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WITWATERSRAND,
JOHANNESBURG

THE POTENTIAL PROPHYLACTIC EFFECT OF MORINGA (*MORINGA OLEIFERA* LAM.) LEAF EXTRACTS AGAINST FRUCTOSE-INDUCED METABOLIC DYSFUNCTION IN EARLY-WEANED FEMALE RATS

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in

Physiology

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DECLARATION

I, Mmahiine Christina Mosana, student number **494404**, declare that the work contained in this thesis is my own, unaided work. This thesis is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg. The work herein has not been submitted before for any degree or examination at any other University. I certify that all the experimental procedures used in this thesis were approved by the Animal Ethics Screening Committee of the University of the Witwatersrand (AESC number: 2020/04/08/B).

Signature of Student _____  _____Date __15 September 2025__

DEDICATION

I solemnly dedicate this thesis to my incredible parents, Maphala Grace Rampheri and Maditsi Johannes Rampheri, whose unwavering support has been the cornerstone of my journey. Their boundless love and invaluable guidance have illuminated my path, shaping me into the person I am today. I am eternally grateful for their sacrifices and encouragement, without which this accomplishment would not have been possible. I am committed to continually making them proud.

Additionally, I extend this dedication to my beloved husband, Lucas Mamule Mosana, whose steadfast support and unwavering belief in my abilities have been my constant motivation. He has been my pillar of strength and my greatest source of encouragement, and for that, I am profoundly thankful. His presence by my side has made all the difference, and I am blessed to have him in my life.

I would also like to express my gratitude to all my family members, including Rosina Molepo, Abokase Mosana, my siblings, and siblings-in-law, for their unyielding belief in me and their continuous encouragement. Their support has been instrumental in my pursuit of excellence.

Lastly, I pay tribute to the cherished memory of my grandparents, whose influence and guidance have left an indelible mark on my journey. In loving memory of:

Kabati Frans Molepo
1929 - 2018

Mamohuba Lidah Molepo
1934 - 2013

I dedicate this achievement to them, honouring their legacy and the profound impact they have had on shaping my aspirations and accomplishments.

Output arising from this research project.

Presentations

1. Mosana, M., Erlwanger, K., Nyakudya, T., & Ndhlala, A. (2022). Preliminary results of prophylactic effects of *Moringa oleifera* hydroethanolic leaf extracts on general metabolomics (biomarkers of health) of female Sprague-Dawley rats, early-weaned on a high fructose solution. *Global Summit on Public Health and Preventive Medicine*, May 26-28, 2022, Munich, Germany.

2. Mosana, M., Nyakudya, T. T., Ndhlala, A. R., & Erlwanger, K. H. (2022). Administration of *Moringa oleifera* hydroethanolic leaf extracts attenuates fructose-induced leptin and adiponectin concentration derangements, and dyslipidaemia, in early-weaned female Sprague-Dawley rats. *Wits Faculty of Health Sciences Biennial Research Day*, September 15, 2022, University of the Witwatersrand, South Africa.

3. Mosana, M., Erlwanger, K., Nyakudya, T., & Ndhlala, A. (2025). Short-term post-weaning *Moringa oleifera* Lam. leaf extracts intervention alleviates fructose-induced renal dysfunction associated with early weaning in female rats. *Physiology Society of Southern Africa Congress*, August 24-27, 2025, North West University, Potchestroom, South Africa.

ABSTRACT

Early weaning has become increasingly common worldwide due to urbanisation, socioeconomic demands, and health reasons. It is estimated that 88% of infants are early-weaned (less than 6 months old) globally. Consequently, early-weaned infants, especially when weaned onto high fructose diets, are at risk of developing disorders like metabolic dysfunction associated with fatty liver disease and kidney disease early in childhood. These metabolic disorders then progress into adulthood, affecting their lifespan and functionality, and are a financial burden to the healthcare system. This study investigated the long-term potential prophylactic effects of *Moringa oleifera* leaf extracts when administered for the first two weeks post-weaning, in female Sprague-Dawley rats early-weaned onto high fructose diets.

The rats were weaned early (18 days old), and fed either standard rat feed, a fructose-rich diet, a normal diet with Moringa extracts, or a combination of high fructose with Moringa extracts for 18 days. The normal control group was weaned at 21 days old. Post weaning, the rats were fed a high-fructose diet or a normal diet for 111 days. The rats were terminated on postnatal day 112. Early weaning with fructose-induced elevated serum concentrations of leptin, low-density lipoproteins (LDL), triglyceride, total cholesterol, and alanine transaminase ($p<0.05$, ANOVA). It also resulted in increased hepatic micro- and macrosteatosis, hypertrophy, inflammation, area fraction of the collagen, peroxisome proliferator-activated receptor alpha (PPARα), and sterol regulatory element-binding protein 1c (SREBP-1c) gene expression ($p<0.05$). Early weaning with fructose decreased serum concentrations of insulin, adiponectin, high-density lipoprotein (HDL), and hepatic carnitine palmitoyltransferase I (CPT-1) gene expression ($p<0.05$).

However, early weaning treatment with Moringa for only two weeks, demonstrated a broad scope of protection against the fructose-induced metabolic dysfunction, specifically hepatic steatosis, and modulated gene expression related to lipogenesis and fatty acid oxidation by decreasing hepatic SREBP-1c, Acetyl-CoA Carboxylase 1 (ACC-1), and fatty acid synthase (FAS) gene expression and increasing CPT-1 and PPARα gene expression ($p<0.05$), with dose-dependent effects observed.

Early weaning alone did not significantly affect kidney mass, relative mass, serum renal function markers (kidney injury molecule-1 (KIM-1), creatinine, or blood urea nitrogen (BUN) levels, but led to increased serum neutrophil gelatinase-associated lipocalin (NGAL) serum concentrations) and kidney glomerular/Bowman's capsule area (BCA) ($p<0.0001$). Fructose caused significant

functional (↑KIM-1, BUN, NGAL; ↓creatinine) and structural kidney damage (enlarged and deformed glomeruli), which Moringa reversed. Dose differences were minimal but indicated slightly better biochemical function with higher doses, but greater histopathological protection with lower doses.

In conclusion, early weaning combined with high fructose consumption resulted in significant metabolic dysfunction, hepatic steatosis, and renal damage in female Sprague-Dawley rats. The short-term supplementation of *Moringa oleifera* leaf extract immediately after early weaning offered long term sustained, multi-organ protection. Given its low cost, accessibility, and translational potential, Moringa represents a promising early-life nutritional strategy to protect against long-term metabolic, hepatic, and renal dysfunction.

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LIST OF ABBREVIATIONS

ACC-1: …….....	Acetyl-CoA Carboxylase 1
ACE: ……	Angiotensin-Converting Enzyme
ACR: ……	Albumin-to-Creatinine Ratio
Actinβ: …….	Beta-Actin (housekeeping gene)
ADP: ……	Adiponectin
Afraction: ……	Area Fraction
AGEs: …….	Advanced Glycation End Products
ALP: ……...	Alkaline Phosphatase
ALT: ……...	Alanine Aminotransferase
ARBs: …….	Angiotensin Receptor Blockers
ARRIVE: ……	Animal Research: Reporting of In Vivo Experiments
AST: ……...	Aspartate Aminotransferase
BCA: ……..	Glomerular/Bowman's Capsule Area
BMI: ……...	Body Mass Index
BPA: ……..	Bisphenol A
BUN: ……..	Blood Urea Nitrogen
CCK: ……...	Cholecystokinin
CCl4: ……...	Carbon Tetrachloride
cDNA: …….	Complementary DNA
CKD: ……	Chronic Kidney Disease
CML: ……	N(ε)-(Carboxymethyl) Lysine
CPT-1: ……..	Carnitine Palmitoyltransferase I
CREA: ……..	Creatinine
CRP: …….	C-Reactive Protein
DAG: ……	Diacylglycerol
DOHaD: ……	Developmental Origins of Health and Disease
EDCs: ……	Endocrine-Disrupting Chemicals
ELISA: ……...	Enzyme-Linked Immunosorbent Assay
ESRD: ……	End-Stage Renal Disease
EWC: …….	Early Weaning Control

EWC: …… Early Weaning Control

EWF: ……. Early Weaning Fructose

EWF: ……. Early Weaning Fructose Only

EWMH: ……. Early Weaning Moringa High Dose Only

EWMHF: ……... Early Weaning Moringa High Dose + Fructose

EWML: …….. Early Weaning Moringa Low Dose Only

EWMLF: …… Early Weaning Moringa Low Dose + Fructose

FAS: ……... Fatty Acid Synthase

FFA: ……... Free Fatty Acids

FIB-4: ……. Fibrosis-4 Index

FS: …….. Fructose Solution

FTO: ……... Fat Mass and Obesity-Associated Gene

GFR: ……... Glomerular Filtration Rate

GGT: ……... Gamma-Glutamyl Transferase

GLP-1: …… Glucagon-like Peptide-1

GLUT-2: ……. Glucose Transporter 2

GLUT-5: ……. Glucose Transporter 5

GSH: ……... Reduced Glutathione

GSK-3β: …….. Glycogen Synthase Kinase-3 Beta

GTA: …… Glomerular Tuft Area

GWAS: …… Genome-Wide Association Studies

H&E: …… Hematoxylin and Eosin

Hb: …… Haemoglobin

HbA1c: ……. Hemoglobin A1c

HCC: …… Hepatocellular Carcinoma

Hct: …….. Haematocrit

HDL: …… High-Density Lipoprotein

HOMA-IR: …….. Homeostatic Model Assessment of Insulin Resistance

IDF: ……. International Diabetes Federation

IFG: ……. Impaired Fasting Glucose

IGT: ……. Impaired Glucose Tolerance

IL-1β: …….. Interleukin-1 Beta

IL-6: …… Interleukin-6

KIM-1: …… Kidney Injury Molecule-1

LDL.....Low-Density Lipoprotein

LEP.....Leptin

LEP/ADP Ratio: …….. Leptin-to-Adiponectin Ratio

LPS.....Lipopolysaccharides

MAFLD.....Metabolic Dysfunction-Associated Fatty Liver Disease

MAKD.....Metabolic-Associated Kidney Dysfunction

MCHC:.....Mean Corpuscular Haemoglobin Concentration

MCP-1: …… Monocyte Chemoattractant Protein-1

MDA .....Malondialdehyde

MEML.....Methanolic Extract of Moringa Leaves

MOE.....*Moringa Oleifera* Ethanolic Extract

mRNA .....Messenger RNA

MT.....Masson's Trichrome

NAFLD .....Non-Alcoholic Fatty Liver Disease

NASH.....Non-Alcoholic Steatohepatitis

NCEP ATP III.....National Cholesterol Education Program Adult Treatment Panel III

NGAL.....Neutrophil Gelatinase-Associated Lipocalin

NO.....Nitric Oxide

Nrf2/HO-1 .....Nuclear Factor Erythroid 2-Related Factor 2/Heme Oxygenase-1

NWC .....Normal Weaning Control

PD18.....Postnatal Day Eighteen

PD21: .....Postnatal Day Twenty-one

PDW.....Plain Drinking Water

PGC-1α.....Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha

PNPLA3: .....Patatin-like Phospholipase Domain Containing 3

PPAR-α .....Peroxisome Proliferator-Activated Receptor Alpha

PVC.....Polyvinyl Chloride

RAAS: ……. Renin-Angiotensin-Aldosterone System

RCA: .....Renal Corpuscular Area

RNA .....Ribonucleic Acid

ROS.....Reactive Oxygen Species

ROS:.....Reactive Oxygen Species

RT-PCR.....Reverse Transcription Polymerase Chain Reaction

SCFAs .....Short-Chain Fatty Acids

SOD.....Superoxide Dismutase

SRC .....Standard Rat Chow

SREBP-1c.....Sterol Regulatory Element-Binding Protein 1c

T2DM.....Type 2 Diabetes Mellitus

TAC.....Total Antioxidant Capacity

TBM .....Terminal Body Mass

TC.....Total Cholesterol

TG .....Triglycerides

TIMP-1: ……. Tissue Inhibitor of Metalloproteinases-1

TNF-α.....Tumor Necrosis Factor-Alpha

U:C Ratio: ……. Urea-to-Creatinine Ratio

UCP-1.....Uncoupling Protein 1

UNICEF .....United Nations International Children's Emergency Fund

VLDL: ……... Very-Low-Density Lipoprotein

WC .....Waist Circumference

WHO .....World Health Organisation

WtBLR: ……. Waist-to-Body Length Ratio

α-SMA.....Alpha-Smooth Muscle Actin

The context and rationale for this study are presented in Chapter 1. The Chapter also emphasises the health risks associated with early weaning through development of metabolic dysfunction, especially metabolic associated fatty liver disease (MAFLD) and renal dysfunction. In this chapter, the goals and objectives of the study are presented, along with the hypothesis that *Moringa oleifera* can help prevent negative consequences associated with early weaning and fructose consumption in early-weaned rats.

CHAPTER ONE:

INTRODUCTION

1.1. Organisation of the thesis

In accordance with the Faculty of Health Sciences style guide for theses, dissertations and research reports, this thesis is presented in the 'divided block' format. It is systematically organised into seven chapters. Each chapter is designed to address specific concepts of the research comprehensively. For the chapters relating to experiments, each has an introduction section, methods, results, discussion and conclusions sections. The structure ensures a logical flow from the introduction to the conclusion, allowing readers to follow the development of the researched concepts and findings systematically. In summary:

Chapter 1: Introduction
The first chapter sets the stage by introducing the background of the study. It explains and describes the significant health challenges caused by metabolic dysfunction, particularly MAFLD and renal dysfunction, which are often linked to dietary habits and early life nutrition. The chapter also presents the aims and objectives of the research, articulating the hypothesis that *Moringa oleifera* can mitigate the adverse effects of a high-fructose diet in early-weaned rats.

Chapter 2: Literature review and justification
The literature review delves into existing research on metabolic dysfunction, obesity, and related health issues. It examines the prevalence and pathophysiology of these conditions, the impact of early life dietary practices, and the potential role of phytochemicals like those found in *Moringa oleifera*. This chapter aims to provide a comprehensive background and highlight gaps that the current study seeks to fill, including the influence of early weaning practices, which are particularly relevant in the South African context. The chapter also provides the justification of the study.

Chapter 3: The effect of strategic administration of *Moringa oleifera* lam. hydroethanolic leaf extracts in the first two weeks post-weaning on fructose-induced metabolic fatty liver disease (MAFLD) in early-weaned female Sprague-Dawley rats.
Chapter 3 focuses on the experimental investigation into metabolic dysfunction and MAFLD in relation to early weaning onto a high fructose diet. It provides background to the study and details the methodology used to assess liver function, lipid content and related biomarkers in

the study rats. The content of the chapter elucidates the impact of the interventions on liver health and the potential protective role of *Moringa oleifera*.

Chapter 4: The effect of *Moringa oleifera* lam. hydroethanolic leaf extracts administered during the first two weeks post-weaning on renal structure and function in fructose-induced early-weaned female Sprague-Dawley rats.

This chapter explores the impact of early weaning and high-fructose diets on renal function and structure. The investigation was carried out by evaluating kidney function through various biomarkers and histological analyses. Additionally, determined the renal impact of *Moringa oleifera* in early-weaned fructose fed rats.

Chapter 5: The impact of hydroethanolic Moringa leaf extract administration for the first two weeks post-weaning and long-term fructose consumption on feed intake, pancreatic morphometry, and haematological parameters in early-weaned female rats.

This chapter investigates the effects of hydroethanolic Moringa leaf extracts and fructose consumption on growth, organ morphometry, and haematological parameters in early-weaned female rats. It explores various aspects of their physiological responses, including feed and fluid consumption patterns, morphometric measurements such as body mass index (BMI) and waist circumference, organ morphometry focusing on pancreas size, and haematological parameters like haemoglobin concentration and haematocrit levels. The study aims to understand how dietary interventions during the early stages of life influence metabolic health and development in rats. Through comprehensive analysis, it sheds light on the potential impacts of these interventions on various physiological parameters, contributing to the broader understanding of dietary influences on metabolic function.

Chapter 6: Conclusions, limitations and directions for future studies

In this chapter, the findings from all of the preceding experimental chapters are synthesized and interpreted considering the broader implications of the results, the potential mechanisms behind the observed effects, and the relevance to human health. It also summarizes the key findings of the research, reaffirming the protective effects of *Moringa oleifera* against metabolic, MAFLD and renal dysfunctions induced by early weaning and high-fructose diets. It emphasizes the significance of early dietary interventions and the potential for phytochemicals to improve long-term health outcomes.

It also addresses the limitations of the study and suggests areas for future research.

Chapter 7: References
All referenced materials are detailed in this chapter.

Additionally, appendices are provided. The manuscript of the research project, along with the research methods and ethical clearance certificates, are all provided in the appendices.

1.2. Introduction

The global burden of metabolic dysfunction, including metabolic dysfunction-associated fatty liver disease (MAFLD, previously termed non-alcoholic fatty liver disease), renal dysfunction, and related disorders, is rising at an alarming rate. These conditions, once largely confined to adults, are increasingly affecting children and adolescents, making them a major public health concern. Several factors, including lifestyle choices, poor dietary habits, sedentary behaviour, and genetic predispositions, are driving this increase (Albuquerque *et al.*, 2017). In South Africa, the rise in childhood obesity, alongside metabolic disorders such as MAFLD, has been particularly striking, with childhood obesity rates now exceeding global averages (Wu *et al.*, 2022). For example, 10% of South African children and 5% of adolescents are affected by metabolic dysfunction, compared to the global rates of 7% and 3%, respectively (Wu *et al.*, 2022). This makes South Africa a prime region for investigating effective interventions for metabolic dysfunction, particularly in its youth population.

A unique challenge in South Africa comes from the combination of rapid urbanisation, socio-economic shifts, and dietary changes. As urbanisation progresses, traditional diets are being replaced by processed, high-sugar/fat foods that are relatively affordable and accessible, leading to a sharp increase in obesity rates among urban youth (Pheiffer *et al.*, 2018). This dietary shift, along with the decline in physical activity, has resulted in a corresponding rise in the incidence of MAFLD, renal dysfunction, insulin resistance, and other metabolic disorders among children and adolescents in South Africa (Muller *et al.*, 2019).

In addition to childhood obesity, early weaning practices have been identified as a key factor contributing to metabolic dysfunction associated illnesses(Barrett *et al.*, 2023). Early

weaning, which is the introduction of solid foods before the recommended age of six months, often involves the introduction of high-fructose containing foods (Zhang et al., 2021). In South Africa, early weaning is common, particularly in urban areas, where socio-economic factors such as maternal employment and limited access to breastfeeding support contribute to this practice (Wrottesley *et al.*, 2021). Early exposure to high fructose, processed foods during weaning has been linked to the early development of metabolic dysfunction, underscoring the need for better nutritional practices during this critical period (Barrett *et al.*, 2023).

When fructose-rich foods are introduced during early weaning, they can have negative effects on metabolic health. Fructose, commonly found in sugary beverages and processed snacks, is known to promote liver fat accumulation, disrupt insulin signalling, and increase the risk of developing metabolic dysfunction (Jensen *et al.*, 2018). Excessive fructose consumption during early weaning not only disrupts liver function but also alters metabolic programming, making children more susceptible to obesity and related metabolic disorders (Rodríguez *et al.*, 2016). In South Africa, this is prominent among children from lower socio-economic backgrounds who rely on these inexpensive, easily accessible high fructose/fats foods (Kimani-Murage *et al.*, 2010).

Given the increasing prevalence of metabolic dysfunction in South African children, following the introduction of high-energy diets during the early postnatal period, it is crucial to explore potential interventions that can mitigate these risks. Nutritional interventions, especially during the weaning phase, offer an opportunity to prevent the onset of conditions such as MAFLD, obesity, renal dysfunction and insulin resistance. One such intervention is the use of *Moringa oleifera*, a plant known for its potent anti-inflammatory, anti-oxidant, and lipid-lowering properties (Luoh *et al.*, 2017a). Moringa has been shown to modulate lipid metabolism, improve insulin sensitivity, and reduce hepatic fat accumulation in adult animal models (Irfan, Khan and Asmawi, 2022). However, there is a gap in the literature regarding the potential of Moringa to prevent metabolic dysfunction, specifically when administered for a short term during the early weaning period only, a phase of developmental plasticity where metabolic programming is still highly adaptable. This presents an opportunity for novel research into the potential of Moringa as a preventative agent against metabolic dysfunction in early life.

Moringa oleifera contains bioactive compounds such as polyphenols, flavonoids, and carotenoids, which have strong anti-oxidant properties that help reduce oxidative stress and inflammation, common features of metabolic dysfunction (Irfan, Khan and Asmawi, 2022). These properties make Moringa a promising candidate for modulating the pathways involved in lipid metabolism, insulin sensitivity, liver fat accumulation, renal injury and inflammation. Despite the growing evidence supporting the benefits of Moringa in experimental models of metabolic dysfunction using adults, its long-term potential to prevent metabolic dysfunction when administered in early life remains under-explored. Given the rising prevalence of metabolic dysfunction due to poor dietary habits most likely compounded by early weaning in South African children in particular and globally, this study aimed to investigate the potential metabolic protective effects of Moringa when administered during the early weaning period. Thus, the study explored whether Moringa could offer a natural, accessible solution for reducing the risk of development of metabolic, hepatic and renal dysfunction.

1.3. Aim and objectives

1.3.1. Aim

The primary aim of this study was to investigate, using an early-weaned rat experimental model of metabolic dysfunction, the potential protective effects of a two-week strategic administration of Moringa hydroethanolic leaf extracts against the development of fructose-induced metabolic dysfunction (including MAFLD, renal dysfunction, and impaired metabolism) later in adulthood.

1.3.2. Objectives

The main objectives of this study were to investigate the effect of early weaning onto a high fructose diet and short-term administration of Moringa hydroethanolic leaf extracts on:

A. Growth performance

i. By assessing terminal body lengths and weekly body mass changes

ii. By measuring food and fluid intake

B. Metabolic dysfunction

i. By assessing serum metabolites; glucose (GLU), lipid profiles (Low density (LDL) and high density (HDL) lipoproteins, triglycerides (TG), free fatty acids (FFA), and total cholesterol (TC)).

ii. By determining concentrations of hormones regulating metabolism insulin (INS), leptin (LEP), and adiponectin (APD).

iii. By assessing visceral fat accumulation as an indicator of obesity

C. Liver structure and function

i. By assessing the development of MAFLD through liver histology (Steatosis score, hepatic inflammation and hypertrophy)

ii. By measuring a serum liver function biomarker; alanine transaminase (ALT).

iii. By assessing molecular processes influencing lipid metabolism by measurement of specific gene expression; Sterol Regulatory-Element-Binding Protein-1c (*SREBP-1c*), Acetyl-CoA Carboxylase-1 (*ACC-1*), Carnitine Palmitoyltransferase I (*CPT-1*), Peroxisome Proliferator-Activated Receptor Alpha (*PPAR-α*), and Fatty Acid Synthase (*FAS*).

D. Kidney structure and function

i. By assessing the development of renal dysfunction through histological assessments of kidney morphology (Bowman's/glomerular capsule area (BCA), renal corpuscular area (RCA) and glomerular tuft area (GTA))

ii. By assessing the development of renal dysfunction through evaluation of serum biomarkers of renal function, namely Creatinine (CREA), Blood Urea Nitrogen (BUN), Kidney Injury Molecule-1 (KIM-1), and Neutrophil Gelatinase-Associated Lipocalin (NGAL).

i. The supplementary chapter was included to investigate the effect of the interventions on waist circumference, waist-to-body length ratio (WtBLR) and calculation of body mass index (BMI) physical morphometry.

ii. Pancreas mass to explore effects on pancreatic morphology, erythrocyte indices, including haematocrit, haemoglobin concentration, and mean corpuscular haemoglobin concentration (MCHC), to identify the possible impact of metabolic dysfunction on erythrocytes.

1.4. Hypotheses

1.4.1. H_0 (Null Hypothesis):

1. Early weaning, high fructose diet and hydroethanolic Moringa leaf extract do not significantly affect growth performance and result in metabolic dysfunction in female Sprague-Dawley rats.
2. Early weaning, high fructose diet and hydroethanolic Moringa leaf extract do not significantly affect hepatic metabolism and structure of female Sprague-Dawley rats.
3. Early weaning, high fructose diet and hydroethanolic Moringa leaf extract do not significantly affect kidney structure and function in female Sprague-Dawley rats.

1.4.2. H_1 (Alternative Hypothesis):

1. Early weaning, a high fructose diet, and hydroethanolic Moringa leaf extract significantly affect growth performance and result in metabolic dysfunction in female Sprague-Dawley rats.
2. Early weaning, a high fructose diet, and hydroethanolic Moringa leaf extract significantly affect liver function in female Sprague-Dawley rats.
3. Early weaning, a high fructose diet, and hydroethanolic Moringa leaf extract significantly affect kidney function in female Sprague-Dawley rats.

The following chapter provides a detailed critical review of the literature relevant to the current study, focusing on neonatal metabolic programming and its long-term metabolic effects in adulthood life. It outlines also the pathogenesis and prevalence of metabolic dysfunction, MAFLD, and renal dysfunction, emphasising the impact of early life nutrition. Additionally, the chapter reviews the current literature on the beneficial effects of *Moringa oleifera* and its phytochemicals on metabolic and general health, identifying gaps that the current study aims to address.

CHAPTER TWO: LITERATURE REVIEW AND JUSTIFICATION

2.1. Introduction

Weaning is an important developmental stage in infant growth, marking the transition from exclusive milk feeding to the introduction of solid foods (Sokhela *et al.*, 2023). This stage significantly influences the long-term metabolic health of individuals, with the types of foods introduced and the timing of weaning playing crucial roles in shaping future health outcomes. Weaning practices are influenced by numerous socio-economic, cultural, and environmental factors (Ravikumar *et al.*, 2022). These factors are critical in determining the risk of developing chronic non-communicable conditions like obesity, type 2 diabetes, MAFLD, and renal dysfunction (Ravikumar *et al.*, 2022). In South Africa, socio-economic pressures, such as maternal employment and limited access to breastfeeding support, have led to early weaning practices that deviate from the recommendations of breast feeding by the World health Organisation (WHO) (Wrottesley *et al.*, 2021). This has contributed to an alarming rise in childhood obesity and associated metabolic disorders, including insulin resistance, liver disease, and renal dysfunction (Soares *et al.*, 2022).

In particular, the introduction of high-fructose diets during early weaning has been shown to significantly impact metabolic programming (Barrett *et al.*, 2023). Fructose, often found in sugary beverages and processed foods, is linked to fat accumulation in the liver, impaired insulin sensitivity, and increased susceptibility to diseases like MAFLD and renal dysfunction (Barrett *et al.*, 2023). Furthermore, early exposure to high-fructose diets during the weaning period can have lasting consequences, predisposing individuals to the above mentioned metabolic disorders (Barrett *et al.*, 2023).

Given these concerns, there is growing interest in exploring natural interventions that may help mitigate the negative metabolic effects of early weaning, particularly those induced by high-fructose diets.

2.2. Weaning and infant development

2.2.1. Introduction to weaning

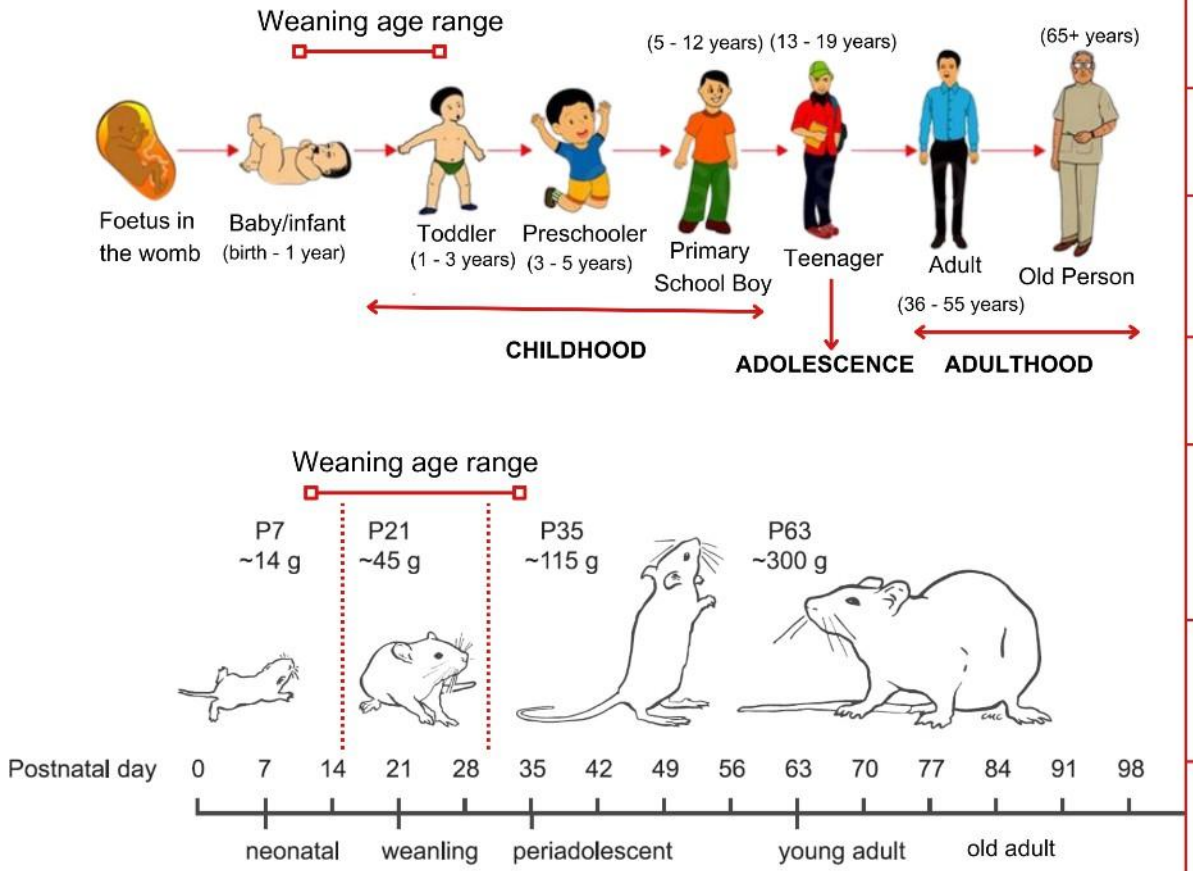
Weaning is defined as the gradual process by which a young mammal shifts from complete dependence on maternal milk or milk substitutes to the consumption of solid food (Bewket

Zeleke *et al.*, 2017). This process is a crucial developmental stage because it marks a significant developmental transitioning from infancy to early independence. In detail, weaning involves a decrease in the frequency and duration of breastfeeding or nursing in mammals, often accompanied by the introduction of complementary foods or formula, depending on the species (Teles Silva *et al.*, 2019). The timing and duration of weaning can vary widely across different species, influenced by factors such as nutritional needs, environmental conditions, and maternal behaviour (Teles Silva *et al.*, 2019). Rats are used as an experimental model to understand human growth and physiological processes in scientific studies, such as the one presented here. Therefore, it is important to compare the weaning stages in rats and humans.

In rats, weaning begins around postnatal day (PD) 14 (2 weeks), though the exact timing can vary depending on factors such as litter size, maternal care, and environmental conditions (Figure 2.1) (Kikusui *et al.*, 2019). Rats are generally fully weaned by day PD 21 to 28 (3 to 4 weeks) and predominantly relying on solid food while still occasionally nursing for comfort (Figure 2.1) (Kikusui *et al.*, 2019). In terms of developmental equivalence, PD 21 in rats, when they are fully weaned, corresponds approximately to the infant stage in humans (around 4 - 6 months), reflecting similar milestones in growth, nutritional independence, and organ development. In contrast, weaning in humans typically starts around 4 to 6 months of age when the infant begins to show signs of readiness for solid food, such as the ability to sit up with support, increased interest in food, nutritional needs, and the ability to swallow (Figure 2.1) (Alles, Eussen and Van Der Beek, 2014; Ganiatsou, Souleles and Papageorgopoulou, 2023). Initially, supplementary foods, such as pureed fruits, vegetables, and cereals, are introduced alongside breast milk or formula (Alles, Eussen and Van Der Beek, 2014). These complementary foods must have key nutrients like iron, vitamin D, and protein to support rapid growth and organ development in infants (Alles, Eussen and Van Der Beek, 2014). However, by 6 to 12 months, human infants continue to rely on breast milk or formula as their primary source of nutrition, with the solid foods gradually incorporated into their diet (Humphrey, 2010). Furthermore, full weaning, where the infant no longer relies on breast milk or formula, usually occurs by 12 to 18 months of age, depending on cultural practices and individual circumstances (Figure 2.1) (Humphrey, 2010). Failure to meet the nutritional needs of infants during the weaning period can results in detrimental effects on their health and development.

As established above, the nutritional needs during the weaning period are crucial in shaping long-term health, which is explained by the Developmental Origins of Health and Disease (DOHaD) framework (Gluckman *et al.*,, 2010; Lacagnina, 2020). This framework explains that early-life nutritional exposures shape the long-term health of an individual, including susceptibility to diseases such as obesity, type 2 diabetes, MAFLD, and renal dysfunction (Gluckman, Hanson and Buklijas, 2010; Lacagnina, 2020). To prevent these illnesses, the World Health Organisation (WHO) has developed guidelines to support optimal weaning practices and ensure that infants receive the necessary nutrition during this critical period of development.

The World Health Organisation (WHO) recommends exclusive breastfeeding for the first six months, followed by the introduction of complementary foods while continuing breastfeeding for up to two years or more (WHO, 2020). Research indicates that when the weaning process aligns with optimal timings and nutritional quality, it supports the correct programming of metabolic systems such as insulin sensitivity and liver function (Sokhela *et al.,* 2023). Conversely, deviations from these recommendations, whether due to socio-economic factors, cultural practices, or insufficient breastfeeding support, can disrupt metabolic programming and increase the risk of chronic diseases later in life (Joshi, Rathore and Deshpande, 2019).



Developmental Stage	Rats (Age)	Humans (Age)
Neonatal	Birth to 10 days	Birth to 1 month
Pre-weaning (Early Development)	10 to 21 days	1 to 4 months
Weaning	21 to 28 days	4 to 6 months
Juvenile (Early Independence)	28 to 42 days (4 to 6 weeks)	6 months to 2 years
Adolescence	6 to 8 weeks to 12 weeks	12 to 18 years
Adulthood	12 weeks onwards	18 years and onward

Figure 2. 1. The developmental stages comparisons in rats and humans, modified image with information taken from McCutcheon and Marinelli, 2009; Humphrey, 2010; Alles, Eussen and Van Der Beek, 2014; Ganiatsou, Souleles and Papageorgopoulou, 2023.

2.2.2. Physiological and anatomical changes during weaning

Weaning is a crucial period not only for nutritional transition but also for significant physiological and anatomical changes in the body. During this phase, the body adapts from a liquid-based diet to the digestion of more complex solid foods, necessitating recalibration of key physiological processes. These physiological processes lead to changes in the gastrointestinal system, oral and chewing mechanisms, digestive enzymes activity, the development of the pancreas, liver, immune system, hormones and neural networks (Crispel *et al.*, 2019; Modina *et al.*, 2019; Kalhoff *et al.*, 2024).

2.2.2.1. Changes in the gastrointestinal system

One of the most important changes occurs in the gastrointestinal system, which matures to efficiently handle the digestion of solid foods (Modina *et al.*, 2019). This is reflected in the elongation of the small intestine and an increase in its surface area, which facilitates better nutrient absorption (Crispel *et al.*, 2019). The stomach also begins to secrete more gastric acid to break down proteins (Modina *et al.*, 2021). The pyloric sphincter which regulates the passage of food from the stomach to the small intestine also matures to better control food flow as solid foods are introduced (Leite *et al.*, 2024). The maturation helps manage the more complex digestion process that occurs during weaning. These adaptations in the gastrointestinal system ensure that the infant can digest a variety of foods, improving nutrient uptake and supporting good metabolic growth.

2.2.2.2. Oral and chewing mechanism development

In infants, the oral cavity undergoes significant anatomical and functional changes during weaning. As the infant transitions to solid foods, the jaw and oral muscles strengthen, enabling effective chewing (Infant and Toddler Forum, 2014). This development is essential for breaking down food into smaller pieces, to make it easy to swallow and digest (Kalhoff *et al.*, 2024). During this phase, the eruption of teeth plays a crucial role as teeth support the ability to chew a variety of food textures and ensure the proper function of the jaw muscles (Cudziło, Pałczyńska and Bednarczyk, 2018). The coordination of chewing, swallowing, and breathing down food becomes increasingly refined as the infant adapts to the new demands of solid food intake (Kalhoff *et al.*, 2024). Additionally, the development of oral motor skills, such as clearing food from a spoon with the upper lip and moving food effectively within the mouth, is crucial for progressing to self-feeding (Kalhoff *et al.*, 2024). These changes in oral

and chewing mechanisms are key to ensuring healthy eating habits because they ensure that the infant can safely and effectively consume a variety of foods without limitations.

