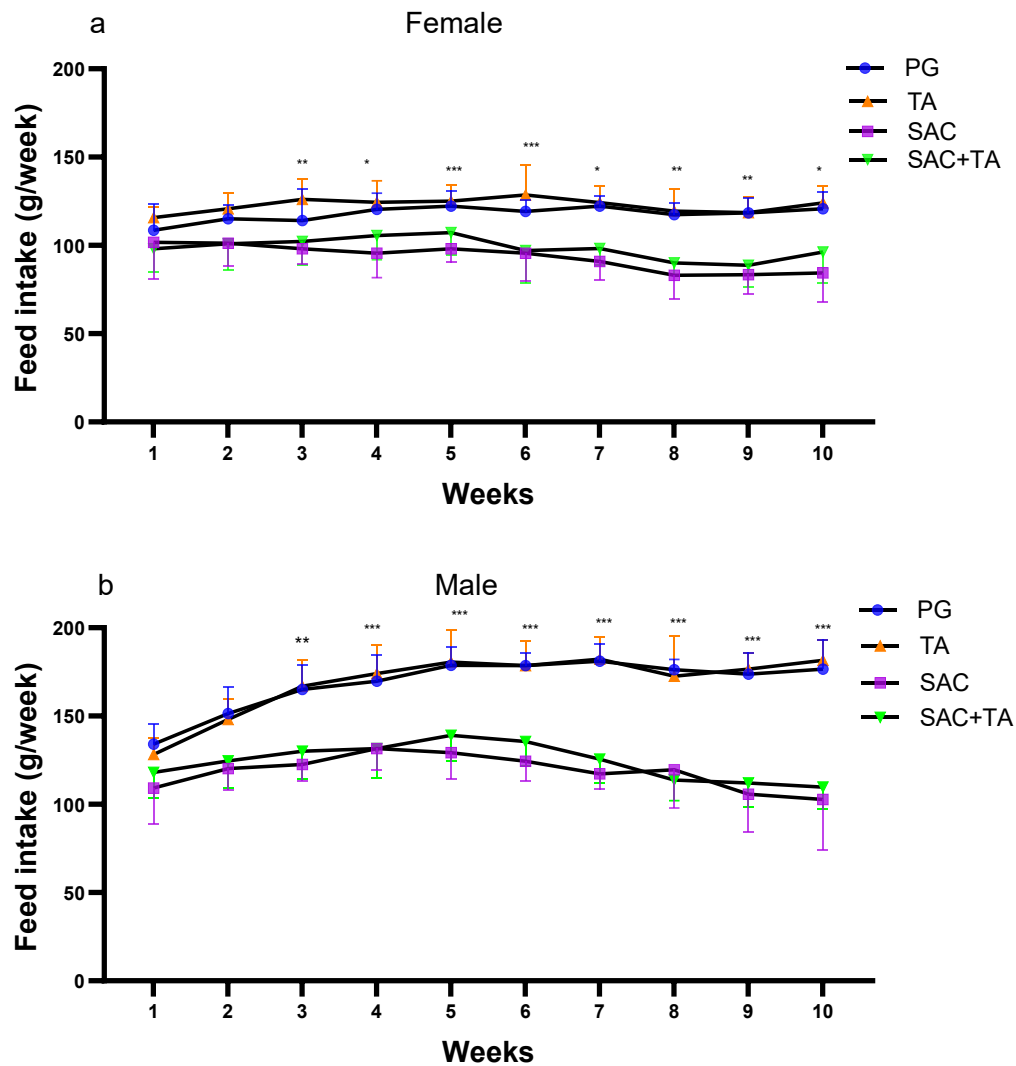


Figure 4.1: Line graphs illustrating the effect of SAC and TA on the weekly feed intake of (a) the female rats and (b) the male rats. From week 3 to the end of the treatment period in both sexes, the rats in the SAC and the SAC+TA groups had reduced feed intake compared to the PG and the TA groups. Data expressed as mean $\pm$ Standard deviation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ANOVA, Tukey's post hoc test. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).

4.2.1 Effect of TA and SAC on body mass gain

The effect of TA and SAC on the initial and terminal body mass of the female rats (a) and the male rats (b) across the treatment groups is shown in Figure 4.2. The initial body masses were similar across the treatment groups in both sexes ($p>0.05$). In both sexes, all treatment groups (PG, TA, SAC and SAC+TA) exhibited a significant increase in body mass from the initial to the terminal stage ($p<0.0001$). However, the body mass was similar across the treatment groups ($p>0.05$) for both sexes at the start or the end of the treatment. In addition, the terminal body mass of the female rats was significantly lower than the male rats in each of the treatment groups (Sex effect: $p<0.001$; treatment effect: $p = 0.5219$; interaction effect: $p = 0.2515$).

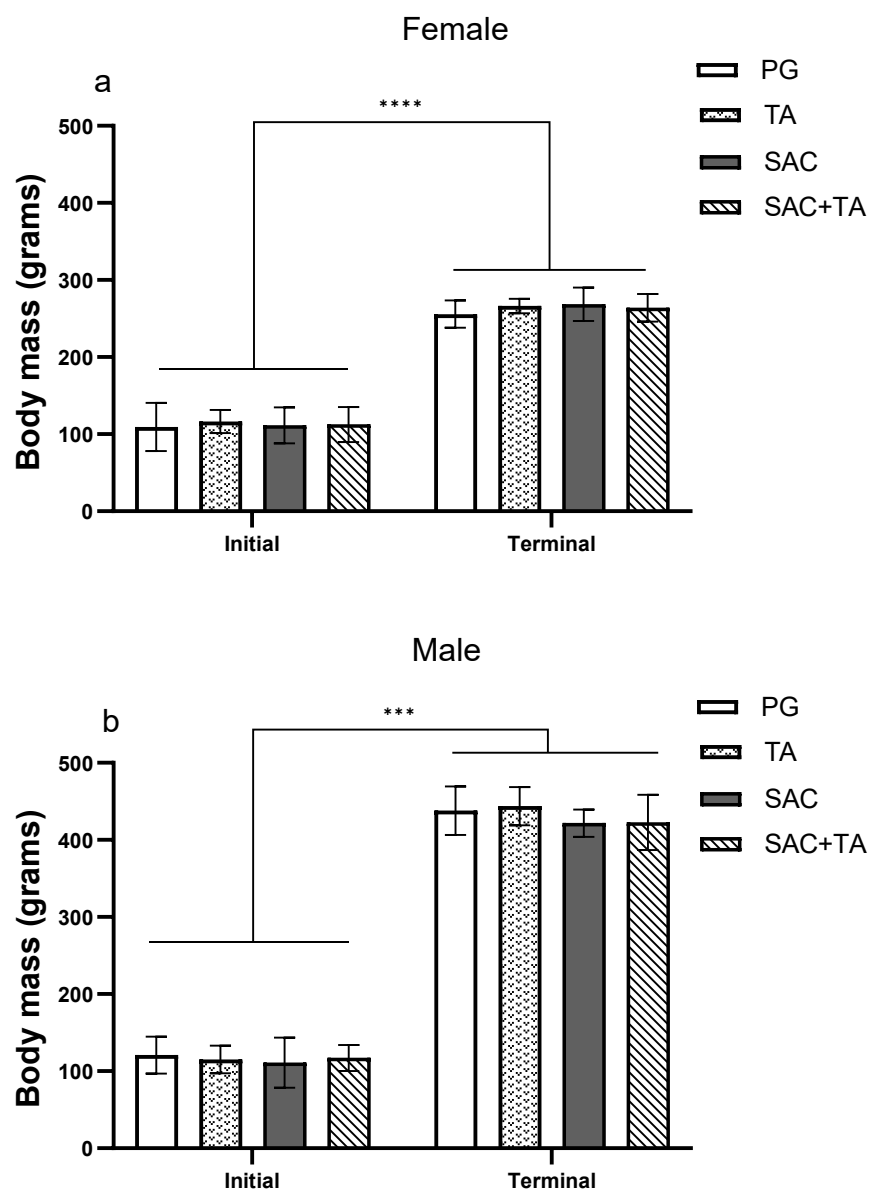


Figure 4.2: Plots illustrating the effect of SAC and TA on the initial and terminal weights of (a) the female and (b) the male rats. All rats grew significantly across the groups in both sexes. *Data expressed as mean $\pm$ Standard deviation, **** = $p < 0.001$ ANOVA, Tukey's post hoc test. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.2.2 Effect of TA and SAC on visceral fat and epididymal fat accumulation

The effect of SAC and TA on fat accumulation in the female and the male rats is shown in Table 4.1. There were significant differences in the absolute visceral fat mass ($p = 0.0374$) and relative visceral fat mass ($p = 0.0468$) of the female rats across the treatment groups. However, Dunn's multiple comparisons did not show any differences within the groups. In the male rats, there was no significant difference in the absolute and relative visceral fat (absolute: $p = 0.8636$; relative: $p = 0.7278$) and epididymal fat (absolute: $p = 0.4524$; relative: $p = 0.9874$) across the groups.

The effect of SAC and TA on the relative visceral fat accumulation of the female rats when compared with the male rats is shown in Figure 4.3. The female rats exhibited a significant increase in relative fat in the TA group ($p = 0.0082$), SAC group ($p = 0.0302$), and the SAC+TA group ($p = 0.0060$) than in the male rats for the same group except for the PG group that had a similar relative visceral fat with the males ($p = 0.5845$). Overall, sex and treatment effects were significantly different, while the interaction effect was not significant (Sex effect: $p < 0.0001$; treatment effect: $p = 0.0399$; interaction effect: $p = 0.4537$).

Table 4.1: A summary of the mean and median values of the absolute and relative visceral fat and epididymal fat for the female and the male rats across the different experimental groups.

	PG		TA		SAC		SAC+TA		P
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
Female (F)									
Visceral fat (g)	6.73 ± 0.83	6.63	8.79 ± 1.40	8.84	8.85 ± 3.05	7.59	9.64 ± 3.07	9.76	0.0374*
Visceral fat (% BW)	2.63 ± 0.30	2.58	3.31 ± 0.55	3.24	3.25 ± 0.84	2.97	3.64 ± 1.11	3.76	0.0468*
Male (M)									
Visceral fat (g)	8.90 ± 1.78	8.79	9.28 ± 2.68	8.90	9.10 ± 2.13	8.90	10.09 ± 3.09	10.21	0.8636
Visceral fat (% BW)	2.03 ± 0.38	1.96	2.76 ± 0.51	1.92	2.15 ± 0.43	2.17	2.38 ± 0.68	2.37	0.7278
Epididymal fat (g)	2.77 ± 0.32	3.90	2.67 ± 0.58	3.50	2.56 ± 0.56	3.30	2.59 ± 0.39	3.25	0.4524
Epididymal fat (% BW)	0.63 ± 0.09	0.64	0.60 ± 0.11	0.58	0.60 ± 0.11	0.57	0.61 ± 0.09	0.60	0.9874

* indicates a significant difference across treatment groups at $p < 0.05$, determined using the Kruskal–Wallis test with Dunn’s post hoc test for all parameters except epididymal fat (%BW), where ANOVA was used.

ANOVA = Analysis of variance, SD= Standard Deviation, PG = plain gelatine ($F = 8$, $M = 8$), TA = TA-50mg/kg and plain gelatine ($F = 8$, $M = 8$), SAC = 20% fructose, 10% alcohol and gelatine ($F = 8$, $M = 7$), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($F = 8$, $M = 8$).

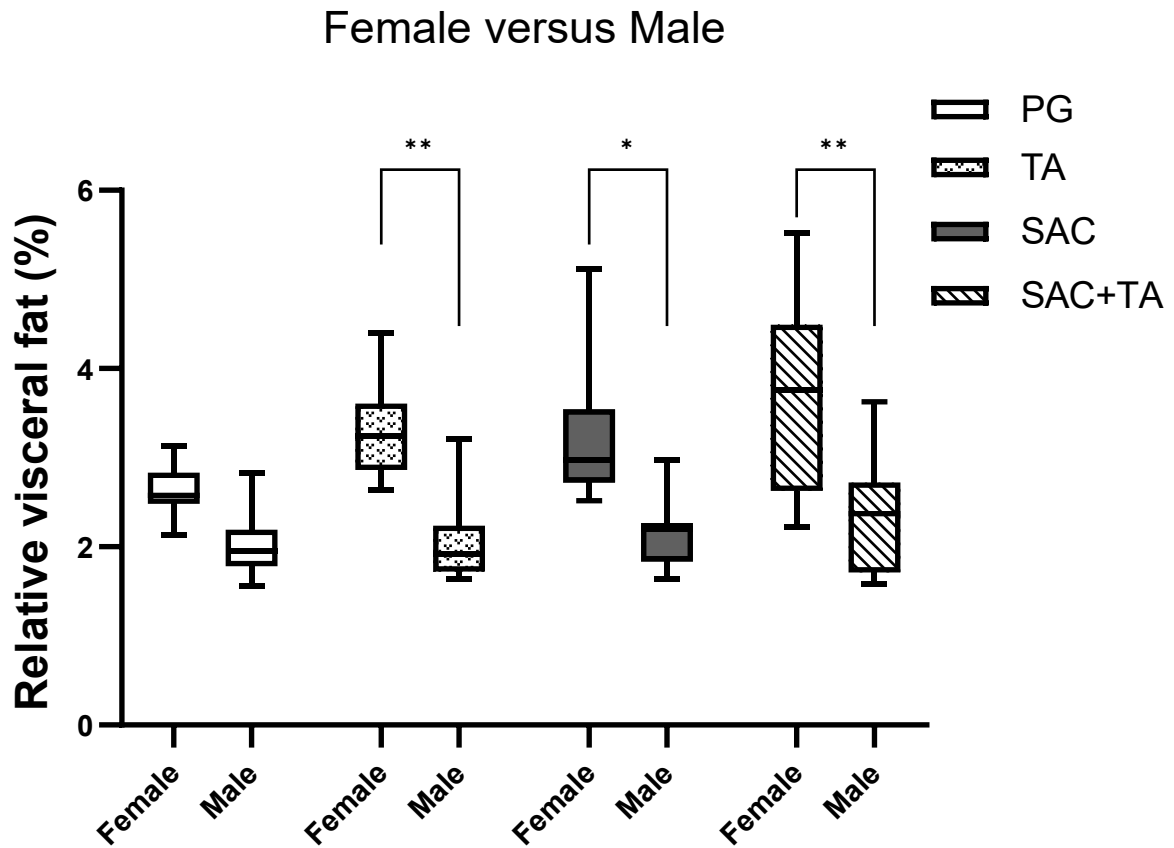


Figure 4.3: A boxplot illustrating the comparison between the effect of SAC and TA on the relative visceral fat of the female rats with that of the male rats. The female rats had significantly higher relative visceral fat than their male counterparts in all the treatment groups except for PG. *Data expressed as median $\pm$ range, * = $p < 0.05$ ** = $p < 0.01$, Two-way ANOVA, Tukey's post hoc test. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.2.3 Effect of TA and SAC on haemoglobin, haematocrit and mean corpuscular haemoglobin concentration (MCHC)

The effect of TA and SAC on the haemoglobin, haematocrit and MCHC of the female and male rats across the treatment groups is shown in Table 4.2.

The haemoglobin concentration was similar across the treatment groups in both sexes of rats (female: $p = 0.3401$; male: $p = 0.5497$). Haemoglobin concentration was not altered by sex and dietary treatment, such that the female rats had similar haemoglobin concentration when compared with their male counterparts in the same treatment group (Sex effect: $p = 0.1799$; treatment effect: $p = 0.6881$). In addition, there was no interaction between sex and dietary treatment (interaction effect: $p = 0.9889$).

The haematocrit concentration was similar across the treatment groups in both sexes of rats (female: $p = 0.4130$; male: $p = 0.4978$). Haematocrit concentration was not altered by sex and dietary treatment, such that the female rats had similar haematocrit concentration when compared with their male counterparts in the same treatment group (Sex effect: $p = 0.3317$; treatment effect: $p = 0.7912$). Also, there was no interaction between sex and dietary treatment (interaction effect: $p = 0.7347$).

The MCHC was similar across the treatment groups in both sexes of rats (female: $p = 0.7394$; male: $p = 0.7720$). MCHC was not altered by sex and dietary treatment, such that the female rats had similar haemoglobin concentration when compared with their male counterparts in the same treatment group (Sex effect: $p = 0.2772$; treatment effect: $p = 0.4401$). Similarly, there was no interaction between sex and dietary treatment (interaction effect: $p = 0.3787$).

Table 4.2: A summary of the mean and median values of the haemoglobin, haematocrit and mean corpuscular haemoglobin concentration (MCHC) for the female and the male rats across the different experimental groups.

	PG		TA		SAC		SAC+TA		P
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
Female (F)									
Haemoglobin (g/dL)	17.94 ± 4.04	19.30	17.96 ± 1.52	18.60	18.54 ± 1.67	18.90	17.9 ± 2.19	18.70	0.3401
Haematocrit (%)	53.88 ± 12.01	58.00	54.00 ± 4.66	56.00	55.63 ± 5.04	56.50	56.25 ± 3.28	56.50	0.4130
MCHC (g/dL)	33.26 ± 0.30	33.28	33.27 ± 0.21	33.21	33.33 ± 0.17	33.33	31.89 ± 3.92	33.21	0.7394
Male (M)									
Haemoglobin (g/dL)	18.8 ± 1.03	18.80	18.64 ± 1.24	18.75	19.37 ± 0.67	19.10	18.31 ± 1.80	18.85	0.5497
Haematocrit (%)	56.38 ± 3.14	56.50	56 ± 3.63	56.00	58.14 ± 2.12	57.00	54.88 ± 5.25	56.50	0.4978
MCHC (g/dL)	33.35 ± 0.15	33.33	33.28 ± 0.15	33.27	33.32 ± 0.14	33.33	33.36 ± 0.23	33.42	0.7720

For each parameter, no significant differences were observed across treatment groups in either sex ($p > 0.05$), based on the Kruskal–Wallis test, except MCHC male, which was analysed using ANOVA.

SD= Standard Deviation, PG = plain gelatine ($F = 8$, $M = 8$), TA = TA-50mg/kg and plain gelatine ($F = 8$, $M = 8$), SAC = 20% fructose, 10% alcohol and gelatine ($F = 8$, $M = 7$), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($F = 8$, $M = 8$).

4.2.4 Effect of TA and SAC on fasting blood glucose, insulin, and HOMA-IR

The effects of TA on fasting blood glucose, insulin, and HOMA-IR are shown in Table 4.3. The fasting blood glucose concentration (FBG) was similar across the treatment group in both sexes (Female: $p = 0.0519$; Male: $p = 0.0816$). The female rats in the TA group had a higher FBG than the male rats in the same group ($p = 0.0388$), while the female and male rats in the other groups had similar FBG (Sex effect: $p = 0.0007$; treatment effect: $p = 0.0834$; interaction effect: $p = 0.0652$).

For insulin concentration, there was no significant difference in the female rats ($p = 0.6530$) and the male rats ($p = 0.8517$) across the treatment groups. The female rats had similar insulin concentration when compared with their male counterparts (Sex effect: $p = 0.3349$; treatment effect: $p = 0.7627$; interaction effect: $p = 0.6691$).

Also, there was no significant difference in the HOMA-IR of the female and male rats across the treatment groups ($p = 0.3005$, $p = 0.3021$, respectively). The female rats had similar HOMA-IR when compared with their male counterparts (Sex effect: $p = 0.0720$; treatment effect: $p = 0.9411$; interaction effect: $p = 0.0736$).

Table 4.3: A summary of the mean values of the fasting blood glucose, the insulin, and the HOMA-IR for the female and the male rats across the different experimental groups.

	PG		TA		SAC		SAC+TA		P
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
Female (F)									
Fasting blood Glucose (mmol/L)	3.73 ± 0.35	3.70	4.14 ± 0.35 [#]	4.10	3.80 ± 0.50	3.60	3.61 ± 0.24	3.60	0.0519
Insulin (pg/mL)	401.60 ± 93.97	399.10	385.80 ± 92.44	380.90	422.50 ± 79.72	388.40	448.10 ± 97.81	484.30	0.6530
HOMA-IR	1.67 ± 0.27	1.69	2.00 ± 0.52	1.97	2.07 ± 0.59	1.88	2.07 ± 0.48	2.22	0.3005
Male (M)									
Fasting blood Glucose (mmol/L)	3.78 ± 0.55	3.90	3.43 ± 0.53 [#]	3.50	3.16 ± 0.58	3.30	3.33 ± 0.31	3.25	0.4424
Insulin (pg/mL)	406.60 ± 110.60	366.70	393.80 ± 80.52	363.80	367.00 ± 32.55	374.10	397.80 ± 80.21	403.10	0.8517
HOMA-IR	1.97 ± 0.65	1.73	1.76 ± 0.55	1.65	1.48 ± 0.19	1.51	1.67 ± 0.27	1.69	0.3021

For each parameter, no significant differences were observed across treatment groups in either sex ($p > 0.05$), based on the One-Way Analysis of variance, except for fasting blood glucose (female), which was analysed using Kruskal-Wallis test.

When the females were compared to the males in the same group, the same superscript symbols (#) indicate a significant difference at $p < 0.05$ using a Two-way Analysis of variance followed by Tukey's post hoc test.

SD= Standard Deviation, PG = plain gelatine (F = 8, M = 8), TA = TA-50mg/kg and plain gelatine (F = 8, M = 8), SAC = 20% fructose, 10% alcohol and gelatine ((F = 8, M = 7), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (F = 8, M = 8).

4.2.5 Effect of TA and SAC on serum TG and HDL-C concentrations and TG/HDL-C ratio

The effect of TA and SAC on TG, HDL-C, and TG/HDL-C ratio is shown in Table 4.4. There was no significant difference in the TG concentration across the treatment groups (Female: $p = 0.3332$; Male: $p = 0.199$) in both sexes. The female rats had similar TG concentrations when compared with their male counterparts (Sex effect: $p = 0.0006$; treatment effect: $p = 0.1757$; interaction effect: $p = 0.3318$). In the female rats, there were no significant differences in the HDL-C concentration across the groups ($p = 0.9326$). However, the male rats had significantly increased HDL-C levels in the SAC group ($p = 0.0405$) and SAC+TA group ($p = 0.0312$) when compared with the PG group. Thus, TA did not alter the SAC-induced increase in HDL-C.

There was no significant difference in the HDL-C concentrations of the female rats when compared with the male rats (Sex effect: $p = 0.6872$; treatment effect: $p = 0.1251$; interaction effect: $p = 0.2241$). There was also no significant difference in the TG/HDL-C ratio across the treatment groups in both sexes of rats (Female: $p = 0.3931$; Male: $p = 0.8517$). While the sex effect ($p = 0.0035$) was significant when the TG/HDL-C ratio of female rats was compared to the corresponding male rats, there was no difference in the treatment effect ($p = 0.8396$) and interaction effect ($p = 0.1132$).

Table 4.4: A summary of the mean values of the serum TG, HDL-C, and TG-HDL-C ratio for the female and the male rats across the different experimental groups.

	PG	TA	SAC	SAC+TA	p
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Female (F)					
TG (mmol/L)	0.49 $\pm$ 0.22	0.74 $\pm$ 0.37	0.75 $\pm$ 0.39	0.72 $\pm$ 0.39	0.3332
HDL-C (mmol/L)	0.34 $\pm$ 0.06	0.36 $\pm$ 0.10	0.35 $\pm$ 0.10	0.33 $\pm$ 0.08	0.1441
TG/HDL-C	1.50 $\pm$ 0.71	2.06 $\pm$ 0.83	2.30 $\pm$ 1.42	2.21 $\pm$ 0.88	0.3921
Male (M)					
TG (mmol/L)	0.93 $\pm$ 0.25	0.82 $\pm$ 0.22	1.02 $\pm$ 0.42	1.20 $\pm$ 0.46	0.1994
HDL-C (mmol/L)	0.29 $\pm$ 0.08 ^{ab}	0.33 $\pm$ 0.08	0.47 $\pm$ 0.20 ^a	0.39 $\pm$ 0.08 ^b	0.0175*
TG/HDL-C	3.45 $\pm$ 1.36	2.62 $\pm$ 1.05	2.34 $\pm$ 1.56	3.07 $\pm$ 1.23	0.3108

Row means with same superscript letters (a, b) indicate a significant difference at $p < 0.05$, ANOVA, Tukey's post hoc test.

SD= Standard Deviation, PG = plain gelatine (F = 8, M = 8), TA = TA-50mg/kg and plain gelatine (F = 8, M = 8), SAC = 20% fructose, 10% alcohol and gelatine ((F = 8, M = 7), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (F = 8, M = 8).

4.3 Liver results

4.3.1 Effect of TA and SAC on liver mass and hepatosomatic index

There was no significant difference in the absolute liver mass of the female ($p = 0.3615$) and the male rats ($p = 0.7204$) across the treatment groups, as shown in Table 4.5. Also, there was no significant difference in liver mass relative to body mass (hepatosomatic index) in both sexes (Female: $p = 0.4739$; Male: $p = 0.1026$).

While the sex effect was significant when the liver mass of the female rats was compared with the male rats, there was no difference in the treatment effect and interaction effects (Sex effect: $p < 0.0001$; treatment effect: $p = 0.5177$; interaction effect: $p = 0.5874$). The same trend was observed when the hepatosomatic index of the female rats was compared to the male rats (Sex effect: $p < 0.0001$; treatment effect: $p = 0.1189$; interaction effect: $p = 0.7803$).

Table 4.5: A summary of the mean values of the liver mass and hepatosomatic index for the female and the male rats across the different experimental groups.

	PG	TA	SAC	SAC+TA	p
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Female (F)					
Liver mass (g)	7.63 $\pm$ 0.57 [#]	8.00 $\pm$ 0.62 ^{\$}	8.33 $\pm$ 1.06 [€]	8.24 $\pm$ 0.95 ^Δ	0.3615
Body mass (g)	255.80 $\pm$ 17.73	266.30 $\pm$ 9.27	268.60 $\pm$ 21.57	263.90 $\pm$ 18.01	0.4823
Hepatosomatic index (%)	2.99 $\pm$ 0.19	3.01 $\pm$ 0.19	3.09 $\pm$ 0.19	3.13 $\pm$ 0.24 [#]	0.4739
Male (M)					
Liver mass (g)	12.00 $\pm$ 1.07 [#]	12.00 $\pm$ 0.76 ^{\$}	12.16 $\pm$ 0.84 [€]	11.97 $\pm$ 1.24 ^Δ	0.7204
Body mass (g)	438.00 $\pm$ 31.36	443.80 $\pm$ 24.77	421.70 $\pm$ 17.73	422.90 $\pm$ 35.85	0.3500
Hepatosomatic index (%)	2.74 $\pm$ 0.11	2.82 $\pm$ 0.12	2.88 $\pm$ 0.09	2.83 $\pm$ 0.11 [#]	0.1026

For each parameter, there was no difference across the treatment groups in both sexes of rats, $p > 0.05$ using One-Way Analysis of variance.

When the females were compared to the males in the same group, the same superscript symbols (#, \$, €, and Δ) indicate a significant difference at $p < 0.05$ using a Two-way Analysis of variance followed by Tukey's post hoc test.

SD= Standard Deviation, PG = plain gelatine (F = 8, M = 8), TA = TA-50mg/kg and plain gelatine (F = 8, M = 8), SAC = 20% fructose, 10% alcohol and gelatine ((F = 8, M = 7), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (F = 8, M = 8).

4.3.2 Effect of TA and SAC on TBARS concentration in the liver

The effect of TA and SAC on TBARS concentration is shown in Figure 4.4. In the female rats (Figure 4.4a), there was a significant difference in the TBARS concentration across the treatment groups ($p = 0.0111$).

The TBARS concentration of the female rats was significantly increased in the TA group ($p = 0.0321$) or the SAC group ($p = 0.0180$) when compared to the PG group. However, the female rats in the SAC+TA group had similar TBARS concentrations when compared to the PG group ($p = 0.6972$).

In the males (Figure 4.4b), there was also a significant difference in the TBARS concentration across the treatment groups ($p = 0.0122$). The TBARS concentration in the SAC group was significantly lower than that of the PG group ($p = 0.0117$). While the TA group ($p = 0.3786$) or the SAC+TA group ($p = 0.0535$) had similar TBARS concentrations when compared to the PG group.

The comparison of the TBARS concentration in both sexes showed significant differences in the sex effect ($p < 0.0001$) and interaction effect ($p = 0.0003$), but there was no significant difference in the treatment effect ($p = 0.2403$).

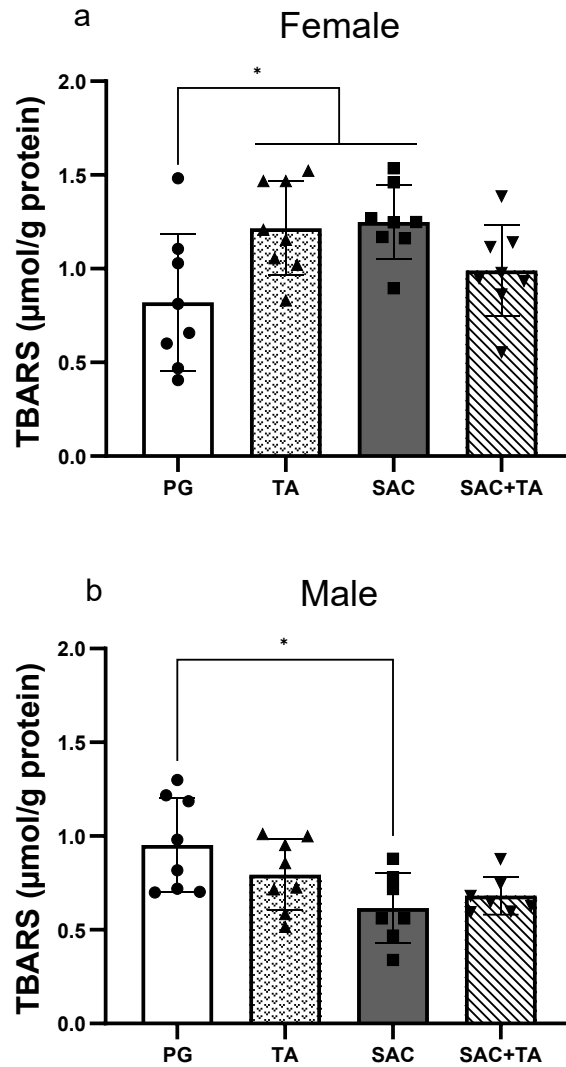


Figure 4.4: Plots showing the effect of SAC and TA on TBARS concentration in the liver for (a) the female and (b) the male rats. The female rats in the TA and SAC groups had higher TBARS concentrations than the PG while the SAC group had lower TBARS concentrations compared to the PG group in the male rats. *Data expressed as mean $\pm$ standard deviation, * = $p < 0.05$ ANOVA, Tukey's post hoc test. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.3.3 Effect of TA and SAC on mRNA expression of markers of metabolism and inflammation in the liver

The effect of TA and SAC on the mRNA expression of selected genes in the liver is shown in Table 4.6. There was no significant difference in the mRNA expression of markers of metabolism (*CYP2E1* and *SREBP-1*), and inflammation (*IL-10*, *NF-κB1*, and *TNF-α*) across the treatment groups ($P > 0.05$ ANOVA) in both sexes of rats.

When the gene expression of the female rats was compared with the male rats, the sex effect, treatment effect and interaction effect were similar for the marker of alcohol metabolism – *CYP2E1* (Sex effect: $p = 0.1890$; treatment effect: $p = 0.2360$; interaction effect: $p = 0.2390$), or the marker of lipid metabolism – *SREBP-1* (Sex effect: $p = 0.3182$; treatment effect: $p = 0.1812$; interaction effect: $p = 0.9298$).

Similarly, the pro-inflammatory markers of the female rats did not show any significant difference when compared to the corresponding male rats: *IL10* (Sex effect: $p = 0.1890$; treatment effect: $p = 0.2361$; interaction effect: $p = 0.2390$), *NF-κB1* (Sex effect: $p = 0.4768$; treatment effect: $p = 0.0983$; interaction effect: $p = 0.9425$) and *TNF-α* (Sex effect: $p = 0.5917$; treatment effect: $p = 0.5520$; interaction effect: $p = 0.6159$).

Table 4.6: A summary of the mean and median values of the mRNA expression levels of some genes in the liver of the female and the male rats across the different experimental groups.

mRNA Expression	PG	TA		SAC		SAC+TA		P	
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	
Female (F)									
<i>CYP2E1</i>	0.93 $\pm$ 0.16	0.95	1.02 $\pm$ 0.39	1.06	1.20 $\pm$ 0.86	0.81	0.92 $\pm$ 0.55	0.68	0.1224
<i>IL-10</i>	1.20 $\pm$ 0.83	0.99	1.21 $\pm$ 0.67	1.13	0.77 $\pm$ 0.28	0.88	0.73 $\pm$ 0.54	0.62	0.5590
<i>NF-κB1</i>	1.03 $\pm$ 0.27	0.99	1.02 $\pm$ 0.22	1.08	1.01 $\pm$ 0.33	1.07	0.75 $\pm$ 0.11	0.75	0.3535
<i>SREBP-1</i>	1.17 $\pm$ 0.70	1.20	1.26 $\pm$ 0.59	1.16	1.09 $\pm$ 0.49	1.12	0.63 $\pm$ 0.34	0.50	0.4030
<i>TNF-α</i>	1.03 $\pm$ 0.28	1.05	1.04 $\pm$ 0.23	1.08	1.30 $\pm$ 0.73	1.20	1.48 $\pm$ 0.80	1.47	0.4191
Male (M)									
<i>CYP2E1</i>	1.57 $\pm$ 0.41	1.37	1.88 $\pm$ 0.69	2.12	1.08 $\pm$ 0.48	1.05	0.72 $\pm$ 0.86	0.53	0.7113
<i>IL-10</i>	1.24 $\pm$ 0.81	1.25	0.59 $\pm$ 0.30	0.56	0.51 $\pm$ 0.04	0.53	0.43 $\pm$ 0.19	0.43	0.5175
<i>NFκB1</i>	1.02 $\pm$ 0.23	0.94	1.13 $\pm$ 0.36	1.12	1.14 $\pm$ 0.26	1.18	0.79 $\pm$ 0.14	0.79	0.2571
<i>SREBP-1</i>	1.13 $\pm$ 0.63	1.02	0.89 $\pm$ 0.41	0.74	0.92 $\pm$ 0.19	0.99	0.46 $\pm$ 0.11	0.46	0.3966
<i>TNF-α</i>	1.04 $\pm$ 0.36	1.07	1.03 $\pm$ 0.52	1.17	1.47 $\pm$ 0.45	1.03	0.87 $\pm$ 0.49	1.02	0.4848

For each parameter, no significant differences were observed across treatment groups in either sex ($p > 0.05$), based on the One-Way Analysis of variance, except for *CYP2E1* female, *IL-10* male, *TNF- α* female, which were analysed using the Kruskal-Wallis test.

SD= Standard Deviation, *CYP2E1*= Cytochrome P450 2E1, *IL-10*= interleukin-10, *NF- κ B1*= Nuclear factor kappa B1, *SREBP-1*= Sterol regulatory element-binding protein-1, *TNF- α* = tumour necrosis factor- α , PG = plain gelatine (F = 4, M = 4), TA = TA-50mg/kg and plain gelatine (F = 4, M = 4), SAC = 20% fructose, 10% alcohol and gelatine (F = 4, M= 3), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (F = 4, M = 2).