2.2.2.3. Enzyme activity

During weaning, when the diet of the infant shifts, digestive enzymes responsible for breaking down carbohydrates, fats, and proteins become more active and efficient (Hao *et al.*, 2021). Enzymes responsible for carbohydrate and fat digestion (amylase and lipase, respectively), activity levels increase in order to facilitate the breakdown of solid foods (Crispel *et al.*, 2019). Proteases such as pepsin and trypsin, responsible for protein digestion, also become more active to enhance the breakdown of proteins in solids (Leite *et al.*, 2024). These enzyme activities are influenced by dietary composition, gastrointestinal pH, and microbial interactions, which together optimises digestion and nutrient absorption (Shi *et al.*, 2022). The gut microbiome also starts to diversify at weaning so that it can further supports enzyme function in the breakdown of complex carbohydrates and proteins to promote an efficient digestive process (Walker, 2015). These physiological changes allow the digestive system to effectively handle a wider variety of nutrients found in solid foods in order to meet the growing nutritional demands of the infant and supporting metabolic growth and development.

2.2.2.4. The development of the pancreas

The pancreas undergoes significant functional changes during weaning to meet the increasing dietary demands. As the infant begins to consume solid foods, the level of digestive enzymes (amylase, lipase, and proteases) production increases. This ensures an effective facilitation of digestion of complex diets (Magistrelli *et al.*, 2009; Bonner-Weir, Aguayo-Mazzucato and Weir, 2016). This is followed by regulation of insulin secretion to ensure that glucose levels from carbohydrates are effectively managed by adapting insulin production to match the dietary shift (Bonner-Weir, Aguayo-Mazzucato and Weir, 2016). These metabolic changes help maintain stable blood glucose levels despite the increased complexity of the dietary choices.

When the pancreas undergoes structural and functional maturation, insulin-producing beta (β) cells mature so that they can secrete insulin in response to glucose (Stolovich-Rain *et al.*, 2015). As such, weaning facilitates a maturation step in pancreatic β cells, to enhance both

their mitogenic and secretory responses to glucose, which is essential for maintaining metabolic balance as the infant adapts to solid food consumption (Stolovich-Rain *et al.*, 2015; Bonner-Weir, Aguayo-Mazzucato and Weir, 2016). These changes are fundamental for metabolic homeostasis and metabolic growth.

2.2.2.5. The development of liver function and metabolism

The liver plays a pivotal role during weaning, as it undergoes maturation to handle the increased metabolic load associated with the introduction of solid foods. This includes enhanced fat metabolism, glucose metabolism, and the regulation of various metabolic processes crucial for maintaining energy balance and supporting growth (Nakagaki *et al.*, 2018). The liver adapts by increasing the activity of key enzymes involved in fatty acid oxidation (Carnitine palmitoyl transferase I (CPT-1) and Acyl-CoA dehydrogenase) and gluconeogenesis (Pyruvate carboxylase, Fructose-1,6-bisphosphatase and Glucose-6-phosphatase) and increases the glycogen storage (Tanimizu, 2022). These functional changes make the liver process and store nutrients derived from solid foods effectively, thereby supporting the energy needs of the body as it transitions from a liquid-based to a solid food diet.

Additionally, the liver also undergoes structural changes to accommodate the demands of nutrient processing. The biliary system, including bile canaliculi and bile ducts, matures to facilitate bile secretion, which is essential for the digestion and absorption of fats (Nakagaki *et al.*, 2018; Tanimizu, 2022). These physiological changes are not only crucial for energy balance and growth but also play a foundational role in the development of long-term metabolic health, as disruptions during this critical developmental stage can lead to challenges in metabolic regulation later in life (Yu *et al.*, 2024).

2.2.2.6. Immune system development

The weaning period also affects the development of the immune system. As the diet shifts, the gut microbiome diversifies (Li *et al.*, 2018). This microbial diversification plays a crucial role in regulating metabolism and supporting immune function (Walker, 2015; Green, Arora and Prakash, 2020). The diversification of gut bacteria helps establish a robust immune system and enables the body to build defence against infections (Li *et al.*, 2018). This process also supports the development of the gut-associated lymphoid tissue (GALT), which is an

essential component of the immune system (Modina *et al.*, 2019). These changes enable the body to overcome challenges posed by solid dietary exposures and external pathogens (Li *et al.*, 2018).

2.2.2.7. Hormonal changes

Weaning triggers significant hormonal changes that are crucial for regulating metabolism and energy balance as the diet shifts from milk to solid foods (Souza, De Moura and Lisboa, 2020). Hormones such as ghrelin, leptin, and insulin adjust to support the growing nutritional demands during weaning (Tanimizu, 2022). Ghrelin stimulates appetite and during weaning it increases food intake, while leptin, a key regulator of energy balance, signals satiety and helps modulate long-term fat storage (Tanimizu, 2022). Insulin plays a pivotal role in regulating blood glucose levels in response to carbohydrate intake to effectively convert glucose into energy (Yu *et al.*, 2024). These hormones work together in a complex feedback system to help manage the increased intake of nutrients from solid foods. As the infant adapts to the nutritional complexity of solid foods, ghrelin, leptin, and insulin help promote efficient nutrient absorption, maintain metabolic stability, and regulating energy homeostasis (Gruppuso and Sanders, 2016). This is essential for supporting the physical growth of the body.

2.2.2.8. Neurodevelopment during weaning

Neurodevelopment is a critical aspect of weaning. During weaning, the brain of the infant must adapt to new sensory inputs introduced by solid foods, such as texture, taste, and smell (Infant and Toddler Forum, 2014). This transition requires the development and strengthening of neural pathways to process these sensory experiences (Gabbianelli and Damiani, 2018). Brain plasticity during weaning enables the infant to integrate sensory and motor functions necessary for activities like chewing, swallowing, and food processing (Kalhoff *et al.*, 2024). This neurodevelopmental process is essential not only for feeding behaviours but also for cognitive growth, as it supports the development of motor skills and the ability to learn and perform complex actions (Kalhoff *et al.*, 2024). During this process, the brain forms and refines neural circuits responsible for controlling the muscles used in feeding and other early developmental tasks (Fico *et al.*, 2023). The ability to adapt and learn new sensory-motor tasks lays the foundation for the infant to develop independent feeding

behaviours and healthy eating habits, both of which are integral for long-term cognitive and social development (Infant and Toddler Forum, 2014; Kalhoff *et al.*, 2024).

Given the significant impact of early-life nutrition on long-term health, it is important to consider how metabolic programming through early nutritional exposures influences the risk of diseases later in life. The timing of weaning, particularly when it occurs too early, can disrupt the natural development of metabolism and immune system explained above. This can increase the risk of chronic health issues such as obesity, insulin resistance, renal and hepatic dysfunction and other metabolic disorders.

2.3. Early weaning and metabolic health

Early weaning is defined as the premature transition from exclusive milk feeding (either breastmilk or formula) to the introduction of solid foods, typically occurring before the recommended age of six months (WHO, 2020). Socio-economic pressures, cultural practices, and environmental factors often influence this transition, which can all impact the metabolic health of the infant in both the short and long-term (Brown *et al.*, 2014). In South Africa, where socio-economic challenges such as maternal employment, poverty, and inadequate access to breastfeeding support are prevalent, early weaning practices are becoming more common, leading to increased risks of metabolic dysfunction, obesity, and other chronic diseases (Vitalis *et al.*, 2021).

2.3.1. Factors contributing to early weaning

Several factors contribute to the timing and quality of weaning. These factors include environmental factors, such as cultural practices, socioeconomic status, sedentary lifestyle and access to healthcare.

2.3.1.1. Cultural practices as a contributor towards early weaning

Cultural practices can influence the timing of weaning. In some communities, traditional beliefs about infant nutrition may encourage the early introduction of solid foods, often before six months of age (Chakona, 2020). For example, in a study among Saudi Arabian mothers, it was reported that parents introduced supplementary foods early because they thought breast milk alone would not provide sufficient nutrition for their infants in the first

six months (Quebu, Murray and Okafor, 2023). In addition, the mothers also interpreted the unexplained weeping and increased appetite of infants as signs that breast milk alone was insufficient to satisfy their hunger (Quebu, Murray and Okafor, 2023). Consequently, the mothers then introduced other liquids and solid foods prematurely as early as 3 to 4 months of age (Quebu, Murray and Okafor, 2023).
This practice, while rooted in cultural norms, contradicts the recommended weaning period practices and increase the risk of metabolic disorders (Budree *et al.*, 2016).

2.3.1.2. Socio-economic status as a cause of early weaning

Socio-economic status also plays a critical role in early weaning practices. Families from lower-income backgrounds may resort to cheap, processed foods, often rich in sugar and unhealthy fats, as their weaning choice of food (Sayed *et al.*, 2020). These foods do not provide essential nutrients needed for healthy metabolic development and they increase the likelihood of developing obesity, insulin resistance, and other metabolic disorders later in life (Vitalis *et al.*, 2021).

2.3.1.3. Employment and sedentary lifestyle and its influence on early weaning

The sedentary lifestyle of many urban South African families contributes to the challenges of maintaining a healthy metabolic profile during the early stages of childhood development (Portela *et al.*, 2015). For example, in South Africa, many working mothers face significant challenges in adhering to the WHO recommendation of exclusive breastfeeding for the first six months (Doherty *et al.*, 2020). Limited maternity leave, inadequate workplace support for breastfeeding often lead to early weaning practices (Teena and Sujatha, 2016). As a result, infants are introduced to solid foods prematurely.

Physical activity plays a vital role in regulating metabolism, supporting insulin sensitivity, and reducing the risk of obesity (Katzmarzyk *et al.*, 2022). However, modern work environments, technological advancements, and a reliance on sedentary forms of entertainment, such as television and video games, have contributed to a decrease in physical activity levels among both children and adults in South Africa (Brumana *et al.*, 2017). For infants and young children, reduced physical activity and lack of movement can further exacerbate the adverse effects of early weaning. The introduction of solid foods and the disruption of normal metabolic programming, compounded by physical inactivity, promotes

an imbalance between calorie intake and energy expenditure (Liu *et al.*, 2020). This imbalance leads to fat storage, particularly visceral fat, which is strongly linked to metabolic diseases like type 2 diabetes mellitus (T2DM) and MAFLD (Park *et al.*, 2020).

2.3.1.4. Lack of access to health care as a cause for early weaning

Access to healthcare is a key factor influencing the timing of weaning, as limited access to healthcare services and breastfeeding support can significantly contribute to early weaning practices (Stuebe *et al.*, 2014). In many low-resource settings, mothers face lack of education on infant nutrition, insufficient access to lactation consultants, and inadequate prenatal and postnatal care (Stuebe *et al.*, 2014; de Holanda and da Silva, 2022). These challenges often result in mothers introducing solid foods or formula before the recommended six months due to lack of knowledge. Without access to professional support, common issues such as breastfeeding difficulties or infant difficult behaviours may be misinterpreted as signs of inadequate nutrition, prompting early supplementation (de Holanda and da Silva, 2022). As such, these barriers and lack of healthcare access can lead to the contribution of early weaning to inadequate health and development.

2.3.2. The effect of early weaning on developmental plasticity

Developmental plasticity refers to the ability of the physiological systems of infants to adapt to early-life environmental conditions (Peixoto *et al.*, 2019). In the context of weaning, this means that the body has a window of opportunity to adjust its metabolism in response to the introduction of new foods (Peixoto *et al.*, 2019). Early weaning can disrupt developmental plasticity by altering the ability of an infant to adapt to nutritional changes, leading to metabolic ill-programming that affects long-term health and results in metabolic dysfunction (Acevedo *et al.*, 2021). Furthermore, by interfering with the natural processes of metabolic programming during the critical stages of development, early weaning may increase the risk of metabolic dysfunction and chronic diseases later in life, aligning with the hypothesis of the Developmental Origins of Health and Disease (DOHaD) (Koemel and Skilton, 2022).

2.3.2.1. Developmental origins of health and disease (DOHaD)

The concept of DOHaD plays a critical role in understanding how early life factors influence long-term health outcomes, particularly in relation to metabolic dysfunction.

The DOHaD framework originated from studies investigating the relationship between early-life conditions and adult-onset diseases (Koemel and Skilton, 2022). Initially proposed based on epidemiological studies, DOHaD has expanded to encompass several biological mechanisms, including epigenetic modifications, that link early developmental factors with long-term health (Koemel and Skilton, 2022). According to the DOHaD hypothesis, exposure to adverse conditions such as poor maternal nutrition, exposure to toxins, or stress during gestation and early childhood can programme physiological systems of the body, increasing the risk of metabolic diseases in later life (Lacagnina, 2020). These early exposures may alter the development of organs and systems such as the pancreas, liver, adipose tissue, and kidneys, affecting energy metabolism, insulin sensitivity, and fat storage, which are key components in the development of metabolic diseases (Lacagnina, 2020).

It is important to note that the DOHaD framework acknowledges that the risk of metabolic dysfunction is not solely determined by genetic inheritance but is heavily influenced by the early life environment too (Koemel and Skilton, 2022).

2.3.3. The effect of early weaning (human studies)

In human studies, early weaning before infants are 4 months old (16 weeks) has been associated with increased risk of infections, including diarrhoea and premature mortality (Fawzy *et al.*, 2011). In addition to infection risks, Early weaning has been shown to result in undernutrition and health issues such as pale skin, tiredness, swollen joints, and upset stomachs in children weaned at 4 months of age (Suliman *et al.*, 2023). Early weaning before six months has also been associated with increased malnutrition and emotional stress for mothers (Alves *et al.*, 2023). In a study where 26% of mothers initiated early weaning, with most of them beginning the process at 4 months of age (29.9%), followed by 25.9% at 5 months, 21.6% at 3 months, and 11.3% starting between 1-2 months, early weaning resulted in a higher risk of allergies (28.9%), obesity (12.7%), respiratory problems (11.9%), and choking (25.5%) in infants (Gahima *et al.*, 2024).

2.3.4. The effects of early weaning (animal studies)

In Wistar rats, early weaning (PD 18) resulted in increased visceral fat accumulation, hyperleptinaemia, and insulin resistance in both male and female offspring (Miranda *et al.*, 2020). The effects of early weaning observed in male Wistar rats included altered

glucocorticoid signalling in adipose tissues, which led to changes in adipogenesis and lipogenesis (Peixoto *et al.*, 2019). Additionally, Wistar rats showed impaired brown adipose tissue (BAT) thermogenesis, with reduced levels of Uncoupling Protein 1 (UCP-1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) proteins, because of early weaning (Soares *et al.*, 2022). In another study, early weaning (PD 16) in rats resulted in morphological changes of the small intestine mucosa, including deeper crypts, a lower villous/crypt ratio, and a smaller villous area at just 21 days post weaning (Crispel *et al.*, 2019). At 90 days, it led to shallower crypts, a greater villous/crypt ratio, and a smaller villous area compared to normally weaned rats, and these changes were linked to differences in food absorption and somatic growth (Crispel *et al.*, 2019). All these are indications of metabolic dysfunction.

2.4. Metabolic dysfunction

2.4.1. Definition of metabolic dysfunction/metabolic syndrome

Abnormalities in metabolic processes are a characteristic of metabolic syndrome, a multi-tissue and multi-organ disorder that affects the liver, heart, kidney and adipose tissue (Figure 2.2) (Vincent, 2017). It is closely linked to disruptions in the metabolism of fat and glucose, which can result in diseases like obesity, cardio metabolic diseases, and metabolic dysfunction associated fatty liver disease (MAFLD) (Eslam, Sanyal, *et al.*, 2020). Impaired insulin signalling, dysregulated lipid homeostasis, chronic inflammation, and elevated pro-thrombotic tendencies are important indicators of metabolic dysfunction and raise the risk of cardiovascular disorders (Al Hashmi *et al.*, 2024).

These risk factors when combined, significantly raise the chance of developing type 2 diabetes and cardiovascular conditions among other diseases shown in Figure 2.2 (ADA, 2022; Davies *et al.*, 2022; Godoy-Matos *et al.*, 2024). The complex interactions between environmental influences, dietary choices, and genetic predisposition all have a role in the development and occurrence of metabolic dysfunction, making a current management strategy and thorough understanding of the condition important.

Considering the constituent components of metabolic dysfunction, it is important to explore how it is diagnosed.

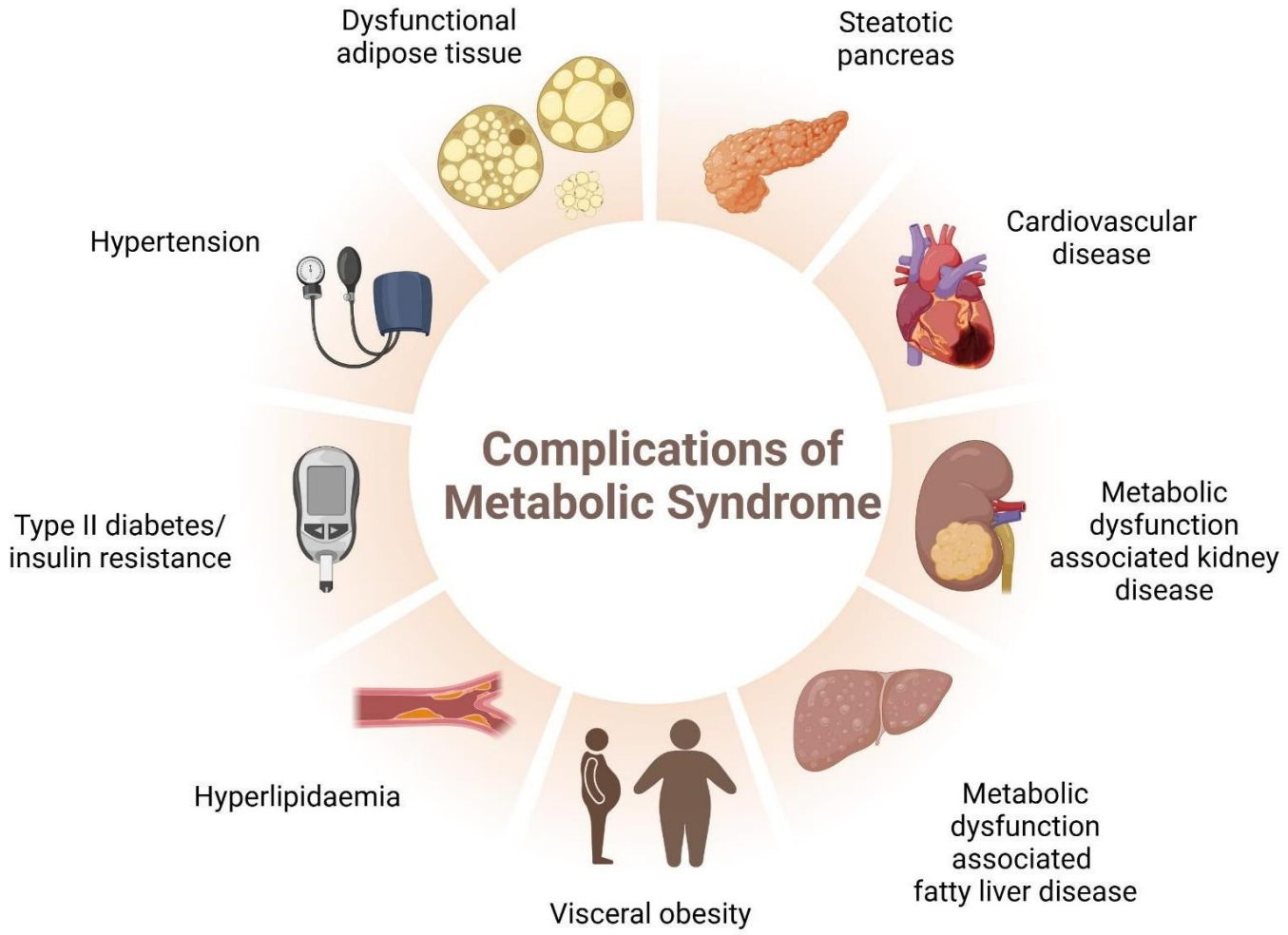


Figure 2. 2. Pathophysiology of metabolic dysfunction associated with metabolic syndrome and its effects on multiple organs, image designed in Biorender.

2.4.2. Diagnosis of metabolic dysfunction associated with metabolic syndrome

A comprehensive clinical assessment is required to diagnose metabolic dysfunction associated with metabolic syndrome, which involves analysing indicators to understand the complex relationships within metabolic pathways. Assessment typically involves measuring fasting glucose levels, insulin sensitivity, and lipid profiles (AHA, 2023). Additional diagnostic methods, such as imaging examinations to assess the location of adipose tissue and markers of inflammation, assist in creating a complete profile (Ko *et al.*, 2023). Additionally, identification of associated comorbidities, including hypertension and cardiovascular risk factors, forms a crucial component of the diagnostic process (Díaz-Ortega *et al.*, 2023). By adopting a comprehensive approach that incorporates both clinical and laboratory analysis, it is possible to obtain a precise diagnosis and provide tailored therapeutic recommendations based on the analysis results.

Numerous methods and standards have been developed to provide a complete diagnosis of metabolic dysfunction associated with metabolic syndrome that is appropriate for diverse groups, including adults and children. Key metabolic risk variables such as visceral obesity, fasting glucose levels, blood pressure, triglycerides, and HDL cholesterol levels are usually at the centre of the diagnostic criteria for metabolic dysfunction (Fahed *et al.*, 2022). However, these standards may vary significantly based on guidelines or group under evaluation.

The guidelines from the World Health Organisation (WHO, 2023a), the National Cholesterol Education Program Adult Treatment Panel III (Lorenzo *et al.*, 2007), and the International Diabetes Federation (IDF, 2005) are the most often cited ones for adults (Table 2.1).
The metabolic dysfunction diagnostic standards for children are specifically tailored to take into account their distinct physiological and developmental traits. Age and sex-specific thresholds are important considerations in the criteria provided by several health organisations, including the WHO, the NCEP, and the IDF (Table 2.1) (Díaz-Ortega *et al.*, 2023). Children and adolescents undergo fast development and hormonal changes that might affect metabolic parameters, making these adjustments essential (Jee, Jumani and Mericq, 2023).

Table 2. 1. Updated diagnostic criteria for metabolic dysfunction associated with metabolic syndrome.

ADULTS			
Criteria	**International Diabetes Federation**	**The national cholesterol education program adult treatment panel III (NCEP ATP III)**	**World health organisation**
Visceral Obesity	Waist circumference ≥94 cm (men), ≥80 cm (women)	Waist circumference ≥102 cm (men), ≥88 cm (women)	Waist-to-hip ratio >0.90 (men), >0.85 (women), BMI >30 kg/m²
Fasting Glucose	≥5.6 mmol/L	≥5.6 mmol/L	IGT, IFG, or diabetes, or insulin resistance measured by HOMA
Blood Pressure	≥130/85 mm Hg or treatment	≥130/85 mm Hg or treatment	≥140/90 mm Hg or antihypertensive medication
Triglycerides	≥1.7 mmol/L	≥1.7 mmol/L	≥1.7 mmol/L
HDL Cholesterol	<1.0 mmol/L (men), <1.3 mmol/L (women)	<1.0 mmol/L (men), <1.3 mmol/L (women)	<0.9 mmol/L (men), <1.0 mmol/L (women)
CHILDREN			
Criteria	**Children** (IDF, 2007; Díaz-Ortega *et al.*, 2023)	(NCEP, 2002; Grundy, 2013)	(WHO, 2023a)
Central Obesity	Waist circumference >90th percentile for age/sex	Waist circumference >90th percentile for age	Waist-to-height ratio >0.5
Fasting Glucose	≥5.6 mmol/L	≥5.6 mmol/L	IGT, IFG, or diabetes
Blood Pressure	≥90th percentile for age/sex or treatment	≥90th percentile for age/sex or treatment	≥90th percentile for age/sex
Triglycerides	≥1.1 mmol/L	≥1.1 mmol/L	≥1.1 mmol/L
HDL Cholesterol	<1.03 mmol/L	<1.03 mmol/L	<1.03 mmol/L

(NCEP, 2002; IDF, 2005, 2007; Lorenzo *et al.*, 2007; Grundy, 2013; Díaz-Ortega *et al.*, 2023; WHO, 2023a)

BMI (Body Mass Index), IGT (Impaired Glucose Tolerance), IFG (Impaired Fasting Glucose), HOMA (Homeostasis Model Assessment), HDL (High-Density Lipoprotein), NCEP (National Cholesterol Education Program), ATP (Adult Treatment Panel), IDF (International Diabetes Federation) and WHO (World Health Organisation).

2.4.3. Prevalence & epidemiology of metabolic syndrome/dysfunction

2.4.3.1. Prevalence of metabolic dysfunction in children globally

Around the world, paediatric metabolic dysfunction has alarming high rates and is strongly associated with the rise in type 2 diabetes and childhood obesity. Over the past several decades, there has been a significant increase in the prevalence of childhood obesity (Rito *et al.*, 2019). In 2020, there were more than 38 million overweight or obese children under the age of five, and in 2015, there were 124 million overweight or obese children between the ages of five and nineteen, up from 18 million in 1975 (WHO, 2023b). Increased rates of metabolic dysfunction, such as insulin resistance, dyslipidaemia, and hypertension, are recorded in younger populations globally as a result of the rise in children obesity (Pappachan, Fernandez and Ashraf, 2024). Sedentary lifestyles, high-calorie diets, and the growing accessibility of processed foods are all contributing reasons.

2.4.3.2. Prevalence of metabolic dysfunction in children in South Africa

Similar to global trends, childhood obesity and metabolic dysfunction are becoming major issues in South Africa (Otitoola, Oldewage-Theron and Egal, 2020). There has been a notable increase in metabolic diseases in children due to the growing prevalence of overweight and obesity (Otitoola, Oldewage-Theron and Egal, 2020). Local research indicates that between 10 and 20 percent of South African children are overweight or obese, with urban regions being more affected by factors related to lifestyle such as diets heavy in processed foods and less physical activity (Otitoola, Oldewage-Theron and Egal, 2020). Given that children are at risk for early-onset metabolic dysfunction, insulin resistance, and other related disorders, this trend is alarming.

2.4.3.3. Prevalence of obesity in adults globally

Global obesity rates have nearly quadrupled since 1975, according to the World Health Organisation (WHO), with over 2.5 billion individuals categorised as overweight and over 890 million obese (WHO, 2023a). In addition to obesity, over 830 million individuals worldwide now suffer from diabetes, which has become more prevalent (WHO, 2024). These disorders have a significant correlation with metabolic dysfunction, and as sedentary lifestyles and high-calorie diets grow more prevalent worldwide, the number of cases is predicted to rise even further (Noubiap *et al.*, 2022).

2.4.3.4. Prevalence of obesity in adults in South Africa

Obesity affects over 30% of adult South Africans, affecting women more than males (Pheiffer *et al.*, 2018. Women in South Africa have a higher prevalence of obesity compared to men, with recent studies indicating that 42.9% of adult women are living with obesity, a figure that is significantly higher than the 18.2% prevalence among men (Case and Menendez, 2009; Global Nutrition Report, 2023). This increased prevalence is partly attributed to inherent biological and physiological characteristics, including hormonal fluctuations, fat distribution patterns, neurobiological responses to food stimuli, and metabolic differences, which collectively make women more vulnerable to excess weight gain and related metabolic complications (Kanter and Caballero, 2012; Guglielmi *et al.*, 2024). According to Zhang *et al.*, (2022), this has led to a rise in dyslipidaemia, hypertension, and type 2 diabetes too because of urbanisation and dietary changes. As a result, public health initiatives are being launched nationwide to address the adult obesity pandemic and the related metabolic dysfunctions (Spearman *et al.*, 2021).

2.4.4. Factors contributing to the development of metabolic dysfunction

2.4.4.1. Biological factors contributing to the development of metabolic dysfunction

The intricate interactions between several biological systems that regulate energy balance, fat accumulation, and metabolic activities lead to metabolic disorders. Many underlying factors, such as hormone imbalance, imbalances in the gut microbiota, inflammation in adipose tissue, and mitochondrial dysfunction, can cause disruptions to these systems (Thomas *et al.*, 2022).

a. Hormonal regulation of leptin and ghrelin

Adipocytes, or fat cells, release the hormone leptin, which instructs the brain, especially the hypothalamus to boost energy expenditure and decrease hunger (Guzzardi *et al.*, 2021). Its main purpose is in satiety which helps prevent overeating (Rohde *et al.*, 2019). Leptin resistance is a disorder that affects people with metabolic dysfunction, particularly those who are obese (Guzzardi *et al.*, 2021). The brain does not respond to the normal satiety signal from high leptin levels, consequently overeating and further fat deposition (Blüher, 2019). However, when energy levels are low, the stomach produces ghrelin that serves as a hunger signal, boosting appetite and food consumption (Hadzhibozheva *et al.*, 2023). Ghrelin controls energy intake in healthy subjects by rising before meals and falling after (Hadzhibozheva *et al.*, 2023). However, even in cases where the body has enough energy

reserves, ghrelin levels in individuals with metabolic dysfunction may continue to rise, leading to an increase in food intake (Blüher, 2019; Hadzhibozheva *et al.*, 2023). A chronic condition of positive energy balance, where more calories are ingested than expended, is caused by the dysregulation of the ghrelin and leptin signals, contributing to weight gain and metabolic abnormalities (Hadzhibozheva et al., 2023).

b. Gut microbiome dysbiosis

The gut microbiome is essential for controlling immunological response, digestion, and energy metabolism (Green, Arora and Prakash, 2020). Dysbiosis, or an imbalance in the makeup of gut microbes, is becoming more widely acknowledged as a major factor in metabolic dysfunction (Green, Arora and Prakash, 2020). Firmicutes and Bacteroidetes, the two main bacterial phyla in the gut, are responsible for the digestion and fermentation of food ingredients (Meale *et al.*, 2017; Li *et al.*, 2022). Increased fat deposition and energy absorption have been associated with a greater Firmicutes to Bacteroidetes ratio, which may lead to obesity and insulin resistance (Li *et al.*, 2022).

Dysbiosis interferes with the normal functioning of the gut in subjects with metabolic disorders, which increases the production of toxic metabolites such as lipopolysaccharides (LPS) (Mostafavi Abdolmaleky and Zhou, 2024). Gram-negative bacteria have a component called LPS that may cross the intestinal barrier and enter the circulation, where it causes a systemic inflammatory response (Mostafavi Abdolmaleky and Zhou, 2024). Metabolic endotoxemia, a persistent low-grade inflammation, impairs insulin signalling and encourages the emergence of insulin resistance and metabolic diseases (O'Toole and Shiels, 2020).

Additionally, the production of advantageous short-chain fatty acids (SCFAs) such as butyrate, which are protective in preserving metabolic balance, is attributed to gut bacteria (He *et al.*, 2020). Research has demonstrated that SCFAs, which are created during the fermentation of dietary fibre, improve insulin sensitivity, lower inflammation, and encourage the metabolism of fat (He *et al.*, 2020). Nevertheless, the generation of SCFAs is reduced in dysbiotic microbiota, which exacerbates metabolic dysfunction (He *et al.*, 2020). As such, probiotic and prebiotic therapies are being investigated as possible treatments for metabolic illnesses including diabetes and obesity (Green, Arora and Prakash, 2020). These interventions attempt to restore the equilibrium of the gut microbiome (Thomas *et al.*, 2022).

c. Adipose tissue dysfunction and inflammation

Adipose tissue is an important endocrine organ that secretes hormones that control metabolism in addition to acting as a storage of energy (Brunt *et al.*, 2015; Frühbeck *et al.*, 2019). However, when there is metabolic dysfunction, fat, especially visceral fat, which encompasses essential organs, stops functioning (Brunt *et al.*, 2015). The capacity of adipose

tissue to store fat is weakened as it grows, particularly in obesity, and this causes pro-inflammatory cytokines like TNF-α, IL-6, and CRP (C-reactive protein) to be released (Fico *et al.*, 2023). According to Frühbeck *et al.*, (2018), these pro-inflammatory chemicals cause a long-term low-grade inflammatory state that interferes with metabolic functions and increases insulin resistance and lipotoxicity.

Lipotoxicity, or the excessive amount of fatty acids and triglycerides infiltrating into non-adipose tissues including the liver, muscles, and pancreas, is another consequence of inflamed adipose tissue (Velarde-Ruiz Velasco *et al.*, 2019). According to Frühbeck *et al.*, (2018), this ectopic fat deposition worsens the function of these organs by increasing insulin resistance, fostering metabolic dysfunction associated fatty liver disease (Figure 2.2) (MAFLD). As compared to subcutaneous fat, which is stored immediately beneath the skin, visceral fat is more metabolically active and prone to inflammation, making its build-up more harmful (McLaughlin *et al.*, 2011).

Adipose tissue malfunction not only results in insulin resistance but also dysregulates the production of hormones, which includes elevated levels of leptin and lowered levels of adiponectin, an anti-inflammatory hormone that enhances insulin sensitivity (Reddy *et al.*, 2019). The inflammatory condition in the adipose tissue attracts immune cells like macrophages, which penetrate fat tissue and intensify inflammation (Reddy *et al.*, 2019). Inflammation and metabolic dysfunction are generated in a vicious cycle by the interaction between immune cells and adipocytes (Reddy *et al.*, 2019).

d. Mitochondrial dysfunction

Mitochondria produce energy required by cells for survival, mostly through oxidative phosphorylation (San-Millán, 2023). Mitochondrial activity is frequently impaired in the setting of metabolic dysfunction, which results in a reduction in energy production and an increase in reactive oxygen species (ROS) (San-Millán, 2023). San-Millán, (2023) further explains that these ROS are potentially harmful substances that lead to oxidative stress, which damages proteins, DNA, and cellular structures. Since insulin-sensitive organs including muscles and liver cells mostly rely on mitochondrial energy to control glucose metabolism, mitochondrial dysfunction and insulin resistance are intimately related (Weiss, Bremer and Lustig, 2013).

The mitochondria are unable to make enough energy when there is a build-up of glucose in the circulation, a defining feature of hyperglycemia, resulting from cells being less effective at utilising glucose (Sifuentes-Franco *et al.*, 2018). Moreover, fatty acid oxidation, which is the mechanism by which the body converts lipids into energy, is impaired by mitochondrial dysfunction (Goedeke *et al.*, 2018). Fats therefore build up in organs such as the liver, which aggravates insulin resistance and metabolic dysfunction and results in metabolic dysfunction associated fatty liver disease (MAFLD) (Rinaldi *et al.*, 2021). This makes lipotoxicity a major factor in the development of metabolic diseases (Velarde-Ruiz Velasco *et al.*, 2019).

Muscle weakness and decreased physical endurance are two other symptoms of mitochondrial dysfunction that are prominent in subjects with metabolic diseases (Fusagawa *et al.*, 2023). Muscle contraction efficiency is compromised during physical exercise due to reduced ATP synthesis resulting from impaired mitochondrial function in muscle cells (Fusagawa *et al.*, 2023). Due to the loop of inactivity and energy imbalance that is created by this decreased energy output, weight gain and metabolic dysfunction are further encouraged (Chen, Zhao and Li, 2023). Therefore, the capacity of the body to maintain metabolic homeostasis deteriorates with a reduction in mitochondrial health, which raises the risk of further developing metabolic disorders (Chen, Zhao and Li, 2023).

2.4.4.2. Epigenetic factors contributing to the development of metabolic dysfunction

Epigenetic changes are modifications in gene expression without alterations in the underlying DNA sequence (Acevedo *et al.*, 2021). These changes are influenced by environmental factors such as diet, physical activity, and exposure to toxins, and they contribute to how the body processes energy and stores fat (Hruby and Hu, 2015). The key processes being DNA methylation, histone modification, and non coding RNAs. Each will be briefly discussed in turn.

a. DNA methylation

DNA methylation is a process in which a methyl group is added to DNA, usually at cytosine-phosphate-guanine (CpG) sites, leading to changes in gene expression (Smith, Chandanathil and Millis, 2023). This epigenetic modification can either enhance or suppress the expression of specific genes that regulate metabolism, fat storage, and insulin sensitivity (Zhou *et al.*,

2020). For example, hyper methylation of genes involved in insulin signalling can reduce their expression, leading to impaired glucose metabolism and insulin resistance (Acevedo *et al.*, 2021). The DNA methylation patterns can be influenced by lifestyle factors such as diet and physical activity, highlighting the dynamic relationship between the environment and gene expression (Acevedo *et al.*, 2021).

In the context of metabolic dysfunction, DNA methylation often affects genes that regulate adipogenesis (the formation of fat cells), energy homeostasis, and lipid metabolism (Loh *et al.*, 2019). Studies have shown that subjects with obesity tend to have different methylation patterns in these genes compared to subjects with normal metabolic function (Loh *et al.*, 2019). These epigenetic changes contribute to the alteration of the ability of the body to store and burn fat, making it more difficult for the body to manage energy balance and avoid fat accumulation (Loh *et al.*, 2019). Environmental factors such as a high-fat diet or prolonged exposure to endocrine-disrupting chemicals (EDCs) can induce abnormal methylation patterns that predispose individuals to metabolic dysfunction (Encarnação *et al.*, 2019). For instance, prenatal exposure to a nutrient-poor environment has been shown to alter methylation patterns in genes associated with glucose and lipid metabolism, increasing the risk of developing obesity and type 2 diabetes later in life (Smith, Chandanathil and Millis, 2023).

Furthermore, changes in DNA methylation can also be reversible with lifestyle interventions such as diet modification and increased physical activity (Acevedo *et al.*, 2021). Research has shown that individuals who adopt a healthier lifestyle, including weight loss and regular exercise, experience changes in DNA methylation patterns that improve insulin sensitivity and metabolic health (Hruby and Hu, 2015). This highlights the importance of early intervention in preventing and managing metabolic dysfunction through lifestyle changes.

b. Histone modifications

Histone modifications represent another important epigenetic mechanism that influences gene expression. Histones are proteins that help package DNA into a compact structure called chromatin (Smith, Chandanathil and Millis, 2023). By adding or removing chemical groups such as acetyl or methyl groups to histones, cells can control how tightly or loosely DNA is wrapped around them, thus regulating gene accessibility (Sifuentes-Franco *et al.*, 2018).

Histone acetylation generally promotes gene expression by relaxing chromatin, while histone deacetylation tightens chromatin and suppresses gene expression (Loh *et al.*, 2019).

In metabolic dysfunction, changes in histone acetylation and methylation patterns affect genes involved in fat metabolism, insulin signalling, and glucose regulation (Zhou *et al.*, 2020). For instance, high fats and sugars diets can promote histone modifications that enhance the expression of genes driving fat accumulation, while simultaneously suppressing genes that regulate fat oxidation (Zhou *et al.*, 2020). Over time, these changes contribute to the storage of excess fat, particularly in visceral tissues, exacerbating insulin resistance and metabolic dysfunction (Zhou *et al.*, 2020).

Environmental factors such as caloric restriction or the intake of bioactive compounds, such as those found in fruits like grapes and vegetables like broccoli, can also induce beneficial histone modifications (Hruby and Hu, 2015; Block and El-Osta, 2017). For example, compounds like resveratrol, found in grapes, have been shown to activate histone acetyltransferases (HATs), which promote the expression of genes that enhance insulin sensitivity and reduce fat accumulation (Hruby and Hu, 2015). This further underscores the interplay between genetics, lifestyle, and metabolic health, with epigenetic modifications being a key mechanism through which environmental factors influence metabolic outcomes.

c. Non-coding RNAs

Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), play critical roles in regulating gene expression in the post-transcription stage (Zhou *et al.*, 2020). Unlike messenger RNAs (mRNAs), which code for proteins, non-coding RNAs regulate the activity of other RNAs by binding to them and either promoting or inhibiting their translation into proteins (Smith, Chandanathil and Millis, 2023). In the context of metabolic dysfunction, miRNAs have been linked to the regulation of genes involved in lipid metabolism, glucose homeostasis, and insulin sensitivity (Zhou *et al.*, 2020).

Specific miRNAs have been shown to suppress the expression of genes involved in fat oxidation (lipolysis), promoting fat storage instead (Mandala *et al.*, 2020). Conversely, some miRNAs can downregulate insulin-signalling pathways, leading to insulin resistance and impaired glucose metabolism (Mandala *et al.*, 2020). These regulatory roles make miRNAs

central to the development of metabolic dysfunction, and they are considered potential biomarkers for diagnosing metabolic dysfunction early (Zhou *et al.*, 2020).

2.4.4.3. Genetic factors contributing to the development of metabolic dysfunction

Genetic predispositions play a big role in determining susceptibility to metabolic dysfunction. These genetic influences affect key metabolic processes such as fat storage, energy utilisation, and insulin sensitivity, increasing the likelihood of developing metabolic dysfunction (Hruby and Hu, 2015). Genome-wide association studies (GWAS) are used to understand the complex mechanisms driving metabolic diseases (Uffelmann *et al.*, 2021).

a. Genome-wide association studies (GWAS)

Genome-wide association studies (GWAS) are large-scale research efforts aimed at identifying genetic variations across the entire genome that are associated with specific traits or diseases (Uffelmann *et al.*, 2021). By comparing the genomes of subjects with and without certain conditions, researchers can locate genetic loci linked to traits such as obesity, fat distribution, and insulin resistance (Uffelmann *et al.*, 2021). One of the most well-studied genetic loci identified through GWAS is the fat mass and obesity-associated gene (FTO gene), which is strongly linked to obesity and increased body mass index (BMI) (Tirthani, Said and Rehman, 2023). Subjects with specific FTO variants have been found to have a higher appetite and reduced satiety, leading to increased caloric intake and fat storage (Uffelmann *et al.*, 2021). This gene is particularly relevant in populations with high access to calorie-dense foods, where genetic susceptibility can lead to rapid weight gain and metabolic imbalances.

Other genes identified through GWAS include those involved in leptin signalling, which regulates hunger and fat storage. Variants in genes such as LEP (leptin) and LEPR (leptin receptor) can disrupt the normal functioning of leptin, leading to leptin resistance (Rohde *et al.*, 2019). Additionally, GWAS has identified genetic variants in insulin-signalling pathways, which are associated with an increased risk of type 2 diabetes (Tirthani, Said and Rehman, 2023). These variants impair the ability of the body to regulate glucose levels and maintain insulin sensitivity (Tirthani, Said and Rehman, 2023).

GWAS has also highlighted genetic variants in sterol regulatory element-binding protein-1c (*SREBP-1c*), acetyl-CoA carboxylase 1 (*ACC-1*), carnitine palmitoyltransferase 1 (*CPT-1*), peroxisome proliferator-activated receptor alpha (*PPAR-α*), and fatty acid synthase (*FAS*) (Deng *et al.*, 2014; Pawlak, Lefebvre and Staels, 2015; Tirthani, Said and Rehman, 2023). These genes are central to lipid metabolism and energy regulation. For example, *SREBP-1c* is involved in the synthesis of fatty acids and cholesterol (Moslehi and Hamidi-Zad, 2018). Variants that over activate this gene can lead to increased lipid accumulation in tissues, contributing to obesity and insulin resistance (Moslehi and Hamidi-Zad, 2018). *ACC-1* plays a key role in fatty acid biosynthesis, and its genetic variants have been associated with altered fat storage and metabolic imbalances (Goedeke *et al.*, 2018). *CPT-1*, essential for the transport of fatty acids into mitochondria for beta-oxidation, has variants linked to impaired fat oxidation and increased fat accumulation (Deng *et al.*, 2014). *PPAR-α* is crucial in regulating lipid metabolism, and genetic variations in this gene are associated with changes in fat oxidation and the risk of developing metabolic diseases (Pawlak, Lefebvre and Staels, 2015). In addition, *FAS* is involved in the synthesis of long-chain fatty acids, and its variants are linked to fat accumulation and metabolic disorders such as metabolic dysfunction associated fatty liver disease (MAFLD) (Dorn *et al.*, 2010).

b. Genetic variants in energy metabolism

Genetic variants that influence energy metabolism play a crucial role in determining how efficiently the body converts food into energy and stores excess calories as fat (Rohde *et al.*, 2019). Polymorphisms in genes that regulate adiponectin, a hormone secreted by adipose tissue, have been associated with impaired glucose metabolism and increased fat accumulation (Quek *et al.*, 2022). Adiponectin enhances insulin sensitivity and promotes fat oxidation, as such subjects with lower levels of adiponectin due to genetic variants are more likely to develop insulin resistance and metabolic dysfunction (Mansour *et al.*, 2023).

c. Heritability and metabolic dysfunction

The heritability of metabolic traits, such as BMI and fat distribution, suggests that genetic factors account for a significant portion of the variance in body weight among individuals (Tirthani, Said and Rehman, 2023). Studies estimate that the heritability of BMI is between 40% and 70%, meaning that genetic factors play a major role in determining how individuals respond to their environment in terms of weight gain and metabolic health (Uffelmann *et al.*,

2021). This heritability is particularly evident in families where obesity and metabolic disorders like T2DM are prevalent across generations (Uffelmann *et al.*, 2021). However, genetic predisposition is not deterministic. Environmental factors, such as diet, physical activity, and exposure to pollutants, interact with genetic predispositions to influence the risk of developing metabolic dysfunction (Smith, Chandanathil and Millis, 2023).

2.4.4.4. Sedentary lifestyle and metabolic dysfunction

A sedentary lifestyle characterised by physical inactivity and high energy diets (high fats or sugar diets) has a profound impact on insulin sensitivity and lipid metabolism, both of which contribute to the development of metabolic dysfunction (Katzmarzyk *et al.*, 2022). Physical inactivity and high energy diets reduce the use of glucose in the body, as muscle tissue becomes less responsive to insulin, leading to hyperglycaemia and generalised insulin resistance (Katzmarzyk *et al.*, 2022). This causes the pancreas to overproduce insulin, eventually exhausting its function and resulting in T2DM (Petersen and Shulman, 2018).

In addition to affecting glucose metabolism, a sedentary lifestyle also disrupts lipid metabolism. Physical inactivity elevates triglyceride levels and low-density lipoprotein (LDL) cholesterol, while reducing high-density lipoprotein (HDL) cholesterol (Feingold, 2020). These changes contribute to dyslipidaemia, which is a key component of metabolic dysfunction, and significantly increase the risk of cardiovascular diseases (Feingold, 2020). Furthermore, excess visceral fat resulting from sedentary behaviour, worsens metabolic health by releasing pro-inflammatory cytokines (Reddy *et al.*, 2019). This pro-inflammatory cytokines which are tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) impair insulin signalling, contributing to insulin resistance and metabolic dysfunction (Reddy *et al.*, 2019).

2.4.4.5. Obesity as a risk factor for the development of MAFLD and nephropathy

Obesity contributes to metabolic dysfunction by creating an imbalance between energy intake and expenditure, leading to excess fat accumulation in several vital organs like the liver and kidney (Powell-Wiley *et al.*, 2021). One of the most common conditions linked to obesity is MAFLD. Similarly, obesity negatively affects kidney function, leading to conditions like obesity-related glomerulopathy (ORG) (Wei *et al.*, 2021). ORG is marked by increased kidney size and accelerated chronic kidney disease (CKD) progression due to the pressure

exerted by the excess adipose tissue on the kidneys (Wei *et al.*, 2021). Obesity-induced hypertension and hyperglycaemia further damage the kidneys, increasing the risk of end-stage renal disease (ESRD) (Wei *et al.*, 2021).

2.5. Metabolic dysfunction-associated fatty liver disease (MAFLD)

2.5.1. Introduction to MAFLD

Metabolic dysfunction-associated fatty liver disease (MAFLD) is a liver condition that has become increasingly recognised in recent years due to its strong association with metabolic dysfunction, obesity, and insulin resistance (Figure 2.3). Introduced as a new nomenclature in 2020, MAFLD replaces the term non-alcoholic fatty liver disease (NAFLD) to better reflect the underlying metabolic abnormalities that drive the disease (Eslam, Sanyal, *et al.*, 2020). MAFLD encompasses a spectrum of liver diseases, ranging from simple steatosis (fat accumulation in the liver) to more advanced stages such as non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Figure 2.3) (Vulchi *et al.*, 2023). The metabolic causes of MAFLD distinguish it from other liver diseases, and it is now regarded as one of the most prevalent liver conditions globally, affecting both adults and children (Vulchi *et al.*, 2023).

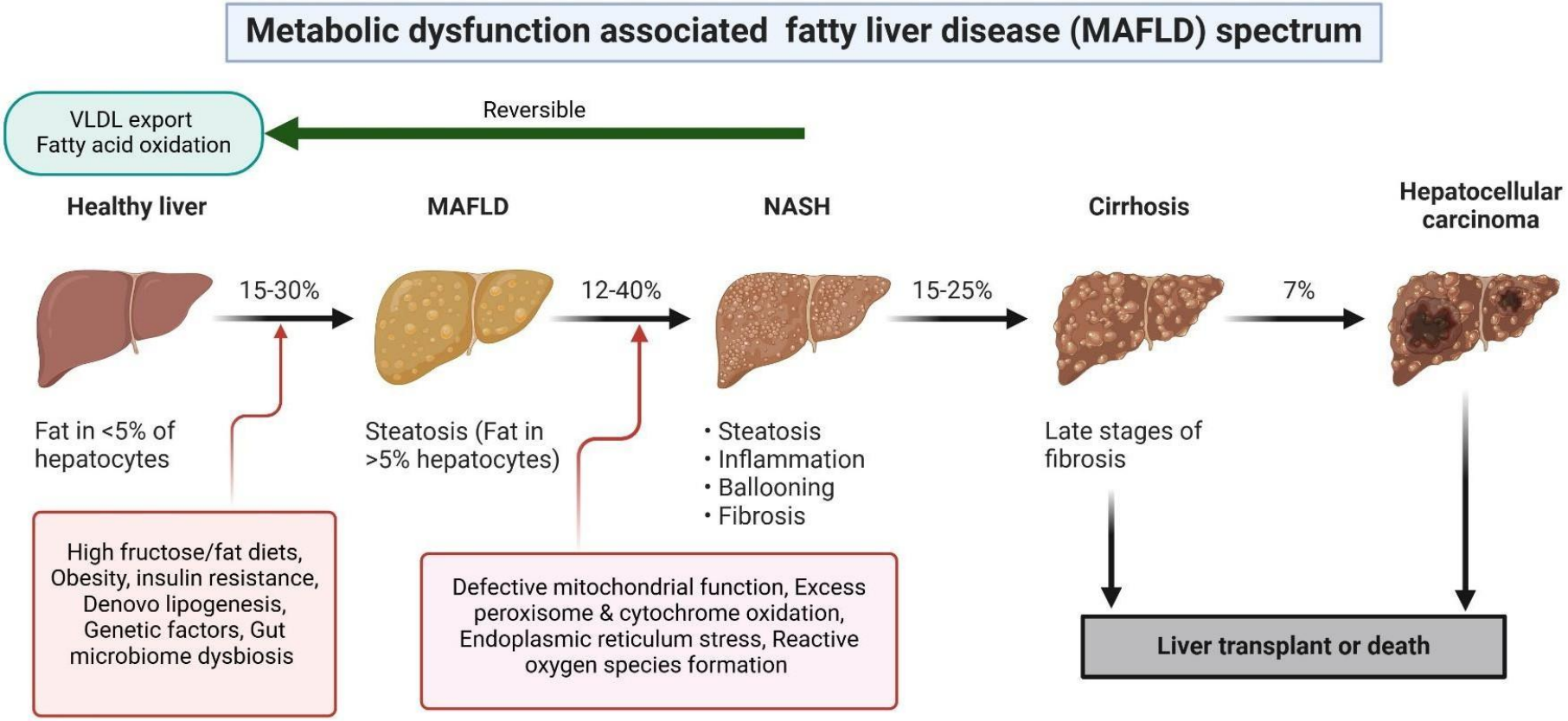


Figure 2. 3. The aetiology and pathogenesis of MAFLD, image accessed in Biorender and modified using information from (Vulchi *et al.*, 2023).

2.5.2. Global prevalence of MAFLD in adults and children

The global prevalence of MAFLD in both children and adults is rising at an alarming rate, driven by the increasing rates of obesity and metabolic dysfunction. It is estimated that between 25% and 35% of the global adult population has MAFLD, with the highest rates observed in regions such as the Middle East and South America (Liu *et al.*, 2021; Sangro *et al.*, 2023) (Figure 2.4). In South Africa, the prevalence of MAFLD is also on the rise, largely due to the growing rates of obesity and T2DM (Figure 2.4).

Childhood cases of MAFLD are also becoming more common, with estimates suggesting that between 3% and 10% of children globally are affected (Ciardullo *et al.*, 2023) (Figure 2.4). The prevalence of MAFLD among children and adolescents in South Africa has surpassed global averages, with an estimated 10% of children and 5% of adolescents affected, compared to the global prevalence of 7% and 3%, respectively (Liu *et al.*, 2021; Ciardullo *et al.*, 2023) (Figure 2.4). This represents a 3% higher prevalence in children and a 2% higher prevalence in adolescents than global averages (Figure 2.4). This alarming rise indicates that South African children are facing a higher burden of MAFLD than their global counterparts, which can be attributed to early weaning and sedentary lifestyles as discussed earlier (Liu *et al.*, 2021).

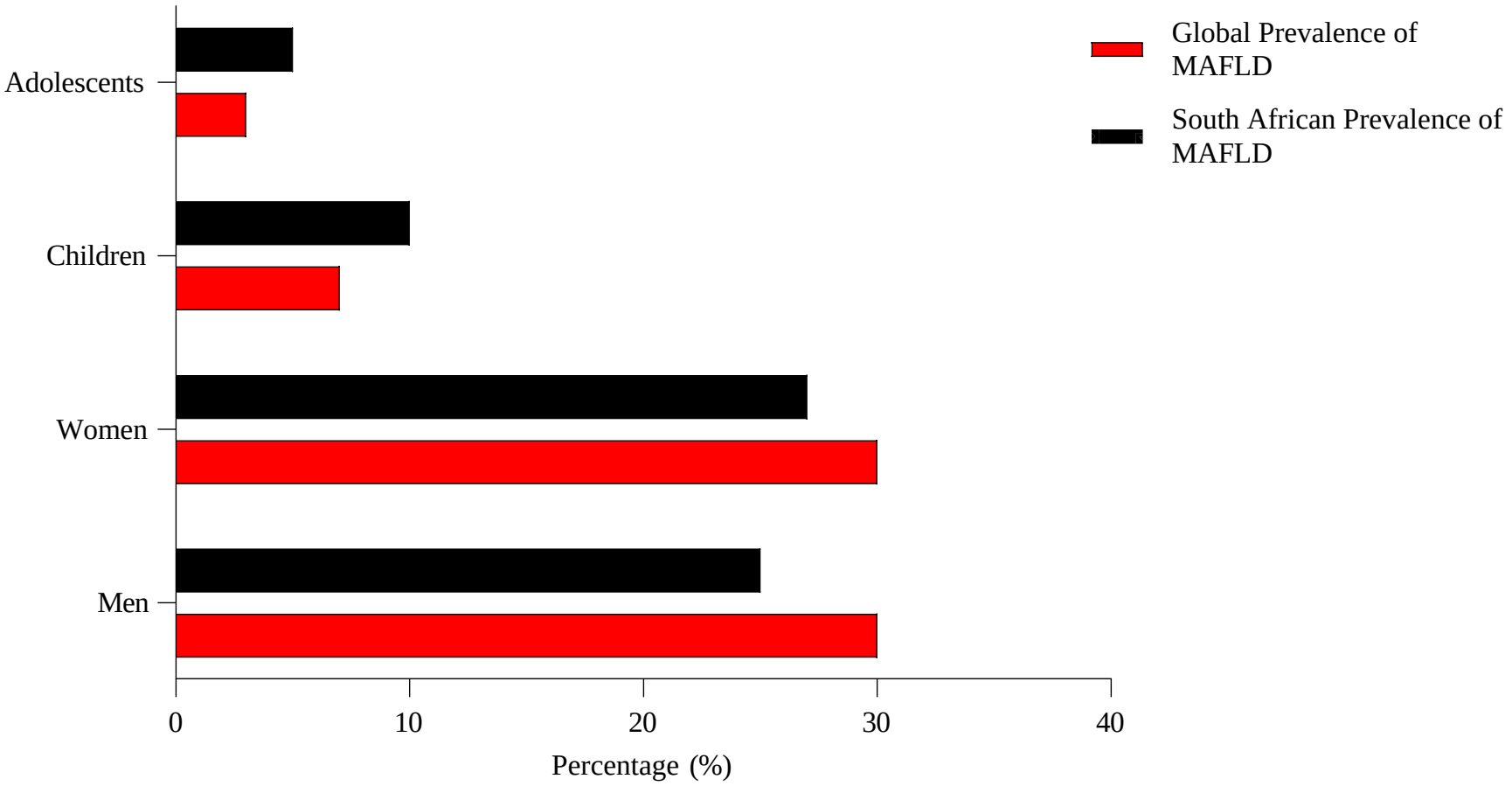


Figure 2. 4. Percentile estimations of prevalence of MAFLD among adult men, women, children, and adolescents globally and in South Africa. Figure generated from statistics by Liu *et al.*, 2021; Ciardullo *et al.*, 2023.

2.5.3. High fructose diets and their role in MAFLD development

One of the most significant contributors to the elevated prevalence of MAFLD is the rise in high consumption of processed foods, sugary drinks, and snacks in early weaning and childhood (Ciardullo *et al.*, 2023). The excessive caloric intake associated with these diets leads to fat accumulation in the liver, contributing to the higher-than-global prevalence of MAFLD among South African youth (Heyman and Abrams, 2017). These high fructose foods are also being fed to early-weaned children.

2.5.3.1. Processed fructose-rich food consumed during weaning and childhood phases

Processed foods and beverages such as sweetened infant food purees, sugary fruit juices, sweetened biscuits, and fizzy drinks form as part of major nutrients in infants and young children and they contain significantly higher levels of fructose compared to natural fruits (Monteiro *et al.*, 2023). During the early stages of weaning, fruits that are soft, easy to digest, and rich in essential nutrients are commonly preferred for weaning infants (Heyman and Abrams, 2017). Natural fruits, such as apple, pear, banana, peach, and apricot are used in their natural form, where mothers often make purees for their babies (Chaudhary and Chaurasia, 2017). These fruits contain natural fructose and glucose, which provide essential sugars for energy and support healthy metabolic development (Anderson *et al.*, 2021). For example, apples (5.74g of fructose per 100g) are often pureed into weaning foods due to their mild sweetness and are a good source of Vitamin C and fibre, supporting immune function and digestive health (Figure 2.5) (Gupta and Gupta, 2018). Similarly, bananas (3.4g of fructose per 100g) are soft, provide quick energy, and contain potassium, which is important for muscle function (Figure 2.5) (Kabeer *et al.*, 2023). Pears (6.73g of fructose per 100g) are also popular due to their soft texture and rich vitamins A and C, contributing to healthy growth and immune support (Figure 2.5) (More, 2013).

However, some of fruit are naturally higher in fructose, for example grapes (7.44g of fructose per 100g) (Figure 2.5) (Hendel, 2020). In contrast, while some fruits like peaches (1.23g of fructose per 100g) and plums (2.01g of fructose per 100g) are not naturally high in fructose when fresh (Figure 2.5) (Hendel, 2020). However, they are commonly processed by drying or pureeing in order to make processed weaning and childhood foods. These processing methods not only concentrate the natural sugars in these fruits but may also involve the addition of extra sugars, making them sweeter and more palatable, which

enhances the taste of commercial weaning and childhood foods (Campbell-ward, 2012). For example dried peaches (7.39g of fructose per 100g) and plum puree (16.24g of fructose per 100g) (Figure 2.5), contain significantly higher levels of fructose due to the concentration of sugars during the drying or pureeing process (Hendel, 2020). The consumption of this processed fructose raises concerns. This is because when fructose is introduced in processed forms, such as dried or pureed, it is absorbed more rapidly by the body and accelerates development of metabolic dysfunctions over time (Monteiro *et al.*, 2023).

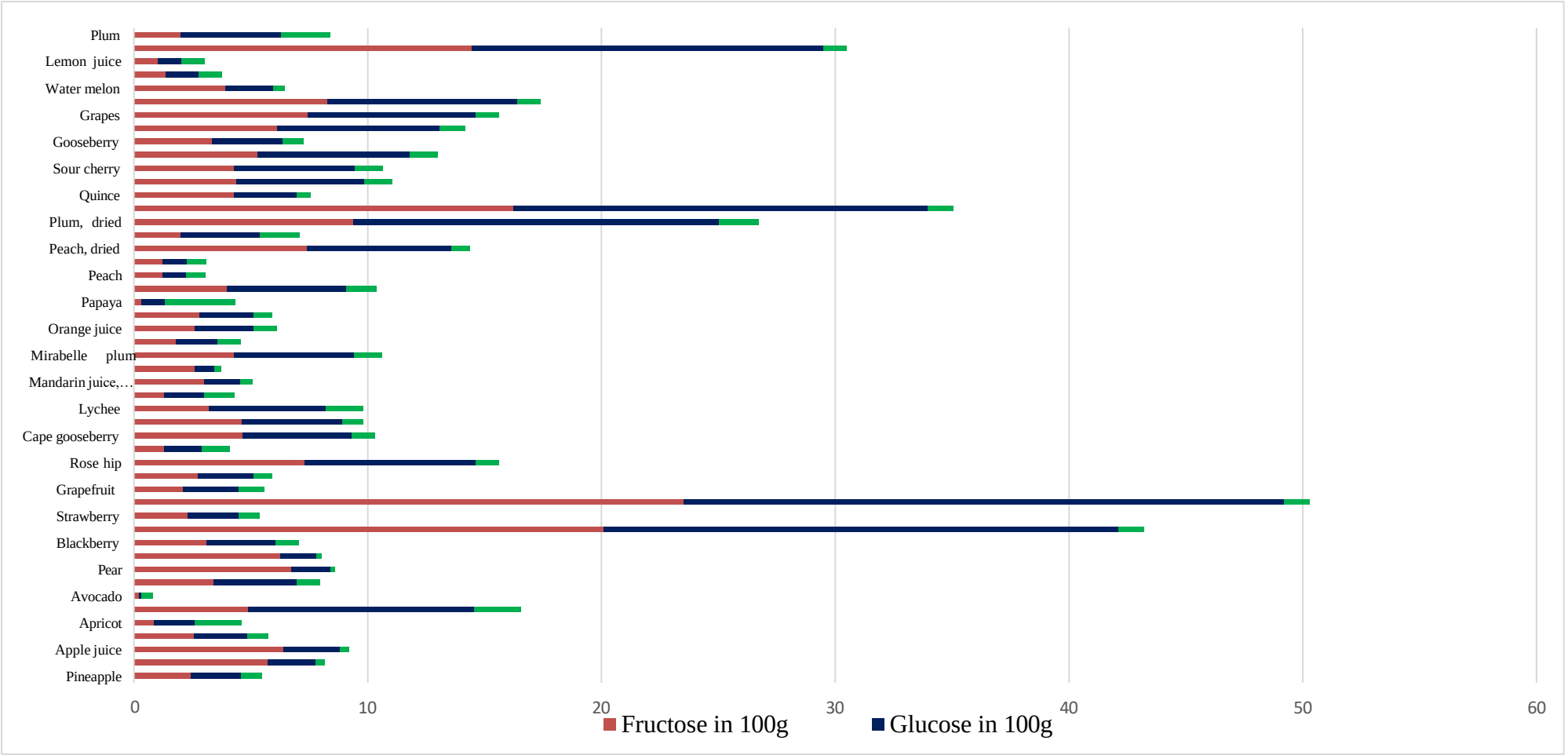


Figure 2. 5. Fructose and glucose content of natural fruits. Graph generated from amounts listed by Hendel, 2020.

2.5.3.2. Fructose metabolism in the liver

The liver is the primary organ responsible for metabolising fructose. Fructose is a monosaccharide (simple sugar) but it differs from glucose in its structure (Figure 2.6). It is a ketose sugar, meaning it has a ketone group (–C=O) on the second carbon atom, unlike glucose, which is an aldose sugar with an aldehyde group (–CHO) at the first carbon (Figure 2.6). It also differs from glucose in that fructose is metabolized primarily in the liver and does not directly stimulate insulin secretion or require insulin for its uptake into cells (Sievenpiper *et al.*, 2014). Once ingested, fructose is absorbed into the bloodstream and transported to the liver, where it undergoes phosphorylation by fructokinase to produce fructose-1-phosphate (Figure 2.6) (Merino *et al.*, 2020). Fructose is further metabolised into glyceraldehyde and dihydroxyacetone phosphate (Figure 2.6), which can enter several metabolic pathways, including glycolysis, gluconeogenesis, and de novo lipogenesis (Merino *et al.*, 2020).

Excessive fructose intake leads to the production of excessive acetyl-CoA, which serves as a precursor for fatty acid synthesis, contributing to the development of hepatic steatosis (fat accumulation in the liver) (Rippe and Angelopoulos, 2015; Merino *et al.*, 2020). This process promotes development of reactive oxygen species (ROS) production, oxidative stress, activation of inflammatory pathways, dyslipidaemia and MAFLD (Crescenzo *et al.*, 2018). These metabolic disturbances can progress into more severe liver diseases, including non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis as shown in Figure 2.3 (Jensen *et al.*, 2018).

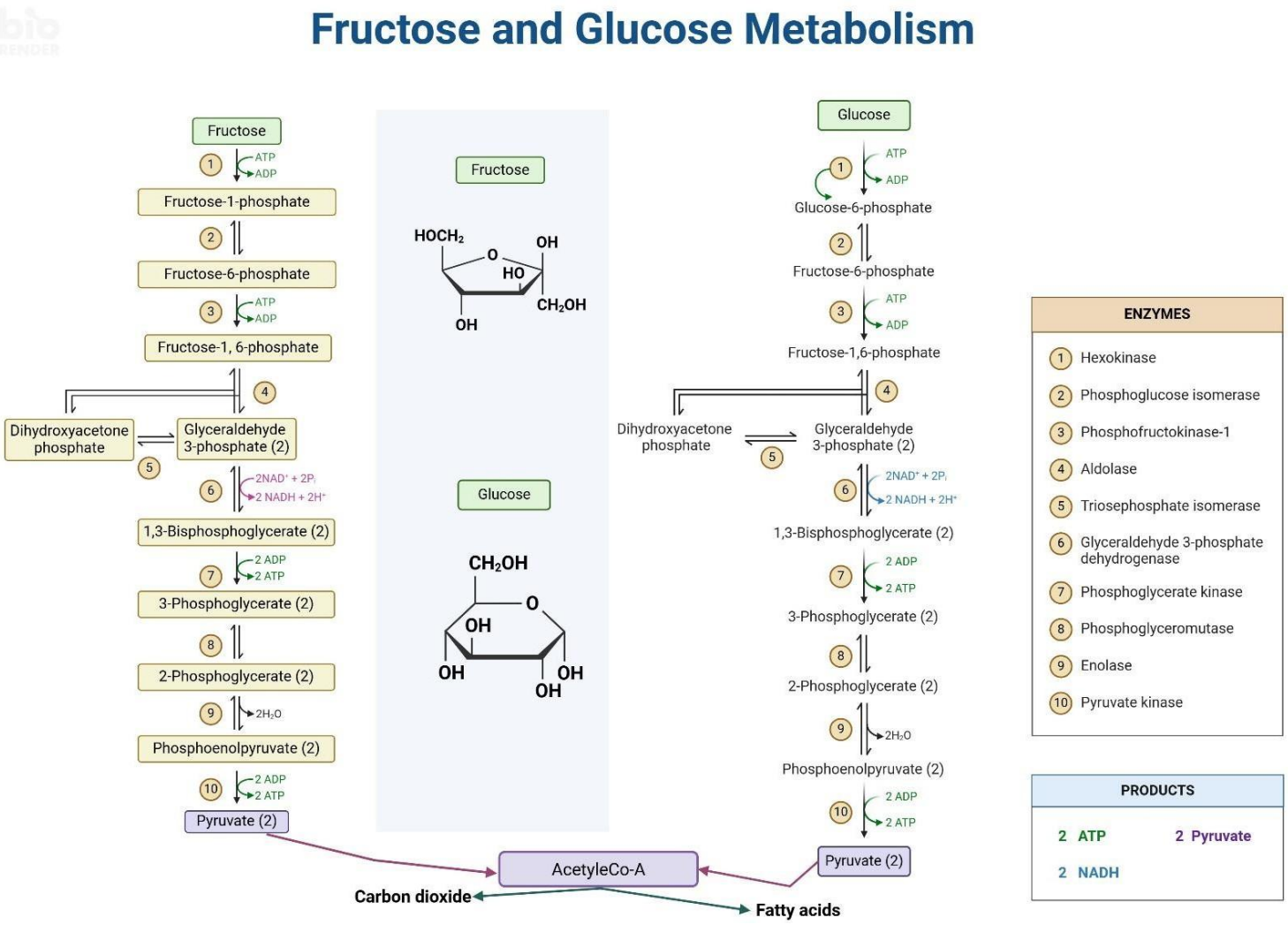


Figure 2. 6. Fructose and Glucose structure and metabolism pathways, image created in Biorender.

2.5.4. Mechanisms of MAFLD development

As explained above, when the liver continues to metabolise excess fructose, it produces more triglycerides (TGs), which are then stored in the hepatocytes (Song *et al.*, 2021). These triglycerides can then form toxic lipid species (Diacylglycerol (DAG), ceramides, and free fatty acids (FFAs)) (Li *et al.*, 2020). The metabolic capacity of the liver then becomes impaired resulting in dyslipidaemia. Dyslipidaemia is characterised by altered lipid profiles such as elevated levels of triglycerides, low-density lipoprotein (LDL) cholesterol, very-low-density lipoprotein (VLDL), and total cholesterol (TC) (Deeb *et al.*, 2018). When the storage capacity in hepatocytes becomes overwhelmed, the liver is unable to clear the excess lipids efficiently (Dajani and Abuhammour, 2016). As a result, fatty acids and triglycerides accumulate in the hepatocytes, leading to the development of hepatic steatosis, the earliest stage of MAFLD (Figure 2.3) (Eslam, Newsome, *et al.*, 2020).

2.5.5. Progression of MAFLD to NASH and fibrosis

With increasing hepatic steatosis, there is an overload of triglycerides that cannot be processed within hepatocytes and this worsens lipotoxicity (Duell *et al.*, 2022). This lipotoxicity further exacerbates liver damage by promoting the production of reactive oxygen species (ROS), a key factor in oxidative stress (García-Berumen *et al.*, 2019). The ROS generated from this excess fat accumulation causes cellular damage to hepatocytes and activate inflammatory pathways which leads to the release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and recruitment of immune cells, accelerating the progression of MAFLD to more severe stages like NASH (Figure 2.3) (García-Berumen *et al.*, 2019). In NASH, inflammation and cellular injury become more prominent, and the liver responds by initiating a fibrotic repair process, leading to fibrosis (Figure 2.3) (Bassal *et al.*, 2022). Over time, continuous inflammation, oxidative stress, and fibrosis can progress to cirrhosis, where the liver becomes severely scarred and loses its functional capacity, ultimately increasing the risk of liver cancer (Figure 2.3) or end-stage liver disease (Bassal *et al.*, 2022).

It is important to note that, while the above dysfunctions occur in the liver, there is also a simultaneous process aimed at removing excess lipids. The liver compensates by packaging triglycerides with cholesterol and proteins to form VLDL particles (Feingold, 2020). This results in increased VLDL secretion into the bloodstream (Feingold, 2020). LDL cholesterol, often referred to as "bad cholesterol," also accumulates in the bloodstream (Feingold, 2020).

This dyslipidaemia contributes to the development of other metabolic dysfunctions like atherosclerosis, insulin resistance, type 2 diabetes mellitus, hypertension and cardiovascular diseases. (Golabi, Owrangi and Younossi, 2024).

2.5.6. Lean MAFLD or lean metabolic dysfunction

Lean MAFLD presents a unique challenge in understanding the full spectrum of the disease. This is because individuals affected by this condition often do not meet the typical obesity criteria based on the parameters in Table 2.2 (Golabi, Owrangi and Younossi, 2024). While MAFLD is strongly associated with obesity, some individuals classified as "lean" (BMI <25 kg/m² in Western populations and <23 kg/m² in Asian populations) still develop the disease, indicating that BMI alone does not fully capture all cases of the condition (Table 2.2) (Golabi, Owrangi and Younossi, 2024). Another study revealed that Lean MAFLD represents around 20% of all MAFLD cases, with individuals having a BMI less than 25 kg/m² in non-Asian populations (Wakabayashi *et al.*, 2024). The prevalence of lean MAFLD in the United States is approximately 7%, but in Asia, it can reach as high as 25% (Golabi, Owrangi and Younossi, 2024). However, when waist circumference is incorporated into the definition of lean MAFLD specifically, measurements less than 102 cm for men and 88 cm for women are seen as the prevalence of "true lean MAFLD" (Osadnik *et al.*, 2022). Contributing factors to lean MAFLD include insulin resistance, visceral obesity, and high intake of fructose and fats (Osadnik *et al.*, 2020). While some individuals with lean MAFLD may not exhibit obesity-related comorbidities, they often present with insulin resistance and elevated plasma triglyceride levels (Golabi, Owrangi and Younossi, 2024). Additionally, lean individuals with MAFLD may face a prognosis similar to that of individuals with obesity (Osadnik *et al.*, 2020). This emphasises the need for comprehensive metabolic evaluations beyond BMI.

Table 2. 2. Differences and similarities (highlighted in grey) between Lean MAFLD and obese MAFLD.