4.3.4 Effect of TA and SAC on the liver cell density

The effect of TA and SAC on liver cell density of the rats across the treatment groups is shown in Figure 4.5. In the female rats (Figure 4.5a), SAC or TA resulted in a 38% reduction in liver cell density ($p < 0.0001$) when compared to the PG, while SAC+TA resulted in a lesser reduction (24%) in liver cell density ($p < 0.0001$) when compared with their control counterparts. In the male rats (Figure 4.5b), the SAC+TA group had a significantly lower liver cell density than the control ($p = 0.0002$) or the TA ($p < 0.0001$). The sex effect, treatment effect and interaction effects were significant ($p < 0.0001$) when the cell density of the female rats was compared to the male rats.

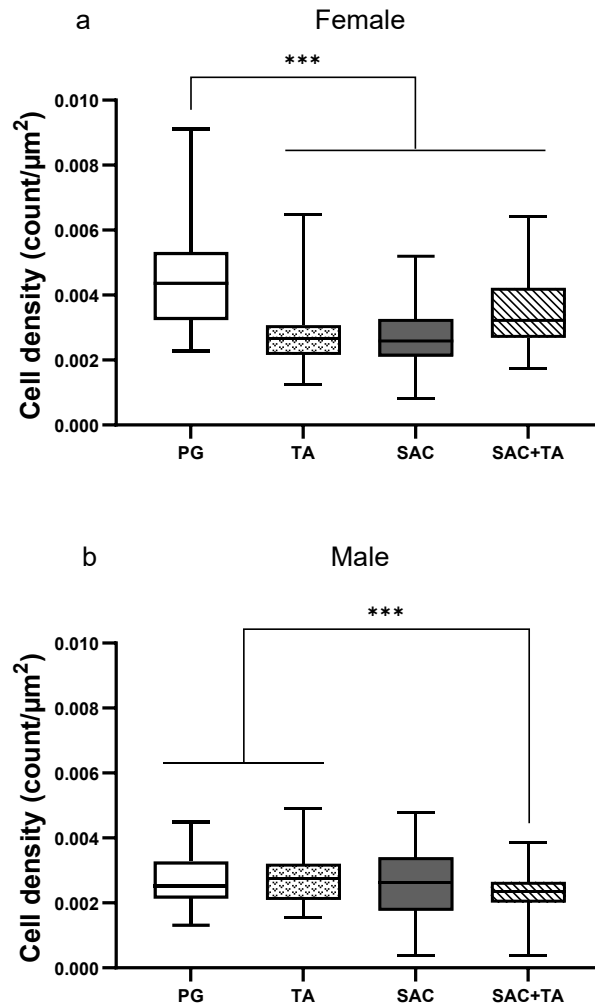


Figure 4.5: Boxplots showing the effect of SAC and TA on the liver cell density of (a) the female and (b) the male rats. The female rats in the TA, SAC, or SAC+TA groups had a reduced cell density when compared to the PG group, while only the male rats in the SAC+TA groups had a reduced cell density when compared to the PG or the TA group. *Data expressed as median with range, *** = $p < 0.001$ Kruskal Wallis, Dunn's post hoc test. PG = plain gelatine (no of images assessed = 167 females, 191 males), TA = TA-50mg/kg and plain gelatine (no of images assessed = 243 females, 138 males), SAC = 20% fructose, 10% alcohol and gelatine (no of images assessed = 137 females, 106 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ((no of images assessed = 204 females, 157 males).*

4.3.5 Effect of TA and SAC on micro and macro vesicular steatosis score

The effect of TA and SAC on micro and macro vesicular steatosis scores is shown in Table 4.7. The representative photomicrographs of the Haematoxylin and Eosin-stained liver sections of (A) the female and (B) the male rats are shown in Figure 4.6.

In the female rats, there was no significant difference in the micro vesicular ($p = 0.4022$) and macro vesicular steatosis scores ($p = 0.3985$) across the treatment groups. In the male rats, the SAC groups had a higher micro vesicular steatosis score compared to the control, SAC group ($p = 0.0088$) and SAC+TA group ($p = 0.0043$). However, the male rats had a similar macro vesicular steatosis score across the treatment groups ($p = 0.6057$).

While the sex and interaction effects were significant when the micro vesicular steatosis scores of the female rats were compared to the male rats (Sex effect: $p < 0.0001$; interaction effect: $p = 0.0441$), there was no difference in the treatment effect ($p = 0.1114$). Also, the sex effects were significant when the macro vesicular steatosis scores of the female rats were compared to the male rats (Sex effect: $p = 0.0024$), however, there was no difference in the interaction ($p = 0.6600$) and treatment effect ($p = 0.5482$). Overall, the female rats had a higher micro and macro vesicular steatosis score compared to the corresponding male rats except for the SAC group.

Table 4.7: A summary of the median and interquartile range values of the steatosis scores for the females and the males across the different experimental groups.

	PG	TA	SAC	SAC+TA	p
	Median (Q1 – Q3)	Median (Q1 – Q3)	Median (Q1 – Q3)	Median (Q1 – Q3)	
Female (F)					
Micro vesicular steatosis	2 (1 – 3) [#]	1 (0 – 2) [€]	2 (0 – 2)	2 (0.25 – 3) ^Δ	0.4022
Macro vesicular steatosis	1 (0 – 1) ^{\$}	0 (0 – 0.75)	0 (0 – 0)	0 (0 – 1) [#]	0.3985
Male (M)					
Micro vesicular steatosis	0 (0 – 1) ^{ab#}	0 (0 – 1) [€]	1 (0.25 – 1.75) ^a	1 (0 – 2) ^{bΔ}	0.0013
Macro vesicular steatosis	0 (0 – 0) ^{\$}	0 (0 – 0)	0 (0 – 0)	0 (0 – 0) [#]	0.6057

For each parameter, the same superscript letters (a, b, c, and d) indicate a significant difference between any two groups at $p < 0.05$ using Kruskal-Wallis followed by Dunn's post hoc test

The same superscript symbols (#, \$, €, and Δ) indicate a significant difference at $p < 0.05$ when the females were compared to the males in the same group using a Two-way Analysis of variance followed by Tukey's post hoc test.

Q1 – Q3 = interquartile range, PG = plain gelatine (no of images assessed, F = 15, M = 18), TA = TA-50mg/kg and plain gelatine (no of images assessed, F = 12, M = 14), SAC = 20% fructose, 10% alcohol and gelatine (no of images assessed, F = 18, M = 12), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (no of images assessed, F = 12, M = 19).

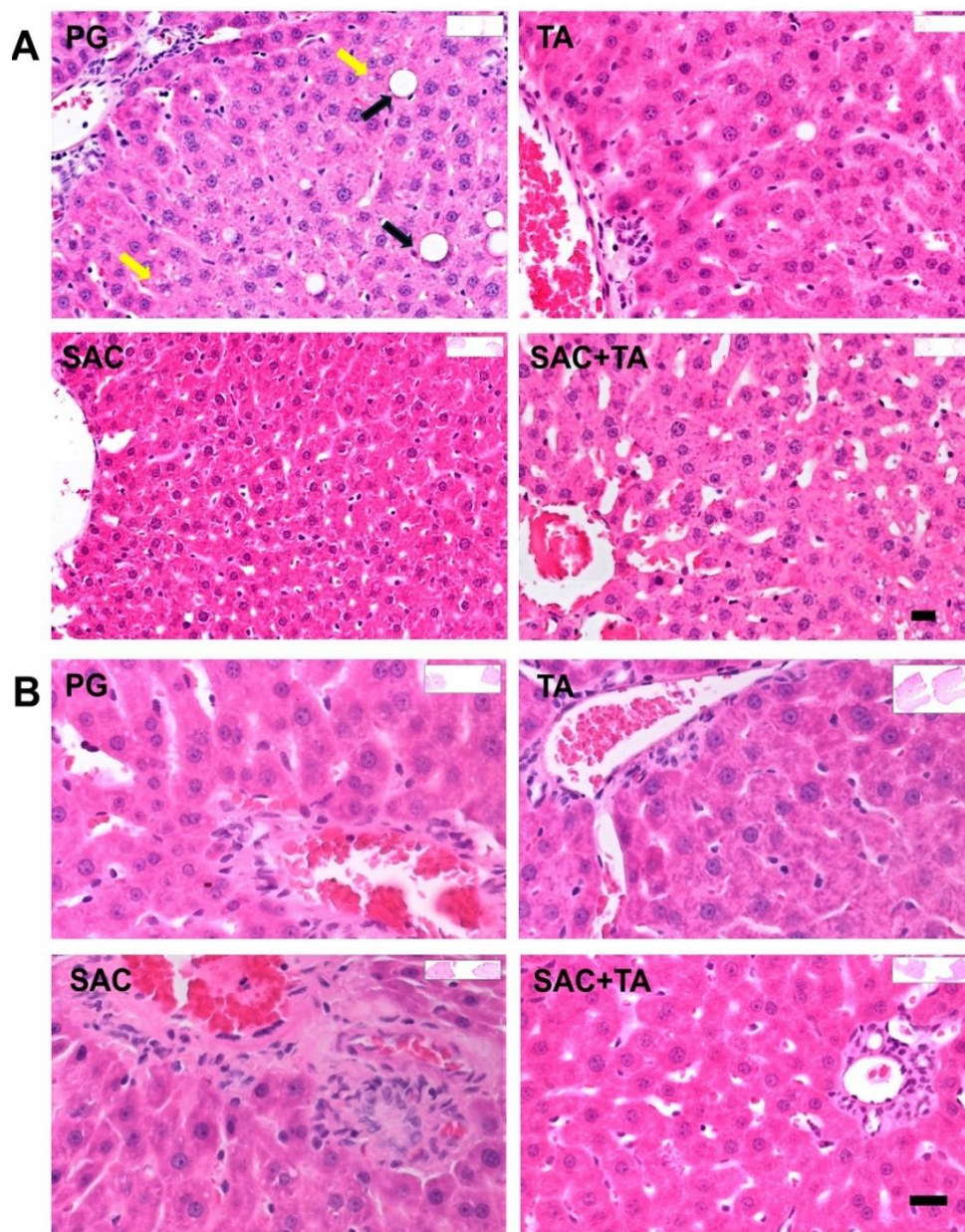


Figure 4.6: Representative photomicrographs of the liver of (A) the female and (B) the male rats. The sections were stained with Haematoxylin and Eosin. The yellow arrows indicate micro steatosis while the black arrows indicate macro steatosis in the liver. Scale bar = 20 μ m. *Image at the top right corner = section map, PG = plain gelatine, TA = TA-50mg/kg and plain gelatine, SAC = 20% fructose, 10% alcohol and gelatine, SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine.*

4.3.6 Effect of TA and SAC on the percentage area of collagen distribution

The effect of TA and SAC on the percentage area of collagen distribution is shown in Figure 4.7. The representative photomicrographs of the Masson trichrome-stained liver sections of (A) the female and (B) the male rats are shown in Figure 4.8.

There was no significant difference in the collagen ratio across the treatment groups ($p = 0.5409$) in the female rats (Figure 4.7a). However, in the male rats (Figure 4.7b), the percentage area of collagen distribution was significantly lower in the TA group ($p = 0.0254$) or the SAC group ($p < 0.0001$) when compared to the PG group. The male rats in the SAC+TA group had a similar percentage area of collagen distribution as that of the PG group ($p = 0.2308$).

There was no difference in sex effect and treatment effect when the percentage area of collagen distribution in the female rats was compared to the corresponding male rats (Sex effect: $p = 0.0641$; treatment effect: $p = 0.0798$), but the interaction effect was significant (interaction effect: $p = 0.0045$).

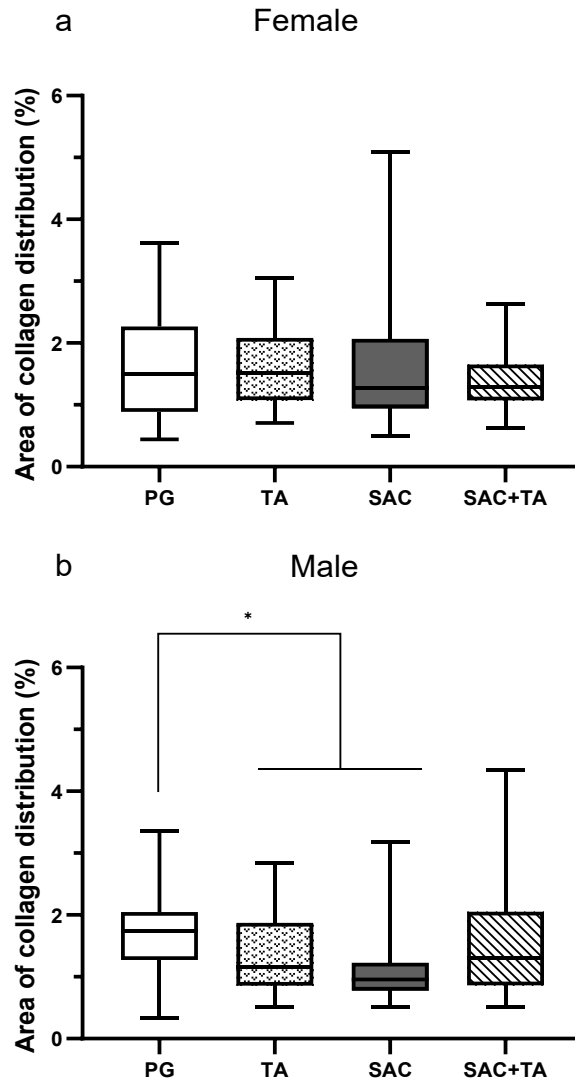


Figure 4.7: Boxplots showing the effect of SAC and TA on the percentage area of liver collagen distribution for (a) the female and (b) the male rats. In the female rats, there was no difference in the percentage area of collagen distribution. While in the male rats, TA and SAC groups had a significantly lower percentage area of collagen distribution. *Data expressed as median with range, *** = $p < 0.001$, Kruskal Wallis, Dunn's post hoc test. PG = plain gelatine (no of images assessed = 167 females, 191 males), TA = TA-50mg/kg and plain gelatine (no of images assessed = 243 females, 138 males), SAC = 20% fructose, 10% alcohol and gelatine (no of images assessed = 137 females, 106 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine (no of images assessed = 204 females, 157 males).*

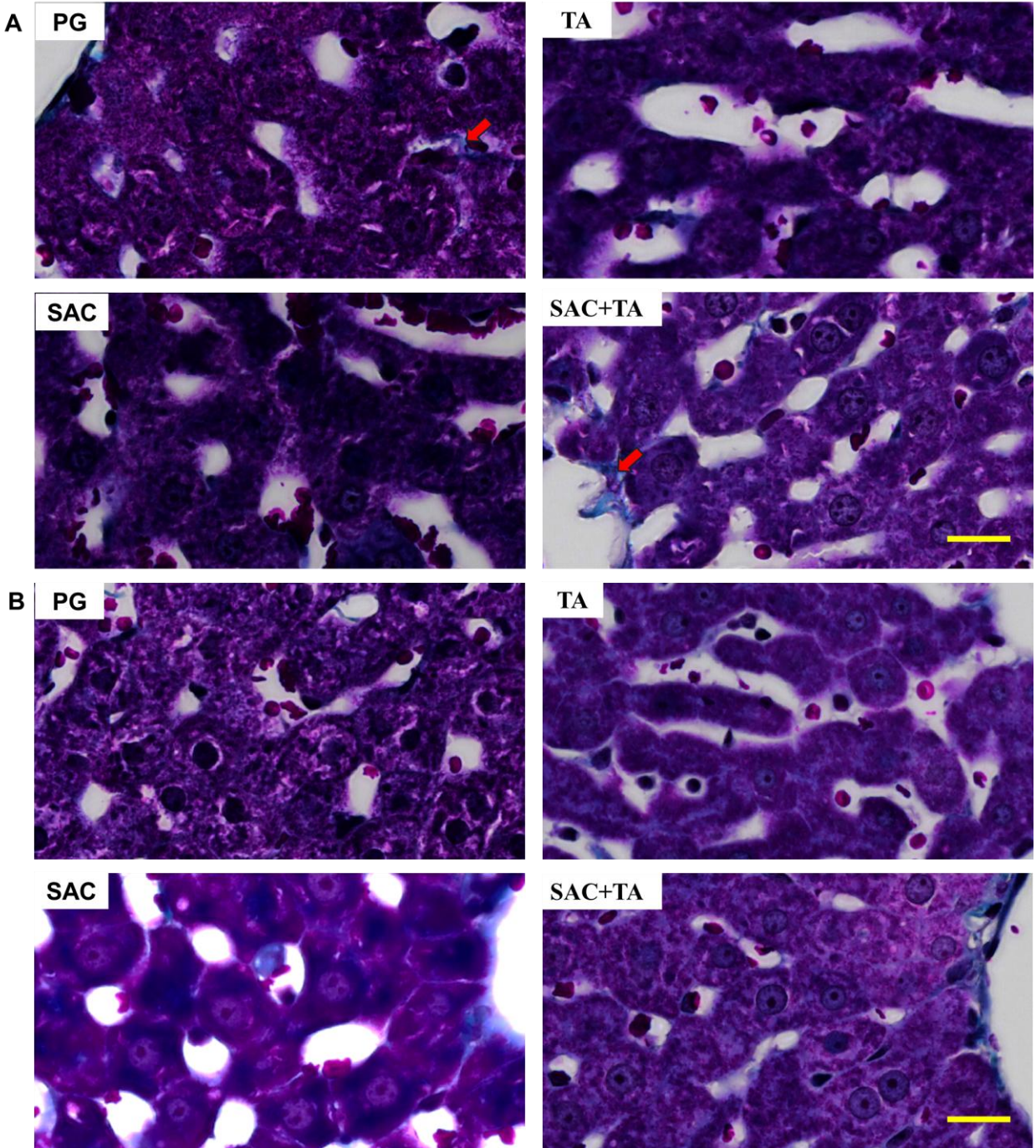


Figure 4.8: Representative photomicrographs of the liver of (A) the female and (B) the male rats. The sections were stained with Masson trichrome. The orange arrows indicate some areas of collagen in the liver. Scale bar = 20 μ m. *PG* = plain gelatine, *TA* = TA-50mg/kg and plain gelatine, *SAC* = 20% fructose, 10% alcohol and gelatine, *SAC+TA* = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine.