Characteristic	Lean MAFLD	Obese MAFLD
Obesity	Individuals may not meet typical obesity criteria (BMI <25 kg/m² in Western populations, <23 kg/m² in Asian populations and <30 kg/m² in African populations)	Strongly associated with obesity (BMI ≥30 kg/m² in Western populations, ≥25 kg/m² in Asian populations and ≥30 kg/m² in African populations)
Visceral fat	Men <102 cm, Women <88 cm	Men >102 cm, Women >88 cm
Fasting glucose	>100 mg/dL or HbA1c >5.7%	>100 mg/dL or HbA1c >5.7%
Insulin resistance	HOMA-IR >2.5	HOMA-IR >2.5
Triglyceride levels	150 mg/dL or >1.7 mmol/L	150 mg/dL or >1.7 mmol/L
Waist circumference	Men <94 cm, Women <80 cm	Men >102 cm, Women >88 cm
Age	Typically diagnosed in individuals <40 years	Typically diagnosed in individuals >40 years
Genetic predisposition	PNPLA3 Gene: Variants like I148M have been associated with an increased risk of developing lean MAFLD FTO Gene: Less likely to show a strong association in lean individuals	PNPLA3 Gene: Strong association between PNPLA3 variants (especially I148M) and MAFLD, especially in individuals with obesity and metabolic dysfunction FTO Gene: Stronger association with obesity, insulin resistance, and increased liver fat accumulation
Typical hepatic steatosis score (level of fibrosis)	0 – 2 (mild fibrosis)	2 – 3 (severe fibrosis/cirrhosis)

Data in the table is generated from (Liang *et al.*, 2014; Osadnik *et al.*, 2020, 2022; Golabi, Owrangi and Younossi, 2024; Wakabayashi *et al.*, 2024). HOMA-IR (Homeostasis Model Assessment of Insulin Resistance), HbA1c (Hemoglobin A1c), PNPLA3 (Patatin-like phospholipase domain containing 3), FTO (Fat Mass and Obesity-Associated Gene), and LDL (Low-Density Lipoprotein).

2.5.7. Financial implications of MAFLD

MAFLD places a significant financial burden on healthcare systems globally, with costs escalating as the disease advances. The total projected cost of NAFLD and its complications, including diabetes, is expected to exceed $1.5 (US dollars) trillion globally over the next two decades (Golabi, Owrangi and Younossi, 2024). In the United States alone, the direct medical costs for managing MAFLD/NASH are estimated at $103 billion annually (Younossi *et al.*, 2016; Kim *et al.*, 2024). This is due to hospitalisations, medical interventions, and the treatment of complications like NASH and liver cirrhosis (Younossi *et al.*, 2016). In Europe, countries such as Germany, France, Italy, and the UK incur approximately $35 billion in annual costs related to MAFLD (Younossi *et al.*, 2016). Although Sub-Saharan Africa lacks comprehensive financial data on MAFLD, studies show that approximately 13.5% of the population is affected, suggesting a rising but underreported economic burden (Spearman *et al.*, 2021). There is a need for early intervention strategies such as lifestyle changes, improved screening, and timely treatment to reduce the long-term costs associated with MAFLD. Indeed there is need for targeted public health efforts to address this growing ill-health issue (Kim *et al.*, 2024).

2.5.8. Diagnosis and prognosis of MAFLD

The diagnosis of MAFLD relies on the detection of hepatic steatosis, either through imaging techniques or liver biopsy, combined with evidence of metabolic dysfunction (Eslam, Newsome, *et al.*, 2020). Liver ultrasound is commonly used to detect fat accumulation in the liver, but more sensitive techniques such as transient elastography and magnetic resonance imaging (MRI) are increasingly being used to assess liver fat content and fibrosis (Ciardullo *et al.*, 2023). The prognosis of MAFLD depends on the stage of the disease. Early-stage MAFLD can often be reversed with lifestyle interventions, while more advanced stages, such as NASH and cirrhosis, are associated with a poorer prognosis and a higher risk of liver-related complications, such as cirrhosis and liver cancer (Bassal *et al.*, 2022). Non-invasive biomarkers and scoring systems, such as the Fibrosis-4 (FIB-4) index and the MAFLD fibrosis score, are often used to estimate the degree of liver fibrosis in patients with MAFLD (Liang *et al.*, 2014; Mansoor *et al.*, 2015). These tools help to identify individuals at higher risk of advanced liver disease without the need for a liver biopsy, which remains the gold standard for assessing liver inflammation and fibrosis, but is invasive and not always feasible for all patients (Bassal *et al.*, 2022).

2.5.9. Prevention and management of MAFLD

Public awareness of MAFLD remains relatively low, despite its growing prevalence. Many individuals with MAFLD are asymptomatic in the early stages, and the condition is often underdiagnosed (Hegarty *et al.*, 2018). The prevention and management of MAFLD focusses on addressing the underlying metabolic dysfunctions associated with it. Lifestyle interventions, such as weight loss, dietary changes, and increased physical activity, are the cornerstone of MAFLD treatment. Studies have shown that a 7% to 10% reduction in body weight can significantly improve liver microstructure, presenting as reduced hepatic steatosis, inflammation, and fibrosis (Park *et al.*, 2020). Increasing public awareness through educational campaigns about the risks of obesity, poor diet, and sedentary lifestyles is essential for preventing the onset of MAFLD, particularly in children and adolescents (Ciardullo *et al.*, 2023).

A study using a non-physiological early weaning (NPEW) model in male Wistar rats found that early weaning led to increased plasma triglycerides, liver triglycerides, and cholesterol levels, which indicated disrupted lipid metabolism, a major characteristic feature of MAFLD (Bertasso *et al.*, 2020). In human studies, involving participants from the China Multi-Ethnic Cohort Study found that males exposed to famine during fetal life had an increased risk of MAFLD in adulthood, highlighting the long-term effects of early-life nutritional stress on liver health (Yin *et al.*, 2024).

If left untreated, MAFLD and obesity can contribute to the development of metabolic-associated kidney dysfunction (MAKD), as the metabolic disturbances such as insulin resistance, dyslipidaemia, chronic inflammation, and oxidative stress associated with MAFLD increase the risk of kidney injury and the progression of chronic kidney disease (Ciardullo *et al.*, 2023).

2.6. Metabolic-associated kidney dysfunction (MAKD)

2.6.1. Introduction to MAKD

Metabolic-associated kidney dysfunction (MAKD) refers to a spectrum of kidney impairments that arise from metabolic disorders such as obesity, type 2 diabetes, hypertension, and dyslipidaemia (Cen *et al.*, 2024). These metabolic conditions lead to a

series of pathophysiological changes that increase the risk of kidney damage and dysfunction (Figure 2.7) (Scurt *et al.*, 2024). MAKD is linked to metabolic dysfunction and MAFLD (Figure 2.7).The rise in the prevalence of metabolic dysfunction, MAFLD and type 2 diabetes mellitus (T2DM) has led to an increased understanding of the interconnectedness between metabolic dysfunctions and kidney health (Scurt *et al.*, 2024). More specifically, MAFLD has been identified as an important risk factor for kidney dysfunction (Wei *et al.*, 2023). The pathophysiology of MAKD involves complex mechanisms including insulin resistance, adiposity, systemic inflammation, and oxidative stress, all of which contribute to kidney injury (Lin *et al.*, 2022). As these metabolic disturbances progress, they exacerbate kidney dysfunction, ultimately leading to chronic kidney disease (CKD) (Tanaka *et al.*, 2023).

The significant link of MAKD and metabolic disorders make it crucial to explore various aspects of the condition further. This includes understanding the global prevalence of MAKD in both adults and children, as well as examining the impact of high fructose diets on the development of MAKD. Additionally, the mechanisms underlying MAKD development, its progression to CKD, fibrosis and end-stage renal disease (ESRD), as well as the financial implications of managing this condition need to be addressed. Moreover, public awareness on MAKD is also important in promoting early detection and intervention. The following sections will go deeper into these critical aspects of MAKD and the pressing need for comprehensive approaches to tackle this growing public health issue.

2.6.2. Global prevalence of MAKD in adults and children

The global prevalence of MAKD remains poorly documented, largely due to the lack of a universally accepted definition and standardised diagnostic criteria (J. Zhou *et al.*, 2023). Additionally, MAKD is often overlooked in studies focusing on specific conditions such as diabetic or hypertensive kidney disease, leading to underreporting. The variability in geographical and socioeconomic factors further complicates accurate global prevalence estimates. MAKD was previously named early kidney disease (EKD) in subjects with metabolic dysfunction and is frequently misclassified as diabetic kidney disease, hypertensive kidney disease, or chronic kidney disease (CKD), which masks its true prevalence (Landecho *et al.*, 2011; J. Zhou *et al.*, 2023). Furthermore, studies have shown that MAKD is highly prevalent among individuals with metabolic dysfunction, MAFLD and T2DM (Landecho *et*

al., 2011; Su *et al.*, 2022) . The presence of multiple metabolic abnormalities, such as hypertension, dyslipidaemia, and obesity, accelerates kidney dysfunction into CKD.

In high-risk adult populations such as those with diabetes, hypertension, and obesity, the prevalence of diabetic kidney disease and CKD is well documented, with CKD affecting 20 to 40% of individuals with diabetes (Afkarian, 2015; Lopez *et al.*, 2022). The global prevalence of chronic kidney disease (CKD) is estimated to affect 8 to 16% of the population, while diabetic renal dysfunction is a leading cause of CKD, affecting 30 to 40% of individuals with type 1 diabetes mellitus and 20 to 30% of those with type 2 diabetes mellitus (Figure 2.7) (Afkarian, 2015; Lopez *et al.*, 2022). Hypertensive renal dysfunction contributes to approximately 25% of CKD cases worldwide, and obesity-related renal dysfunction affects 5 to 10% of obese individuals (Figure 2.7) (Mhundwa, Joubert and Mofokeng, 2023).

The prevalence of kidney-related conditions in children is concerning as well, with CKD affecting 0.1 to 0.35% of the paediatric population (Figure 2.7) (Forbes and Selewski, 2025). Diabetic kidney disease (DKD) impacts 30% of children with type 1 diabetes and 10 to 20% of those with type 2 diabetes (Figure 2.7) (Afkarian, 2015; Lopez *et al.*, 2022). Hypertensive renal dysfunction is present in 10 to 20% of children with hypertension globally (Figure 2.7) (Lilova, 2005; Shatat, Becton and Woroniecki, 2019). Furthermore, over 10% of obese children experience obesity-related kidney dysfunction (Figure 2.7) (Yim and Yoo, 2021). End-stage renal disease (ESRD) in children is rare, with a prevalence of 0.0012 to 0.0014%, but is a serious concern in high-risk groups such as those with congenital abnormalities (Ku *et al.*, 2017).

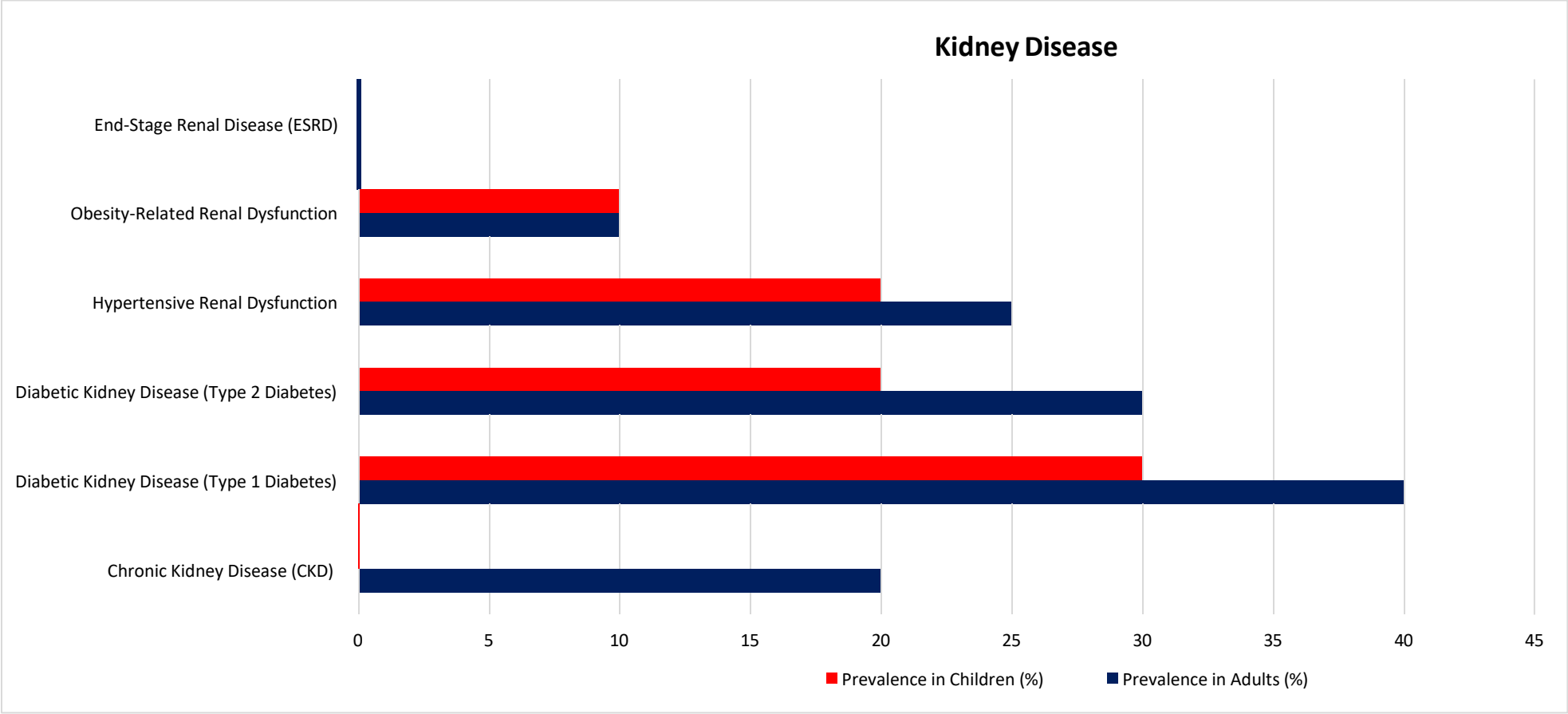


Figure 2. 7. Global Prevalence of kidney disease related to metabolic disorders, graph generated with statistics from Afkarian, 2015; Lopez *et al.*, 2022; Mhundwa, Joubert and Mofokeng, 2023**.**

2.6.3. High fructose diets and their role in MAKD development and progression to CKD and ESRD

The kidneys play a crucial role in the metabolism and excretion of fructose. After ingestion of a high fructose diet, fructose is absorbed in the small intestine, then it enters the bloodstream and transported to various organs, including the kidney (Nakagawa *et al.*, 2020). It is then filtered through the kidneys, where it triggers an increase in uric acid production (Nakagawa *et al.*, 2020). Elevated uric acid levels have been associated with glomerular hypertension and endothelial dysfunction, both of which contribute to renal damage (Lee *et al.*, 2016). Fructose metabolism in the kidneys causes cellular hypertrophy, proliferation, and inflammation (Figure 2.8), which is characterised by the increased expression of inflammatory biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL) and monocyte chemoattractant protein-1 (MCP-1) (Nakagawa *et al.*, 2020).

2.6.1.1. Fructose metabolism in the kidney

In the kidney, about 10 to 15% of the total circulating fructose is filtered through the glomeruli (Nakagawa *et al.*, 2020). It will then be taken by the renal cells in the proximal tubules through specific transporters (Glucose Transporter 5 (GLUT-5) and Glucose Transporter 2 (GLUT-2)) (Kalimuthu *et al.*, 2024). Fructokinase then converts fructose into fructose-1-phosphate, which is then broken down by aldolase B to create dihydroxyacetone phosphate (DHAP) and glyceraldehyde (Nakagawa and Kang, 2021). These metabolites are involved in various metabolic pathways, including glycolysis and the synthesis of fatty acids (Nakagawa *et al.*, 2020). Through this metabolic process, uric acid is produced as a by-product when fructose-1-phosphate is broken down (Nakagawa and Kang, 2021).

2.6.1.2. Effects of high fructose diets on kidney health

Excess fructose metabolism leads to elevated levels of uric acid in the kidney (Toyoda *et al.*, 2018). Elevated levels of this uric acid in the kidneys contribute to increased oxidative stress and inflammation (Figure 2.8), leading to endothelial dysfunction and glomerular hypertension (Johnson, Sanchez-Lozada and Nakagawa, 2010; Khitan and Kim, 2013). These can lead to renal cell hypertrophy (enlargement), cellular proliferation, and inflammation, altering kidney function (Figure 2.8) (Prasad, Jha and Keerti, 2022). Because of this inflammation, various biomarkers of kidney injury, such as neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1), are upregulated (Lim *et al.*, 2022).

Additionally, creatinine and blood urea nitrogen (BUN) are also increased (Kohl *et al.*, 2020). These markers indicate a reduction in kidney clearance and glomerular filtration rate (GFR), both signs of kidney dysfunction (Thongprayoon, Cheungpasitporn and Kashani, 2016).

The inflammatory processes also contribute to the progression of proteinuria, glomerulosclerosis, and other forms of chronic kidney disease (CKD) (Flisiński *et al.*, 2022). Chronic high fructose consumption can alter the pH and oxalate content of urine, increasing the risk of kidney stones (Johnson *et al.*, 2018). The kidneys become more prone to the formation of stones (uroliths) due to changes in urine composition (Johnson *et al.*, 2018). Additionally, a high fructose intake alters the pH and oxalate content of urine, increasing the risk of kidney stones and further exacerbating renal damage (Johnson et al., 2018). With a prolonged high fructose consumption, the kidneys may suffer from ongoing inflammation, fibrosis (scarring), and a decline in renal function eventually leading to chronic kidney disease (CKD) (Nakagawa and Kang, 2021). Ultimately, the combined effects of increased uric acid, inflammation, oxidative stress, proteinuria, fibrosis, and reduced GFR result in End-stage renal disease (ESRD) (Figure 2.8), where the kidneys are no longer able to function effectively to remove waste products from the body (Résimont *et al.*, 2022).

2.6.4. Financial implications of MAKD

MAKD significantly affects global economies, primarily through the escalating costs of managing its progressed severe forms chronic kidney disease CKD and ESRD. In South Africa, the cost of dialysis is particularly high, with annual costs per patient for haemodialysis estimated at US$ 25,888 and for peritoneal dialysis at US$ 31,106 (Malatji, Wamukuo and Hyera, 2019). Despite the growing prevalence of CKD, access to renal replacement therapy remains limited in the public healthcare sector, with some patients unable to access necessary treatments due to these prohibitive costs (Malatji, Wamukuo and Hyera, 2019). In sub-Saharan Africa, the annual cost of managing ESRD ranges from US$ 7,370 to US$ 42,800 per patient, which exceeds the gross national product per capita of many African nations, placing an enormous strain on both families and healthcare systems (Agada-Amade *et al.*, 2023).

In the United States, for instance, CKD accounts for a quarter of Medicare spending, approximately US$ 76.8 billion annually (L. Zhang *et al.*, 2022). In China, the economic

burden of CKD is projected to rise from US$ 179 billion in 2019 to US$ 198 billion by 2025 (L. Zhang *et al.*, 2022). In addition, projections indicate that by 2032 countries like the United States, Brazil, the United Kingdom, Spain, Germany, the Netherlands, China, and Australia will be affected by CKD (AstraZeneca, 2024). The financial burden in these countries in terms of the annual costs of renal replacement therapies, including dialysis and transplants, are anticipated to reach approximately US$ 186 billion by 2032 (AstraZeneca, 2024) . This projection in healthcare expenditure highlights that there is urgent need for effective prevention and management strategies to mitigate the economic impact of MAKD globally.

The high cost of treatment and lack of adequate healthcare infrastructure make CKD management challenging, leading to catastrophic health expenditures and exacerbating poverty for many affected families. These statistics highlight the urgent need for affordable treatment options, improved healthcare access, and sustainable financial strategies to address the growing burden of CKD in these regions.

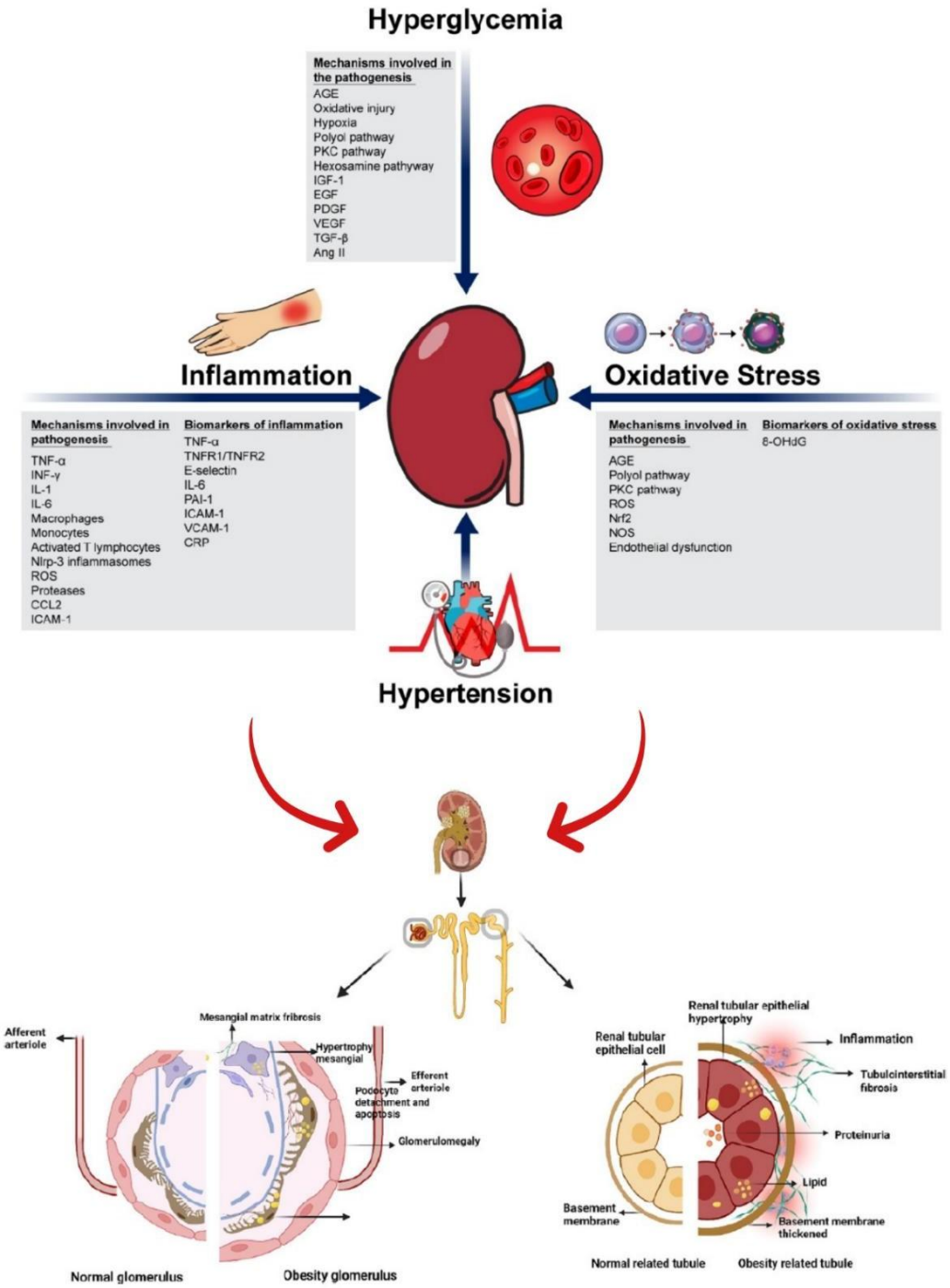


Figure 2. 8. Pathophysiological mechanisms of metabolic associated kidney dysfunction (MAKD). Image modified from Jung and Yoo, 2022; Jiang *et al.*, 2023.

2.6.5. Diagnosis and prognosis of MAKD

The diagnosis of MAKD begins with a thorough patient history and clinical evaluation, focusing on risk factors such as obesity, hypertension, and type 2 diabetes mellitus (Muntean, Starcea and Banescu, 2022). (Muntean, Starcea and Banescu, 2022)Urine analysis plays a crucial role in diagnosing MAKD, with the presence of albumin indicating kidney damage (Lopez *et al.*, 2022). Microalbuminuria in the early stages can progress to macroalbuminuria. The albumin-to-creatinine ratio (ACR) in a urine sample is used to quantify the albumin level (Scurt *et al.*, 2024). Serum creatinine and the calculation of GFR help assess kidney function (Résimont *et al.*, 2022). Reduced GFR indicates kidney impairment (Résimont *et al.*, 2022). Imaging techniques, such as kidney ultrasound, may be used to rule out structural issues, while biomarkers like KIM-1 and NGAL help identify early kidney injury (Zdziechowska *et al.*, 2020; Deng, Zhao and Gong, 2021).

2.6.6. Prevention and management of MAKD

An effective ways to prevent metabolic associated kidney diseases is via public awareness. Public awareness of kidney diseases is critically low, which negatively affects early detection and effective prevention strategies (Prasad, Jha and Keerti, 2022). In many regions, particularly in low- and middle-income countries, there is a general lack of education regarding the link between metabolic disorders such as obesity, diabetes, and hypertension, and kidney disease (Prasad, Jha and Keerti, 2022). As a result, individuals are often unaware of the risks associated with poor metabolic health and the impact these conditions can have on kidney function. Despite the rising prevalence of conditions such as type 2 diabetes and obesity, public health campaigns focusing specifically on MAKD and kidney health remain scarce. This lack of awareness contributes to delayed diagnosis, where kidney dysfunction is often detected only in its advanced stages, when intervention options are limited (Devarajan, 2010). Efforts to raise public awareness through targeted education, community-based screening, and early cost-effective interventions are crucial in addressing the growing burden of MAKD. Such initiatives could not only reduce the incidence of kidney disease but also mitigate the broader socioeconomic impacts of untreated renal dysfunction, especially in early childhood.

The management of MAKD in children and adolescents requires a comprehensive approach that includes glycaemic control, blood pressure management, and early intervention with

renal-protective cost effective short-term therapies. Maintaining healthy blood glucose levels is the essential for preventing the progression of renal dysfunction, as poor glycaemic control is the most significant risk factor for kidney damage (Pecoits-Filho *et al.*, 2016). Regular monitoring of HbA1c levels and prompt adjustments to insulin therapy are essential in reducing the risk of renal dysfunction in children with diabetes mellitus (Pecoits-Filho *et al.*, 2016).

Blood pressure control is also critical in managing MAKD, particularly given the role of hypertension in accelerating kidney damage (Do Val *et al.*, 2019). Angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are frequently used to reduce blood pressure and provide renal-protective effects by inhibiting the RAAS pathway (Fularski *et al.*, 2024). These medications not only lower blood pressure but also reduce proteinuria, thereby slowing the progression of renal dysfunction (Fularski *et al.*, 2024). Lifestyle modifications like regular physical activity and a balanced diet, play an important role in managing MAKD as well (Neumiller, Burnier and Hoogeveen, 2022). Early screening and intervention are vital in preventing the progression of MAKD to ESRD, which would otherwise necessitate dialysis or kidney transplantation, both of which are associated with significant morbidity and a reduced quality of life (Fularski *et al.*, 2024).

2.7. Targeting the early life phases for management of metabolic diseases

The early stages of life, particularly infancy and childhood, represent critical windows of developmental plasticity where nutritional and environmental exposures can shape long-term health outcomes (Vickers, 2014; Wells, 2014; Quek *et al.*, 2022). The concept of developmental plasticity refers to the ability of an organism to adapt to its environment during sensitive periods of growth, which can result in permanent physiological and metabolic changes (Langley-Evans, 2015). Targeting these early life phases for the management of metabolic diseases such as obesity, T2DM, MAFLD and MAKD is therefore a promising strategy for preventing the onset of these conditions in adulthood.

Traditional treatment approaches to metabolic dysfunction, including pharmacological medications, behavioural therapy, and lifestyle modifications, are often expensive, time-consuming, and require long-term adherence (American Diabetes Association, 2016).

Moreover, many pharmaceutical treatments have undesirable side effects that discourage individuals from maintaining their treatment regimens (Bramante, Lee and Gudzune, 2017). This highlights the need for alternative strategies that are not only more sustainable but also preventive in nature. Early-life interventions, particularly those focused on nutrition during critical periods of growth, offer a proactive solution for managing and preventing metabolic diseases.

2.7.1. Weaning period as a potential target for interventions

As already established, the weaning period is crucial for shaping long-term metabolic health. Early weaning onto nutrient-rich, whole-food diets can promote healthy metabolic programming (Kabeer *et al.,* 2023). Animal studies, particularly in rats, show that early weaning onto high-fat or high-fructose diets leads to insulin resistance and fat accumulation, highlighting the impact of food choices during this critical period (Bellamkonda *et al.*, 2018; Pietrobon, Miranda, *et al.*, 2020; Song *et al.*, 2021). A study on rats found that omega-3 fatty acid supplementation during weaning improved insulin sensitivity and reduced fat accumulation (Al Hashmi *et al.,* 2024). Other rodents studies also revealed that non-pharmacological nutrient-rich diets like Quercetin and Moringa extracts programmed for good metabolic heath when administered at weaning (Das *et al.*, 2013; Peixoto-Silva *et al.*, 2017; Tshingani *et al.*, 2017; Irfan, Khan and Asmawi, 2020; El-Shehawi *et al.*, 2021).
In both animal and human studies, diets rich in phytochemicals, such as polyphenols, have also demonstrated benefits in preventing metabolic diseases by enhancing insulin sensitivity and reducing fat deposition (Villarruel-López *et al.,* 2018; Zheng *et al.*, 2020; Federico *et al.*, 2021). These findings underscore the importance of early nutritional interventions to prevent the onset of metabolic diseases and promote lifelong metabolic health.

Therefore, the next section explores the potential of *Moringa oleifera,* a plant rich in phytochemicals, as a therapeutic intervention during early developmental phases to combat metabolic dysfunction. *Moringa oleifera* has been studied for its potential health benefits, including anti-inflammatory, oxidant and analgesic properties among other protective properties (Saleem, Saleem and Akhtar, 2020; El-Shehawi *et al.,* 2021). Research involving rodent models has demonstrated that Moringa protected against insulin resistant, dyslipidaemia, type 2 diabetes, oxidative stress, MAFLD and kidney injury (Villarruel-López

et al., 2018; Mohamed, Ahmed and El Sayed, 2019; Muhammad *et al.*, 2019; Akinrinde *et al.*, 2020; Zhang *et al.*, 2024).

2.8. *Moringa oleifera*

2.8.1. *Moring oleifera* origin

Moringa oleifera Lam, commonly known as Moringa, has its origins rooted in tropical and subtropical regions across the globe (Figure 2.9) (Peñalver *et al.*, 2022). This drought-tolerant species has spread effectively over several continents, where it thrives in conditions that are typical of high temperatures and abundant sunlight (Peñalver *et al.*, 2022). Its cultivation has become widespread. Originally endemic to regions such as South Asia and Northern Africa, Moringa species have extended beyond their native geographical range, emerging as valuable and resilient contributors to the biodiversity of various ecosystems (Omodanisi *et al.*, 2017). Its adaptability to diverse climatic conditions has facilitated its incorporation into local cultures and traditional medicinal practices, establishing it as a globally recognized botanical resource (Mehwish *et al.*, 2020).

2.8.2. Pharmacological properties of Moringa

Moringa oleifera has remarkable pharmacological potential, earning its reputation as a versatile and valuable resource in traditional medicine (Mehwish *et al.*, 2020). The spectrum of its properties is large and includes potent anti-inflammatory, anti-microbial, anti-cancer, anti-hyperlipidaemia, anti-diabetic, anti-ulcer, analgesic, anti-fertility, anti-convulsant, anti-pyretic, anti-epileptic, anti-spasmodic, and diuretic effects (Figure 2.9) (Biswas *et al.*, 2012; Luoh *et al.*, 2017a). The extensive range of pharmacological activities exhibited by this plant underscores its versatility, rendering it a subject of increasing interest in both traditional and contemporary medical practices. The diverse bioactive compounds present in Moringa contribute to its multifaceted therapeutic potential, establishing it as a botanical resource with numerous health benefits (Table 2.3) (Mehwish *et al.*, 2020; Wijayanti *et al.*, 2023).

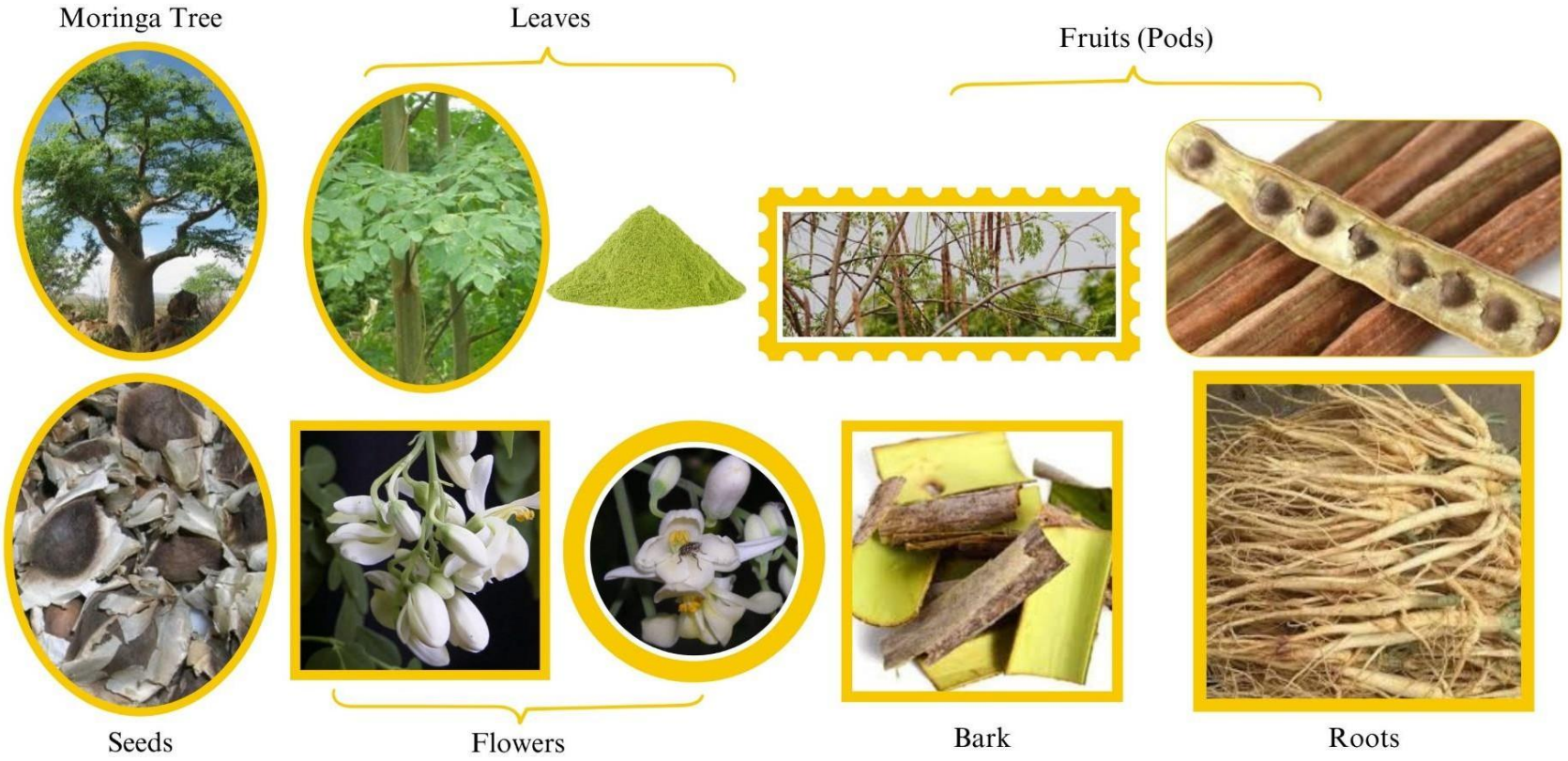


Figure 2. 9. The Moringa plant and its various therapeutic properties of all its parts, images generated in Canva.