4.4 Kidney results

4.4.1 Effect of TA and SAC on absolute kidney mass and kidney index

There was no significant difference in the absolute kidney mass of the female rats ($p = 0.4897$) and male rats ($p = 0.7738$) across the treatment groups, as shown in Table 4.8. Also, there was no significant difference in kidney indices in both sexes (female: $p = 0.7621$; male: $p = 0.7082$).

Two-way ANOVA showed a significant main effect of sex on absolute kidney mass when the female rats were compared with the male rats, such that absolute kidney mass was lower in females than in males. However, absolute kidney mass was not altered by dietary treatment, and there was no interaction between sex and dietary treatment (Sex effect: $p < 0.0001$; treatment effect: $p = 0.2311$; interaction effect: $p = 0.2154$). While the same trend was observed when the kidney index of the female rats was compared to the male rats, the females had similar kidney index with the males (Sex effect: $p = 0.0183$; treatment effect: $p = 0.9941$; interaction effect: $p = 0.4961$).

Table 4.8: A summary of the mean and median values of the absolute kidney mass and kidney index for the female and the male rats across the different experimental groups.

	PG		TA		SAC		SAC+TA		p
	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	Mean $\pm$ SD	Median	
Female (F)									
Kidney mass (g)	1.57 $\pm$ 0.07 [#]	1.56	1.65 $\pm$ 0.09 ^{\$}	1.62	1.61 $\pm$ 0.14 [€]	1.55	1.60 $\pm$ 0.09 ^Δ	1.60	0.4897
Body mass (g)	255.8 $\pm$ 17.73	254.50	266.30 $\pm$ 9.27	265.00	268.60 $\pm$ 21.57	261.50	263.90 $\pm$ 18.01	263.50	0.9998
Kidney index (%)	0.61 $\pm$ 0.03	0.62	0.62 $\pm$ 0.04	0.62	0.60 $\pm$ 0.03	0.57	0.61 $\pm$ 0.04	0.58	0.7702
Male (M)									
Kidney mass (g)	2.58 $\pm$ 0.14 [#]	2.55	2.57 $\pm$ 0.16 ^{\$}	2.57	2.52 $\pm$ 0.19 [€]	2.52	2.49 $\pm$ 0.25 ^Δ	2.50	0.7738
Body mass (g)	438.00 $\pm$ 31.36	426.00	443.80 $\pm$ 24.77	439.00	421.70 $\pm$ 17.73	414.00	422.90 $\pm$ 35.85	423.00	0.9999
Kidney index (%)	0.59 $\pm$ 0.02	0.59	0.58 $\pm$ 0.02	0.58	0.60 $\pm$ 0.04	0.59	0.59 $\pm$ 0.05	0.59	0.7082

For each parameter, no significant differences were observed across treatment groups in either sex ($p > 0.05$), based on One-Way Analysis of variance, except for Kidney index (female and male), which were analysed using Kruskal-Wallis test.

When the females were compared to the males in the same group, the same superscript symbols (#, \$, €, and Δ) indicate a significant difference at $p < 0.05$ using a Two-way Analysis of variance followed by Tukey's post hoc test.

SD= Standard Deviation, PG = plain gelatine ($F = 8$, $M = 8$), TA = TA-50mg/kg and plain gelatine ($F = 8$, $M = 8$), SAC = 20% fructose, 10% alcohol and gelatine ($F = 8$, $M = 7$), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($F = 8$, $M = 8$).

4.4.2 Effect of TA and SAC on serum KIM-1 concentration

The effect of TA and SAC on the serum KIM-1 concentration of the female (a) and male rats (b) across the treatment groups is shown in Figure 4.9. The KIM-1 concentration was similar across the treatment groups in both sexes of rats (female: $p = 0.3413$; male: $p = 0.1275$). KIM-1 concentration was not altered by sex and dietary treatment, such that the female rats had similar KIM-1 concentration when compared with their male counterparts across the treatment groups (Sex effect: $p = 0.8121$; treatment effect: $p = 0.3459$). Also, there was no interaction between sex and dietary treatment (interaction effect: $p = 0.1325$).

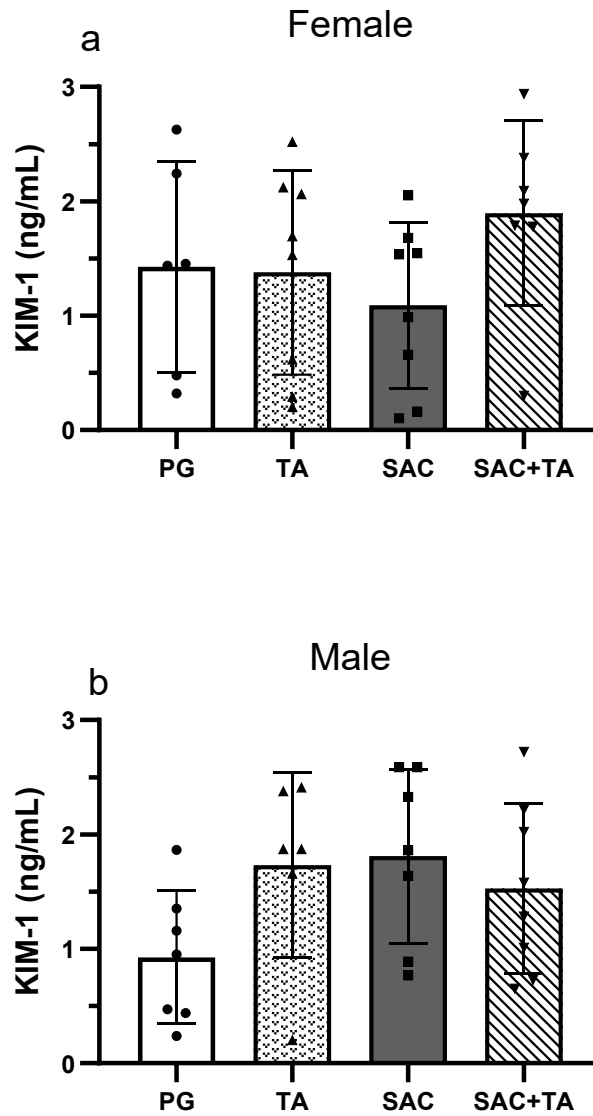


Figure 4.9: Plots showing the effect of SAC and TA on the KIM-1 concentration of the female rats (a) and the male rats (b). There were no differences in the KIM-1 concentrations across the groups in both sexes of rats. *Data expressed as mean $\pm$ Standard deviation, $p > 0.05$ ANOVA. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.4.3 Effect of TA and SAC on serum NGAL concentration

The effect of TA and SAC on the serum NGAL concentration of the female (a) and male rats (b) across the treatment groups is shown in Figure 4.10. The NGAL concentration was similar across the treatment groups in both sexes of rats (female: $p = 0.3792$; male: $p = 0.5795$). Two-way ANOVA showed that NGAL concentration was not altered by sex and dietary treatment, such that the female rats had similar NGAL concentration when compared with their male counterparts across the treatment groups (Sex effect: $p = 0.6081$; treatment effect: $p = 0.7365$). Similarly, there was no interaction between sex and dietary treatment (interaction effect: $p = 0.2683$).

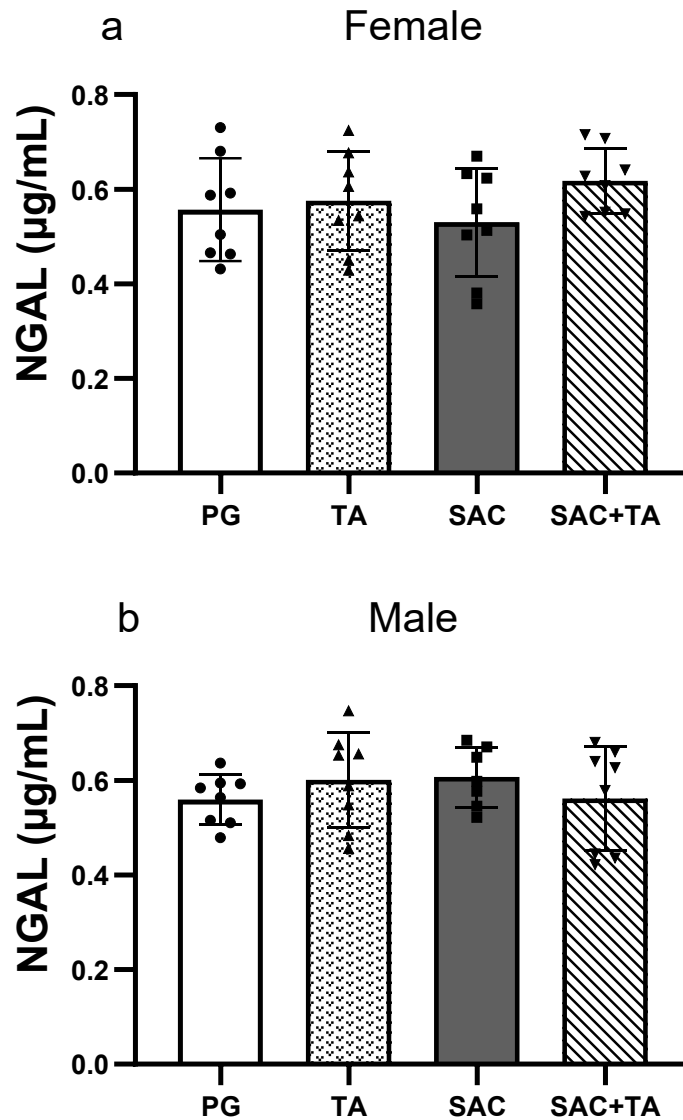


Figure 4.10: Plots showing the effect of SAC and TA on the NGAL concentration of the female rats (a) and the male rats (b). There were no differences in the NGAL concentrations across the groups in both sexes of rats. *Data expressed as mean $\pm$ Standard deviation, $p > 0.05$ ANOVA. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.4.4 Effect of TA and SAC on kidney TBARS concentration

The effect of TA against the effect of SAC on kidney TBARS concentration of the female (a) and male rats (b) is shown in Figure 4.11. There was no significant difference in the kidney TBARS concentration across the treatment groups in both sexes (females: $p = 0.7408$; males: $p = 0.6822$). Kidney TBARS concentration was altered by sex, such that females had higher TBARS levels than males (Sex effect: $p = 0.0139$). However, TBARS levels were not altered by dietary treatment and there was no interaction between sex and dietary treatment (treatment effect: $p = 0.7677$; interaction effect: $p = 0.6518$).

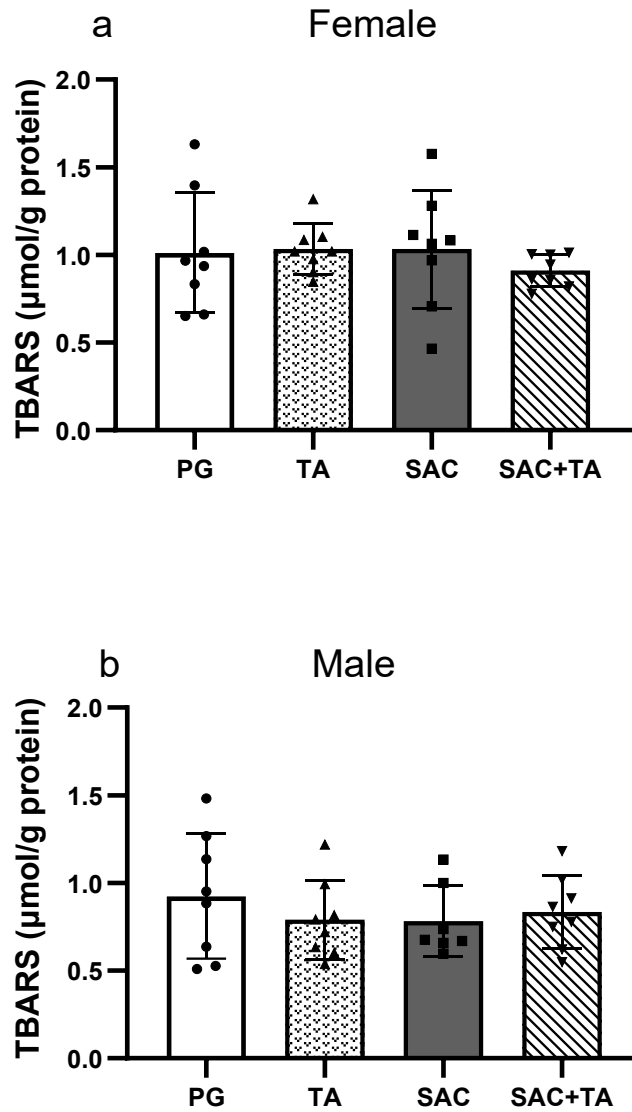


Figure 4.11: Plots showing the effect of SAC and TA on the kidney TBARS concentration of the female rats (a) and the male rats (b). There were no differences in the TBARS concentration across the groups in both sexes of rats. *Data expressed as mean $\pm$ Standard deviation, $p > 0.05$ ANOVA. ANOVA = Analysis of variance, PG = plain gelatine ($n = 8$ females, 8 males), TA = TA-50mg/kg and plain gelatine ($n = 8$ females, 8 males), SAC = 20% fructose, 10% alcohol and gelatine ($n = 8$ females, 7 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ($n = 8$ females, 8 males).*

4.4.5 Effect of TA and SAC on kidney morphometry

The effect of TA against the effect of SAC on urinary space, Bowman's capsule area, and glomerular tuft area are shown in Table 4.9. The representative photomicrographs of the Haematoxylin and Eosin-stained kidney sections of (A) the female and (B) the male rats are shown in Figure 4.12.

In the females, the urinary space was significantly higher in the TA and the SAC group when compared to the PG group ($p < 0.0001$). In the males, the urinary space of the SAC group was significantly lower than the other treatment groups ($p < 0.001$).

Females in the TA group had significantly higher Bowman's capsule area than PG ($p < 0.001$). However, there was no difference in Bowman's capsule area across the treatment groups in the males ($p > 0.05$).

Similarly, the female rats in the TA ($p = 0.0027$) and the SAC+TA ($p = 0.0366$) groups had significant increases in the glomeruli tuft area compared to PG group. However, the male rats in the TA group had significantly lower glomeruli tuft area than the PG ($p = 0.0348$), SAC ($p < 0.001$), or SAC+TA ($p = 0.0493$) group.

Furthermore, two-way ANOVA showed that urinary space was not altered by sex and dietary treatment, such that females had similar urinary space to males (sex effect: $p = 0.1021$; treatment effect: $p = 0.1240$). However, there was a significant interaction between sex and dietary treatment ($p < 0.0001$). Bowman's capsular area was altered by sex but not by dietary treatment, such that females had lower Bowman's capsule area than males (sex effect: $p = 0.0003$; treatment: $p = 0.5735$). However, there was a significant interaction between sex and diet (interaction effect: $p < 0.0001$). Similarly, the glomeruli area was altered by sex but not by dietary treatment, such that females had lower glomeruli area than males (sex effect: $p < 0.0001$; treatment effect: $p = 0.2692$). There was significant interaction between sex and diet (interaction effect: $p < 0.0001$).

Table 4.9: A summary of the mean and median values of the kidney morphological area for the female and the male rats across the different experimental groups.

	PG		TA		SAC		SAC+TA		P
	Mean $\pm$ SD ($\times 10^3$)	Median ($\times 10^3$)	Mean $\pm$ SD ($\times 10^3$)	Median ($\times 10^3$)	Mean $\pm$ SD ($\times 10^3$)	Median ($\times 10^3$)	Mean $\pm$ SD ($\times 10^3$)	Median ($\times 10^3$)	
Female (F)									
Bowman's capsule area (μm^2)	5.14 $\pm$ 1.61 ^{a#}	5.12	6.14 $\pm$ 1.5 ^{a€}	6.19	5.54 $\pm$ 1.59 ^d	5.51	5.77 $\pm$ 1.65 ^{\$}	5.59	0.0011
Glomeruli area (μm^2)	3.76 $\pm$ 1.25 ^{\$}	3.80 ^{ab}	4.46 $\pm$ 1.38	4.52 ^a	3.78 $\pm$ 1.12 [#]	3.81 ^c	4.28 $\pm$ 1.45	4.13 ^{bc}	0.0002
Urinary space (μm^2)	1.38 $\pm$ 0.73	1.27 ^{ab}	1.68 $\pm$ 0.60	1.66 ^a	1.76 $\pm$ 0.86	1.57 ^b	1.49 $\pm$ 0.59	1.46	0.0017
Male (M)									
Bowman's capsule area (μm^2)	6.80 $\pm$ 1.81 [#]	6.65	6.23 $\pm$ 1.64 [€]	5.946	6.53 $\pm$ 1.77 ^d	6.19	6.57 $\pm$ 1.72 ^{\$}	6.46	0.1823
Glomeruli area (μm^2)	4.86 $\pm$ 1.23 ^{\$}	4.69 ^a	4.44 $\pm$ 1.33	4.42 ^{abc}	5.24 $\pm$ 1.36 [#]	5.22 ^b	4.87 $\pm$ 1.54	4.80 ^c	0.0139
Urinary space (μm^2)	1.94 $\pm$ 1.07	1.71 ^a	1.79 $\pm$ 0.71	1.69 ^b	1.29 $\pm$ 0.74	1.05 ^{abc}	1.70 $\pm$ 0.81	1.56 ^c	<0.0001

For each parameter, the same superscript letters (a, b, c, and d) indicate a significant difference between any two groups at $p < 0.05$ based on Kruskal-Wallis followed by Dunn's post hoc test, except for Bowmann's capsule area (female and male), which were analysed using One-Way Analysis of variance followed by Tukey's post hoc test.

The same superscript symbols (#, \$, €, and Δ) indicate a significant difference at $p < 0.05$ when the females were compared to the males in the same group using a Two-way Analysis of variance followed by Tukey's post hoc test.

SD = Standard deviation; PG = plain gelatine (no of images assessed: F = 94 , M = 90), TA = TA-50mg/kg and plain gelatine (no of images assessed: F= 68, M= 86), SAC = 20% fructose, 10% alcohol and gelatine (no of images assessed: F =113, M= 55), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ((no of images assessed: F= 70, M = 127).

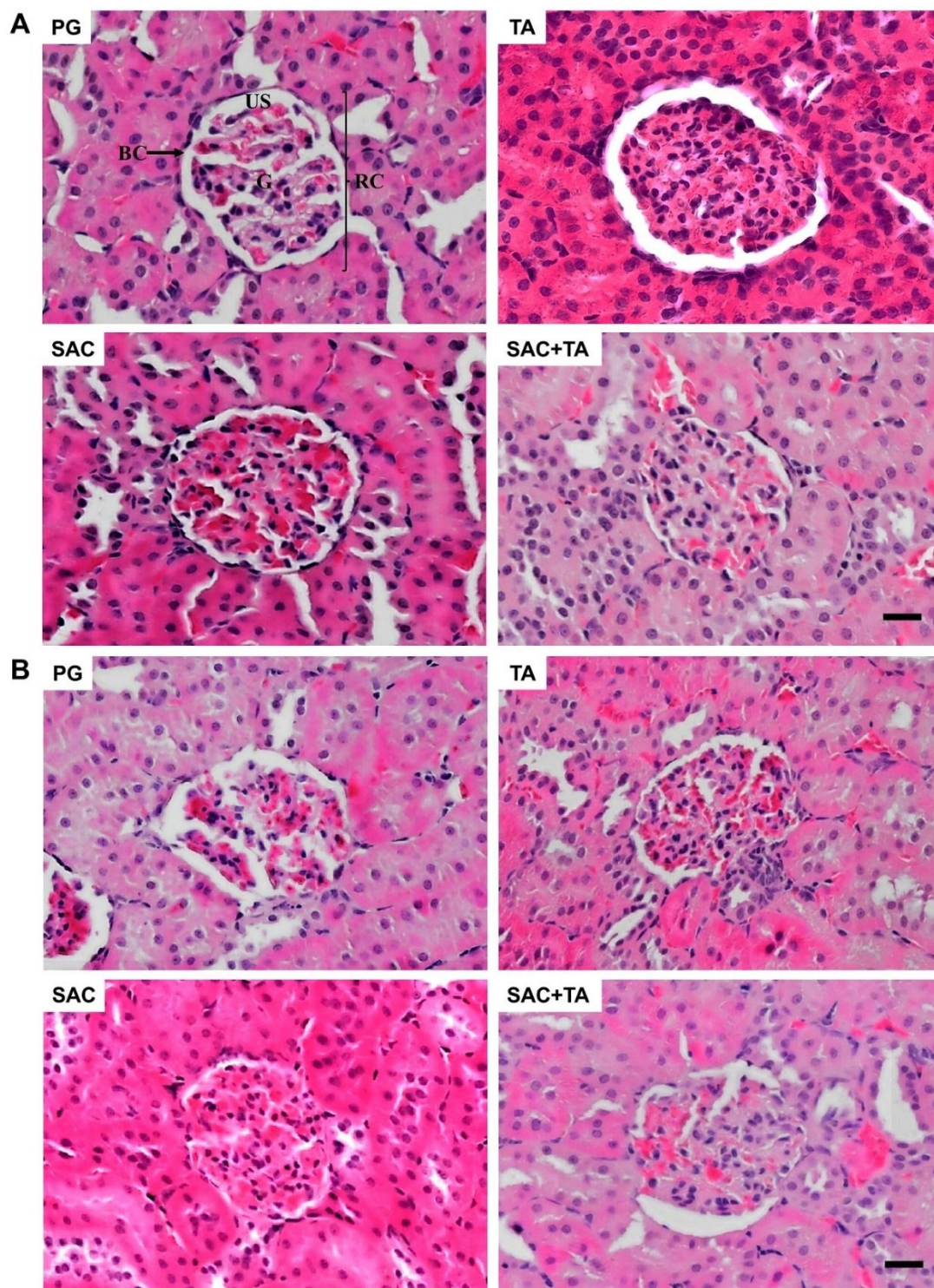


Figure 4.12: Representative photomicrographs of the kidney of (A) the female and (B) the male rats. The sections were stained with Haematoxylin and Eosin. Scale bar = 20 μ m. The renal corpuscles (RC) consist of the Bowman's capsule (BC), which encloses the glomerulus (G), with the urinary space (US) separating them. *PG* = plain gelatine, *TA* = TA-50mg/kg and plain gelatine, *SAC* = 20% fructose, 10% alcohol and gelatine, *SAC+TA* = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine.

4.4.6 Effect of TA and SAC on the percentage area of collagen distribution

The effect of TA against the effect of SAC on the percentage area of collagen distribution is shown in Figure 4.13. The representative photomicrographs of the kidney sections of (A) the female and (B) the male rats stained with Masson trichrome is shown in Figure 4.14.

In the females (Figure 4.12a), there were significant increases in the collagen ratio of the SAC or SAC+TA groups when compared with the PG or TA groups ($p < 0.001$). Also, the female SAC+TA group had significantly higher collagen distribution than SAC alone ($p = 0.0038$). Similarly, in the male rats (Figure 4.12b), the percentage area of collagen distribution was significantly higher in the TA, SAC, or SAC+TA groups when compared to the PG group ($p < 0.001$). The male rats in the SAC or SAC+TA groups had a significantly higher percentage area of collagen distribution than the TA group ($p < 0.001$). However, the male rats in the SAC+TA group had a significantly reduced percentage area of collagen distribution than the SAC group ($p = 0.0353$).

Two-way ANOVA revealed that the percentage area of collagen distribution was altered by sex as well as diet (Sex effect: $p = 0.0001$; treatment effect: $p < 0.0001$), and there was significant interaction between sex and diet (interaction effect: $p < 0.0001$).

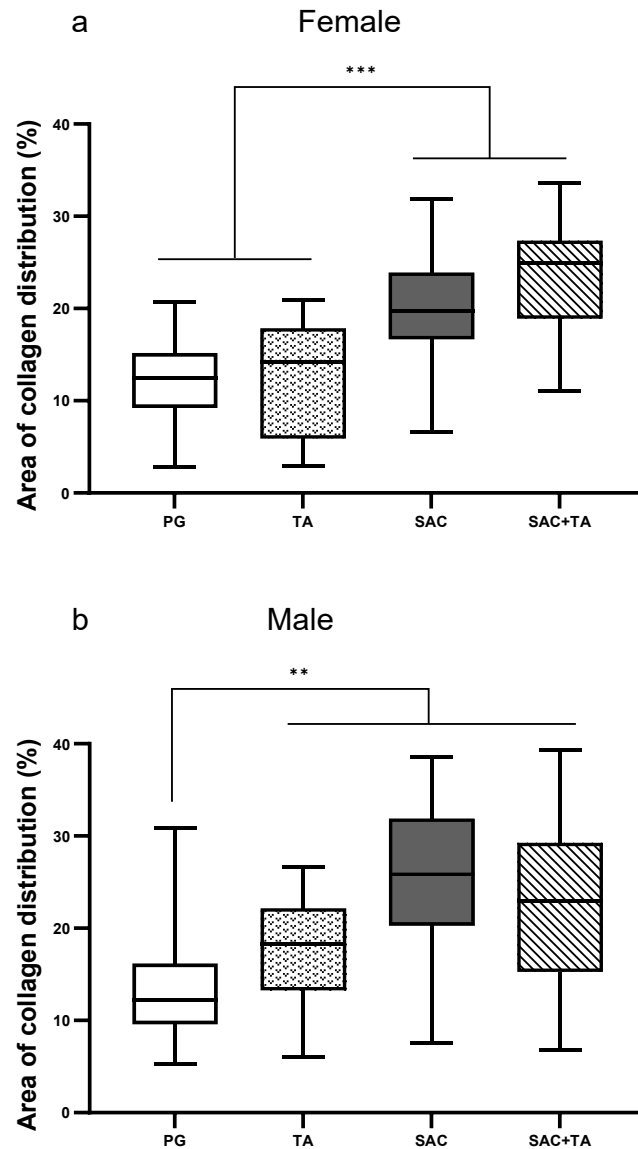


Figure 4.13: Box plots showing the effect of SAC and TA on the percentage area of kidney collagen distribution for (a) the female and (b) the male rats. In the female rats, SAC and SAC+TA had a higher percentage area of collagen distribution than PG and TA. While in the male rats, TA SAC, and SAC+TA groups had a significantly higher percentage area of collagen distribution than PG. Data expressed as median with range, *** = $p < 0.001$, ** = $p < 0.01$, Kruskal Wallis, Dunn's post hoc test. PG = plain gelatine (no of images assessed = 93 females, 73 males), TA = TA-50mg/kg and plain gelatine (no of images assessed = 75 females, 75 males), SAC = 20% fructose, 10% alcohol, and gelatine (no of images assessed = 96 females, 75 males), SAC+TA = 20% fructose, 10% alcohol, TA-50mg/kg and gelatine ((no of images assessed = 75 females, 105 males).

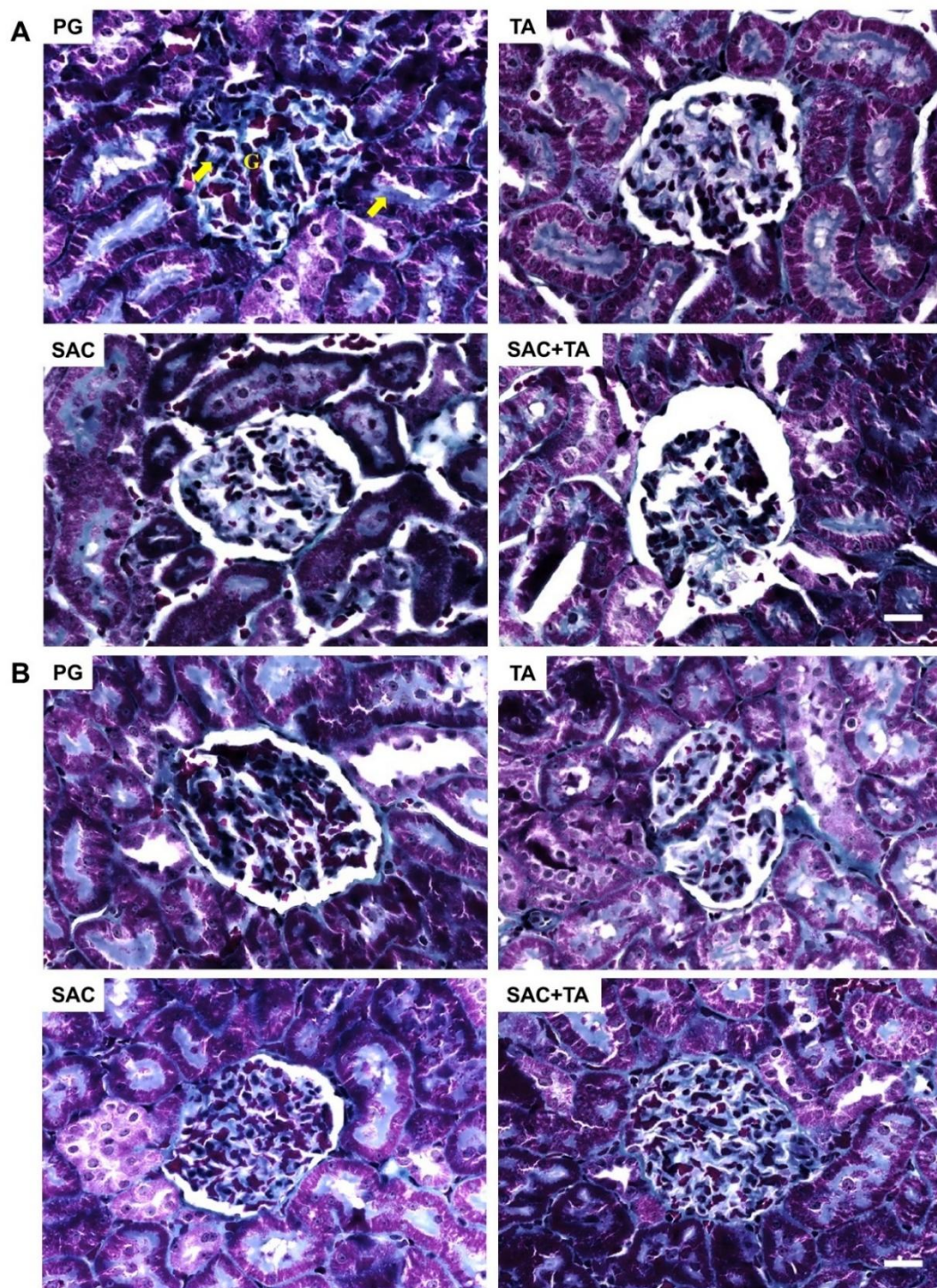


Figure 4.14: Representative photomicrographs of the kidney of (A) the female and (B) the male rats. The sections were stained with Masson trichrome. The yellow arrows indicate some areas of collagen in the kidney. Scale bar = 20 μ m. *PG* = plain gelatine, *TA* = TA-50mg/kg and plain gelatine, *SAC* = 20% fructose, 10% alcohol and gelatine, *SAC+TA* = 20% fructose, 10% alcohol, TA--50mg/kg and gelatine.

The next chapter analyses and contextualises the findings, comparing them with existing literature and expanding on them based on the research results. It examines the potential role of TA in alleviating metabolic, hepatic, and renal impairments linked to SAC consumption, providing insights into the biological mechanisms behind these effects.

CHAPTER FIVE

DISCUSSION

Aim and summary of findings

This study examined the effects of voluntary TA and SAC (in gelatine) consumption on the metabolic, hepatic and renal health of adolescent female and male Sprague-Dawley rats. SAC reduced feed intake in both sexes without affecting body mass gain or visceral fat accumulation. Neither TA nor SAC influenced fasting blood glucose, insulin concentration, or HOMA-IR in either sex. While serum triglyceride levels and TG/HDL-C ratios remained unaffected, moderate intake of SAC increased HDL-C in male rats. There was no impact on liver mass, hepatosomatic index, or liver mRNA expression of specific markers of metabolism and inflammation. However, female rats' livers showed increased TBARS concentrations with TA and SAC consumption, while males in the SAC group had decreased TBARS levels. Histological analyses indicated reduced hepatocyte density due to TA or SAC in the females, and only with combined TA and SAC consumption in the males. While male rats had higher micro steatosis scores from SAC consumption, females exhibited higher micro and macro steatosis scores than males. Also, collagen distribution was unaffected in the females but was reduced in the TA alone or SAC alone in the males.

There were no changes in absolute kidney mass or kidney index across the treatment groups in both sexes, suggesting that moderate intake of SAC and TA does not affect kidney mass. Although females exhibited lower absolute kidney mass, they had similar kidney index when compared with males. There were no differences in circulating KIM-1 and NGAL concentrations between groups, indicating the absence of proximal tubule injury or general renal damage from these dietary interventions. The kidney tissue lipid peroxidation measured as TBARS (oxidative stress marker) was not affected by SAC consumption in either sex, although females had higher TBARS concentrations than males. Renal glomeruli morphometry revealed increased urinary space in females consuming TA and SAC, while males consuming SAC experienced a decrease in urinary space. Additionally, TA, alone or combined with SAC, increased glomerular tuft area in females but caused glomerular atrophy in males. Sex differences were observed in the glomerular tuft and Bowman's capsule areas, with females showing smaller area measurements compared to males; however, urinary space was similar between sexes. SAC consumption, with or without TA, increased renal fibrosis in both sexes.

Notably, TA exacerbated SAC-induced fibrosis more in females than in males. These findings are discussed in detail in the following subsections of the discussion.

Feed intake

Rats consuming SAC with or without TA had reduced feed intake compared to the control or TA-only groups, likely due to the appetite-suppressing effects of alcohol (Bach, Koopmann and Kiefer, 2021) and sugar (de Oliveira *et al.*, 2020). Alcohol (Bach, Koopmann and Kiefer, 2021) and high-sugar diets (de Oliveira *et al.*, 2020) increase leptin secretion, a hormone that reduces feed intake by acting on the hypothalamus. Alcohol provides 7 kcal/g of energy, which has little nutritional content (Brenes *et al.*, 2021), while fructose provides 3.6 kcal/g (Mattar *et al.*, 2022), comparable to the rat feed energy content of 3.6 kcal/g, which includes all the necessary nutrients. Consequently, the higher energy content of alcohol reduced the rats' feed intake to balance their total energy needs (Kwon *et al.*, 2024). This aligns with a previous report where 10% ethanol reduced feed intake by increasing the satiety-inducing hormone, cholecystokinin (Richardson, E Rumsey and Read, 1990).

TA alone did not affect feed intake, contradicting reports of increased appetite in rats fed Western diets high in TA (Chung *et al.* 2019). In this study, the TA dose was much lower (0.005%) than that used by Chung *et al.* (2019), possibly accounting for the different effects of TA on appetite. When combined with SAC, TA did not alter the reduction in feed intake, indicating that the appetite suppression was most likely due to SAC alone, not TA. TA's potential bitterness, which could have discouraged feeding or lowered appetite in the rats (Osakabe *et al.*, 2024) was probably also masked by the fructose in the gelatine mixtures, resulting in the normal appetite of the TA group. Alterations in appetite and diet can lead to body mass changes.