Table 2. 3. Bioactive compounds (phytochemicals) of *Moringa oleifera.*

Nutrient Category	**Specific Nutrients**	**Health Benefits and Functional Roles**	**Approximate Quantity per 100g (Fresh Leaves)**
Vitamins	Vitamin A (β-carotene), Vitamin C, Vitamin E, Vitamin B1 (Thiamine), Vitamin B2 (Riboflavin), Vitamin B3 (Niacin), Vitamin B6 (Pyridoxine), Vitamin B9 (Folate), Vitamin K, Vitamin D, Vitamin B12	Vitamin A (β-carotene) supports vision and immune function, Vitamin C boosts immunity and promotes skin health, Vitamin E acts as an antioxidant protecting cells, Vitamin K is essential for blood clotting, and B-vitamins (B1, B2, B3, B6, B9) support energy metabolism and nerve function. Vitamin D supports bone health, and Vitamin B12 is important for red blood cell production and nerve function.	Vitamin A (β-carotene): 6,780 µg (113% Daily Value (DV)), Vitamin C: 220 mg (367% DV), Vitamin E: 4.0 mg (27% DV), Vitamin D: Trace, Vitamin B12: Trace
Proteins	Essential amino acids: Leucine, Isoleucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine; Non-essential amino acids	Moringa provides all nine essential amino acids necessary for muscle repair, enzyme function, and neurotransmitter synthesis. It supports tissue growth and repair and aids in protein synthesis.	Protein: 2.1 g
Minerals	Calcium, Potassium, Iron, Magnesium, Phosphorus, Zinc, Copper, Manganese, Selenium, Sodium, Molybdenum, Chromium	Calcium supports bone and dental health, Magnesium regulates muscle and nerve function, Iron prevents anaemia, Potassium helps manage blood pressure, and Zinc, Copper, Manganese, Selenium play key	Calcium: 185 mg (19% DV), Potassium: 337 mg (10% DV), Iron: 4.0 mg (22% DV), Magnesium: 42 mg (11% DV), Sodium: Trace, Molybdenum: Trace, Chromium: Trace

		roles in immune function, metabolism, and antioxidant defence. Sodium helps maintain fluid balance and nerve function. Molybdenum supports enzyme activity, and Chromium aids in macronutrient metabolism.	
Phytochemicals	Flavonoids (e.g., Quercetin, Kaempferol), Phenolic acids, Glucosinolates, Isothiocyanates, Alkaloids, Tannins, Saponins, Triterpenoids, Nitrates	Flavonoids like Quercetin and Kaempferol have anti-inflammatory, antioxidant, and anticancer effects. Glucosinolates and Isothiocyanates may reduce the risk of cancer, while Alkaloids, Tannins, and Saponins have antibacterial and antifungal properties. Triterpenoids have anti-inflammatory and anticancer effects, and Nitrates support cardiovascular health by enhancing blood flow.	Quantitative data not commonly available for each specific phytochemical.
Antioxidants	Quercetin, Kaempferol, Chlorogenic acid, Caffeic acid, β-carotene, Vitamin C, Vitamin E, Polyphenols	These antioxidants reduce oxidative stress, protect cells from damage, and may lower the risk of chronic diseases like heart disease, cancer, and diabetes. Quercetin is also anti-inflammatory, and β-carotene supports eye health.	Quantitative data for antioxidants is often part of total Vitamin and flavonoid content.

Other Nutrients	Omega-3 and Omega-6 fatty acids, fibre, Carbohydrates, Sugars, Starch, Water	Omega-3 and Omega-6 fatty acids are essential for brain function and reducing inflammation. Fibre supports digestive health and helps maintain healthy cholesterol levels, while Carbohydrates provide energy for daily functions. Sugars and Starch provide quick energy, and Water contributes to hydration.	Omega-3: 0.13 g, Omega-6: 0.58 g, fibre: 2.0 g, Carbohydrates: 8.28 g, Water: 77-80%

(Luoh *et al.*, 2017a; Vergara-Jimenez, Almatrafi and Fernandez, 2017). DV (Daily Value) and β (Beta).

2.8.2.1. Anti-inflammatory properties

The anti-inflammatory effects exhibited by Moringa can be attributed to specific phytochemicals namely, quercetin, chlorogenic acid, and beta- (Luoh *et al.*, 2017a). These anti-inflammatory properties make Moringa a promising candidate for managing inflammatory conditions, offering a natural and holistic approach to health. For example, Moringa has been shown to reduce levels of tumor necrosis factor (TNF-α) and macrophage migrating inhibitory factor (MIF) in Bisphenol A (BPA)-induced hepatotoxicity in Wistar adult rats (Abd-Elnaby *et al.*, 2022).

2.8.2.2. Anti-hyperlipidaemic properties

The anti-hyperlipidaemic properties of Moringa emphasise its potential in addressing lipid-related disorders. Phytochemicals found in Moringa, specifically, beta-sitosterol and 4-[α-(L-rhamnosyloxy) benzyl]-o-methyl thiocarbamate (trans) have been shown to regulate lipid levels, presenting Moringa as a valuable asset in the management of conditions associated with abnormal lipid metabolism (Thakur *et al.*, 2024). In vivo testing of the crude hydroethanolic extract of Moringa leaf extracts revealed anti-hyperlipidaemic properties by preventing the high-fructose diet-induced liver lipid accumulation and steatosis in both female and male Sprague Dawley rats without compromising liver function (Setyawati *et al.*, 2022). In a study by (Irfan, Khan and Asmawi, 2020) Moringa leaf extract exhibited anti-metabolic dysfunction properties by significantly mitigating features such as increased LDL, visceral fat, and liver weight in male Sprague Dawley rats fed a high-fat diet and 20% fructose, compared to rats receiving a normal matched diet without other treatments.

2.8.2.3. Anti-diabetic properties

Moringa poses anti-diabetic effects which are attributed to moringine, moringinine, quercetin, chlorogenic acid, beta-sitosterol, kaempferol, and glucosinolate (Thakur *et al.*, 2024). These bioactive substances contribute to the ability of Moringa to lower blood glucose levels and improve insulin sensitivity (Prajapati *et al.*, 2022). Moringa ethanolic extract was able to down-regulate the mRNA expression of leptin and resistin, while up-regulating adiponectin gene expression in obese female Wistar rats (Metwally *et al.*, 2017). These changes in adipokine expression were associated with improvements in glucose levels and insulin resistance status, indicating how Moringa was able to mitigate the symptoms of diabetes by directly targeting adipokines in visceral adipose tissue (Metwally *et al.*, 2017). Furthermore,

Moringa leaf extract demonstrated anti-diabetic properties by reducing glucose and insulin levels, improving insulin sensitivity, increasing peroxisome proliferator-activated receptor gamma (*PPARγ)* levels, and decreasing inflammatory cytokines in type 2 diabetic male Wistar rats induced by high-fat diet and streptozotocin (Anwer *et al.*, 2021).

2.8.2.4. Hepatoprotective properties

Moringa is known for its hepatoprotective qualities, via biological properties of its constituent flavonoids like quercetin and kaempferol (Monraz-Méndez *et al.*, 2022). In a study by (El-bakry *et al.*, 2016), Moringa leaf extract demonstrated hepatoprotective properties against liver injury induced by carbon tetrachloride (CCl4) by mitigating oxidative stress and enhancing antioxidant defences in female albino rats. Specifically, treatment with the extract reduced the levels of malondialdehyde (MDA), a marker of lipid peroxidation, and increased the activity of antioxidant parameters such as total antioxidant capacity (TAC), blood reduced glutathione (GSH), and enzymes such as superoxide dismutase (SOD) and catalase. Additionally, the extract attenuated the elevation of liver enzymes induced by CCl4, indicating protection against hepatocellular damage (El-bakry *et al.*, 2016). In another study, Moringa ethanol leaf extract demonstrated hepatoprotective effects against liver fibrosis of male Wistar rats induced with chronic carbon tetrachloride (CCl4) administration (Supriono *et al.*, 2020). This was revealed by reduced fibrotic changes, reduced expression of collagen-1, α-smooth muscle actin (α-SMA), and tissue inhibitors of metalloproteinases-1 (TIMP-1) (Supriono *et al.*, 2020).

2.8.2.5. Anti-obesity properties

Moringa has potential therapeutic effects in the management of obesity and its related complications (El-Shehawi *et al.*, 2021). Phytochemicals like beta-sitosterol contribute to its effects on lipid metabolism, influencing body weight regulation and providing a holistic approach to addressing obesity-related concerns (Biswas *et al.*, 2012). The methanolic leaf extract of Moringa (MEML) administered to obese male Wistar rats resulted in a significant reduction in body weight gain compared to the high-fat diet (HFD) group (Mabrouki *et al.*, 2020). Specifically, rats treated with MEML exhibited alleviation of abnormalities in serum biochemical parameters associated with obesity (Mabrouki *et al.*, 2020). In male Wistar albino rats fed a high-fat diet (HFD), the treatment of Moringa ethanolic leaf extract (MOE)

substantially decreased increases in body weight and visceral fat mass (El-Shehawi *et al.*, 2021).

2.8.2.6. Reno-protective properties

Moringa has demonstrated protective effects on renal function, attributable to its diverse phytochemical profile. Quercetin, chlorogenic acid, kaempferol, beta-sitosterol, glucosinolates, tannins, zinc, vitamin C, and iron in Moringa contribute to maintaining renal health, expanding its spectrum of health benefits to include the protection and support of kidney function (Faheem, Amina, Iqbal and Faheem, 2023). The administration of Moringa seed extract significantly improved kidney function in male Wistar rats maintained on a high-fat and high-fructose (HFHF) diet (Putri *et al.*, 2023). The Moringa seed extract reduced serum creatinine levels, improved overall kidney structure, and increased the expression of superoxide dismutase (SOD) (Putri *et al.*, 2023). Moringa seed extract reduced blood glucose levels, oxidative stress, and kidney tissue damage while improving overall kidney function in rats fed a high-fat diet and with streptozotocin-induced diabetic renal dysfunction (Wen *et al.*, 2022). Furthermore, the Moringa seed extract also activated glycogen synthase kinase-3beta (GSK-3β) and nuclear factor erythroid-derived 2-related factor 2/heme oxygenase-1 (Nrf2/HO-1) pathways, leading to antioxidant and anti-renal fibrosis activities and delaying the progression of diabetic renal dysfunction (Wen *et al.*, 2022).

2.8.2.7. Other protective properties

Beyond its well-documented pharmacological and phytochemical properties, Moringa also had additional properties of potential health benefits. The aqueous extract of Moringa roots emerges as a source of estrogenic and progestational activity, showing hormonal regulatory properties (Mehwish *et al.*, 2020). This discovery expands the relevance of Moring as an intervention option, particularly in women's health. Moreover, the antispasmodic activity of the roots of Moringa adds another value to its therapeutic benefits, indicating potential applications in addressing spasmodic conditions (Islam *et al.*, 2021).

Moringa aqueous leaf extracts revealed capacity to inhibit the formation of fluorescent and non-fluorescent advanced glycation end products (AGEs), including N(ε)-(carboxymethyl) lysine (CML), and significantly reduced fructosamine levels induced by reducing monosaccharides-induced protein glycation (Nunthanawanich *et al.*, 2016). Additionally,

Moringa aqueous leaf extracts exhibited a dose-dependent inhibition of protein oxidation, which was shown by decreased protein carbonyl content and preservation of protein thiol groups (Nunthanawanich *et al.*, 2016). This inhibition holds significant potential in preventing complications associated with diabetes, as AGEs are implicated in various diabetic complications. Furthermore, the traditional uses of pods, rich in fibre content, for addressing stomach-related issues and intestinal problems, is evidence that Moringa has gastrointestinal benefits (Zvinorova *et al.*, 2015). Moringa seed powder have also been shown to reduce arsenic levels in the blood, liver, and kidneys, which means that Moringa has potential in tackling environmental toxins (Dhakad *et al.*, 2019).

Furthermore, the healing potential of Moringa extends to chronic gastric ulcers induced by acetic acid, as evidenced by extracts from its leaves and fruits (Dhakad *et al.*, 2019). This aligns with traditional medicinal uses and underscores the gastro-protective properties of Moringa. These diverse and multifaceted benefits position Moringa as a cost effective ethno-medicinal resource with the potential to address a spectrum of health challenge.

2.9. Justification of the study

Early weaning results in reduced mother-infant contact and the early introduction of high-fructose or high-energy diets, which negatively influence their metabolic development immediately and throughout their lifespan, often leading to a rapid increase in childhood obesity that can progress into adulthood, placing a burden on public health systems. There is a paucity of research on the potential prophylactic properties of medicinal plants and their phytochemical constituents, against diet-induced metabolic dysfunction in early-weaned animals on a high fructose diet. The present study is designed to investigate the potential protective effect of hydroethanolic Moringa leaf extracts administered during the early weaning period against the development of metabolic disorders later on in life in female Sprague Dawley rats.

Chapter 3 provides a comprehensive exploration of the experimental investigation into general metabolic dysfunction and metabolic dysfunction associated fatty liver disease (MAFLD) induced by early weaning and a high-fructose diet in rats. This chapter details the study's rationale, objectives, methodologies, and findings, aiming to explain in details the potential protective role of *Moringa oleifera* against these conditions.

CHAPTER THREE: THE EFFECT OF STRATEGIC ADMINISTRATION OF *MORINGA OLEIFERA* LAM. HYDROETHANOLIC LEAF EXTRACTS IN THE FIRST TWO WEEKS POST-WEANING ON FRUCTOSE-INDUCED METABOLIC FATTY LIVER DISEASE (MAFLD) IN EARLY-WEANED FE-MALE SPRAGUE-DAWLEY RATS

3.1. Introduction

The World Health Organisation (WHO) reported that between 2015 and 2020, only 44% of infants aged from 0 to 6 months were exclusively breastfed globally (WHO, 2020). This indicates that 56% of infants were early-weaned. Early weaning is the introduction of solid food to an infant before the prescribed weaning age and can have a significant impact on the health and developmental progress of the infant (Souza, De Moura and Lisboa, 2020). The WHO recommends exclusive breastfeeding of infants throughout the first six months of their lives (Lechosa-Muñiz *et al.*, 2021). However, it is alarming that complementary foods have been introduced into the diets of infants as early as six weeks postnatally, despite the WHO guidelines (Budree *et al.*, 2016).

Socioeconomic, cultural and health related factors have been documented as major contributors to early weaning (Budree *et al.*, 2016). Poverty and food insecurity plays a major role in decisions caregivers have to make in infant feeding. Maternal employment and inadequate workplace support for breastfeeding also contribute to early cessation, with many mothers returning to work due to unpaid or partial maternity leave (Sayed *et al.*, 2020). Additionally, cultural beliefs and familial advice, particularly from grandmothers, often encourage mixed feeding or the early introduction of solid foods, despite WHO or health care recommendations (Wrottesley *et al.*, 2021). There is a misconception that breastmilk is nutritionally insufficient to satisfy infants or for their growth as well, which contributes to the practice of early weaning (Budree *et al.*, 2016). According to Budree et al. (Budree *et al.*, 2016), 54% of infants consume high-fructose beverages like fruit juices and soft drinks, candy and pastries, before they turn a year old (Budree *et al.*, 2016).

Early weaning alone has significant consequences for the metabolic health of infants, including impaired growth, weakened immune system, and impaired metabolic programming, but when combined with high-fructose diets, these effects are exacerbated, leading to increased risks of obesity, insulin resistance, and long-term metabolic disorders (Souza, De Moura and Lisboa, 2020). Quek et al. (Quek *et al.*, 2022) indicated that maternal, and postnatal factors such as early dietary disruptions could potentially lead to liver fat accumulation and metabolic dysfunction, even without the presence of excessive adiposity (Quek *et al.*, 2022). Therefore, although not directly addressing early weaning, the study reinforces the idea that early

nutritional interventions, such as breastfeeding length, may be protective against metabolic disorders, later in life (Quek *et al.*, 2022). These risks from early weaning and high-fructose diets create a critical pathway to the development of metabolic-associated fatty liver disease (MAFLD) (Mandala *et al.*, 2020). This is further supported by Xu et al. (Xu *et al.*, 2022) who reported that early-life dietary disruptions may contribute to the development of lean/non-obese MAFLD, particularly by influencing liver fat accumulation and insulin resistance, even in the absence of significant obesity (Xu *et al.*, 2022). This is because early dietary disruptions and metabolic imbalances significantly increase susceptibility to lipid accumulation and inflammation in the liver (Mandala *et al.*, 2020). MAFLD is characterised by an excessive build-up of lipids in the liver cells (Harring *et al.*, 2023) in the absence of heavy consumption of alcohol.

There is a high global prevalence of MAFLD, exceeding 40% worldwide, representing approximately 3.12 billion people (Herrera-Marcos *et al.*, 2023). Even more alarming is that approximately 10% of MAFLD cases involve children (Ferrer *et al.*, 2023). Projections suggest that by 2030, MAFLD cases could escalate by 97% (Bolatimi *et al.*, 2023). A meta-analysis by Balakrishnan and colleagues found that MAFLD is more common in men than women, worldwide (Balakrishnan, 2021). However, women are more susceptible than men are to advanced phases of the condition, such as non-alcoholic steatohepatitis (NASH) and fibrosis, and women are more impacted in older age groups (Balakrishnan, 2021). Women have a 37% increased risk of advanced fibrosis (Balakrishnan, 2021). This highlights the need for sex-specific research on MAFLD in women. The increasing prevalence of MAFLD requires urgent attention to address this pressing public health concern.

The pathogenesis of MAFLD centres on glucose, lipid, and cholesterol metabolism, including inflammation and fibrotic progression, eventually leading to liver injury and potential progression to NASH, cirrhosis, and hepatocellular carcinoma (HCC) (Jensen *et al.*, 2018). These pathological processes are influenced by various factors such as insulin resistance, adipokines, oxidative stress, and gut microbiota dysbiosis, creating a complex interplay in the development and progression of MAFLD (Jegatheesan and De Bandt, 2017).

Although some countries have acknowledged the potential risks linked to the early introduction of fruit juice to infants and have implemented measures to prohibit or limit its use, numerous other countries have yet to embrace such regulatory actions (Heyman and Abrams, 2017). The

introduction of fruit juice during the weaning process, often leads to early exposure to high fructose diets. There is a well-established correlation between elevated consumption of fructose and the development of metabolic diseases, such as obesity and insulin resistance (Barrett *et al.*, 2023). The aforementioned risk factors contribute to the pathogenesis of MAFLD.
It is crucial to understand the complex link between premature cessation of breastfeeding, consumption of diets high in fructose, and the maintenance of metabolic health (Lechosa-Muñiz *et al.*, 2021).

There is no pharmacotherapy that has been approved to treat MAFLD and its associated metabolic derangements (M. Yan *et al.*, 2023). However, existing interventions emphasise dietary and lifestyle changes for maintaining healthy body weight and preventing metabolic derangements (Ferrer *et al.*, 2023). There is however, increasing interest in the strategic use of medicinal plants and their derived phytochemicals during critical windows of development, such as developmental plastic periods or the weaning phase, to influence metabolic programming and prevent poor health outcomes associated with unhealthy lifestyle choices (Sathiya, Manoharan and Rajarajeshwari, 2019; Mukonowenzou *et al.*, 2021).

The Moringa tree (*Moringa oleifera),* is highly regarded for its numerous health benefits. It's leaves contain a wide array of vitamins, minerals and proteins (Gopalakrishnan, Doriya and Kumar, 2016; Kim, Choi and Shin, 2020; Sokhela, Govender and Siwela, 2023). They are also rich in phytochemicals including flavonoids and polyphenols (Pareek *et al.*, 2023). Moringa has a long history of usage in traditional medicine (Luoh *et al.*, 2017b). In animal studies using rats and mice, administration of Moringa for durations ranging from 7 days to 90 days have demonstrated its ability to treat cardio-metabolic conditions such as hypertension, diabetes, and hypercholesterolemia (Jaja-Chimedza *et al.*, 2018; Abd-Elnaby *et al.*, 2022; Irfan, Khan and Asmawi, 2022; Monraz-Méndez *et al.*, 2022). Its long-term administration has also been shown to prevent and treat MAFLD (Gopalakrishnan, Doriya and Kumar, 2016; El-Shehawi *et al.*, 2021; Monraz-Méndez *et al.*, 2022). Additionally, in human studies consumption of Moringa for durations between 2 to 16 weeks have been shown to reduce glucose levels, inflammation and lipotoxicity (Phimarn *et al.*, 2021; Faheem, Amina, Iqbal and Faheem, 2023). Thus the effects of Moringa on metabolic function are well-researched in both human and animal studies. However, long-term consumption of Moringa for its health benefits may not be financially sustainable, especially in resource limited communities. Therefore, there remains

a critical need to investigate the impact of short-term Moringa administration during developmental plastic periods, such as the weaning phase, in order to determine whether it can program for good long-term metabolic health into adulthood.

Given the adverse effects of early weaning and the inclusion of high fructose foods in the early weaning diets, we hypothesised that strategic short term intervention with Moringa hydroethanolic leaf extracts administered to early-weaned rats, in the first two weeks postweaning, can protect against development of MAFLD and high fructose diet induced metabolic derangements later in adulthood.

3.2. Methods and Materials

3.2.1. Study setting

The study was conducted at the University of the Witwatersrand's Research Animal Facility (WRAF) in Johannesburg, South Africa. This study was designed in alignment with the ARRIVE guidelines (du Sert *et al.*, 2020). Ethical clearance for the study (reference: 2020/08/04/B) was granted by the Animal Research Ethics Committee of the University of the Witwatersrand.

3.2.2. Sources of *Moringa oleifera* leaves and preparation of extracts

Fresh *Moringa oleifera* Lam. leaves (Cultivar PKM1) were harvested and collected at the Agricultural Research Council's Moringa farm in Pretoria (GPS coordinates 25°59"S 28°35"E), South Africa. The leaves were collected during November 2019, during late spring season. A voucher specimen of Moringa was prepared and sent to the University of the Witwatersrand's C.E. Moss Herbarium for identification and verification. A voucher specimen (specimen number: J103704).

Moringa leaves were dried in an oven (Salvis®, Salvis Lab, Rotkreuz, Schweiz, Switzerland) at 50°C until a constant dry weight and then ground into powder. The leaf powder was extracted (1:20 w/v) with 70% aqueous ethanol (v/v) in an ultrasonic bath (Scientific Engineering Inc, Salina, Kansas, USA) for 1 hour to make a Moringa hydroethanolic leaf extract. The extract was filtered through Whatman No.1 (50 mm) (Whatman™, No. 4 size 150 mm Ø, Hebei, China) under a vacuum (Esco Technologies, Horsham, Pennsylvania, USA), concentrated

under pressure using a rotatory evaporator (Buchi Labortechnik AG, Flawil, Switzeland) at 30°C and air-dried at room temperature. The hydroethanolic extracts were stored in air-tight containers at room temperature in the dark before use.

3.2.3. Study animals and husbandry

Female Sprague Dawley pups (*Rattus norvegicus*) from eleven dams, meeting the inclusion criteria of giving birth to a litter of 8-12 pups were selected for the study. The rats were housed in polycarbon cages (Hebei Rital Metal Products Co., Ltd, Shijiazhuang, China) with wood shavings and shredded paper for bedding. Short sections of PVC pipes were placed in the cages for environmental enrichment. The room temperature was maintained at 25 ± 2 °C with a light and dark cycle of 12 hours (lights on from 07:00 to 19:00) with adequate ventilation. The rat pups had *ad libitum* access to commercially sourced rat chow (SRC) (LabChef Nutrition hub, Stellenbosch, South Africa) and drinking fluids. The nutritional component of the rat chow (g/kg) used in this study includes protein (220), fats (50), Fibre (40), moisture (100), linoleic acid (12), calcium (12), phosphorus (7.5), vitamin E (100) and vitamins (IU/kg) A (16000) and D (2000) and crude oils and fats (De Las Heras *et al.*, 2009).

3.2.4. Experimental design

This interventional prospective experimental study consisted of two experimental stages (see Figure 3.1). **Stage 1** was the intervention during either the early weaning period from PD (post-natal day) 18 up to PD35 or normal weaned (PD21) for the control group which was also included. **Stage 2** was the post-weaning intervention from PD36 to PD111.

Pups were randomly allocated to seven treatment groups (n=8 each) using the stratified randomization method (Bespalov, Martin and Steckler, 2020), namely: **Group 1:** Normal Weaning Negative Control (NWC) (pups were weaned at PD21), in Stage 1 the pups were fed standard rat chow (SRC) + plain gelatine cubes and plain drinking water. In Stage 2, they were given water to drink. **Group 2:** Negative control (EWC), in Stage 1 the pups were fed standard rat chow (SRC) + plain gelatine cubes and plain drinking water. In Stage 2, they were given water. **Group 3:** Metabolic derangements were induced (EWF), where in Stage 1, pups were fed SRC + plain gelatine cubes, with 20% (w/v) fructose solution to drink. In Stage 2, they received 20% (w/v) fructose solution as drinking fluid. **Group 4:** the Moringa only low dose control (EWML), in Stage 1, pups received SRC + 50 mg/kg Moringa extract in gelatine cubes for 18 days only and plain drinking water. In Stage 2, they received water to drink and Moringa administration was ceased. **Group 5:** the Moringa only high dose control (EWMH), in Stage 1, pups received SRC + 500 mg/kg Moringa hydroethanolic extract in gelatine cubes for 18

days only and plain drinking water. In Stage 2, they received water. **Group 6:** Low dose Moringa hydroethanolic extract with fructose intervention (EWMLF), In Stage 1, the rats received 50 mg/kg Moringa extract in gelatine cubes + SRC for 18 days only and drank 20% (w/v) fructose solution. In Stage 2, they received 20% (w/v) fructose solution to drink. **Group 7:** High dose Moringa hydroethanolic extract with fructose intervention (EWMHF), In Stage 1, pups were given 500 mg/kg Moringa hydroethanolic extract in gelatine cubes for 18 days only and 20% (w/v) fructose solution to drink, in Stage 2, they continued on 20% (w/v) fructose solution as drinking fluid. All rats in stage 2 had *ad libitum* access to SRC. Moringa doses used in this study were as previously used by (Mapfumo *et al.*, 2019).

Gelatine cubes were prepared by weighing 8 g of gelatine, 8 g of sugar, and 2 g of Bovril and mixing into 100 mL of water to blend the components into a homogeneous mixture. The Moringa extract was added to provide the required doses before allowing the gelatine to set in 1-2 ml ice cube trays.

In stage 2, all the pups were then housed in pairs (two female rats from the same group per cage) from PD36 to the end of the experiment on PD111. This was for socialisation purposes from a welfare perspective and to facilitate oestrous synchronisation through the Whitten effect, whereby the presence of other females or exposure to male pheromones can align reproductive cycles (Mahjabeen *et al.*, 2018). This helps reduce variability in sex-hormone-dependent metabolic and renal responses.

The experimental design and timeline for the study is shown in Figure 3.1 below.

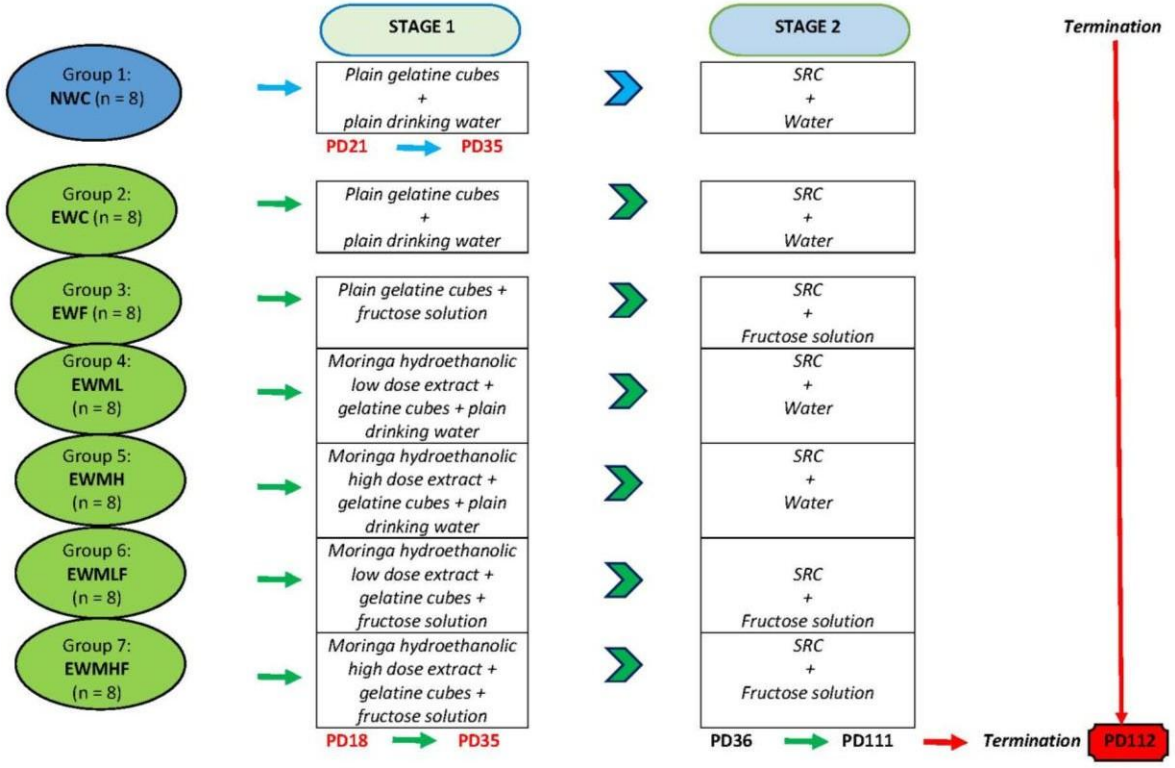


Figure 3. 1. Timeline of the experiment, groups and dietary supplements per Stage. Group 1 was weaned at the normal recommended weaning age of 21 days.

NWC (Normal weaning control group), EWC (Early weaning control group), EWF (Early weaning with 20% fructose solution), EWML (Early weaning with Moringa low dose only), EWMH (Early weaning with Moringa high dose only), EWMLF (Early weaning with Moringa low dose and fructose solution), EWMHF (Early weaning with Moringa high dose and fructose solution), SRC (Standard rat chow) and PD (Postpartum) ((PD21 is normal weaning), while groups 2 – 7 were weaned early at PD18).

3.2.5. Body mass measurement

Rats were weighed daily for welfare monitoring and for adjusted Moringa dose in gelatine cubes in Stage 1 and subsequently, twice a week in stage 2. Feed intake and fructose solution consumption were measured twice a week.

3.2.6. Terminal procedures

Following an overnight fast (with access to plain drinking water) 0.1 ml of blood was collected from the tail vein following a pinprick and was used to measure fasting blood glucose using a glucometer (Contour Plus Bayer Health Care, Isando, South Africa). The rats were then euthanized with an overdose (150 mg/kg i.p.) of sodium pentobarbitone (Merck, Darmstadt, Germany).

More blood was collected through cardiac puncture into heparin-coated and serum separator tubes. The collected blood samples were centrifuged (Hettich EBA 2400, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) for 15 min at 4000 × g at 20 °C and the harvested serum and serum stored at - 20 °C until use.

A midline incision was made through the abdomen wall and the visceral fat and liver were dissected out and weighed. The right lobe of the liver was stored in 10% phosphate-buffered formalin for later processing for histomorphometric measurements. A sample of the left lobe of the liver was preserved in RNA-later (Life Technologies Corp, Van Allen Way Carlsbad, USA) and stored at - 80°C for later molecular analyses.

3.2.7. Measurement of health outcomes associated with MAFLD

3.2.7.1. Liver sample processing for histomorphometry

The formalin preserved liver tissue samples were routinely processed (Leica TP1020 Automatic Benchtop Tissue Processor; Leica instruments, Wetzlar, Germany), mounted onto blocks and sectioned them at 5 µm, then stained with haematoxylin and eosin (H&E) or Masson's trichrome (MT). The sections were viewed using a Leica DM750 microscope (Leica Microsystems (Schweiz) AG, Max Schmidheiny Strasse, Heerbrugg, Switzerland), images were taken with an AU-300-HD excelis lite camera (New York Microscope Company, Hicksville, NY, USA) for further quantitative MAFLD scoring.

3.2.7.2. Liver histology and scoring for MAFLD

The histological features were scored according to (Liang *et al.*, 2014). Liver images taken at 40× magnification were opened in ImageJ software (ImageJ, version 1.51, Bethesda, USA) to determine area (A) and area fraction ($A_{fraction}$) of each liver section according to (Schneider, Rasband and Eliceiri, 2012).

3.2.8. Determination of serum concentrations of metabolic hormones and computation of HOMA-IR

Serum insulin, leptin, and adiponectin levels were determined using rat-specific ELISA kits (Elabscience®, Houston, Texas, USA). The assessment of glycaemic control was conducted using the Homeostatic model assessment index (HOMA-IR), with insulin concentrations converted to µU/L for HOMA-IR calculations. The formula used to determine HOMA-IR was; HOMA-IR = [glucose (mmol/L) × insulin (µU/L)]/22.5 (Minh *et al.*, 2021).

3.2.9. Determination of the hepatic function biomarker alanine aminotransferase (ALT)

Circulating Alanine transaminase (ALT) levels were assessed from 10µL of serum using an automated clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., Westbrook, USA) as recommended by the manufacturer's protocol.

3.2.10. Determination of the serum concentration of lipids (triglycerides (TG) total cholesterol (TC) circulating Lipids (LDL & HDL))

Rat-specific ELISA kits (Elabscience® biochemical assay kit, Houston, Texas, USA) were used to measure serum TG, TC, LDL and HDL. The colour intensity was measured using a spectrophotometer with a 450 nm filter microplate reader (EMCLAB Instruments GmbH, Duisburg, Germany). The standard curves for LDL, HDL, TC, and TG exhibited correlation coefficient (R^2) values of 1.0000, 0.993, 0.995, and 0.994, respectively, indicating excellent linearity.

3.2.11. Gene expression profiling in determining hepatic regulation and metabolism of fatty acids and lipids

3.2.11.1. RNA extraction

The stored liver tissue samples (50 mg) were ground using a mortar and pestle in liquid nitrogen (Afrox, Sandton, Johannesburg, South Africa). An Aurum^{TM} total RNA mini extraction kit

(BioRad, Hercules, California, USA) was used to extract RNA by following the protocol described by (Kemfack *et al.*, 2022).

3.2.11.2. cDNA synthesis

LunaScript supermix (Inqaba biotec, Johannesburg, South Africa) was used to synthesise double stranded cDNA from the obtained RNA sample (1 µg) according to the manufacturer's protocol and run on a thermal cycler (PxE0.2, Thermofisher Scientific, Walthan, Massachusetts, USA).

3.2.11.3. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

After cDNA was synthesised, the samples were analysed using a LightCycler 96 application software and instrument (Roche diagnostics, Basel, Switzerland). The RT-qPCR instrument analysed gene expression of the selected genes of interest (Table 3.1) in triplicate using a SensiMix SYBER No-ROX kit (Bioline, London, United Kingdom) and the following cycling protocol: denaturation at 95°C for 5 seconds, annealing at 62°C for 10 seconds and extension at 72°C for 15 seconds. The expression ratio of genes were then calculated relative to the housekeeping gene (*Actinβ*) using the $2^{-\Delta\Delta CT}$ method.

Table 3. 1. Primers of rat genes used in this study.

Housekeeping genes	**Forward Primer**	**Reverse Primer**	**Melting Temperature (°C)**	**Annealing Temperature (°C)**
Gene of reference				
Actinβ	GCTAACAGTCCGCCTAGAAGCA	GTCATCACCATCGGGCAATGAG	59.3	54.3
Genes of Interest				
SREBP-1c	CGACACCACCAGCATCAACCACG	GCAGCCCATTCATCAGCCAGACC	60.2	55.2
ACC	TGCTGAATATATCCTCACGGAGCT	CGACGTTTCGGACAAGATGAGT	58.1	53.1
CPT-1	ACGCCGTGAAGTATAACCCT	CCAAAAATCGCTTGTCCCTT	59.7	54.7
PPAR-α	GATACCACTATGGAGTCCACGCA	GCCGAAAGAAGCCCTTG	60.4	55.4
FAS	GCTTTGCTGCCGTGTCCTTCT	GTGTCTGCTGGGGTCCTCTT	59.5	54.5

Abbreviations: *Actinβ* (beta-actin), *SREBp-1c* (Sterol regulatory-element-binding protein-1c), *ACC* (Acetyl-CoA carboxylase), *CPT-1* (Carnitine palmitoyltransferase I), *PPAR-α* (Peroxisome proliferator-activated receptor alpha) and *FAS* (Fatty acid synthase). The primer sequence for FAS was adopted from the previously published work of (He Nam *et al.*, 2019) for forward sequence and (Ascencio *et al.*, 2004) for reverse primer, while the remaining primers (CPT-1, ACC, SREBP-1c, PPAR-α, and Actinβ) were independently designed using Primer-BLAST from sequences obtained from the NCBI GenBank database, with accession numbers NM_017085, NM_017008, NM_001107013, NM_012675, and NM_031144, respectively. Quantitative validation was automatically done in the instrument used for analysis, the LightCycler® 96 Instrument and its software. Primer efficiency for all designed primers (CPT-1, ACC, SREBP-1c, PPAR-α, Actinβ, and FAS) was assessed using the LightCycler® 96 Instrument (Roche Life Science) along with the LightCycler® 96 Application Software as well. Standard curves were generated from serial dilutions of cDNA, and amplification efficiency (E) was calculated using the slope of the standard curve. The software uses the Second Derivative Maximum (SDM) method to determine crossing point (Cp) values for accurate quantification. Melting curve analysis was also performed to confirm primer specificity, and all primers exhibited efficiencies within the acceptable range of 90–110%, indicating reliable and reproducible amplification. Since this is an automated process performed by the instrument and software, the detailed calculations are not included in the standard methodology of this thesis. Primer-specific annealing temperatures were applied as determined automatically by the LightCycler® 96 software based on the melting temperatures of each primer pair.