Body mass

Dietary modification directly impacts body mass gain (Yannakoulia *et al.*, 2019). Therefore, we examined the rats' body mass gain during the study period. Despite expectations, the consumption of SAC did not result in increased body mass in rats, with similar body mass observed across all groups. This finding aligns with earlier studies where alcohol consumption did not affect body mass (Rasineni *et al.*, 2021). Although a high fructose diet was previously reported to increase body mass in Wistar rats receiving 20% fructose for 12 weeks (Kumar *et al.*, 2021), this was not the case in the present study. The balance between energy intake from

alcohol (Wilson and Matschinsky, 2020)–and fructose (Chiavaroli *et al.*, 2023) and energy expenditure likely resulted in similar body mass across the groups. TA also did not impact body mass gain, consistent with previous studies on its effects at low doses (Song *et al.*, 2021; Yu *et al.*, 2021). Contradictory reports suggesting TA reduces body mass (Nakamura *et al.*, 2001; Chung *et al.*, 2019; J. Wang *et al.*, 2023)–used higher doses than the present study.

Several factors influence body mass beyond feed intake, these include genetics (Loos and Yeo, 2022), age (Ghasemi, Jeddi and Kashfi, 2021), physical activity (L. Li *et al.*, 2021), and physiological factors like metabolic diseases (Kim, 2021). Obesity can be hereditary and hence influenced by genes, some of these genes are expressed in the central nervous system (Loos and Yeo, 2022). For example, the genetic impacts of leptin can cause extreme hunger and obesity in the ob/ob mouse or Zucker fatty rat (Zhong *et al.*, 2020). Similarly, age has been shown to impact susceptibility to obesity (Catalano *et al.*, 2005). In the present study, young Sprague-Dawley rats which had normal growth trajectories and were in their rapid growth phase were used. They likely channelled feed intake and calories into their growth to meet their high basal metabolic demands (Ghasemi, Jeddi and Kashfi, 2021). Although physical activity can affect metabolism and body mass (Bourdier *et al.*, 2023), the rats in this study were not subjected to forced exercise.

Visceral fat

Since body mass alone does not indicate the relative presence and distribution of body fat (Thomas *et al.*, 2013) and obesity comorbidities (Zhang *et al.*, 2023), visceral and epididymal fat accumulation were measured. Our findings showed no difference in visceral fat accumulation across groups in both the female and male rats and no differences in absolute or relative epididymal fat mass in males. This suggests that neither TA nor SAC consumption impacted visceral obesity and epididymal fat accumulation. While a high fructose diet is linked to increased abdominal fat (Ramos, Batista and Albuquerque, 2017), chronic alcohol consumption has been shown to inhibit visceral fat accumulation (Zhang *et al.*, 2015; Rasineni *et al.*, 2021). This finding of the current study could be due to the counter effects of combined consumption of alcohol and fructose or a balance in total energy intake from alcohol's appetite-regulating effects (Kovačević *et al.*, 2021) as well as the age of the rats (rapid growth phase).

Contrary to our findings, higher doses of TA have been reported to reduce high-fat diet-induced visceral fat accumulation (Kim *et al.*, 2008) and epididymal fat (Yu, Fan and Yang, 2024), but this effect was not observed with the reduced dose used in our study. Additionally, a sex

difference was noted in the current study, whereby females had a significantly higher percentage of relative visceral fat than males in all treatment groups except for the control. Females are at higher risk of abdominal obesity under obesogenic environments, which is due to low testosterone and high oestrogen levels promoting visceral fat (Zong *et al.*, 2020). This explains the higher relative visceral fat in females compared to males. Obesity and visceral fat accumulation are intricately linked to anaemia, prompting us to assess haematological parameters (Hiremath *et al.*, 2023).

Blood parameters

Haematological assessment can provide insights into the health of an individual (Zhanyi Zhang *et al.*, 2022). Our findings indicate that TA and SAC had no significant impact on blood indices, as haemoglobin (Hb), haematocrit (HCT), and mean corpuscular haemoglobin concentration (MCHC) remained consistent across all groups in both sexes of rats.

Alcohol consumption (5 g/kg) has been reported to induce haematological alterations, including anaemia, in mice by reducing HCT, Hb, and MCHC levels (Kipchumba *et al.*, 2023). However, these effects were observed at higher doses than the one used in our study, suggesting a dose-dependent effect. The anaemia-inducing impact of alcohol has been attributed to impaired intestinal absorption of essential hematopoietic nutrients and the activation of reactive oxygen species (ROS) during ethanol metabolism (Ferrao, Ali and Mehta, 2022).

Similarly, a previous study on fructose's effects on blood parameters found that while haemoglobin levels remained unchanged, MCHC decreased in broiler chickens (Altuğ, Şen and Kabakçı, 2024). This reduction was associated with increased haematocrit levels, which remained unchanged in the present study and elevated mean corpuscular volume (MCV), which was not measured. The observed differences may also be due to species-specific variations.

TA at high doses (10 g/kg) has been reported to induce anaemia in rats by reducing iron absorption (Ugur Calis *et al.*, 2016), but such an effect was not found in our study. Likewise, TA supplementation (125–1000 mg/kg) decreased haematocrit and haemoglobin levels in pigs (Lee *et al.*, 2010), whereas the 50 mg/kg dose used in our study had no impact on Hb, HCT, or MCHC. These findings suggest that the haematological effects of TA are dose dependent.

FBG, Insulin and HOMA-IR

We evaluated the effects of TA and SAC on glucose homeostasis. Our findings revealed no significant differences in fasting blood glucose (FBG) or insulin levels among the groups,

suggesting that neither TA nor SAC had a measurable impact on glucose homeostasis. This aligns with other studies showing no significant impact of sugar (de Oliveira *et al.*, 2020) or alcohol (Rasineni *et al.*, 2016) consumption on hyperglycaemia risk. However, it is also important to note that high-sugar diets can be a risk factor for type 2 diabetes mellitus (Veit *et al.*, 2022). The impact of high sugar on glycaemic control is thus impacted by factors including age, duration of consumption and quantity consumed as well as the type of sugar (Ma *et al.*, 2022).

While alcohol-fed rats in previous studies had decreased insulin levels due to altered pancreatic functions (Rasineni *et al.*, 2016), this was not the case, probably due to the lower alcohol dose used in the present study. Chronic alcohol consumption is linked to glucose intolerance and insulin resistance (Kim and Kim, 2012), but our study found no effect of moderate SAC intake on HOMA-IR in either sex. Additionally, TA reduced insulin levels in high-fat diet-fed mice (J. Fang *et al.*, 2022), suggesting possible species differences when compared to the present study.

TG, HDL-C and TG/HDL-C ratio

To understand the potential impact of the interventions on lipid homeostasis, the effects of TA and SAC on serum lipid concentration were determined. The present study found that the triglyceride (TG) concentrations and the TG/HDL-C ratio were not negatively impacted by TA or SAC consumption. Alcohol consumption, known to upregulate triglyceride production (Rasineni *et al.*, 2021), did not increase TG levels in the young rats used in the present study, which aligns with the previous findings that low-to-moderate alcohol intake has protective effects on lipid metabolism (Justice *et al.*, 2019). While some studies reported increased TG levels because of the consumption of high-fructose diets (Milutinović *et al.*, 2020), the present findings were consistent with the study that reported no significant difference in TG concentrations of young rats consuming diets enriched with fructose (De Castro *et al.*, 2013). Additionally, high fructose diets have been reported to increase TG/HDL-C ratios due to oxidative stress (Surour, Ramadhan and Khudair, 2022), but this relationship was not detected in the present study. The present findings on the TG/HDL-C ratio align with the HOMA-IR results, indicating that insulin resistance was not induced.

Notably, SAC increased HDL-C concentrations in the male rats, but no difference was observed in the female rats. Previous studies on the effect of fructose on HDL-C in male rats were contradictory. Benado *et al.* (2004) reported an increase, while a decrease at higher doses was reported by Elseweidy *et al.* (2022). The present study thus suggests that moderate fructose consumption may benefit lipid status by increasing HDL-C concentrations in males. Moderate consumption of alcohol, on the other hand, has been shown to increase the HDL-C concentration of male rats (Kwon *et al.*, 2024). Taken together, the impact of fructose and alcohol could explain the increased concentration of HDL-C in the male rats (unlike in the females) following the moderate consumption of SAC. A previous study has reported that TA alone did not affect serum HDL-C at 100 mg/kg, 200 mg/kg and 400 mg/kg in mice (Wang *et al.*, 2023) which is consistent with the present findings that used a lower dose in rats.

Furthermore, assessing interventions in preclinical studies necessitates a thorough evaluation of organ structure and function (Sellers *et al.*, 2007). The liver and kidneys are among the primary target organs affected by alcohol and high-sugar diet-induced metabolic dysfunction. These are discussed in turn.

The Liver

Liver mass

Alterations in organ mass are widely acknowledged as a key sign of chemically induced damage to organs (Lazic, Semenova and Williams, 2020). This prompted the investigation into the effects of TA and SAC intake on liver mass. Liver mass can be influenced by factors such as age, diet, disease, and body mass (Kromrey *et al.*, 2018), as well as the accumulation of substrates, inflammation, and toxins (Qing *et al.*, 2022). The present findings showed that liver mass was not impacted by TA and SAC intake in both sexes. This is consistent with reports by Taoto *et al.* (2024), who showed that the administration of alcohol in rats at a dose of 40% w/v for 8 weeks had no impact on the liver mass. On the other hand, Rasineni *et al.* (2021) reported that a Lieber-DeCarli alcohol liquid diet (containing 36% ethanol) for 8 weeks increased the liver mass of Wistar rats (Rasineni *et al.*, 2021).

Additionally, while high fructose consumption can increase liver mass via increased steatosis (Mengesha, Sekaran and Mehare, 2021) results from the present study are consistent with the earlier findings that a high fructose diet did not impact both absolute and relative liver mass

(Milutinović *et al.*, 2020). The lack of effect of the combined consumption of fructose and alcohol on the liver mass indicates that there was no negative impact of SAC on the liver mass. Additionally, a study reported that dietary supplementation with 1% TA reduced liver mass in rats (Kim *et al.*, 2008), but this higher dose contrasts with our findings.

Liver TBARS

The present study examined the effects of SAC and TA on liver TBARS concentration, a marker of oxidative stress. Sexually dimorphic responses were observed in the TBARS concentrations in the liver tissues. In female rats, TBARS concentrations were elevated in both the TA-alone and SAC groups compared to the PG group. In contrast, male rats showed reduced hepatic TBARS levels in the SAC group, while the TA group exhibited levels comparable to the PG group. This indicates that oxidative stress was induced in the female rats that consumed TA or SAC alone but was decreased in the male rats that consumed SAC alone. The reduced TBARS levels observed in males treated with SAC may be linked to the earlier reported elevation in HDL-C levels in this group.

Additionally, both sexes of rats consuming SAC supplemented with TA showed TBARS levels statistically indistinguishable from the PG group, indicating a normalising effect of the treatment. This suggests that TA supplementation appeared to mitigate the impact of SAC on oxidative stress in both sexes. This can be attributed to the oxidative stress-reducing capacity of TA, as reported in the literature (Gülçin *et al.*, 2010; de Veras, da Silva and Cabral Ribeiro, 2021). These antioxidant properties of TA are mediated, in part, by its inhibition of enzymes such as glutathione S-transferase and NAD(P)H:quinone oxidoreductase 1 (NQO1), which are involved in the activation of xenobiotic compounds (Karakurt and Adalı, 2011).

Supporting the present findings of significant sex and interaction effects on TBARS levels, a clinical trial on alcoholics revealed that females were more susceptible to alcohol-induced lipid peroxidation than males (Lonappan *et al.*, 2024). Female alcoholics had increased lipid peroxidation, evidenced by higher TBARS levels, which were attributed to elevated products of alcohol and lipid metabolism (Lonappan *et al.*, 2024). Notably, TA supplementation prevented the SAC-induced increase in hepatic oxidative stress in the females, likely due to its antioxidant potential to inhibit reactive oxygen species (ROS) production (Bona *et al.*, 2022).

Hepatic gene expression of metabolism (CYP2E1 and SREBP-1)

To better understand the mechanisms underlying metabolic-associated liver disease and lipid peroxidation, the expression of genes related to alcohol metabolism (Cytochrome P450 2E1, *CYP2E1*) and lipid metabolism (Sterol regulatory element-binding protein-1, *SREBP-1*) were examined. Our study found no differences in *CYP2E1* concentrations across the groups in both sexes. While a report indicates that chronic alcohol consumption does not affect *CYP2E1* levels (Buyco *et al.*, 2023), another reported a dose-dependent increase in *CYP2E1* expression with high alcohol consumption (Sandoval *et al.*, 2022). The null findings reported by Buyco *et al.* (2023) were attributed to a diminished cellular stress response during the early stages of alcohol-induced metabolic dysfunction. A similar explanation may apply to the present study, where voluntary moderate SAC consumption did not significantly induce the microsomal ethanol-oxidising system (MEOS).

Although *SREBP-1*, a key regulator of hepatic lipid metabolism, is typically upregulated in response to high-fructose diets (Ferré, Phan and Foulfelle, 2021) and alcohol consumption (Yu *et al.*, 2022), as expected with SAC intake in the present study, no significant changes in *SREBP-1* mRNA expression were observed. Also, TA did not impact *SREBP-1* mRNA expression in either sex. This finding aligns with previous studies showing that TA at 50 mg/kg dose had no effect on liver mRNA levels of *SREBP-1* in mice on a high-fat diet but inhibited its expression at 150 mg/kg (J. Fang *et al.*, 2022), indicating a dose-dependent effect.

Furthermore, no sex differences were observed in the expression of *CYP2E1* and *SREBP-1*. The unchanged expression of these genes, despite differences in oxidative stress, may be due to the activation of alternative oxidative stress pathways, such as NF-E2–related factor (Nrf2) or peroxisome proliferator–activated receptor alpha (PPAR α), (Li *et al.*, 2023), which were not examined in the present study.

Hepatic gene expression of inflammatory markers (IL-10, NF- κ B1 and TNF- α)

The effect of TA and SAC on inflammatory cytokines [Interleukin-10 (*IL-10*), Nuclear factor kappa B1 (*NF- κ B1*), and Tumour necrosis factor- α (*TNF- α*)] in the liver were examined. The present findings showed that the mRNA expression levels of *IL-10*, *NF- κ B1*, and *TNF- α* were also similar across the treatment groups, suggesting that neither SAC nor TA, induced hepatic inflammation. A previous study reported a dose-dependent decrease in *IL-10* concentration and an increase in *TNF- α* levels in Sprague Dawley rats on high fructose diet for 20 weeks (Wang

et al., 2020) which was not the case in the present study that used a lower fructose dose and a shorter treatment duration. Reports from other studies are also consistent with the present findings, showing that hepatic *IL-10* and *TNF-α* levels were not affected by fructose-enriched beverages (Melo *et al.*, 2021) and 10% ethanol (Kołota *et al.*, 2020).

Although higher alcohol concentrations (40% w/v) have been reported to increase *TNF-α* expression (Li *et al.*, 2018), this was not noted in the present study. The differences may be due to the lower alcohol concentration (10% w/v) used in the present study. Additionally, the TA at a 50 mg/kg dose did not affect *TNF-α* levels in mice on a high-fat diet, but it reduced them at a 150 mg/kg dose, which is consistent with the present findings and suggests a dose-dependent effect.

NF-κB, which regulates the expression of pro-inflammatory genes such as *TNF-α* and *IL-10* (Nowak and Relja, 2020), showed similar levels across the treatment groups. This indicates that TA and SAC did not impact *NF-κB* expression and could be linked to their lack of effect on the expression of pro-inflammatory genes (*TNF-α* and *IL-10*). Previous studies reported an increase in *NF-κB* mRNA expression in rats consuming high doses of ethanol (Li *et al.*, 2018) or fructose (Iskender *et al.*, 2022), but these doses were higher than those used in the present study. The 10% ethanol dose used in this study was selected to represent a low-to-moderate level, ensuring consistent intake through a voluntary consumption method. Based on the formula by Reagan-Shaw *et al.* (2008), this is equivalent to approximately 32 g of ethanol per day in humans.

Overall, the similarities in mRNA expression of metabolism and inflammation-related genes across treatment groups could explain the lack of impact on liver mass, suggesting that inflammation was not induced. However, the mRNA expression findings may be limited by sample size, tissue collection timing, assay sensitivity, or mRNA–protein discrepancies and may not fully exclude inflammatory effects. Similarly, the absence of sex differences in inflammatory gene expression, despite observed differences in oxidative stress markers, may reflect the limitations previously outlined.

Liver cell density

Histological analysis, which is the gold standard for diagnosing liver pathology (Tannapfel *et al.*, 2011), was used to examine the impact of the interventions on hepatic architecture. Female rats in all treatment groups exhibited lower liver cell density compared to the control, while the

male rats that consumed SAC supplemented with TA showed a significantly reduced liver cell density when compared to the control or the TA alone. Voluntary SAC consumption had a greater negative impact on female rats, reducing hepatocyte density more than in males. This suggests increased cellular injury in the hepatic structure due to the combined effects of TA and SAC in this sex group. While high fructose diets can reduce hepatocyte count due to altered liver triglyceride and cholesterol levels (Carvalho *et al.*, 2021), TG levels were not altered in the present study.

Reports suggest that increased expression of proliferating cell nuclear antigen (*PCNA*) after ethanol exposure leads to improved hepatocyte proliferation in male mice more than in female mice (Li *et al.*, 2019), potentially explaining sex differences observed in this study, though hepatocyte proliferation was not assessed. Females are more prone to alcoholic liver disease due to differences in alcohol metabolism and production of gastric and liver alcohol dehydrogenase, influenced by oestrogen activation of Kupffer cells and loss of hepatoprotective genes (Sayaf *et al.*, 2022). Also, women experience more severe alcohol liver injury progression than men, even with lesser alcohol exposure (Eagon and Mandell, 2010). Other molecular mechanisms influencing sex-specific responses to alcohol consumption include higher expression of *caspase-3* and *CYP2E1* in females (Li *et al.*, 2019). Although this study did not evaluate the pro-apoptotic enzyme *caspase-3*, the *CYP2E1* level was unaffected by sex.

Literature on the effect of TA on liver cell density in rodents is scant, but an *in vitro* study showed increased albumin synthesis in hepatocyte cultures with TA (Chen *et al.*, 2023). Similarly, TA suppressed the expression of enzymes responsible for hepatic cell death, caspase-1 and caspase-3, thereby inhibiting hepatocyte apoptosis in broilers (Niu *et al.*, 2022). The exact reasons for the reduction in hepatocyte density when SAC was supplemented with TA remain uncertain, suggesting a need for further research on the impact of TA on apoptotic markers.

Hepatic micro and macro vesicular steatosis

High-sugar diets have been implicated in the development of metabolic-associated fatty liver disease (Drożdż *et al.*, 2021). This can be assessed by visualisation of microscopic lipid droplets via histology. Micro steatosis presents with multiple small lipid droplets without nuclear displacement, while macro steatosis features large lipid globules displacing the nucleus (Tien *et al.*, 2021). Although micro and macro vesicular steatosis scores were similar across

the groups in female rats, the scores were higher in the females than in the males. This may be due to the higher visceral fat accumulation in the females. Consistent with the present findings, female rats on a control diet developed mild steatosis, while the male rats showed no pathological alterations (Kono *et al.*, 2000; Nanji *et al.*, 2002; Li *et al.*, 2019).

Alcohol consumption also increased the severity of steatosis in females compared to males in these previous studies. These sex differences were attributed to the upregulation of Cluster of differentiation 14 (*CD14*) proteins due to the ability of alcohol to induce the activation of Kupffer cells leading to the activation of the *NF- κ B* pathway and the upregulation of *TNF- α* in females. However, in the present study, *NF- κ B* and *TNF- α* genes were not significantly impacted by the interventions. Additionally, the upregulation of Heat shock proteins (*HSPs*) in male mice conferred hepatic protection by preventing the unfolding and misfolding of proteins due to ethanol exposure (Li *et al.*, 2019). Also, the greater severity of steatosis in females may have contributed to their reduced hepatocyte density, as noted in the literature (Walsh *et al.*, 2004).

Further supporting these sex differences, a study showed that adolescent female Wistar rats consuming a high fructose diet for 10 weeks had higher micro and macro vesicular scores, while the males had a mild micro steatosis (Hyer *et al.*, 2019). While SREBP1 levels were not impacted by fructose in the present study, long-term consumption of fructose can activate the *de novo* lipogenesis pathway via SREBP1-dependent or independent mechanisms (Yao *et al.*, 2023). In the male rats, SAC consumption resulted in higher micro-vesicular scores than the control or the TA groups, but similar macro-vesicular steatosis scores across the groups. This suggests that SAC consumption led to early stages of fatty liver development in the male rats but to a lesser severity than in the females. This could be attributed to the protective role of increased levels of circulating HDL-C against hepatic steatosis (Hamaguchi *et al.*, 2012).

Hepatic collagen distribution

Inactive lipids can cause hepatic injury, triggering the activation of hepatic stellate cells responsible for fibrogenesis (Garbuzenko, 2022). Thus, quantification of collagen accumulation in the Masson trichrome-stained liver sections to determine the impact of the interventions on liver fibrosis was performed. A sexually dimorphic response was observed, wherein moderate consumption of SAC reduced collagen formation in the male rats, while the female rats exhibited similar collagen distribution across the treatment groups. Collagen, crucial for liver tissue homeostasis, can become detrimental depending on its location and

distribution (Karsdal *et al.*, 2017, 2020). Despite reports on alcohol increasing collagen formation and degradation (Thiele *et al.*, 2021), this study found otherwise. Similar findings to the present study are seen in a previous study where moderate consumption of alcohol led to a reduction in collagen formation in male rats on a high-fat, high-cholesterol diet (Sun *et al.*, 2018). Another study found no fibrosis but steatosis in male rats consuming 15% fructose for 16 weeks (Fredriksen, Boardman and Meen, 2024).

Additionally, male rats in the TA group showed reduced collagen formation, consistent with earlier studies indicating that TA decreases carbon tetrachloride-induced liver fibrosis homeostasis by inhibiting the activities of hepatic stellate cells (HSCs) (Chu *et al.*, 2016). Similarly, TA possesses the ability to crosslink with collagen and modify its structure (Baldwin and Booth, 2022). There is a need to explore these mechanisms further.

The Kidney

Kidney mass

The lack of differences in absolute kidney mass across the treatment groups in both male and female rats and the similarity in kidney indices indicate that moderate intake of SAC and TA has no harmful impact on kidney mass in the rats used for this study. Similarly, previous research on alcohol alone demonstrated that rats receiving 10% ethanol for 12 weeks exhibited no changes in absolute or relative kidney mass compared to controls (Brzóska *et al.*, 2003). However, a higher dose of 20% ethanol over 12 weeks resulted in decreased kidney mass (Leal *et al.*, 2017), suggesting a dose-dependent effect of alcohol consumption. Similarly, a study on sugar intake found that a 20% fructose diet over 9 weeks did not alter kidney mass in rats (Milutinović *et al.*, 2020), which aligns with our current findings.

Furthermore, a study on the effects of dietary supplementation with TA (0.25% to 2%) on kidney mass found no differences compared to the control group (Barszcz *et al.*, 2018), supporting our finding that a low dose of TA poses no detrimental effect on kidney mass. This suggests that the interventions did not result in grossly observable impacts on the kidney mass. Similarly, the lack of effect of moderate sweetened alcohol or tannic acid intake on kidney mass may reflect the antioxidant properties of TA (Baldwin and Booth, 2022) or adaptive responses to renal injury by tubular epithelial cells (Li *et al.*, 2024).

A reduction in kidney mass would have been suggestive of atrophy of the kidneys, possibly due to toxicity or degenerative processes (Zhou *et al.*, 2021). On the contrary, an increase in kidney mass could have been indicative of hyperplasia, hypertrophy or inflammation (Jiang *et al.*, 2023). Sex differences were observed in absolute kidney mass, with males exhibiting higher values than females. It is well recognised that males tend to have larger kidneys than females due to their larger body size and mass (Dinca *et al.*, 2023). When the kidney mass was indexed to body mass, the females and males had similar kidney indices, indicating that the sex difference in absolute kidney mass was due to higher body mass in males. A lack of change in organ mass following an intervention does not suggest an absence of structural and functional pathology. Therefore, assays of specific biomarkers of kidney function and renal histomorphometry were performed.

Serum KIM-1 and NGAL

KIM-1 and NGAL are sensitive diagnostic biomarkers for acute renal injury (Atere *et al.*, 2021). KIM-1 is a specific marker for proximal tubule injury, with its production upregulated in proximal tubule cells following kidney injury, whereas NGAL upregulation indicates general renal injury (Kohl *et al.*, 2020). Our results showed no differences in KIM-1 and NGAL concentrations across the four treatment groups in both sexes of rats. Similarly, KIM-1 and NGAL did not differ between sexes. The lack of changes in serum KIM-1 and NGAL levels may reflect the absence of overt kidney damage, which would otherwise be expected to affect kidney mass.

There is limited literature on the combined effects of alcohol and fructose on these kidney injury markers. However, studies examining their individual effects have shown that alcohol consumption (6.4% v/v) for 4 weeks increased urinary KIM-1 and NGAL levels in rats. (Latchoumycandane, Nagy and McIntyre, 2014). It is noteworthy that KIM-1 is more reliably detected in urine than in serum (El-Esawy *et al.*, 2020), which may explain the absence of differences in our serum-based study. This suggests that mild renal injury may have gone undetected using the serum biomarkers employed. Additionally, the earlier study included a single binge dose of ethanol (31.5%) following a moderate dose of 5% ethanol, hence providing a high dose which could have caused acute injury, whereas ethanol administration in the current study was moderate and voluntary and less likely to induce acute kidney injury.

A previous study reported no differences in KIM-1 concentrations in mice fed a 30% fructose diet for 16 weeks compared to controls (Meléndez-Salcido *et al.*, 2024) which is consistent with our findings. Similarly, TA administration at 50 mg/kg did not affect KIM-1 expression in rats (Yesilkent and Ceylan, 2022). The direct effects of fructose on NGAL concentrations remain underexplored, but tungsten-based nanodots synthesised from TA and phosphotungstic acid reduced NGAL concentrations in renal tissue homogenates of mice with acute kidney injury (Huang *et al.*, 2023). This reduction was attributed to the ability of nanoparticles to penetrate renal tubular cells and maintain mitochondrial integrity and the protective capabilities of TA. The major difference from the previous study was the mode of TA administration: while the earlier study used nanoparticles, the current study utilised dietary supplementation of TA in its natural form.

Kidney TBARS

To examine whether the interventions increased oxidative stress on the kidneys as measured by lipid peroxidation, TBARS were measured in renal tissue homogenates. This study found no differences in kidney TBARS concentrations across treatment groups in either sex. These findings align with the results for kidney mass, KIM-1, and NGAL, suggesting no significant renal changes due to the interventions. Chronic alcohol consumption has been shown to increase kidney TBARS levels in adult Wistar rats after 12 weeks (Das *et al.*, 2008), but elevated lipid peroxidation was absent in our study, possibly due to a shorter study duration. Similarly, previous research demonstrated that high-fructose diets (65%) elevated TBARS levels compared to controls (Choi *et al.*, 2011); however, the fructose dose used in that study was higher than the 20% dose used in the present study.

In line with our findings, TA administration at 30 mg/kg resulted in kidney TBARS concentrations like those of controls (de Moraes Meine *et al.*, 2020). However, TA caused a dose-dependent decrease in cisplatin-induced TBARS production in rat kidneys (Akinyemi *et al.*, 2015). Similarly, tungsten-based nanodots synthesised from TA and phosphotungstic acid reduced TBARS concentrations in renal tissue homogenates of mice with acute kidney injury (Huang *et al.*, 2023), an effect attributed to the ability of nanoparticles to mitigate oxidative stress. The inhibitory effect of TA observed in previous studies was not evident in our study, likely due to differences in dose and mode of administration.

It is worthy of note that females exhibited higher kidney TBARS concentrations than males, which may be linked to increased lipid peroxidation induced by alcohol (Nanji *et al.*, 2002)

and fructose (Seraphim *et al.*, 2017) consumption in young adult females. Although oestrogen enhances renal mitochondrial activity and may offer protection against oxidative stress, this advantage is reportedly counterbalanced by the larger kidney mass observed in males (Sultanova *et al.*, 2020).

Renal glomerular morphometry

It is not uncommon to find that interventions do not alter the gross morphology of target organs and cause functional damage, but in the early stages, they can cause structural alterations at a microscopic level, which can then progress to more overt clinical manifestations with time. Consequently, a quantitative histological assessment of renal morphology was performed. The focus was on physiologically and functionally important parameters, namely, urinary space area, Bowman's capsule, and glomerular tuft areas.

Urinary space

Renal glomerular morphometry was altered in females consuming TA and SAC, with increased urinary space observed. In contrast, SAC consumption decreased urinary space in males. A recent study reported that a 20% fructose diet caused dilation of the urinary space, suggestive of glomerular hyperfiltration (Sarmiento *et al.*, 2025). While glomerular hyperfiltration helps maintain renal function (Kanbay *et al.*, 2024), it is a potential risk factor for early kidney damage in metabolic conditions (Czupryniak *et al.*, 2024). Additionally, increased urinary space may indicate tubular hypertension, a protective mechanism against elevated blood pressure resulting from glomerular injury (Tobar *et al.*, 2013). Previous studies documented increased urinary space due to alcohol consumption (Leal *et al.*, 2017).

Bowman's capsule and Glomerular tuft areas

In the current study, TA administration increased Bowman's capsule area in females, with no significant effects from SAC alone or in combination with TA in both sexes. Females showed a smaller Bowman's capsule area than males, likely due to their lower kidney mass (Dinca *et al.*, 2023).

While TA alone or in combination with SAC increased glomerular tuft area in females, the combination did not exacerbate this effect. In males, TA alone reduced glomerular tuft area, whereas its combination with SAC appeared to counteract this reduction, mitigating glomerular atrophy. SAC alone had no significant effect on glomerular tuft area in either sex. Although alcohol consumption has been associated with glomerular atrophy (Leal *et al.*, 2017), prior

studies on high-fructose diets align with our findings, showing no differences in glomerular tuft area and suggesting no glomerular dysfunction from fructose consumption (Nakayama *et al.*, 2010). The discrepancies between our findings and previous studies on the effects of alcohol may stem from longer durations (24–32 weeks) and higher ethanol doses (20%) used in earlier research.

TA appeared to induce glomerular hypertrophy in females and glomerular atrophy in males. Although kidney function markers such as KIM-1 and NGAL remained unchanged in this study, the observed glomerular hypertrophy may indicate a potential acute decline in renal function (Kashif *et al.*, 2021). The observed sex differences may be attributed to the greater glomerular mass in males, which enhances kidney functional reserve, offers protection against hyperfiltration-related injuries, and lowers the risk of glomerular capillary hypertension (Neugarten *et al.*, 2002). This protective effect may help account for the lower levels of kidney peroxidation observed in males in the current study.

Despite reports that TA may protect the kidney glomerular architecture from nephrotoxic substances (Israa *et al.*, 2019; Nasreldin *et al.*, 2022), this protective effect was not observed at the lower TA dose used in our study. Sex differences were also noted in measurements of glomerular tuft areas, with females exhibiting smaller areas than males. These differences could be attributed to the lower kidney mass in females (Dinca *et al.*, 2023).

Renal collagen distribution

Our study found that SAC consumption, with or without TA supplementation, increased collagen distribution in the kidneys, an indicator of fibrosis, in both males and females. This finding is consistent with the literature reporting the profibrotic effects of fructose and alcohol. Excess fructose in the kidney activates glycolysis, producing biosynthetic substrates that promote inflammation and fibrosis (Nakagawa and Kang, 2021). Similarly, chronic alcohol consumption has been shown to increase glomerular and peritubular fibrosis by activating *NOX*-mediated oxidative stress pathways (Sönmez *et al.*, 2012). In our study, we detected a profibrotic effect associated with combined sugar and alcohol consumption. SAC consumption resulted in greater collagen distribution in males compared to females, which may be linked to the reduced urinary space observed in this treatment group.

Notably, TA supplementation increased fibrosis in females more than in males. This could be due to heightened susceptibility to alcohol-induced kidney injury in females, which activates

the integrin $\beta1/JNK$ signalling pathway (Zhan *et al.*, 2024), leading to cell apoptosis—a precursor to kidney fibrosis (Portilla, 2014). TA alone was also associated with increased fibrosis in males. While the underlying mechanism remains unclear, it may be linked to TA's known ability to crosslink with collagen fibres (Sarker *et al.*, 2023), potentially promoting fibrotic tissue formation. Although TA is reported to have antifibrotic properties (Reed *et al.*, 2019), this effect was not found in our study, as TA showed limited efficacy in preventing the fibrosis caused by SAC consumption. Reed *et al.* (2019) administered 10 mg/kg of TA intraperitoneally to C57BL/6 mice for 14 days to treat bleomycin-induced pulmonary fibrosis. The differences observed compared to our study may be attributed to variations in the mode of administration and the species used.

The next chapter concludes the thesis by highlighting the study objectives and the key findings, emphasising their significance, and discussing study limitations and recommendations for future research directions.

CHAPTER SIX

CONCLUSION

The present study investigated the potential protective effect of TA, administered as a dietary supplement during adolescence, on the development of metabolic disorders associated with combined alcohol and sugar intake in female and male Sprague Dawley rats. A voluntary SAC consumption model was employed, using gelatine as the vehicle.

The first specific objective assessed whether TA could protect against the harmful effects of SAC on metabolic health and growth indicators. While SAC reduced feed intake in both sexes, it did not significantly affect visceral fat, glucose regulation, triglycerides, or haematological parameters. Notably, sex-specific differences emerged: females were more susceptible to SAC-induced visceral obesity, and TA did not prevent this fat accumulation. Additionally, SAC raised HDL-C levels in males but not in females. Taken together, the H_0 that adolescent TA supplementation does not influence metabolic outcomes associated with voluntary SAC intake in male and female Sprague Dawley rats was not rejected.

The second specific objective evaluated the protective potential of TA against the adverse effects of SAC on liver function and integrity. Key findings showed that neither TA nor SAC significantly affected absolute liver mass, hepatosomatic index, or the mRNA expression of metabolic and inflammatory liver markers (CYP2E1, SREBP-1, IL-10, NF- κ B1, and TNF- α).

Sex-specific differences were evident. SAC increased liver lipid peroxidation (TBARS) and reduced hepatocyte density in females but not in males. While SAC elevated hepatic steatosis in males, females exhibited generally higher steatosis scores overall. Interestingly, TA or SAC alone appeared to protect against liver fibrosis in males, but this effect was absent when both were combined and was not observed in females. Based on these findings, the H_0 that adolescent supplementation with TA does not attenuate liver-related alterations induced by voluntary SAC consumption in female and male Sprague Dawley rats was not rejected.

The third specific objective evaluated the protective potential of TA against the adverse effects of SAC on renal function and nephropathy. SAC consumption did not significantly affect absolute or relative kidney mass, oxidative stress (TBARS), or kidney injury markers (KIM-1

and NGAL). TA also had no impact on these biochemical parameters. However, females showed higher renal lipid peroxidation than males.

Despite no major changes in functional biomarkers, SAC with or without TA altered glomerular morphology and increased renal fibrosis in both sexes. Females exhibited signs of glomerular hyperfiltration, indicating a sex-specific susceptibility. Importantly, TA did not prevent SAC-induced lipid peroxidation, fibrosis, or glomerular alterations, suggesting limited renal protective effects in this context. In summary, the H_0 that dietary TA supplementation during adolescence does not prevent renal dysfunction associated with voluntary SAC intake in male and female Sprague Dawley rats was not rejected.