3.2.12. Statistical analysis

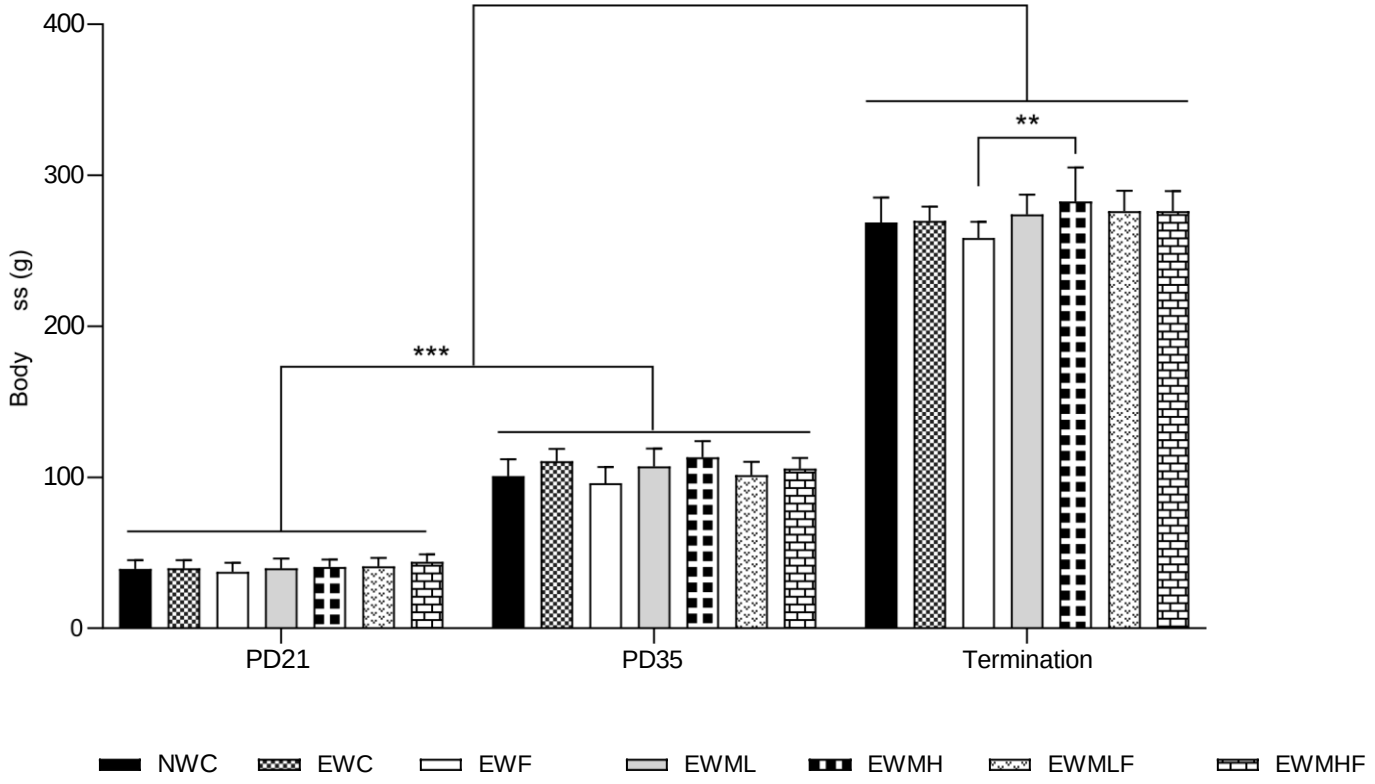
GraphPad Prism 8 statistical software (GraphPad Software Inc, San Diego, USA) was used for data analysis. Assessment of normality was performed using a Shapiro–Wilk test. All data comparisons were subject to analysis of variance (ANOVA) for normally distributed data, followed by Tukey's multiple comparisons test to determine differences between groups. For data that were not normally distributed, the Kruskal–Wallis test followed by Dunn's post-hoc multiple comparisons test was applied. Normally distributed data are presented as mean ± SD, while histology scores for MAFLD are presented as median (25th percentile, 75th percentile). Statistical significance was accepted at $p < 0.05$.

3.3. Results

3.3.1. The effect of hydroethanolic Moringa leaf extract and fructose consumption on body masses at weaning, termination and terminal visceral fat mass of early-weaned female rats

Early weaning in rats did have a significant impact in body masses between treatment groups at PD21 ($p > 0.05$; Figure 3.2A). However, all rats increased in body mass from PD21 to PD35 and PD112 ($p < 0.0001$; Figure 3.2A). Fructose-fed rats were lighter than those fed Moringa low dose only at termination ($p < 0.05$). No significant differences in terminal visceral fat mass were observed ($p > 0.05$; Figure 3.2B).

A


400
300
200
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0
Body ss (g)
***
**
PD21
PD35
Termination
NWC
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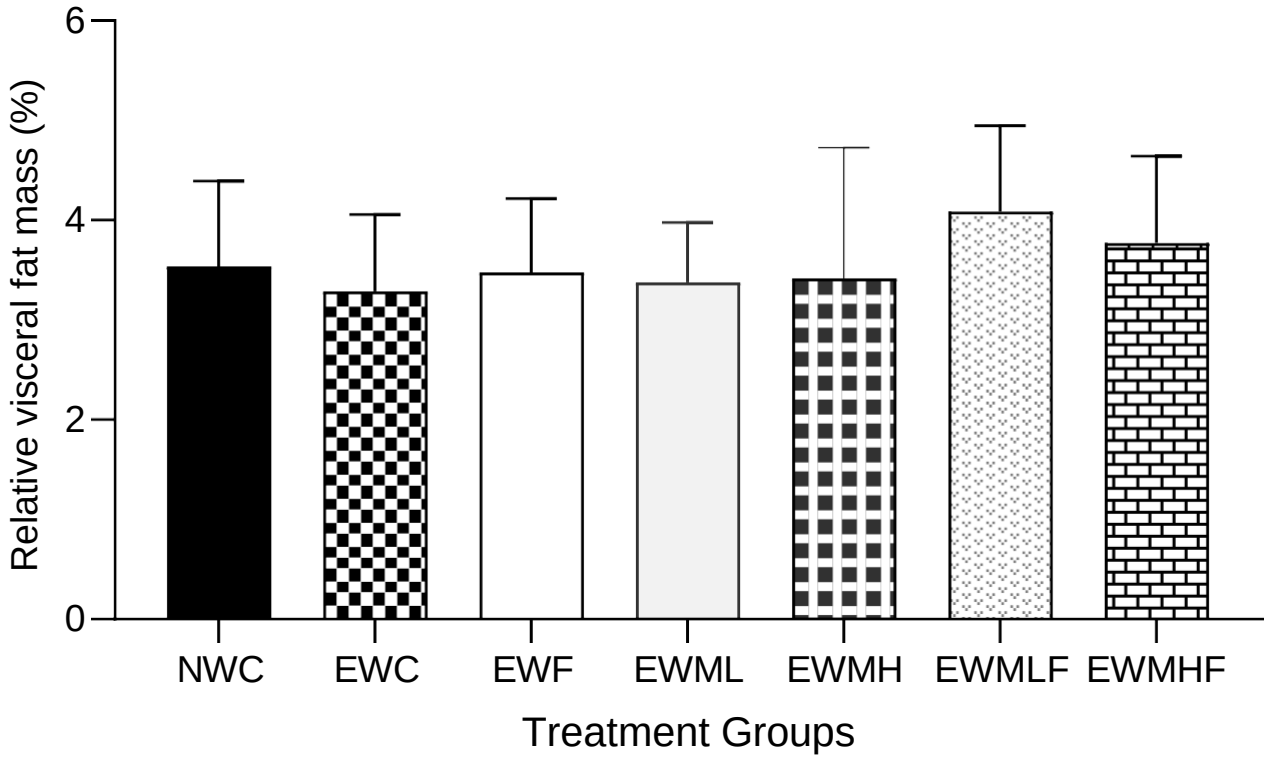


Figure 3. 2. A. Effect of Moringa hydroethanolic leaf extract on body masses at induction, weaning and termination in fructose fed female rats and EWF vs EWMH at Termination, PD 112. **B.** The effect of hydroethanolic Moringa leaf extract and fructose consumption on terminal visceral fat mass among the treatment groups at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (** is $p < 0.01$ and *** is $p < 0.001$) (n=8 for all groups). NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water until PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.3.2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on the absolute and relative liver mass of early-weaned rats

There were no significant differences in the absolute and relative masses of the liver of the rats across the treatment groups of the study ($p > 0.05$; Table 3.2).

Table 3. 2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on absolute mass and mass relative to body mass of the liver at Termination, PD 112.

Treatment Groups	Liver (g)	Liver (%TBM)
NWC	7.90 ± 0.61[a]	2.94 ± 0.15[a]
EWC	8.46 ± 1.27[a]	3.13 ± 0.39[a]
EWF	8.28 ± 0.51[a]	3.21 ± 0.19[a]
EWML	8.15 ± 0.57[a]	2.98 ± 0.19[a]
EWMH	8.25 ± 0.81[a]	2.92 ± 0.12[a]
EWMLF	8.73 ± 0.68[a]	3.16 ± 0.17[a]
EWMHF	8.30 ± 0.72[a]	3.00 ± 0.18[a]

All data are presented as mean ± standard deviation. NB: %TBM = organ mass relative to terminal body mass. n=8 per group. [a] Significant difference is represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.3.3. The effects of Moringa leaf extracts and fructose consumption on fasting blood glucose, serum insulin concentration and HOMA-IR index in early-weaned female Sprague-Dawley rats

Early weaning with or without Moringa and/or fructose did not significantly affect blood glucose concentration compared to normal weaning ($p > 0.05$; Table 3.3). However, high doses of Moringa with fructose significantly lowered blood glucose levels compared to normal weaning ($p < 0.05$; Table 3.3).

Early weaning with fructose significantly reduced insulin levels in rats compared to normal weaning ($p < 0.05$; Table 3.3), while Moringa had no significant impact insulin levels, regardless of dose. There was also no significant impact of the interventions on HOMA-IR ($p > 0.05$; Table 3.3).

Table 3. 3. The effects of Moringa oleifera leaf extracts and fructose consumption on fasting blood glucose, serum insulin concentration and HOMA-IR index in early-weaned female Sprague-Dawley rats at Termination, PD 112.

Parameter	Glucose (mmol/L)	Insulin (ng/mL)	HOMA-IR
NWC	4.16 ± 0.21^{abc}	0.13 ± 0.02^{a}	0.55 ± 0.01^{a}
EWC	4.58 ± 0.58^{a}	0.13 ± 0.018^{a}	0.61 ± 0.01^{a}
EWF	4.46 ± 0.37^{ab}	0.09 ± 0.01^{b}	0.38 ± 0.01^{a}
EWML	4.26 ± 0.24^{abc}	0.12 ± 0.01^{ab}	0.53 ± 0.003^{a}
EWMH	3.96 ± 0.27^{bc}	0.11 ± 0.02^{ab}	0.45 ± 0.01^{a}
EWMLF	3.98 ± 0.35^{bc}	0.12 ± 0.03^{ab}	0.47 ± 0.01^{a}
EWMHF	3.80 ± 0.32^{c}	0.11 ± 0.03^{ab}	0.43 ± 0.01^{a}

All data are presented as mean ± standard deviation significant difference. ^{a,b,c} Significant difference is represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. n = 8 per group. Glucose: EWC vs EWMH ($p=0.02$; $p < 0.05$), EWC vs EWMLF ($p=0.03$; $p < 0.05$), EWC vs EWMHF ($p=0.001$; $p < 0.01$) and EWF vs EWMHF ($p=0.01$; $p < 0.05$). Insulin: NWC vs EWF ($p=0.02$; $p < 0.05$) and EWC vs EWF ($p=0.009$; $p < 0.01$). NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. HOMA-IR is homeostasis model assessment-estimated insulin resistance.

3.3.4. The effect of Moringa leaf extracts and fructose consumption on serum alanine transaminase (ALT) concentration in early-weaned female Sprague-Dawley rats

Early weaning with fructose significantly increased ALT serum concentration compared to normal weaning ($p < 0.05$; Figure 3.3), while Moringa low and high dose diets prevented this increase compared to normal reference concentrations (20 – 61 U/L) (IDEXX, 2019) (Figure 3.3).

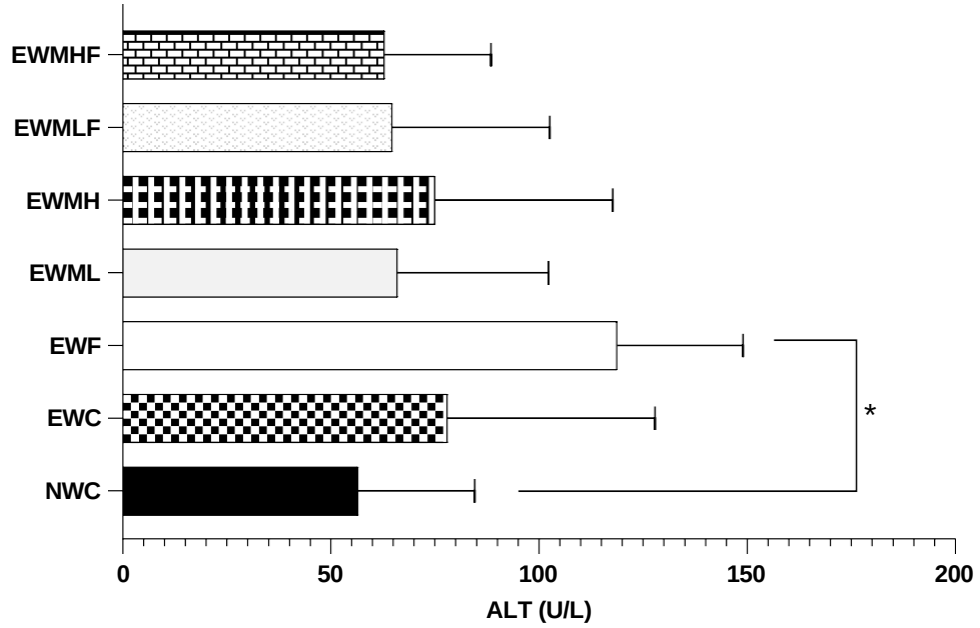


Figure 3. 3. The effect of Moringa leaf extracts and fructose consumption on serum alanine transaminase (ALT) concentration in early-weaned female Sprague-Dawley rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (* is $p < 0.05$). n=8 for all groups. NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.3.5. The effects of Moringa leaf extracts and fructose consumption on leptin, adiponectin concentrations and leptin/adiponectin ratio in early-weaned female Sprague-Dawley rats

Early weaning with or without fructose significantly increased leptin levels ($p < 0.01$; Table 3.4), but Moringa high and low dose with fructose significantly decreased leptin levels ($p < 0.05$; Table 3.4), suggesting Moringa prevented the early weaning fructose-induced leptin increase.

The administration of fructose or a low dose of Moringa during early weaning resulted in a significant reduction in circulating adiponectin levels ($p < 0.05$; Table 3.4). Conversely, the high dose of Moringa prevented the drop in adiponectin caused by fructose ($p < 0.01$; Table 3.4).

Early weaning significantly increased the leptin-adiponectin ratio compared to normal weaning, but only with or without fructose and/or Moringa low dose ($p < 0.05$; Table 3.4). Moringa high dose (EWMHF) significantly decreased the fructose-induced increase in leptin-adiponectin ratio ($p > 0.0001$; Table 3.4).

Table 3. 4. The effects of Moringa leaf extracts and fructose consumption on LEP, ADP and LEP/ADP Ratio concentrations in early-weaned female Sprague-Dawley rats at Termination, PD 112.

Parameter	LEP (ng/mL)	ADP (ng/mL)	LEP/ADP RATIO
NWC	0.95 ± 0.31^{a}	4.25 ± 1.52^{a}	0.25 ± 0.10^{a}
EWC	1.41 ± 0.17^{bc}	2.98 ± 0.50^{ab}	0.50 ± 0.12^{bc}
EWF	1.54 ± 0.22^{b}	2.63 ± 0.31^{bc}	0.58 ± 0.09^{b}
EWML	1.16 ± 0.20^{ac}	2.85 ± 0.39^{bc}	0.43 ± 0.12^{c}
EWMH	1.06 ± 0.16^{a}	3.74 ± 0.65^{ac}	0.30 ± 0.06^{a}
EWMLF	1.13 ± 0.09^{ac}	2.69 ± 0.36^{bc}	0.43 ± 0.08^{b}
EWMHF	1.06 ± 0.25^{a}	3.30 ± 0.39^{ac}	0.32 ± 0.09^{a}

All data are presented as mean ± standard deviation. ^{a,b,c} Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. n = 8 per group. NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

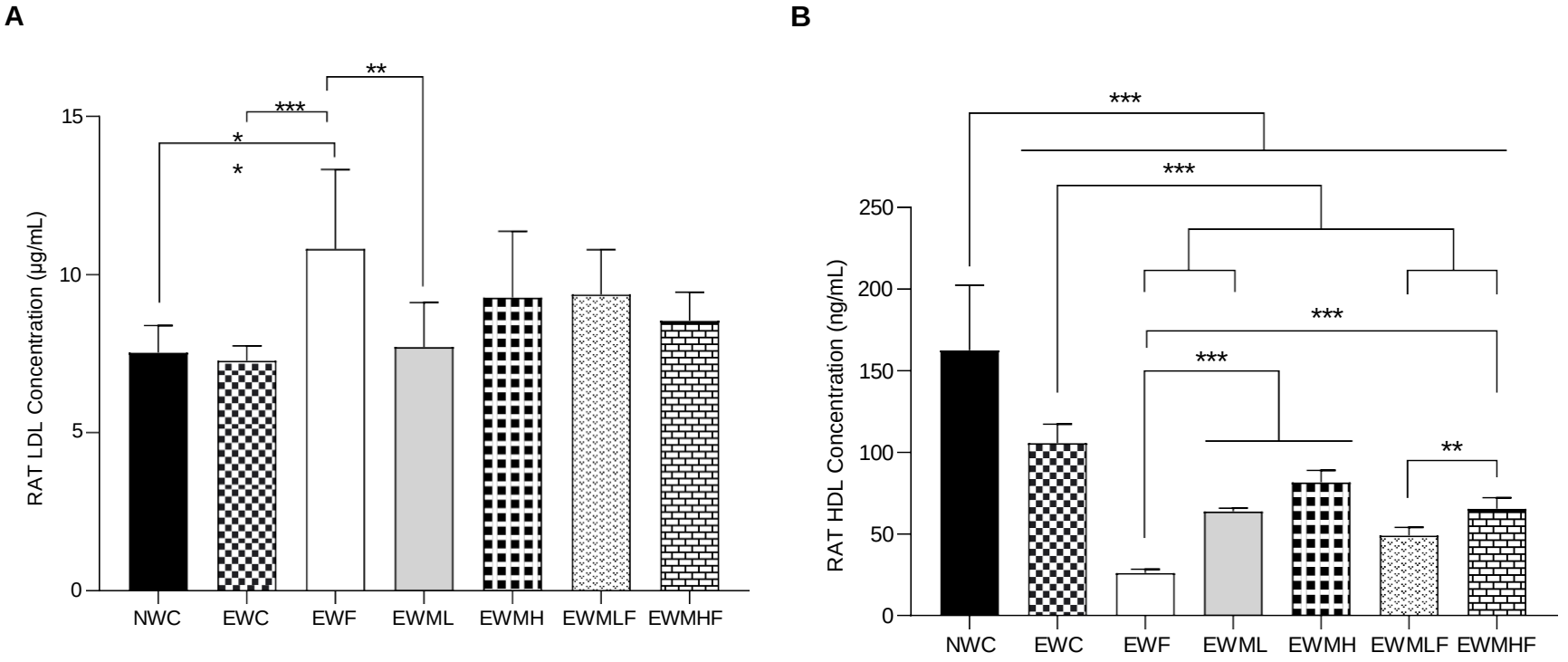
3.3.6. The effects of Moringa leaf extracts and fructose consumption on lipid profile of early-weaned female Sprague-Dawley rats

Early weaning with fructose significantly increased circulating LDL concentration ($p < 0.001$; Figure 3.4A), while Moringa low or high doses prevented this increase, with low dose being most significant ($p < 0.01$; Figure 3.4A).

Early weaning with Moringa low, high dose, or fructose significantly decreased HDL concentration in rats compared to normal weaning ($p < 0.0001$; Figure 3.4B), with the reduction being most distinct in rats fed a high fructose diet (Figure 3.4B). The reduction in early weaning with a high dose of Moringa was less significant than that of the high fructose diet (EWF), as shown in Figure 3.4B.

Early weaning with fructose only and fructose with a low or high dose of Moringa only significantly increased concentration of circulating TG levels compared to normal and early weaning on a normal diet ($p < 0.05$; Figure 3.4C). Early weaning with Moringa low dose had significantly reduced TG levels compared to early weaning with fructose ($p < 0.001$; Figure 3.4C). Both Moringa low or high doses with fructose failed to prevent the fructose induced increase in TG levels, as they resulted in a significant increase in TG concentrations ($p < 0.05$; Figure 3.4C).

Early weaning with fructose significantly increased TC levels compared to normal weaning with a normal diet ($p < 0.05$; Figure 3.4D). There was no observed effect on TC concentrations when early weaning with either a low or high dose of Moringa, or a normal diet (Figure 3.4D). Moringa low and high dose reduced the fructose induced increase in TC levels by about 1% (Figure 3.4D).


A
B
RAT LDL Concentration (µg/mL)
15
10
5
0
*
*
***
**
NWC
EWC
EWF
EWML
EWMH
EWMLF
EWMHF
RAT HDL Concentration (ng/mL)
250
200
150
100
50
0
***
***
***
***
**
NWC
EWC
EWF
EWML
EWMH
EWMLF
EWMHF

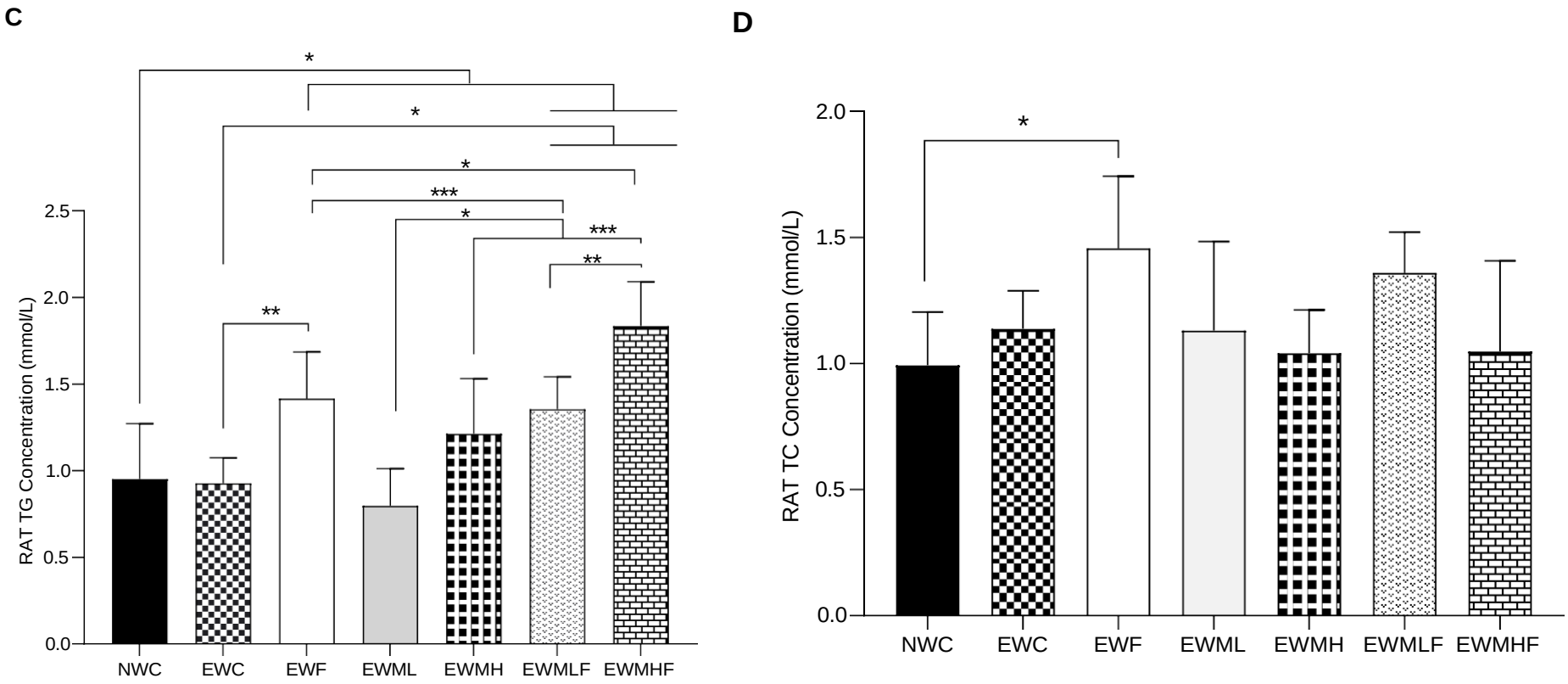


Figure 3. 4. The effects of Moringa leaf extracts and fructose consumption on circulating lipid profiles at Termination, PD 112. **A.** The effects of Moringa leaf extracts and fructose consumption on circulating LDL concentrations in early-weaned female Sprague-Dawley rats. **B.** The effects of Moringa oleifera leaf extracts and fructose consumption on circulating HDL concentrations in early-weaned female Sprague-Dawley rats. **C.** The effects of hydroethanolic Moringa leaf extracts and fructose consumption on circulating concentration of triglyceride (TG) in early-weaned female Sprague-Dawley rats. n = 8. **D.** The effects of hydroethanolic Moringa oleifera leaf extracts and fructose consumption on circulating concentration of total cholesterol (TC) in early-weaned female Sprague-Dawley rats. n = 7.

All data are presented as mean ± standard deviation. Significant difference (* is $p < 0.05$, ** is $p < 0.01$ and *** is $p < 0.001$) (n=8 for all groups). NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.3.7. The effects of hydroethanolic Moringa leaf extracts and fructose consumption on hepatic histology of early-weaned female Sprague-Dawley rats

Representative photomicrographs of female rats' liver sections stained with H&E for histology from each of the experimental groups are shown in Figure 3.5 (NWC to EWMHF). There was no observed fat deposition in the liver of rats weaned normally compared to those weaned early on a normal diet (Figure 3.5; NWC and EWC Images). Early weaning with fructose led to distinct liver fat deposition (Figure 3.5 EWF Image). Administration of Moringa during early weaning was able to reverse the fructose induced liver fat deposition, especially in low doses (Figure 3.5; EWMLF and EWMHF).

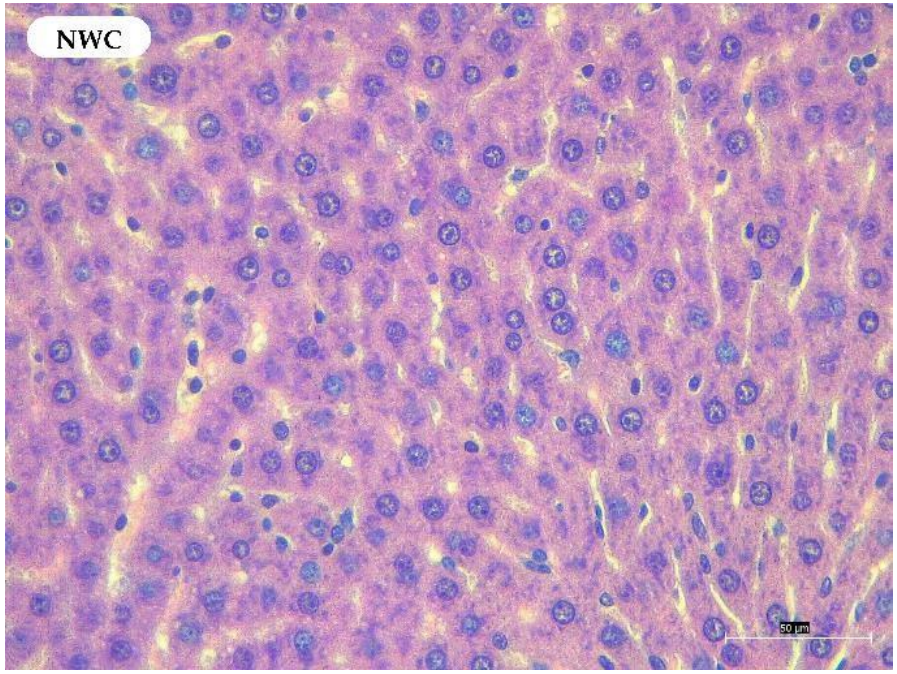
Microsteatosis scores increased significantly in early weaning on a normal diet, fructose, or Moringa low dose compared to normal weaning ($p < 0.01$, $p < 0.0001$ and $p < 0.05$; Table 3.5). However, administration of Moringa high dose with fructose prevented microsteatosis ($p < 0.05$; Table 3.5).

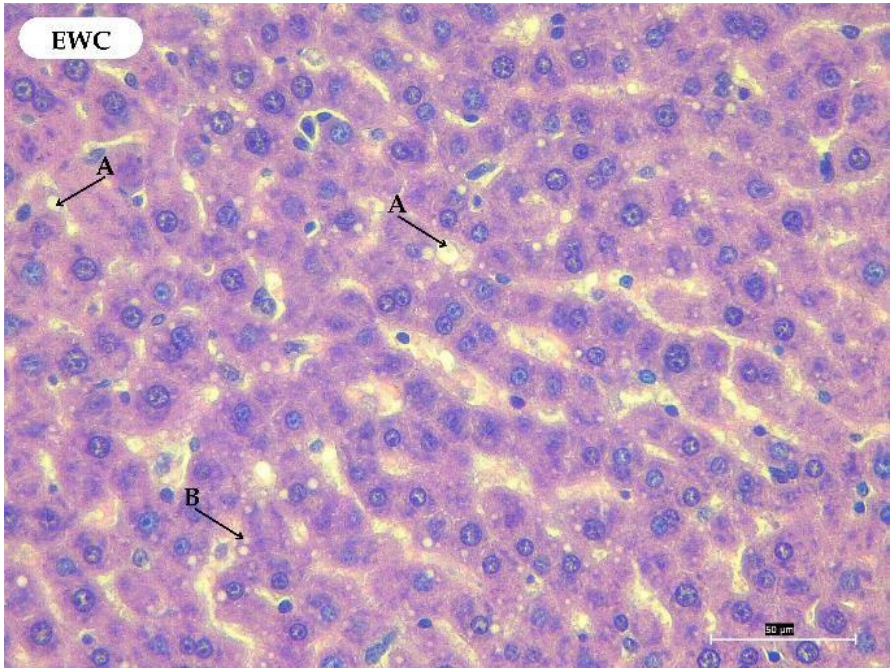
Early weaning with a normal diet or fructose significantly increased macrosteatosis scores in rats compared to normal weaning ($p < 0.05$, $p < 0.0001$; Table 3.5). However, a decrease was observed in macrosteatosis scores from rats weaned early on low and high Moringa doses with fructose ($p < 0.05$; Table 3.5). Both low and high Moringa doses significantly prevented the rise in microsteatosis induced by fructose administration.

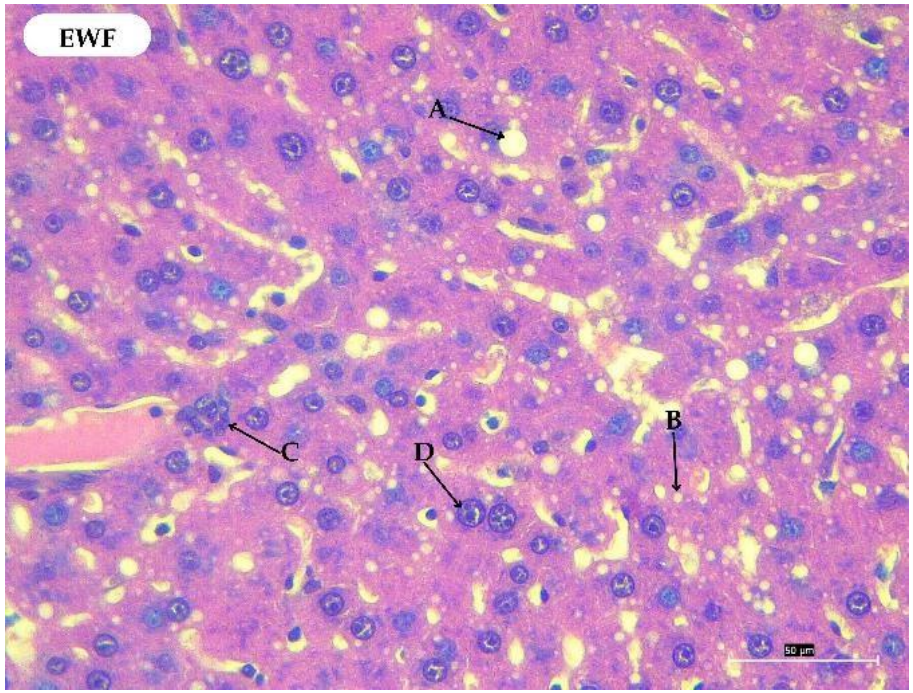
Hypertrophy scores from rats fed a normal diet and those fed fructose at early weaning were significantly increased when compared to rats weaned early on a normal diet ($p < 0.001$, $p < 0.001$; Table 3.5). In contrast, early weaning with Moringa low dose and with Moringa high dose with fructose resulted in a significant decrease in hypertrophy scores when compared to early weaning with a normal diet ($p < 0.01$; Table 3.5).

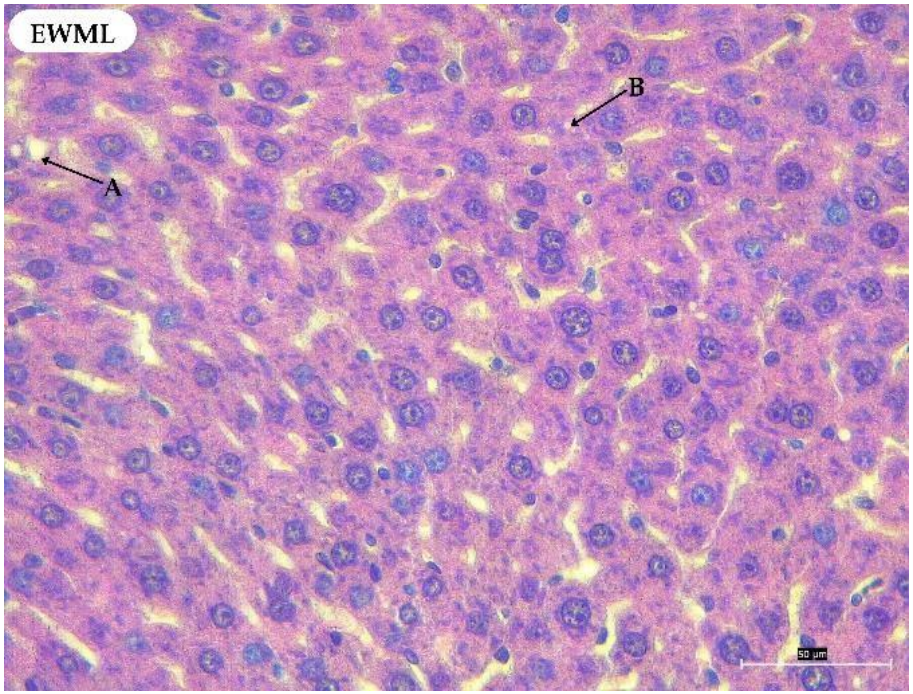
Early weaning with fructose significantly increased inflammation scores compared to either normal or early weaning with a normal diet ($p < 0.0001$; Table 3.5). Liver inflammation scores where significantly lower ($p < 0.01$) in rats early-weaned on Moringa, regardless of dose (Table 3.5). The increase in inflammation scores caused by early weaning with fructose was notably prevented by administration of Moringa in early weaning ($p < 0.001$; Table 3.5). The area fraction of the collagen in the liver was significantly increased in rats early-weaned on fructose, Moringa low and high doses only compared to those normal weaned on a normal diet ($p < 0.05$; Table 3.5). Moreover, early weaning with fructose when compared with early

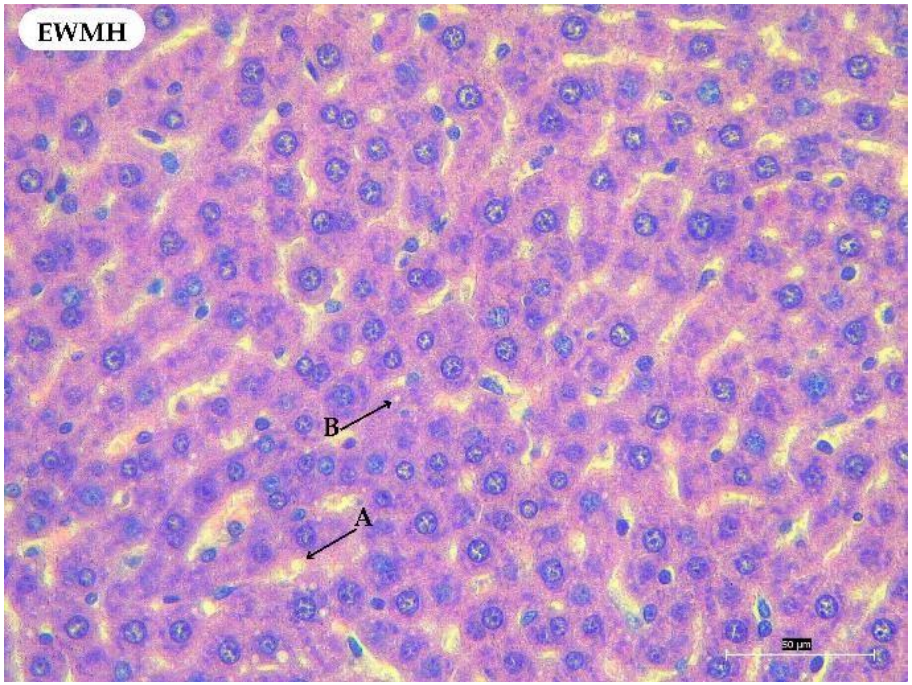
weaning with a normal diet also significantly increased area fraction of collagen ($p < 0.01$; Table 3.5). The early weaning with fructose-induced increase in collagen area fraction was prevented by the administered both doses of Moringa ($p < 0.05$; Table 3.5).