Females were more susceptible to the adverse effects of SAC, including increased visceral fat accumulation, hepatic steatosis, hepatic fibrosis, lipid peroxidation, reduced hepatocyte density, glomerular hyperfiltration, and renal fibrosis. This clear sexual dimorphism underscores the importance of considering sex differences in disease susceptibility and progression. Although moderate voluntary consumption of alcohol in gelatine did not cause overt metabolic dysfunction in rats, where abnormalities were observed, TA at 50 mg/kg body mass offered limited protection, improving only hepatic oxidative stress in males.

In conclusion, SAC poses health risks to both sexes, with females appearing more vulnerable. This heightened sensitivity may stem from sex-specific interactions between metabolic dysregulation and oxidative stress, particularly in the liver and kidneys. Females exhibited greater lipid peroxidation, indicating increased oxidative damage, which likely contributed to more pronounced hepatic and renal effects. In contrast, males exhibited lower oxidative stress and a modest antioxidant response to TA. These findings suggest that adolescent girls who consume SAC may face a higher risk of metabolic, hepatic, and renal dysfunction. Further research is needed to clarify the mechanisms behind these sex-specific effects.

Limitations and recommendations for future studies

- Voluntary consumption of SAC using gelatine as a vehicle did not induce certain components of metabolic syndrome, such as visceral obesity, hyperglycaemia, and dyslipidaemia. Although the study mimicked the consumption of ‘jello shots’, there is a need for further studies on SAC, using different administration paradigms.

- Similarly, the small sample size ($n = 8$ per group) may have limited the study's power to detect interaction between the sex and treatment effects. Future research should consider using a larger sample size to better assess sex-specific responses.
- The small sample size for molecular analyses reduced statistical power, potentially contributing to the lack of significant findings in the molecular endpoints. Future studies with larger cohorts are needed to confirm these findings.
- Another limitation of this study is the lack of measurement of fluid consumption, as changes in fluid balance could have provided valuable insights into metabolic and renal dysregulation.
- This study did not quantify ethanol retention, relying on the findings of Peris et al. (2006) to assume content preservation. Future research should directly measure retention, as evaporation may impact dosing consistency.
- Additionally, renal function tests could have been expanded to include urinalysis, offering a more comprehensive understanding of the observed kidney impairments.
- The absence of hepatic tissue lipid profiling also restricted the study's ability to explore the link between lipid metabolism and liver steatosis development.
- An additional limitation of the study is the absence of assessment of the circulating leptin concentration, which may play a role in appetite regulation influenced by SAC consumption and TA supplementation. This was not measured and is therefore recommended for investigation in future studies.
- Furthermore, future research should examine other molecular mechanisms underlying sex-specific responses to SAC consumption, particularly the expression of pro-apoptotic enzymes *caspase-1* and *caspase-3*.
- Investigating additional pathways—such as Nrf2 (an oxidative stress modulator), mitochondrial markers (including mitochondrial DNA damage and mass), and hormonal regulators (corticotropin-releasing factor [CRF], adrenocorticotrophic hormone [ACTH], cortisol, and corticosterone), would further enhance understanding of the mechanisms underlying SAC-induced effects.
- Building on these insights, future studies should investigate higher doses of TA to better assess its potential protective role against metabolic disorders caused by SAC intake. This recommendation is based on the observation that TA, at a dose of 50 mg/kg body weight, did not produce significant protective effects across most of the measured parameters.

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APPENDICES

Appendix I: Plagiarism Declaration



PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Olanipekun Toluwase Eniola (Student number: 2111582) am a student, registered for the degree of PhD in the academic year 2021.

I hereby declare the following:

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.

I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.

I have followed the required conventions in referencing the thoughts and ideas of others.

I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: 

Date: 25/08/2025

Appendix II: Animal Ethics Clearance Certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)



STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/11/06/C

APPLICANT: Mrs T Olanipekun

School: School of Physiology; Department: Location: WRAF

PROJECT TITLE: Tannic acid as a dietary supplement for protection against metabolic disorders caused by sweetened alcohol Intake in Sprague Dawley Rats

Category: C; Species and Numbers involved: 32X, 42-day old, Female, Sprague-Dawley Rats and 32X, 42-day old, Male Sprague-Dawley Rats

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 29 Nov 2022. This approval remains valid until 26 Jan 2025 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed:  _____ Date: ____30/01/2023____

(Chairperson of the AREC)

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

Signed:  _____ Date: _30 January 2023_____

Director Wits Research Animal Facility (WRAF): Dr Kim Jardine (Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor surname» Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

Appendix III: Modification and extension of study certificate 1



AREC 2022 AM&E

Please note that only electronically completed applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

ANIMAL RESEARCH ETHICS COMMITTEE registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, [reg. no. AREC -101210-002](#)

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

Principal Investigator (PI) Name: Toluwase Olanipekun

School / Department: School of Physiology

Email Address: 2111582@students.wits.ac.za

Experiment to be modified / extended AREC NO: 2022/11/06/C

Original AREC Clearance Certificate Number	2022	11	06/ C
Clearance Certificate Expiry date	26 January 2025		
Number of previous AM&Es	None		

Project Title: Tannic acid as a dietary supplement for protection against metabolic disorders caused by sweetened alcohol Intake in Sprague Dawley Rats

No. Species

f.	Number and species of animals <u>originally approved</u>	64	Sprague Dawley Rats
g.	Number of additional animals previously <u>approved on AM&Es</u>	None	

i.	Number of animals used and currently involved in the study to date	64	Sprague Dawley Rats
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j. Specific details of Adaptations/Modification/ Extension:

Change in Fructose concentration from 10% (w/v) to 20% (w/v) fructose

Inclusion of Co-worker

Collection of brain and oral epithelium from rats.

k. Motivation for Adaptations/Modification/ Extension:

We would like to increase the fructose concentration from 10% to 20% in the gelatine offered to the rats. This is to ensure that we accomplish metabolic outcomes. Previous studies have shown that

20% (w/v) fructose-induced all the components of metabolic syndrome (Dupas et al., 2017; Ibraheem et al., 2014). Thus, we can fully investigate the protective potential of tannic acid.

The change in fructose concentration will affect 32 animals, 16 rats in Group 3 (Sweetened Alcohol group) and 16 rats in Group 4 (Sweetened Alcohol with Tannic Acid group). These animals have been receiving 10% fructose in gelatine to date (the first 5 weeks of the study). The proposed administration of the 20% fructose in gelatine is for the last four weeks of the study, as the total duration of administration is 10 weeks. Therefore, they will be on 10% fructose for 6 weeks and 20% for 4 weeks. The 10-week duration overall, remains unchanged from the previously approved duration.

Dr Olatunbosun Olaleye (Phone 011 717 2214), a lecturer in the School of Oral Health Sciences (Wits) would like to collaborate with us and use tissues from the head region of the rats when terminated.

We would like to carry out a histological assessment of oral mucosa histologically by investigating/ observing and reporting the differences in histological appearance induced by the intake of sweetened alcohol. Furthermore, since alcohol can affect brain structure and function, we would like to observe the regions of the brain that are usually affected by alcohol exposure. The regions to be targeted are the hippocampus, cerebral cortex, brain stem, and pituitary. With basic histological staining such as Nissl body and gold staining for astrocytes (Malungo et al., 2022).

Use of the tissues will result in a reduction of animals as the study does not need to be repeated specifically to study the impact of sweetened alcohol and tannic acid on cephalic structures.

All previously approved interventions for the study remain unchanged.

References

Dupas, J., Feray, A., Goanvec, C., Guernec, A., Samson, N., Bougaran, P., Guerrero, F., Mansourati, J., 2017. Metabolic Syndrome and Hypertension Resulting from Fructose Enriched Diet in Wistar Rats. *Biomed Res. Int.* 2017.

Ibraheem, Z.O., Basir, R., Aljobory, A.K., Ibrahim, O.E., Alsumaidae, A., Yam, M.F., 2014. Impact of gentamicin coadministration along with high fructose feeding on progression of renal failure and metabolic syndrome in sprague-dawley rats. *Biomed Res. Int.* 2014.

Malungo, I.B., Mokale, R., Bertelsen, M.F., Manger, P.R., 2022. Cholinergic, catecholaminergic, serotonergic, and orexinergic neuronal populations in the brain of the lesser hedgehog tenrec (*Echinops telfairi*). *Anat. Rec.* 844–878.

Date: 8 May 2023 PI Signature:



RECOMMENDATIONS:

Approval for the modification to increase the fructose concentration to 20% in the gelatin cubes for rats in groups 3 and 4, (total n=32) as detailed above.

Approval for the collection of additional tissues (Brain and oral epithelium) upon termination of animals.

Approval for the addition of co-worker, Dr O. Olaleye, to the study.

Date: 11 May 2023 Signature:



Deputy Chairperson, AREC

Appendix IV: Modification and extension of study certificate 2



AREC 2023 AM&E

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG ANIMAL RESEARCH ETHICS COMMITTEE registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, [reg. no. AREC -101210-002](#)

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

Principal Investigator (PI) Name: Toluwase Olanipekun

School / Department: School of Physiology

Email Address: 2111582@students.wits.ac.za

Experiment to be modified / extended AREC NO: 2022/11/06/C

Original AREC Clearance Certificate Number	2022	11	06/ C
Clearance Certificate Expiry date	26 January 2025		
Number of previous AM&Es	1		

Project Title: Tannic acid as a dietary supplement for protection against metabolic disorders caused by sweetened alcohol Intake in Sprague Dawley Rats

No. Species

f.	Number and species of animals <u>originally approved</u>	64	Sprague Dawley Rats
g.	Number of additional animals previously <u>approved on AM&Es</u>	None	

i.	Number of animals used and currently involved in the study to date	64	Sprague Dawley Rats
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Specific details of Adaptations/Modification/ Extension:

Inclusion of Co-worker.

Motivation for Adaptations/Modification/ Extension:

We would like to include Dr Ashmeetha Manilall (Phone 011 717 2456, email: Ashmeetha.Manilall@wits.ac.za) a Researcher in the School of Physiology (Wits) as a co-worker on the study. She will provide technical and intellectual input into the molecular studies on the liver samples we collected from the rats.



Date: 15 September 2023 PI Signature:

RECOMMENDATIONS:

Approval for the addition of Dr Manilall as co-worker to the study.



Date: 18 September 2023

Signature: Deputy Chairperson, AREC

Appendix V: Modification and extension of study certificate 3



AREC 2024 AM&E **Please note that only electronically completed applications will be accepted.**

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG ANIMAL RESEARCH ETHICS COMMITTEE

*registered as Animal Research Ethics Committee (AREC) with the South African National Health Research Ethics Council (NHREC) of the National Dept. of Health, **reg. no. AREC - 101210-002***

APPLICATION FOR ADAPTATIONS OR MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

- a. Principal Investigator (PI) Name: OLANIPEKUN TOLUWASE
- b. School / Department: School of Physiology
- c. Email Address: 2111582@students.wits.ac.za

Original AREC Clearance Certificate Number	2022	11	06/ C
Clearance Certificate Expiry date	26 January 2025		
Number of previous AM&Es	Two		

- d. Experiment to be modified / extended **AREC NO: 2022/11/06/C**
- e. Project Title: Tannic acid as a dietary supplement for protection against metabolic disorders caused by sweetened alcohol Intake in Sprague Dawley Rats

	No.	Species
f. Number and species of animals <u>originally approved</u>	64	Sprague Dawley Rats
g. Number of additional animals previously <u>approved on AM&Es</u>	None	
i. Number of animals used and currently involved in the study to date	64	

- j. Specific details of Adaptations/Modification/ Extension:

Extension of ethics clearance certificate expiry date

k. Motivation for Adaptations/Modification/ Extension:

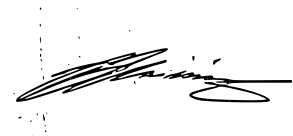
The laboratory analysis for the study is ongoing and will extend beyond the clearance certificate expiry date.

Date: 06 November 2024

PI Signature:



RECOMMENDATIONS:



Approve the extension of the study to 26 January 2027.

Date: 08 November 2024

Signature: Chairperson, AREC

Appendix VI: Post-mortem result

Central Animal Service	REPORTS #: R-VR.NR001 VETERINARY RECORD: NECROPSY REPORT	UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
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POST MORTEM EXAMINATION: (to be completed by Veterinarian):

PI NAME: T. Olanipekun	AREC#: 2022/11/06/C	DATE: 02/06/2023
	Animal ID and Gender: #24, male	
BODY CONDITION: fair		
BODY MASS: not available		

PM FINDINGS & SAMPLES TAKEN:

Skin: alopecia, ulceration and crusting of dermis on right cervical area , moderately cyanotic
Muscle: nothing abnormal detected
Small intestine: Severely congested mesenteric blood vessels, diffuse inflammation of small intestine
Large intestine: severely congested colonic blood vessels, diffuse inflammation of large intestine
Stomach: small amount of ingesta present
Liver: severely congested and enlarged
Spleen: moderately congested
Pancreas: nothing abnormal detected
Kidneys: nothing abnormal detected
Reproductive organs: nothing abnormal detected
Lungs: severe diffuse atelectasis and congestion
Heart: Severely congested coronary blood vessels, mild concentric hypertrophy of heart
Trachea: nothing abnormal detected
Brain: Not examined
Other comments: Showed stereotypical scratching behaviour a few weeks ago that resulted in the formation of an area of moist ulcerative dermatitis on the right neck area. It responded well to treatment but the pruritis flared up again towards the end of the week despite topical and systemic cortisone treatment. It also suddenly lost 10% of its body weight and it was therefore terminated for humane reasons.

Click or tap here to enter text.

SAMPLE PROCESSING: REPORT ID: Euthanised due to weight loss and stereotypical behaviour (underlying compromised cardiovascular system)

REPORT PREPARED BY: Dr AP van der Merwe		DATE: 05 June 2023
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DG ☐

DP ☐ I ☐ E ☐ N ☐ X ☐

Developed by: M Costello Checked by: Add Name Click or tap to enter a date. Version #: Choose an item. AREC Approval: Click or tap to enter a date.

* DG – Disease Genetic Predisposition
DP – Disease Pathogenic Cause

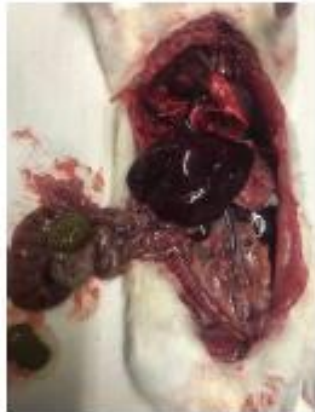


Fig.1. Severely congested carcass

Appendix VII: Summary of results

F = Female, M = Male, ↑ = increase, ↓ = decrease, - = no difference, √ = difference, NA= not applicable

		PG	TA	SAC	SAC+TA	Sex effect (F vs M)	Treatment effect	Interaction effect
Feed intake	F	↑	↑	↓	↓	NA	NA	NA
	M	↑	↑	↓	↓			
Body mass	F	-	-	-	-	↓	-	-
	M	-	-	-	-			
Visceral fat	F	-	-	-	-	↑	√	-
	M	-	-	-	-			
Epididymal fat	M	-	-	-	-	NA	NA	NA
Haemoglobin	F	-	-	-	-	-	-	-
	M	-	-	-	-			
Haematocrit	F	-	-	-	-	-	-	-
	M	-	-	-	-			
MCHC	F	-	-	-	-	-	-	-
	M	-	-	-	-			
Glucose	F	-	↑	-	↓	↑	-	-
	M	-	-	-	-			
Insulin	F	-	-	-	-	-	-	-
	M	-	-	-	-			
HOMA-IR	F	-	-	-	-	-	-	-
	M	-	-	-	-			
TG	F	-	-	-	-	-	-	-
	M	-	-	-	-			
HDL-C	F	-	-	-	-	-	-	-
	M	↓	-	↑	↑			
TG/HDL-C ratio	F	-	-	-	-	-	-	-
	M	-	-	-	-			

Liver results								
		PG	TA	SAC	SAC+TA	Sex effect (F vs M)	Treatment effect	Interaction effect
Liver mass (absolute)	F	-	-	-	-	↓	-	-
	M	-	-	-	-			
Hepatosomatic index	F	-	-	-	-	↑	-	-
	M	-	-	-	-			
Liver TBARS	F	↓	↑	↑	-	↑	-	√
	M	↑	-	↓	-			
CYP2E1	F	-	-	-	-	-	-	-
	M	-	-	-	-			
SREBP-1	F	-	-	-	-	-	-	-
	M	-	-	-	-			
IL-10	F	-	-	-	-	-	-	-
	M	-	-	-	-			
NF-kB1	F	-	-	-	-	-	-	-
	M	-	-	-	-			
TNF-α	F	-	-	-	-	-	-	-
	M	-	-	-	-			
Liver cell density	F	↑	↓	↓	↓	↑	√	√
	M	↑	↑	-	↓			
Micro-steatosis	F	-	-	-	-	↑	-	√
	M	↓	-	↑	↑			
Macro-steatosis	F	-	-	-	-	↑	-	-
	M	-	-	-	-			
Collagen distribution	F	-	-	-	-	-	-	√
	M	↑	↓	↓	-			
Kidney Results								
		PG	TA	SAC	SAC+TA	Sex effect (F vs M)	Treatment effect	Interaction effect
Kidney mass	F	-	-	-	-	↓	-	-
	M	-	-	-	-			

Kidney index	F	-	-	-	-	↑	-	-
	M	-	-	-	-			
KIM-1	F	-	-	-	-	-	-	-
	M	-	-	-	-			
NGAL	F	-	-	-	-	-	-	-
	M	-	-	-	-			
TBARS	F	-	-	-	-	↑	-	-
	M	-	-	-	-			
Urinary space	F	↓	↑	↑	-	-	-	√
	M	↑	↑	↓	↑			
Bowman's capsule	F	↓	↑	-	-	↓	-	√
	M	-	-	-	-			
Glomeruli area	F	↓	↑	-	↑	↓	-	√
	M	↑	↓	↑	↑			
Collagen distribution	F	↓	↓	↑	↑	↓	√	√
	M	↓	↑	↑	↑			