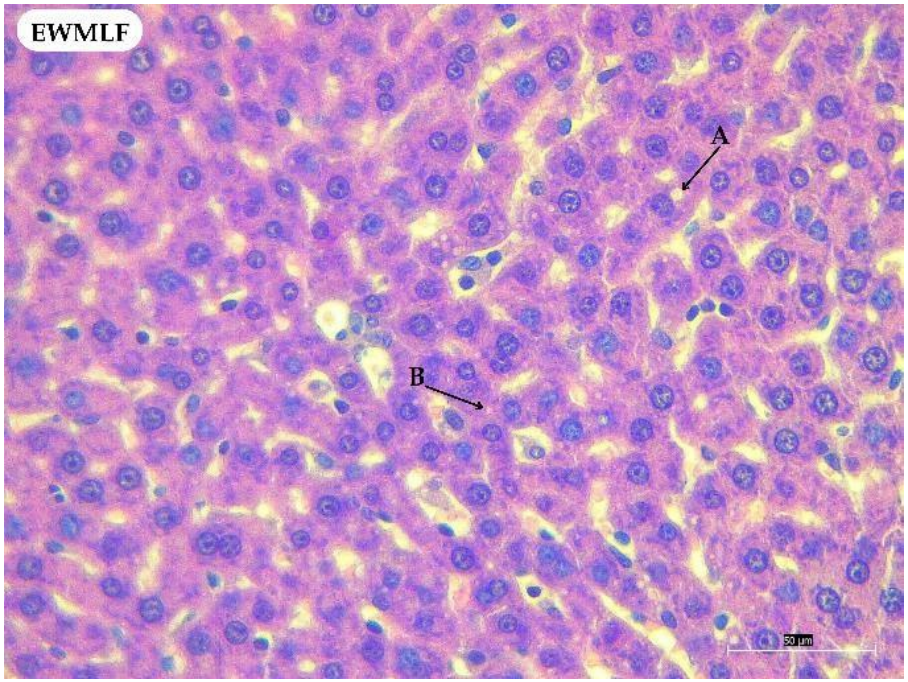

NWC
50 µm


EWC
A
A
B
50 µm


EWF
A
B
C
D
50 µm


EWML
A
B
50 µm


EWMH
B
A
50 µm


EWMLF
A
B
50 µm

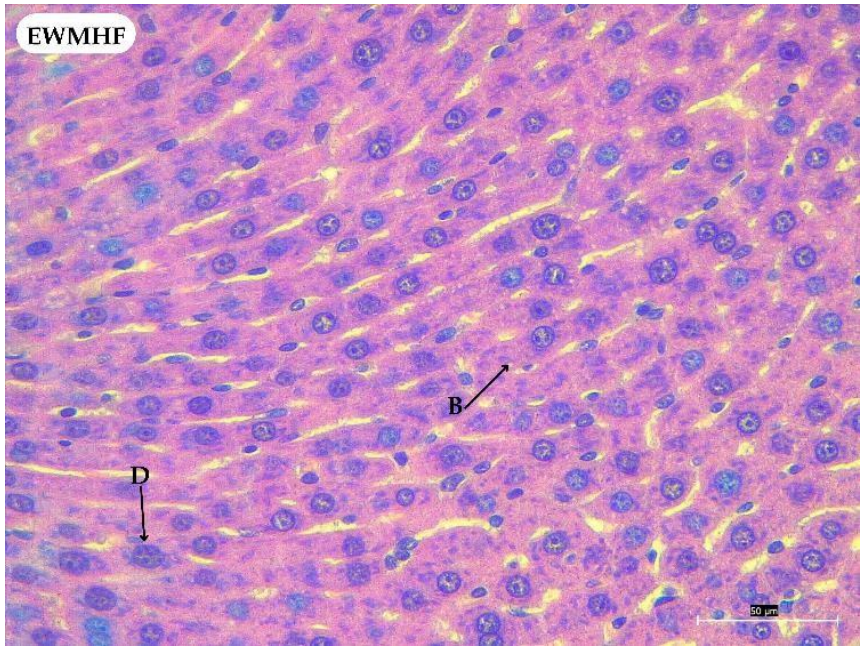


Figure 3. 5. The effects of hydroethanolic Moringa leaf extracts and fructose consumption on hepatic histomorphology in early-weaned female Sprague-Dawley rats at Termination, PD 112.

H&E stain, 40× Magnification. Scale Bar is 50 µm. Black arrows indicate; A= Macrosteatosis, B= Microsteatosis, C= Hypertrophy and D= Inflammation.

NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

Table 3. 5. MAFLD scores of macrosteatosis, microsteatosis, hypertrophy, and Inflammation of rat liver and, hepatic area fraction of collagen across the different experimental groups at Termination, PD 112.

	Microsteatosis	Macrosteatosis	Hypertrophy	Inflammation	Area Fraction of Collagen (%)
	Histology Scores				
NWC	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	0 (0, 0)[a]	3.45 (2.64, 4.54)[a]
EWC	2 (0, 2)[bc]	1 (0, 1)[b]	1 (0, 1)[bc]	0 (0, 0)[a]	4.7 (3.06, 5.44)[a]
EWF	2 (2, 3)[b]	2 (2, 3)[c]	1 (0, 1)[c]	1 (1, 1)[b]	9.18 (7.13, 10.03)[b]
EWML	1 (0, 1)[c]	0 (0, 1)[ab]	0 (0, 1)[ac]	0 (0, 1)[a]	6.90 (5.47, 7.81)[ab]
EWMH	0 (0, 1)[ac]	0 (0, 0)[ab]	0 (0, 0)[a]	0 (0, 1)[a]	7.00 (6.34, 7.90)[ab]
EWMLF	0 (0, 0)[ac]	0 (0, 1)[ab]	0 (0, 0)[a]	0 (0, 0.25)[a]	5.13 (4.48, 5.70)[a]
EWMHF	0 (0, 0)[a]	0 (0, 1)[a]	0 (0, 0)[a]	0 (0, 0.25)[a]	5.16 (3.67, 6.35)[a]

All data are presented as median (25% percentile, 75% percentile). n = 18 per group (number of images scored). [a,b,c] Significant difference is represented by groups with different letters and non significance is represented by at least one similar letter among the groups in the same row. NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.3.8. The effects of hydroethanolic Moringa leaf extracts and fructose consumption on expression of genes involved in hepatic regulation and metabolism of fatty acids and lipids in early-weaned female Sprague-Dawley rats

Early weaning with a fructose diet or with Moringa high dose significantly increased the relative mRNA expression of *ACC-1* compared to normal weaning ($p < 0.04$, $p < 0.003$; Figure 3.6A). Moringa low and high doses prevented the fructose induced increase in the relative mRNA expression of *ACC-1* ($p < 0.004$, $p < 0.0006$; Figure 3.6A). Overall, early weaning with Moringa high dose only resulted in significantly lower relative mRNA expression of *ACC-1* compared with normal or early weaning with a normal diet or fructose or low Moringa dose diets ($p < 0.0001$; Figure 3.6A).

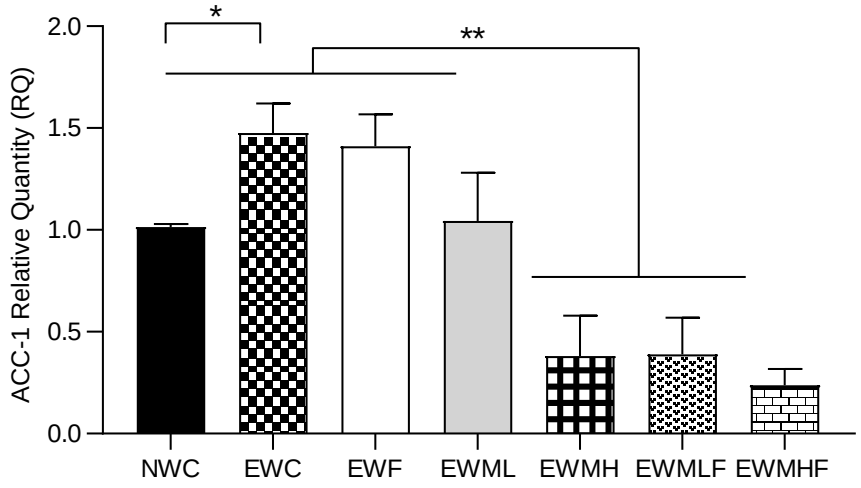
Early weaning with a normal diet or fructose significantly decreased the relative mRNA expression of *PPAR-α* compared to normal weaning ($p < 0.0005$, $p < 0.0002$; Figure 3.6B). However, early weaning with Moringa low or high diets only did not have any significant impact on the relative mRNA expression of *PPAR-α* compared to normal weaning ($p > 0.05$; Figure 3.6B). Early weaning with a low and high Moringa diet significantly increased the relative mRNA expression of *PPAR-α* when compared to early weaning with a normal or fructose diet ($p < 0.0006$, $p < 0.0001$; Figure 3.6B). Both Moringa low and high doses managed to significantly increase the fructose induced decrease of relative mRNA expression of *PPAR-α* activity ($p < 0.0001$; Figure 3.6B).

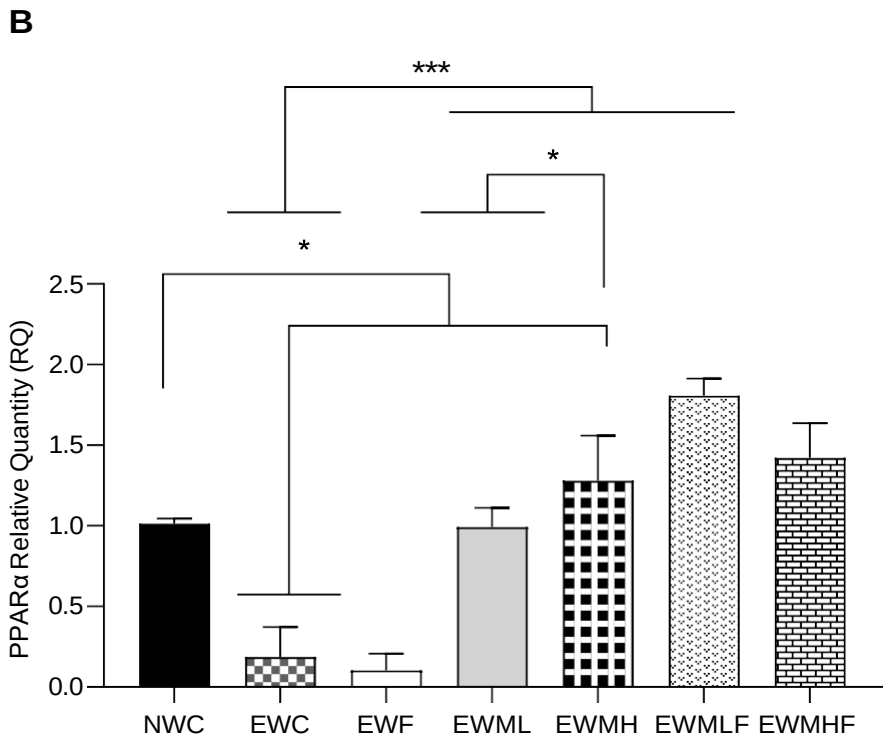
CPT-1 relative mRNA expression was significantly decreased by early weaning alone and with fructose diet when compared with normal weaning ($p < 0.004$, $p < 0.0006$; Figure 3.6C). Early weaning with Moringa low dose only had no effect on the relative mRNA expression of *CPT-1* when compared with normal weaning with a normal diet ($p > 0.05$; Figure 3.6C). Moringa low dose failed to prevent the fructose induced decrease of relative mRNA expression of *CPT-1* in early-weaned rats ($p > 0.05$; Figure 3.6C). Moringa high dose significantly increased relative mRNA expression of *CPT-1* when compared to normal ($p < 0.04$) or early weaning alone ($p < 0.0001$) or fructose ($p < 0.0001$) diet (Figure 3.6C). Whereas, early weaning with Moringa high dose and fructose significantly increased *CPT-1* relative mRNA expression and was able to prevent the fructose induced decrease in relative mRNA expression of *CPT-1* (Figure 3.6C).

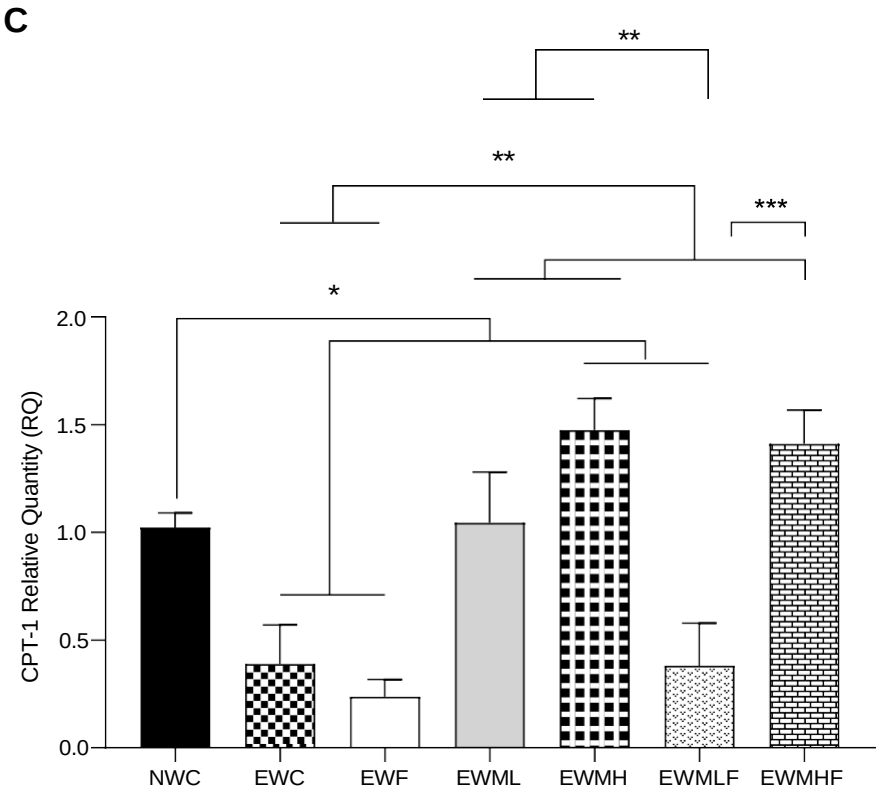
Early weaning had no significant impact on the relative mRNA expression of *FAS* irrespective of diet when compared to normal weaning ($p > 0.05$; Figure 3.6D). Early weaning with Moringa high dose only resulted in significantly lower relative mRNA expression of *FAS* compared with early weaning on a normal diet ($p < 0.03$; Figure 3.6D). Early weaning with fructose significantly increased the relative mRNA expression of *FAS* when compared to early weaning with either Moringa dosages ($p < 0.01$, $p < 0.002$; Figure 3.6D). Moringa dosages significantly reduced the fructose induced increase in the relative mRNA expression of *FAS* ($p < 0.02$, $p < 0.002$; Figure 3.6D).

There were no significant difference in *SREBP-1c* relative mRNA expression between normal weaning and early weaning on a normal or with Moringa diets ($p > 0.05$; Figure 3.6E). Early weaning with fructose significantly increased the relative mRNA expression of *SREBP-1c* when compared with normal weaning and early weaning with either a normal diet, or Moringa dosages ($p < 0.05$; Figure 3.6E). Moringa low and high diets significantly reduced the fructose induced increase in the relative mRNA expression of *SREBP-1c* activity ($p < 0.006$, $p < 0.001$; Figure 3.6E).

A


*
**
2.0
1.5
1.0
0.5
0.0
ACC-1 Relative Quantity (RQ)
NWC
EWC
EWF
EWML
EWMH
EWMLF
EWMHF


B
***
*
*
PPARα Relative Quantity (RQ)
2.5
2.0
1.5
1.0
0.5
0.0
NWC
EWC
EWF
EWML
EWMH
EWMLF
EWMHF


C
**
**
***
*
CPT-1 Relative Quantity (RQ)
2.0
1.5
1.0
0.5
0.0
NWC
EWC
EWF
EWML
EWMH
EWMLF
EWMHF

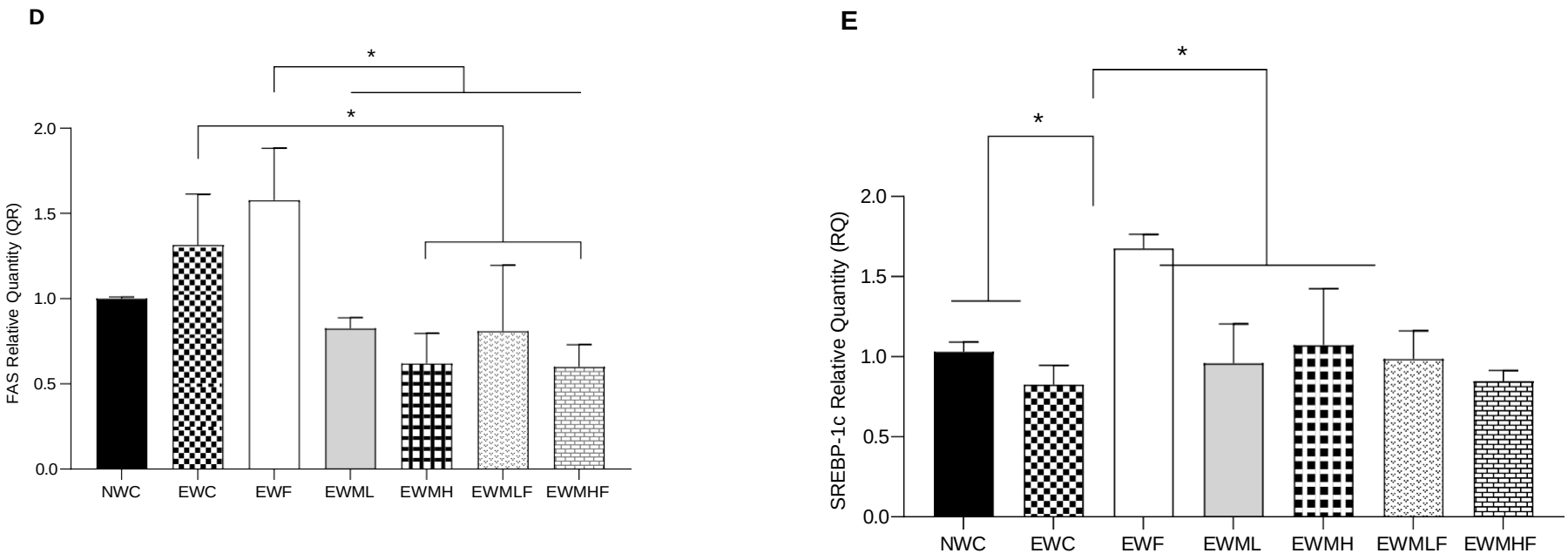


Figure 3. 6. The effects of hydroethanolic Moringa leaf extracts and fructose consumption on hepatic gene expression in early-weaned female Sprague-Dawley rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (* is $p < 0.05$, ** is $p < 0.01$ and *** is $p < 0.001$). NWC (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. EWC (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. EWF = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. EWML = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMH = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. EWMLF = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. EWMHF = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

3.4. Discussion

This study investigated the potential of two-week hydroethanolic Moringa oleifera leaf extracts administration during early weaning to protect against development of poor metabolic health outcomes in adulthood in female rats weaned onto and sustained on a high fructose diet. The results revealed that early weaning alone did not significantly alter body masses, but it resulted in elevated serum leptin levels, hepatic pathology (steatosis, hypertrophy and inflammation) and also elevated hepatic *SREBP-1c* mRNA expression, while it decreased serum HDL levels and hepatic *PPAR-α* mRNA expression. Early weaning onto fructose diet (EWF) did not alter body mass at termination, but it resulted in a significant elevation of circulating leptin, LDL, TG, TC, ALT serum concentrations, all assessed liver histopathological features associated with MAFLD (Steatosis, hypertrophy, inflammation and area fraction of collagen) and *SREBP-1c*. Another finding is that EWF significantly decreased serum insulin, adiponectin and HDL levels, and also hepatic *SREBP-1c* and *ACC-1* mRNA expression. The short-term administration of Moringa extract post-weaning did not show significant variations in body mass, but resulted in significantly increased circulating adiponectin and HDL levels and hepatic *CPT-1* and *PPAR-α* mRNA expression at termination. However, there was decreased circulating leptin, LDL, TG, TC, ALT levels and all assessed liver histopathological features ((Steatosis, hypertrophy, inflammation and area fraction of collagen), as well as hepatic *SREBP-1c*, *ACC-1* and *FAS* mRNA expressions.

The study found no significant differences in body mass and visceral fat accretion among the experimental groups. A possible explanation for visceral fat non-significant results might be that the timing of early weaning on postnatal day 18 may not have been sufficient to induce significant changes in visceral fat mass. In contrast to these findings is that previous studies have shown significant changes in visceral fat mass with early weaning models implemented before PD 18, suggesting that the timing of interventions during the early postnatal period can impact adipose tissue development (Peixoto-Silva *et al.*, 2017; Miranda *et al.*, 2020). The 20% fructose is high enough to stimulate energy production metabolic pathways, such as the glycolysis pathway, when administered at early weaning (Ipsen, Lykkesfeldt and Tveden-Nyborg, 2018). As such, during critical windows of development like early weaning, energy

can be used towards tissue development and rapid growth instead of being stored as visceral fat tissue (Hall *et al.*, 2012). This could be the reason why visceral fat was insignificant across the treatment groups in this study.

Early weaning, with or without fructose, did not significantly affect fasting blood glucose levels. However, the administration of Moringa for just two weeks during early weaning was able to program the metabolic system for improved health in adulthood by significantly lowering fasting blood glucose levels, highlighting its potential antidiabetic metabolic programming effects. Moringa has previously been demonstrated in adult rats to have glucose lowering (antidiabetic) effects (Gupta *et al.*, 2012). In the current study, early weaning with fructose significantly reduced circulating insulin levels. Similarly, an early weaning study in Wistar rats found that early weaning onto fructose decreased insulin levels (Pietrobon, Bertasso, *et al.*, 2020). The observed reduction in circulating insulin levels without a corresponding increase in fasting blood glucose levels may be attributed to several factors. Fructose metabolism does not directly stimulate insulin secretion; instead, it is primarily metabolised in the liver through insulin-independent pathways, allowing for the maintenance of blood glucose levels despite reduced insulin levels. Additionally, early weaning can induce metabolic adaptations that enhance insulin sensitivity in peripheral tissues, contributing to normal glucose homeostasis even in the presence of lower insulin levels.The reduction in insulin levels caused by fructose can be attributed to various mechanisms including insulin-independent uptake, hepatic metabolism, uric acid production, leptin resistance, and inflammation (Hannou *et al.*, 2018; Tamimi, Ahmad and Qinna, 2021). The observed decrease in insulin levels during weaning suggests a potential association between fructose exposure and altered insulin regulation. The reduced serum insulin, adiponectin and increased inflammation scores, suggest a potential disruption in pancreatic insulin secretion and response to stimuli, even though there was no direct measurement of pancreatic islet cell damage. Research has shown that insulin resistance and inflammation could contribute to morphological and pathological changes in pancreatic β-cell by producing pathology such as hyperplasia and hypertrophy, which may further impair insulin secretion capacity (Guney and Akar, 2023). Additionally, these mechanisms collectively influence metabolic programming by altering insulin signalling pathways, promoting hepatic lipid accumulation, and inducing chronic inflammation, which disrupts long-term glucose regulation (Lodge, Dykes and Kennedy, 2024). However, the administration of Moringa for two weeks at weaning did not significantly affect insulin levels, indicating normal glucose-insulin regulation. The anti-

inflammatory effects of Moringa have been reported to protect against pancreatic islet cells damage and overall pancreatic function (farghly *et al.*, 2023). Visceral obesity is generally associated with insulin resistance. However, in this study there was no significant difference in visceral fat across all groups. Therefore, this could explain similarities observed in HOMA-IR.

Early weaning with or without fructose significantly elevated leptin levels, adiponectin concentrations, and administration of high doses of Moringa post weaning counteracted the early weaning and fructose induced increase. Early weaning significantly increased leptin-adiponectin ratio, but only with or without fructose and/or Moringa low dose. Similarly, the leptin-adiponectin ratio was found to be high in rats fed a high fat diet (De Las Heras *et al.*, 2009) and in human subjects with metabolic dysfunction (Norata *et al.*, 2007; Zurita-Cruz *et al.*, 2023). Moringa high dose administered for two weeks post weaning, significantly decreased the fructose-induced increase in leptin-adiponectin ratio at termination.

The concept of lean/non-obese NAFLD (non-alcoholic fatty liver disease) is critical when interpreting metabolic disorders such as MAFLD, in the absence of significant visceral obesity, such as was observed in this study. (Xu *et al.*, (2022) state that in individuals with lean NAFLD, liver fat accumulation and accompanying metabolic disruptions, including insulin resistance and inflammation, can occur despite the lack of excessive adiposity (Xu *et al.*, 2022). This condition presents with features similar to those observed in obese MAFLD, such as liver steatosis, yet occurs in individuals without the typical clinical manifestation of obesity (Xu *et al.*, 2022). The results of this study highlighted the intricate links between genes involved in hepatic lipid metabolism and the observed changes in lipid profiles and the development or severity of MAFLD. The major regulators of fatty acid synthesis, lipid oxidation, and cholesterol metabolism that were found to be impacted by two weeks administration of Moringa post weaning and long-term fructose consumption were *ACC-1*, *PPAR-α*, *CPT-1*, *FAS*, and *SREBP-1c*. Early weaning alone and with fructose increased mRNA expression of *ACC-1*. *ACC-1* plays a role in the synthesis of fatty acids and has been linked to the observed changes in elevated LDL and reduced HDL levels, as well as overall cholesterol levels resulting from EWF (Goedeke *et al.*, 2018). This suggests elevated *ACC-1* activity increased fatty acid synthesis, leading to higher LDL cholesterol levels. However, it also affected HDL cholesterol metabolism, potentially decreasing HDL cholesterol levels (Goedeke *et al.*, 2018). Moreover, increased ACC-1 activity likely contributed to elevated total cholesterol levels by promoting LDL synthesis, leading to dyslipidaemia. However,

Moringa, when administered for two weeks post weaning, was able to prevent the early weaning and long-term fructose induced elevation in LDL and decrease in HDL at termination (adulthood). These findings were also reported in a study where Moringa was administered for four weeks to obese rats (El-Shehawi *et al.*, 2021).

This study found that early weaning onto a long-term fructose diet (EWF) induced a decreased mRNA expression of *PPAR-α* and *CPT-1*, both of which are transcription factors involved in fatty acid oxidation and lipid metabolism (Ipsen, Lykkesfeldt and Tveden-Nyborg, 2018). A reduction in *CPT-1* leads to impaired oxidation, reduced clearance of triglycerides from hepatocytes, and increased triglyceride accumulation in the liver (Pawlak, Lefebvre and Staels, 2015). This dysregulation in lipid metabolism likely contributed to the elevation of triglyceride levels in the EWF group. This lipid metabolism impairment then intensifies the severity of MAFLD, particularly in the presence of fructose, which Moringa reversed, when administered for eight weeks in a study by (Attakpa *et al.*, 2017).\

The elevated mRNA expression of *FAS* and *SREBP-1c* in early weaning, either alone (for *FAS* only) or with fructose, suggests increased de novo lipogenesis, leading to elevated levels of TG, TC and LDL observed in the EWF group. This upregulation of mRNA expression of *FAS* and *SREBP-1c* promotes fatty acid synthesis, resulting in triglyceride accumulation, cholesterol synthesis, and LDL particle production, thereby contributing to fructose-induced dyslipidaemia and MAFLD (Al Hashmi *et al.*, 2024). Moringa supplementation for two weeks post weaning, decreased the long-term fructose-induced *FAS* and *SREBP-1c* increase, which aligns with the observed reduction in microsteatosis and macrosteatosis scores. Similar findings were reported by (Xie *et al.*, 2018), were they investigated the effect of long-term Moringa petroleum ether extract on lipid accumulation in mice.

Some limitations of the current study are that, even though it revealed that Moringa improved metabolic health, the exact mechanisms by which it achieved these effects in tissues beyond the liver and possibly the role of gut microbiota and oxidative stress were not explored. There were also no direct measurements of pancreatic islet cell damage, despite the observed reduced serum insulin levels. The study also did not identify the specific bioactive compounds in Moringa responsible for the observed effects. The aforementioned should be explored further in future studies.

3.5. Conclusions

In conclusion, the results of this study reveal that early weaning alone or with a high fructose diet resulted in metabolic dysfunction and MAFLD. The results also reveal that it was through mechanisms such as increased lipogenesis, impaired fatty acid oxidation, elevated hepatic inflammation, dysregulated lipid metabolism, and impaired insulin sensitivity, all of which contribute to hepatic fat accumulation, liver damage, and metabolic disturbances. However, Moringa administration for two weeks post-weaning showed significant protective effects by improving insulin sensitivity, reducing hepatic inflammation, stimulating fatty acid oxidation, lowering serum glucose and cholesterol levels, and inhibiting fat synthesis, collectively protecting against metabolic dysfunction and MAFLD.

The study further revealed that just two weeks of Moringa administration post-weaning had long-lasting metabolic benefits into adulthood, demonstrating its ability to program the metabolic system during a critical period of developmental plasticity and to protect against future metabolic diseases. This important discovery suggests that Moringa could be a practical, easily accessible, cost-effective intervention, especially for resource-poor communities, for preventing long-term metabolic diseases and ease healthcare burdens associated with early weaning (onto high fructose diets). Future research should aim to identify the is key active phytochemicals in Moringa that can be further developed to target strategic therapies to prevent poor metabolic outcomes associated with early weaning.

Having established the liver as a primary target organ affected by early weaning and high-fructose consumption, it is critical to extend the investigation to the kidney, another key organ susceptible to metabolic dysfunction. Metabolic disturbances such as insulin resistance, dyslipidaemia, and systemic inflammation demonstrated in the liver are known to impose stress on renal structures, contributing to early renal injury, altered glomerular morphology, and impaired renal function. Indeed, MAFLD and renal dysfunction are often mechanistically interconnected, as hepatic lipid accumulation, oxidative stress, and inflammatory mediators can exacerbate kidney injury through the hepato-renal axis. Therefore, assessing biomarkers of renal function and renal microstructure in early-weaned rats fed a high-fructose diet allows for a comprehensive evaluation of systemic metabolic dysfunction and provides insight into whether post-weaning Moringa supplementation can confer protective effects beyond the liver, mitigating the broader multi-organ consequences of early-life nutritional insults.

CHAPTER 4: THE EFFECT OF *MORINGA OLEIFERA* LAM. HYDROETHANOLIC LEAF EXTRACTS ADMINISTERED DURING THE FIRST TWO WEEKS POST-WEANING ON RENAL STRUCTURE AND FUNCTION IN FEMALE SPRAGUE-DAWLEY RATS

4.1. Introduction

Metabolic-associated kidney disease (MAKD) has been documented as one of the biggest health complications associated with both metabolic dysfunction, type 1 (T1DM) and type 2 (T2DM) diabetes mellitus, leading to end-stage kidney disease (ESKD) (Sun *et al.*, 2022; Wu *et al.*, 2022). For this reason, MAKD refers to renal dysfunction and kidney damage caused by a prolonged exposure to insulin resistance, impaired glucose metabolism, dyslipidaemia, inflammation and oxidative stress (Nüsken *et al.*, 2020). This shows that metabolic abnormalities associated with both MAFLD and T2DM are the highest contributors to the development of MAKD. The International Diabetes Federation (IDF) reports that one in every three children and adolescents with diabetic mellitus will develop MAKD at some point in their lifetime (Sun *et al.*, 2022). This is a concerning prediction, as global projections indicate that by 2045 approximately 783 million adults will be living with diabetes (IDF, 2021). Projections also indicate that 383 million children will be living with T2DM by 2035, (Pappachan, Fernandez and Ashraf, 2024). This highlights the escalating burden of T2DM in children and adolescent populations. As of 2025, the global prevalence of T2DM among children and adolescents continues to rise, reflecting a significant public health concern.

A systematic analysis of the 2021 global burden of disease study revealed that there were approximately 1.2 million adolescents aged 10 - 24 years living with T2DM worldwide (Pappachan, Fernandez and Ashraf, 2024). The increase in T2DM cases has been associated with the rise in comorbidities such as obesity, insulin resistance, hypertension, cardiovascular diseases, hyperlipidaemia and hypercholesterolaemia among others (Neumiller, Burnier and Hoogeveen, 2022).

In children with MAKD, the above-mentioned co-morbidities are often linked to impaired renal function (Do Val *et al.*, 2019). Research has shown that after the onset of T2DM, there are structural changes in the kidney, specifically, thickening of the glomerular basement membrane and mesangial expansion (Muntean, Starcea and Banescu, 2022). These changes occur within 1.5 to 5 years of the clinically silent phase after T2DM onset (Muntean, Starcea and Banescu, 2022). The changes develop at different rates depending on which other co-morbidities the subject may be suffering from. In most subjects with MAKD, cardiovascular complications, such as atherosclerosis, increase the risk of myocardial infarction and stroke,

both of which are common in chronic kidney disease (Do Val *et al.*, 2019). These structural changes in the kidney, coupled with the progression of associated comorbidities, underscore the importance of understanding the potential role of early-life factors, such as weaning practices, which can significantly influence long-term renal health and contribute to the onset of conditions like MAKD.

A significant contributing factor to the prevalence of MAKD is early weaning practices (Nüsken et al., 2020). Early weaning, particularly onto high fructose diets is detrimental to the fragile and developing gastrointestinal tract and the urinary system of infants (Abouhegazy *et al.*, 2019). When introduced during early weaning, fructose is metabolised primarily in the liver, where it can promote fat accumulation and impair insulin sensitivity (Tamimi, Ahmad and Qinna, 2021). These metabolic changes may indirectly contribute to hyperglycaemia which poses a significant risk to renal health, as chronic exposure to elevated blood sugar and insulin resistance can lead to kidney damage, including reduced renal function and structural harm over time (Hannou *et al.*, 2018; Sifuentes-Franco *et al.*, 2018).

Moreover, fructose metabolism increases oxidative stress by generating reactive oxygen species (ROS), which are known to cause cellular damage, particularly in renal cells, leading to potential kidney dysfunction over time (Hannou *et al.*, 2018; Sifuentes-Franco *et al.*, 2018). Additionally, fructose induces renal cell inflammation through mechanisms involving uric acid production, oxidative stress, endoplasmic reticulum (ER) stress, activation of inflammatory pathways, fat accumulation, impaired gut barrier function, and immune cell activation (Sifuentes-Franco *et al.*, 2018). Together, these processes drive inflammation and contribute to significant structural and functional damage in kidney tissues, including glomerular injury and fibrosis (Oraby *et al.*, 2019). Understanding the mechanisms of how fructose metabolism and early weaning negatively affect kidney function and structure is highly dependent on both animal and human studies, which shows evidence that further reinforces the connection between early weaning with high fructose diets and the high risk of developing MAKD.

Animal studies have shown a high incidence of MAKD in rats subjected to early weaning combined with a high fructose diet (O'brien, Sakowski and Feldman, 2014; Toyoda *et al.*, 2018; Preguiça *et al.*, 2020). Similarly, human studies have found an association between early weaning and elevated urine protein levels, which is a key marker of kidney damage and

a precursor to MAKD (Résimont *et al.*, 2022; X. hong Zhou *et al.*, 2023). Thus, dietary choices can worsen renal damage. This highlights the urgent need for increased awareness and proactive measures to address the complex relationship between early weaning practices and the development of MAKD in children. This is because early weaning practices can be influenced by environmental factors and socioeconomic statuses.

Environmental factors and socioeconomic status play a significant role in the development and progression of non-communicable diseases, including MAKD, particularly in children and adolescents (Guariguata and Jeyaseelan, 2019). Low-income households face an increased risk of MAKD due to limited dietary knowledge and the prevalence of commercially available high-fructose foods (Guariguata and Jeyaseelan, 2019). The limited availability of healthy food choices in low-income areas worsens health inequalities, highlighting the need for targeted interventions to improve the accessibility and affordability of healthy nutritious diets (Anderson *et al.*, 2021). Given these socioeconomic challenges, addressing the rising prevalence of MAKD requires not only social interventions but also a focus on innovative, natural treatment options that can prevent and mitigate kidney damage from an early age at low costs.

The exploration of plant-based treatments offers promising avenues for addressing diseases such as MAKD. *Moringa oleifera* is one plant that has shown great significance in potential to provide such treatment, as several health beneficial biological activities (Pareek *et al.*, 2023). Scientific studies using rodent models have demonstrated that Moringa has anti-inflammation, anti-oxidation, and anti-insulin resistance properties that can protect against kidney damage (Akinrinde *et al.*, 2020; Akter *et al.*, 2021; Putri *et al.*, 2023). Given these findings, incorporating Moringa into early weaning diets could potentially contribute to preventing renal dysfunction. If early dietary introduction of Moringa can programme for long-term positive renal health, that would contribute to a strategic mitigation intervention to promote renal health.

The aim of this component of the study was to investigate the potential effect of Moringa hydroethanolic leaf extracts, administered for two weeks during the early weaning stage, against fructose-induced renal dysfunction in early-weaned female rats.
The objectives were to assess the impact of early weaning, fructose consumption, and a two weeks post-weaning Moringa hydroethanolic leaf extract administration on:

1. Gross kidney morphometry (determining the kidney mass at the end of the intervention) and assessing kidney structural damage through histological evaluations of the renal corpuscle area (RCA), glomerular tuft area (GTA), and glomerular/Bowman's capsule area (BCA).
2. kidney injury/function biomarkers, specifically plasma concentrations of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) using ELISA techniques and serum creatinine (CREA) and blood urea nitrogen (BUN) and the urea: creatinine (U: C) ratio using a Veterinary clinical chemistry analyser.

4.2. Methods and materials

4.2.1. Study setting, study animals and husbandry

The study setting and design are as described in chapter 3, section 3.2.1 and 3.2.3, with the unique aspect of this chapter focusing on the collection of kidney tissue samples and serum stored from previous procedures, for renal health investigations.

At the termination stage of the study, as outlined in Chapter 3, Section 3.2.6, additionally, from each rat, the kidneys were dissected out and, weighed to determine the absolute mass of the kidneys. The mass of the kidneys relative to body mass was computed by dividing combined mass of the kidneys by terminal body weight of the rats, then multiplied by 100 to express as a percentage;

$$Relative\ kidney\ mass = \frac{\text{Kidney mass (g)}}{Body\ mass\ (g)}\ x\ 100$$

They kidney tissues were preserved/fixed in 10% buffered formalin for histopathological analysis to evaluate fructose-induced pathological changes.

4.2.2. Kidney histomorphometry

4.2.2.1. Kidney sample processing for histomorphometry

The kidney tissue samples, which had been kept in 10% buffered formalin, were processed using an advanced enclosed tissue processor (Leica Biosystems, Deer Park, USA). Key steps included dehydration with alcohol, clearing with xylene. Afterwards, the kidney samples

were embedded in paraffin wax and sectioned at a thickness of 5 µm using a microtome (Leica instruments, Wetzlar, Germany). Subsequently, the tissues were stained with Haematoxylin and Eosin (H & E) or Mason trichrome (MT) in order to evaluate their histological characteristics. The H&E staining method was used to assess the general tissue morphology of the kidney and MT staining was used to identify fibrosis in the kidney tissue. After staining, the sections were mounted on glass slides (Gemeindestraße 4, Bad Wildungen, Germany) and covered with cover slips (Gemeindestraße 4, Bad Wildungen, Germany) using a mounting medium (Waltham, Massachusetts, United States) to preserve the tissue and allow for clear microscopic examination.

A Leica DM750 microscope (Leica Microsystems (Schweiz) AG, Max Schmidheiny Strasse, Heerbrugg, Switzerland) with an AU-300-HD excelis lite camera (New York Microscope Company, Hicksville, New York, USA) was utilised to take photomicrographs of kidney sections at a magnification of 10 × and 40 ×. Four images were taken per slide.

The obtained photomicrographs were analysed using ImageJ software (version 1.51, Bethesda, Maryland, USA), in order to obtain the area measurements of structural components of the kidney mentioned above. Firstly, the photomicrographs scale in ImageJ was adjusted to 20 µm to match the scale embedded on the photomicrographs from the microscope to ensure accurate measurements.
The renal corpuscle area (RCA) and the glomerular tuft area (GTA) were then measured, and the following formula was used to measure the glomerular/Bowman's capsule area (BCA) (Kotyk *et al.*, 2016):
$BCA\ (\mu m^2) = RCA\ (\mu m^2) - GTA\ (\mu m^2)$

The assessments were done with the assessor blinded to the rat identities. To ensure blinding, the kidney tissue samples and corresponding data were assigned random identification codes that did not reveal any information about the treatment groups or individual rats. The codes were generated and assigned by a separate researcher, who was not involved in the tissue analysis. The assessor was provided only with these coded samples, ensuring that the rat identities and treatment allocations remained unknown throughout the evaluation process.

4.2.2.2. Determination of plasma concentrations of biomarkers of kidney injury and function

a. KIM-1 and NGAL

The concentrations of kidney injury markers (KIM-1 and NGAL) were measured using rat-specific ELISA kits from Elabscience® biochemical assay kit (Houston, Texas, USA). The assays were performed according to the instructions provided by the manufacturer (Appendix 3).

Frozen serum samples (collected during terminal procedures) were stored at –80 °C until analysis, then defrosted and diluted before being placed in 96-well plates coated with antibodies specific to KIM-1 or NGAL. The biomarkers were incubated to allow binding to the specific antibodies, and any unbound compounds were subsequently removed through washing. After this step, enzyme-conjugated secondary antibodies were added to bind to the primary antibodies, facilitating detection of the biomarkers. Following additional incubation and washing, substrate solution was applied to each well of the biomarkers. The reaction was stopped based on manufacturer's guidelines, and absorbance was measured at 450 nm using a UV/VIS spectrophotometer (EMCLAB Instruments GmbH, Duisburg, Germany). Standard curves were created for intrapolation of the unknown sample concentrations. The standard curves for KIM-1 and NGAL exhibited correlation coefficient (R^2) values of 0.925 and 0.999, respectively, indicating excellent linearity.

b. Creatinine (CREA) and Urea (BUN)

The levels of CREA and BUN in the serum were measured using an automated clinical chemistry analyser (IDEXX VetTest® Clinical Chemistry Analyser, IDEXX Laboratories Inc., Maine, USA) according to the manufacturer's instructions (Appendix 1).

The Urea-to-Creatinine (U: C) ratio was calculated for each sample by dividing the urea concentration (mg/dL) by the creatinine concentration (mg/dL). This ratio was used as an indicator of renal function, providing insights into possible kidney dysfunction across the different treatment groups.

4.2.3. Statistical analyses

Statistical analysis of all kidney parameters were performed using GraphPad Prism version 8 (GraphPad Software Inc, San Diego, USA). The normality of the data was assessed using the Shapiro-Wilk test. All normally distributed data were presented as mean ± standard deviation (SD), and one-way analysis of variance (ANOVA) was performed to compare the differences between groups, followed by Tukey's *post-hoc* test for multiple comparisons.

Statistical significance was set at $p < 0.05$, and results were reported as 'ns' for not significant. Additionally, the kidney biomarkers data were analysed to identify significant

correlations between the biomarkers within and across the experimental groups. This was achieved using a Pearson's correlation coefficient (r) test. Data were interpreted to have a correlation when the $p < 0.05$ and the correlation coefficient (r) indicated a significant relationship, with values closer to +1 (positive) or -1 (negative) suggesting a stronger association between the biomarkers.

4.3. Results

4.3.1. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on the absolute and relative kidney mass of early-weaned rats

There were no significant differences in the absolute and relative kidney mass among the treatment groups ($p > 0.05$; Table 4.1).

Table 4. 1. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on absolute mass and mass relative to body mass of the kidneys at Termination, PD 112.

Treatment Groups	Kidneys (g)	Kidneys (%TBM)
NWC	1.85 ± 0.18^{a}	0.69 ± 0.03^{a}
EWC	1.89 ± 0.14^{a}	0.70 ± 0.05^{a}
EWF	1.88 ± 0.16^{a}	0.73 ± 0.05^{a}
EWML	1.84 ± 0.42^{a}	0.67 ± 0.15^{a}
EWMH	2.10 ± 0.19^{a}	0.74 ± 0.04^{a}
EWMLF	1.97 ± 0.20^{a}	0.72 ± 0.05^{a}
EWMHF	2.00 ± 0.07^{a}	0.72 ± 0.06^{a}

All data are presented as mean ± standard deviation. [a] Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. NB: %TBM = kidney mass as a percentage of terminal body mass. n = 8 per group. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

4.3.2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on kidney renal corpuscular area (RCA), glomerular tuft area (GTA) and Bowman's capsule area (BCA) of early-weaned rats

The histomorphometric analysis of the kidney showed that early weaning, both alone and with fructose, resulted in a significantly enlarged renal corpuscular area (RCA) and glomerular tuft area (GTA) compared to normal weaning (NWC) ($p < 0.001$; Table 4.2). However, early weaning alone did not affect glomerular/Bowman's capsule area (BCA) ($p > 0.05$; Table 4.2) and only fructose consumption resulted in significantly enlarged BCA ($p < 0.05$; Table 4.2) when compared to normal weaning. Fructose consumption resulted in a prominent enlarged RCA, GTA and BCA compared to early weaning with a normal diet, although only RCA was significant ($p < 0.05$; Table 4.2). Early weaning with Moringa low dose alone significantly reduced the RCA and BCA compared to normal weaning ($p < 0.01$; Table 4.2). However, early weaning with Moringa high dose alone, did not affect GTA and BCA, but significantly reduced RCA ($p < 0.05$; Table 4.2). Administration of Moringa at early weaning was able to mitigate the fructose-induced enlargements of RCA, GTA and BCA ($p < 0.01$; Table 4.2).

Photomicrographs of kidney tissue from rats' representative of each group are presented (Figure 4.1) to visually highlight the histopathological findings as a result of the interventions.

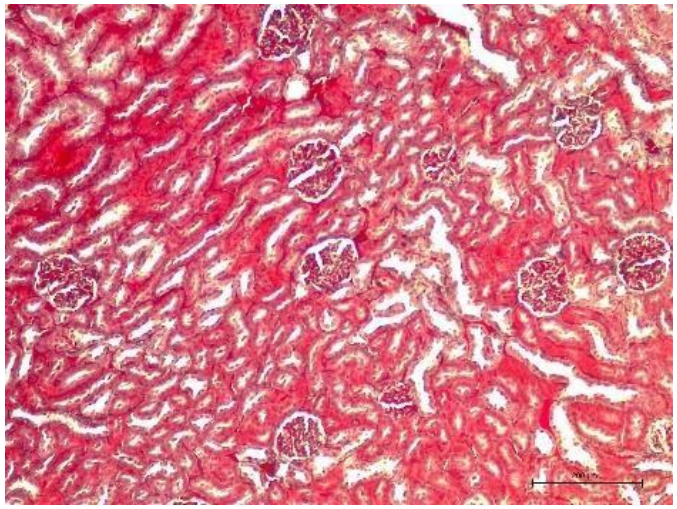
Table 4. 2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on Kidney RCA, GTA and BCA of early-weaned rats at Termination, PD 112.

GROUP	RCA (μm^2)	GTA (μm^2)	BCA (μm^2)
NWC	6115 ± 608.30^{a}	2348 ± 310.10^{ad}	3767 ± 539.20^{ad}
EWC	7853 ± 1084.00^{b}	3826 ± 1096.00^{bc}	4028 ± 550.70^{ac}
EWF	9234 ± 521.50^{c}	4324 ± 805.20^{b}	4910 ± 1045.00^{bc}
EWML	4341 ± 1398.00^{d}	2010 ± 662.20^{ad}	2330 ± 809.00^{e}
EWMH	4537 ± 962.00^{de}	1745 ± 419.50^{a}	2792 ± 793.30^{de}
EWMLF	5889 ± 362.10^{ae}	2960 ± 138.90^{cd}	2929 ± 261.30^{def}
EWMHF	5904 ± 844.90^{ae}	2445 ± 948.50^{ad}	3459 ± 490.50^{af}

All data are presented as mean ± standard deviation significant difference. ^{a,b,c,d,e,f} Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups. n = 8 per group. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

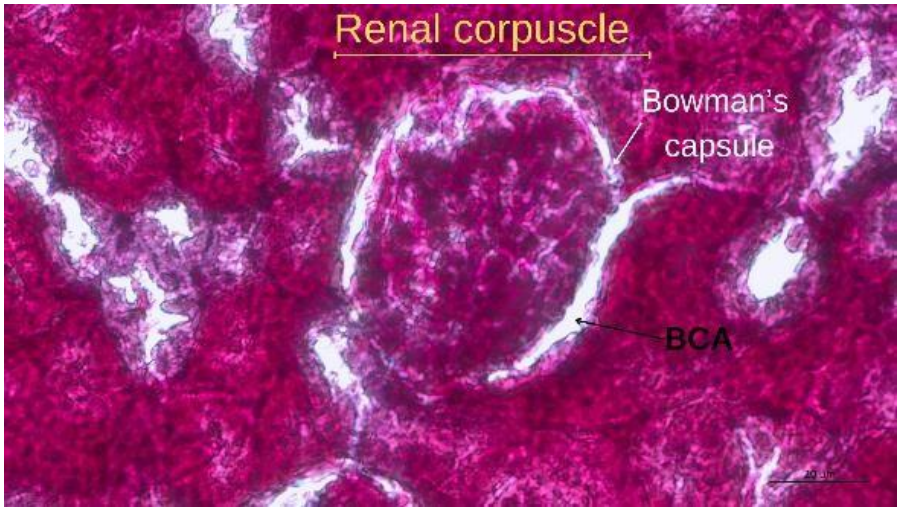
Kidney MT Image 10x

NWC

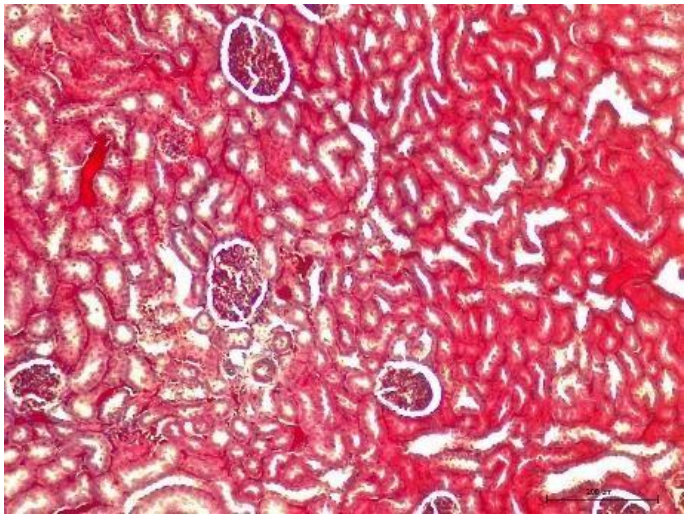


Kidney MT Image 40x

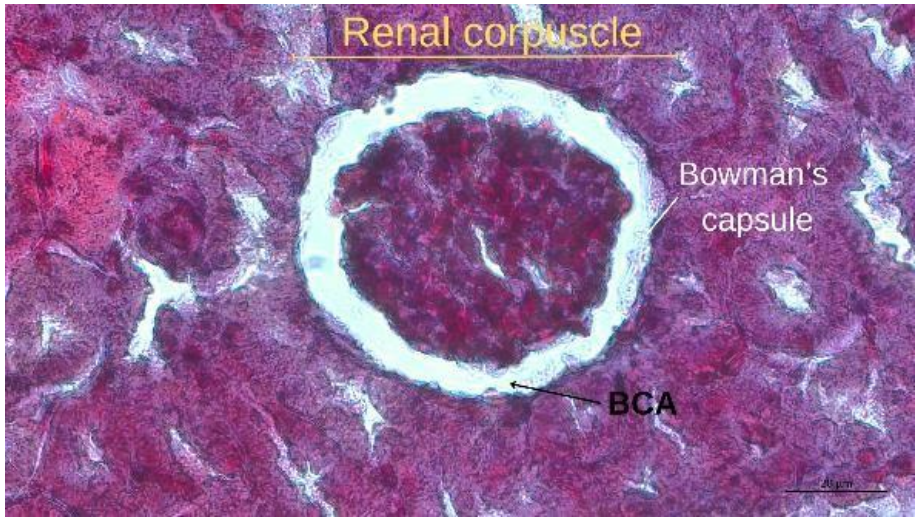
NWC



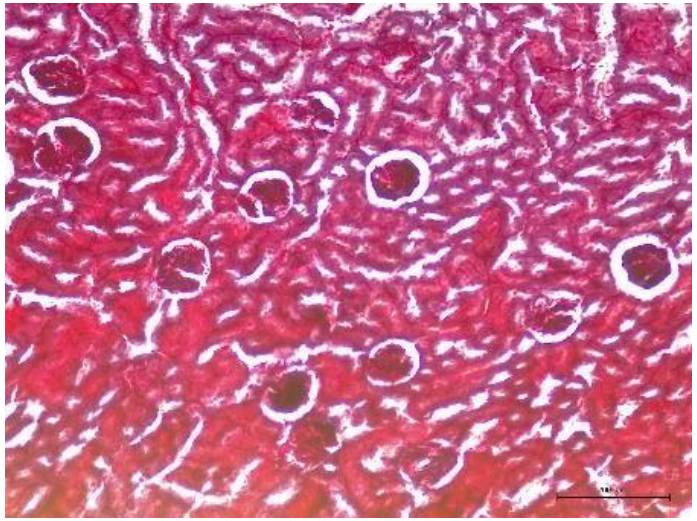
EWC



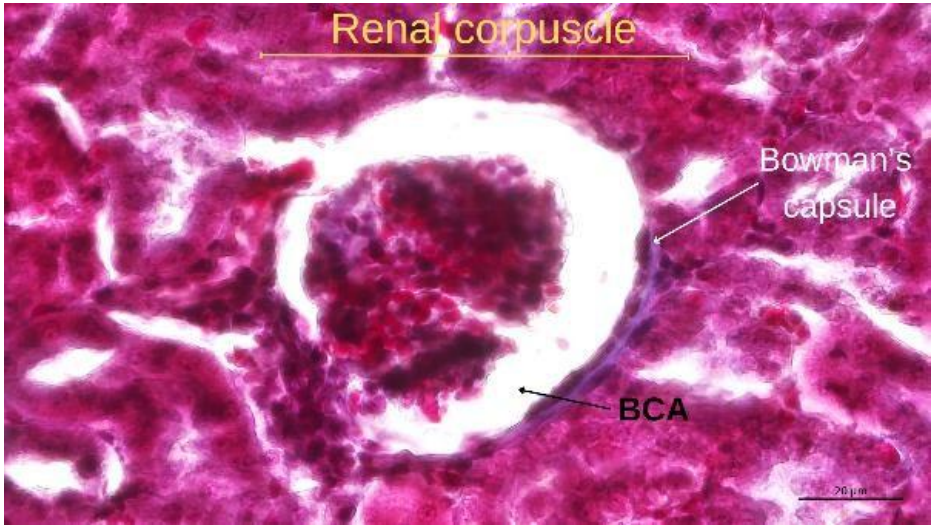
EWC



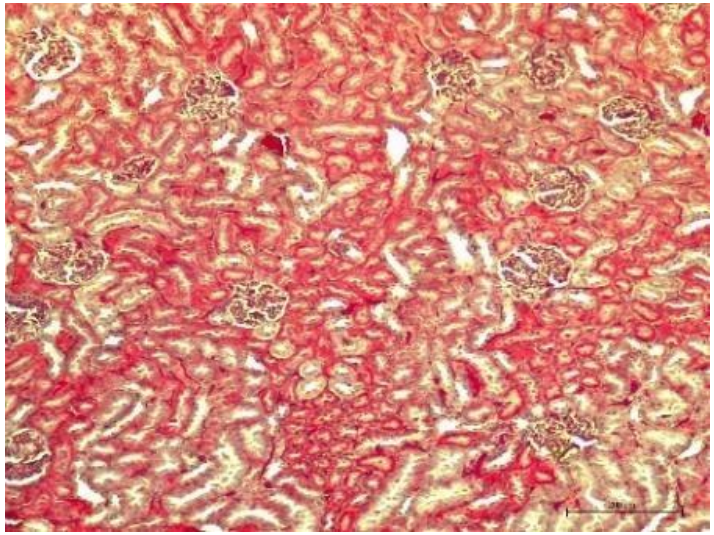
EWF



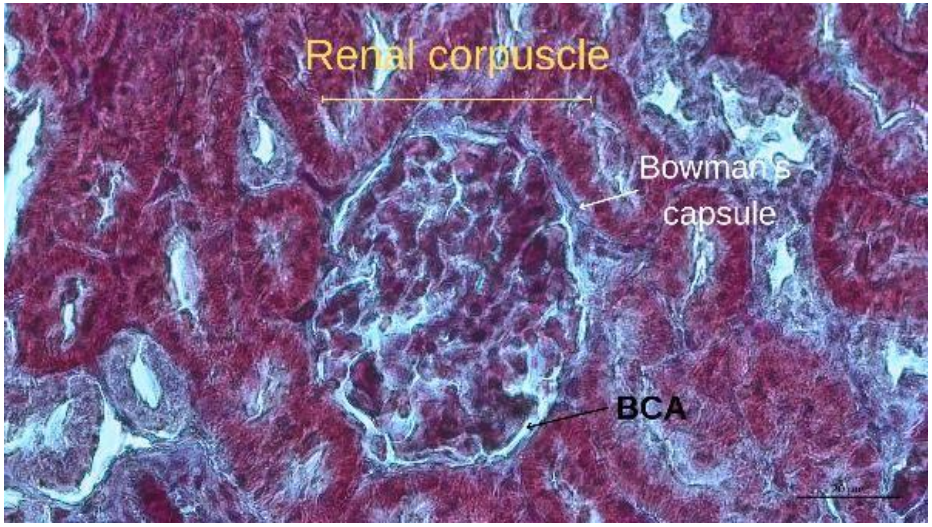
EWF



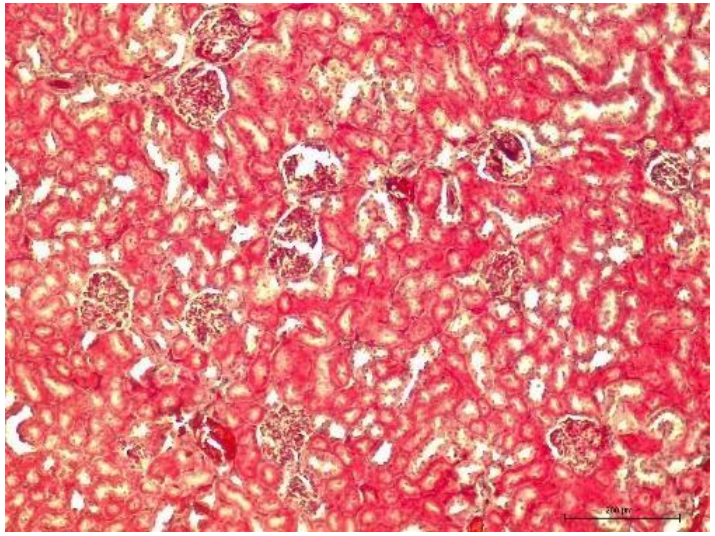
EWML



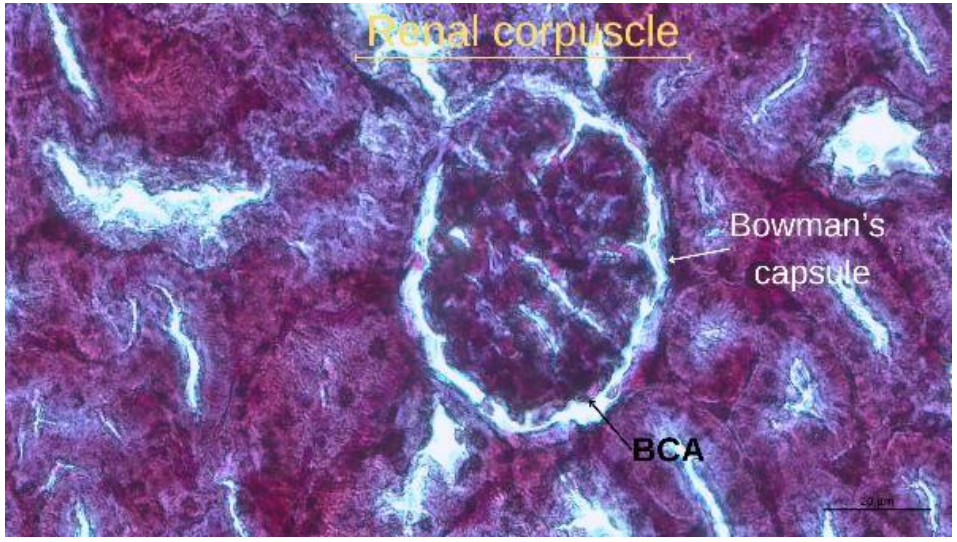
EWML



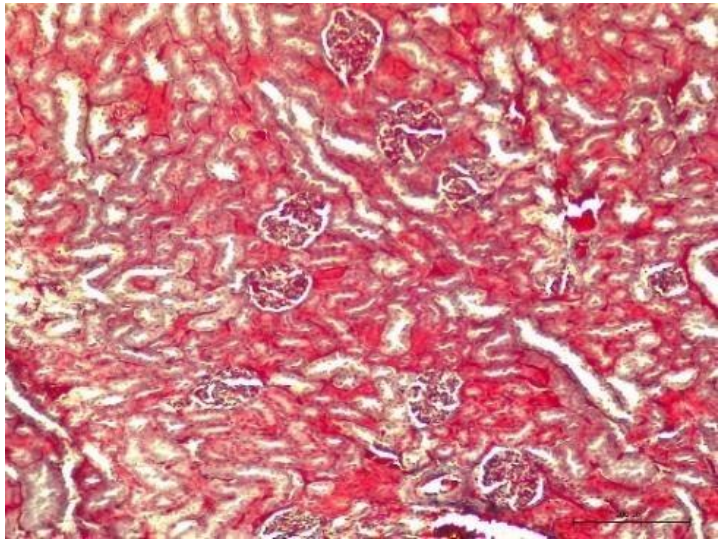
EWMH



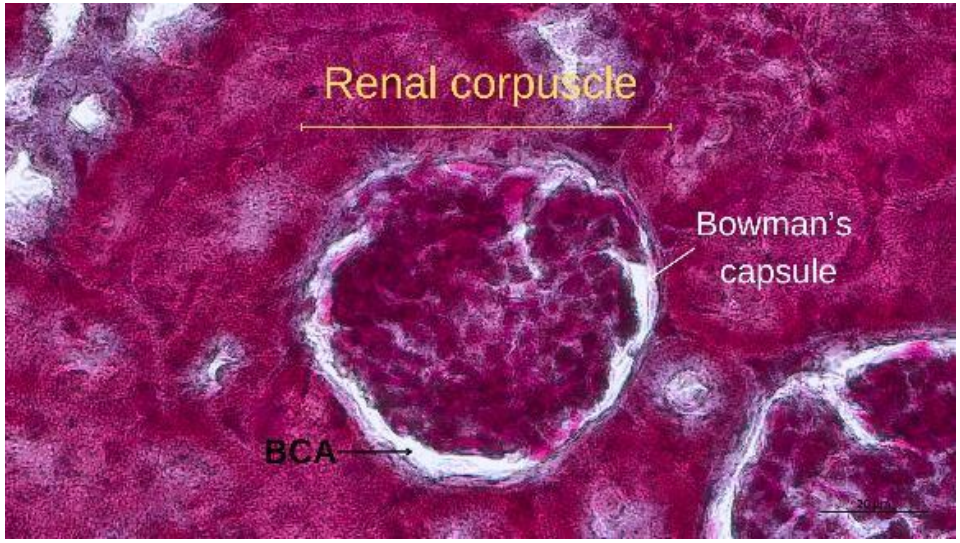
EWMH



EWMLF



EWMLF



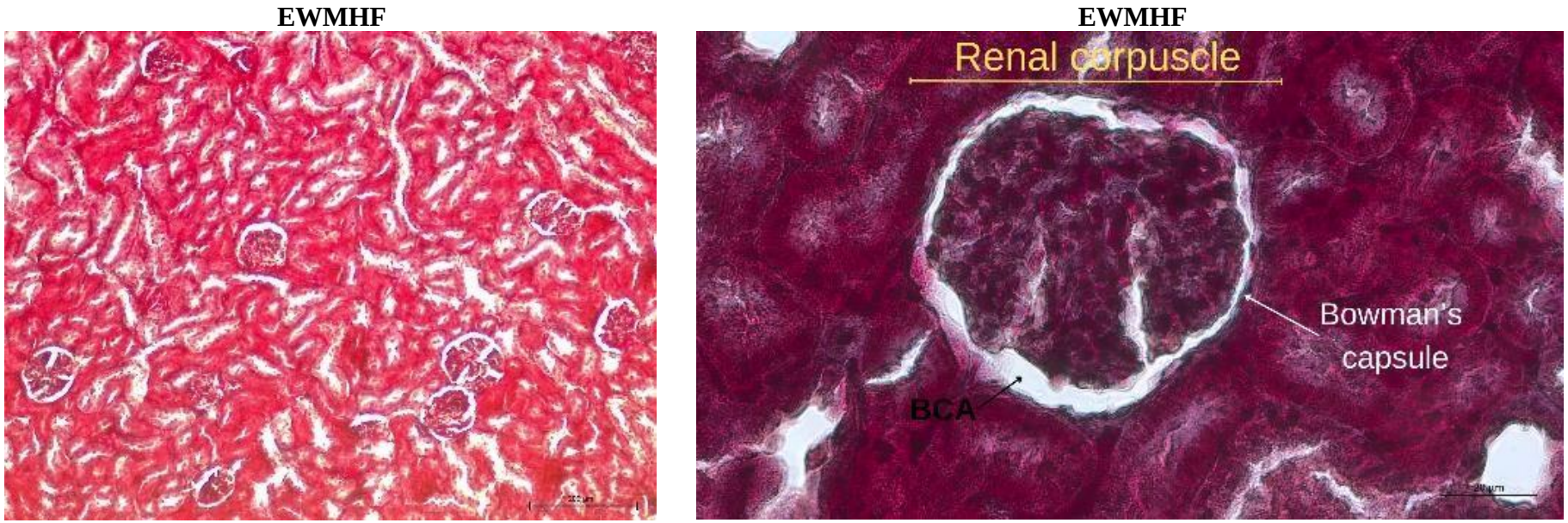


Figure 4. 1. The kidney MT photomicrographs showing the effect of hydroethanolic Moringa leaf extracts and fructose consumption on renal microstructure of early-weaned rats at Termination, PD 112.

Panels on the left show images at x10 while those on the right show images at 40× magnification. n = one for all groups. BCA is Bowman's capsule area. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. Scale bar length for images taken at 10x magnification is 50 µm and for 40x images is 20µm.

4.3.3. The effect of Moringa leaf extracts and fructose consumption on serum KIM-1 and NGAL concentrations in early-weaned female Sprague-Dawley rats.

Early weaning had no significant effect on serum KIM-1 concentration when compared to normal weaning ($p > 0.05$; Figure 4.2). However, early weaning with both Moringa high dose only and fructose only diets significantly increased serum KIM-1 concentration compared to normal weaning ($p < 0.01$, $p < 0.0001$; Figure 4.2). Early weaning with fructose alone led to the most distinct increase compared to all treatment diets ($p < 0.0001$; Figure 4.2). The two weeks administration of a low dose of Moringa post weaning did not significantly affect serum KIM-1 concentrations compared to both normal and early weaning alone ($p > 0.05$; Figure 4.2). In contrast, the high dose of Moringa led to a significant increase in KIM-1 concentrations compared to normal and weaning alone ($p < 0.01$; Figure 4.2). However, it was not significantly increased compared to early weaning alone ($p > 0.05$; Figure 4.2). Moringa high dose only diet significantly increased KIM-1 serum concentrations compared to Moringa low dose only and Moringa high dose with fructose diets ($p < 0.05$; $p < 0.01$; Figure 4.2). Both doses of Moringa prevented the fructose-induced increase in KIM-1 concentration ($p < 0.0001$; Figure 4.2).

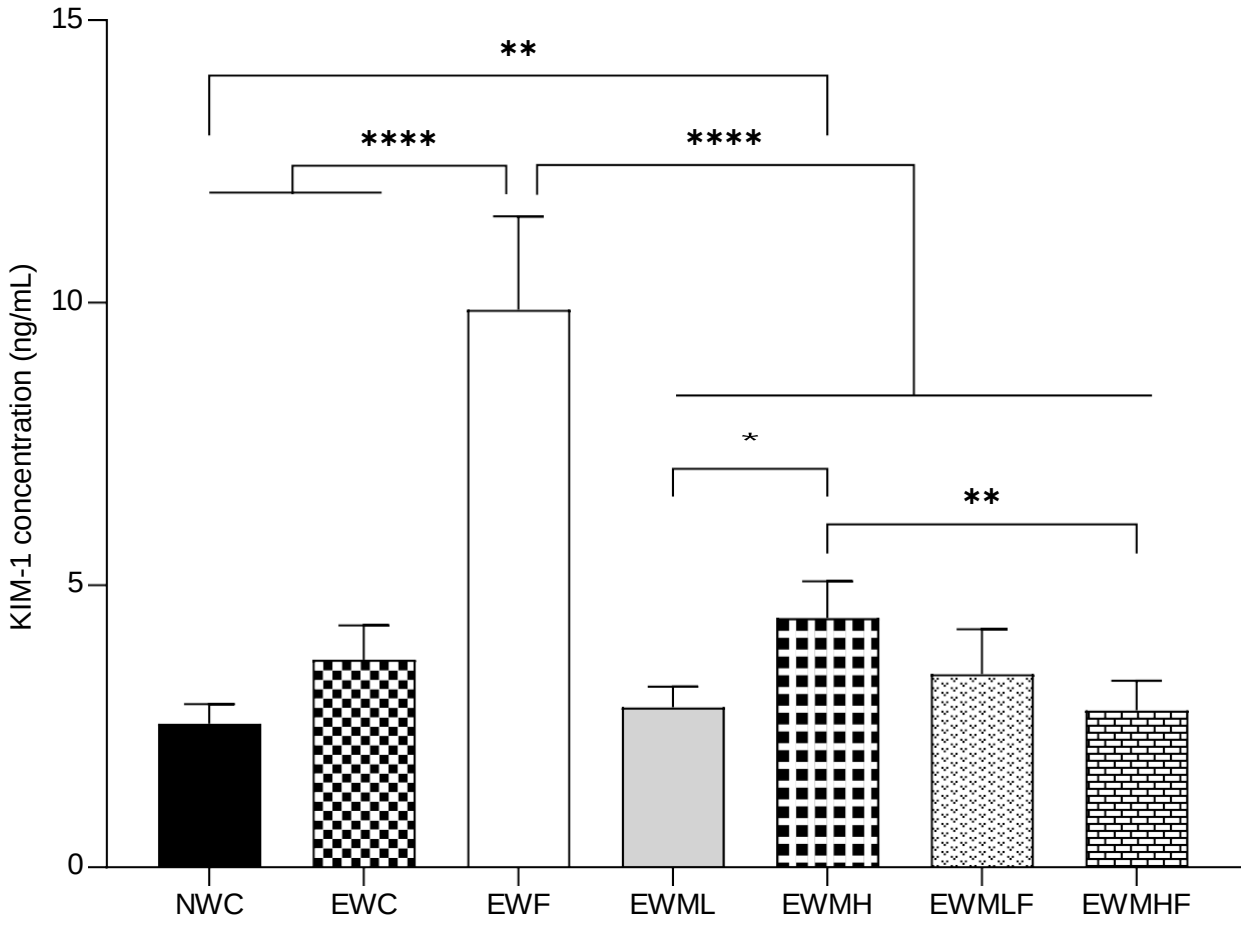


Figure 4. 2. The effect of Moringa leaf extracts and fructose consumption on serum KIM-1 concentration in early-weaned female Sprague-Dawley rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (* is $p < 0.05$, ** is $p < 0.01$ and **** is $p < 0.0001$), (n = 8 for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

Early weaning alone or with fructose and Moringa high dose only significantly increased serum NGAL concentration compared to normal weaning ($p < 0.0001$, $p < 0.0001$ and $p < 0.01$; Figure 4.3). Both early weaning alone and with fructose significantly increased serum NGAL concentrations compared to all treatment groups ($p < 0.0001$; Figure 4.3). The fructose-induced increase in NGAL concentration was more distinct compared to all treatment groups ($p < 0.0001$; Figure 4.3). Both the administration of Moringa at low and high doses two weeks post weaning, were able to prevent the fructose-induced increase in serum NGAL concentration ($p < 0.0001$; Figure 4.3). However, Moringa high dose had a much more potent effect on preventing the fructose-induced increase in NGAL concentration, as it resulted in significantly lower NGAL concentration than the low dose of Moringa (Figure 4.3). Additionally, the Moringa high dose diet (EWMHF) resulted in serum concentration of NGAL that was significantly lower than all the other diets of the study ($p < 0.01$; Figure 4.3).

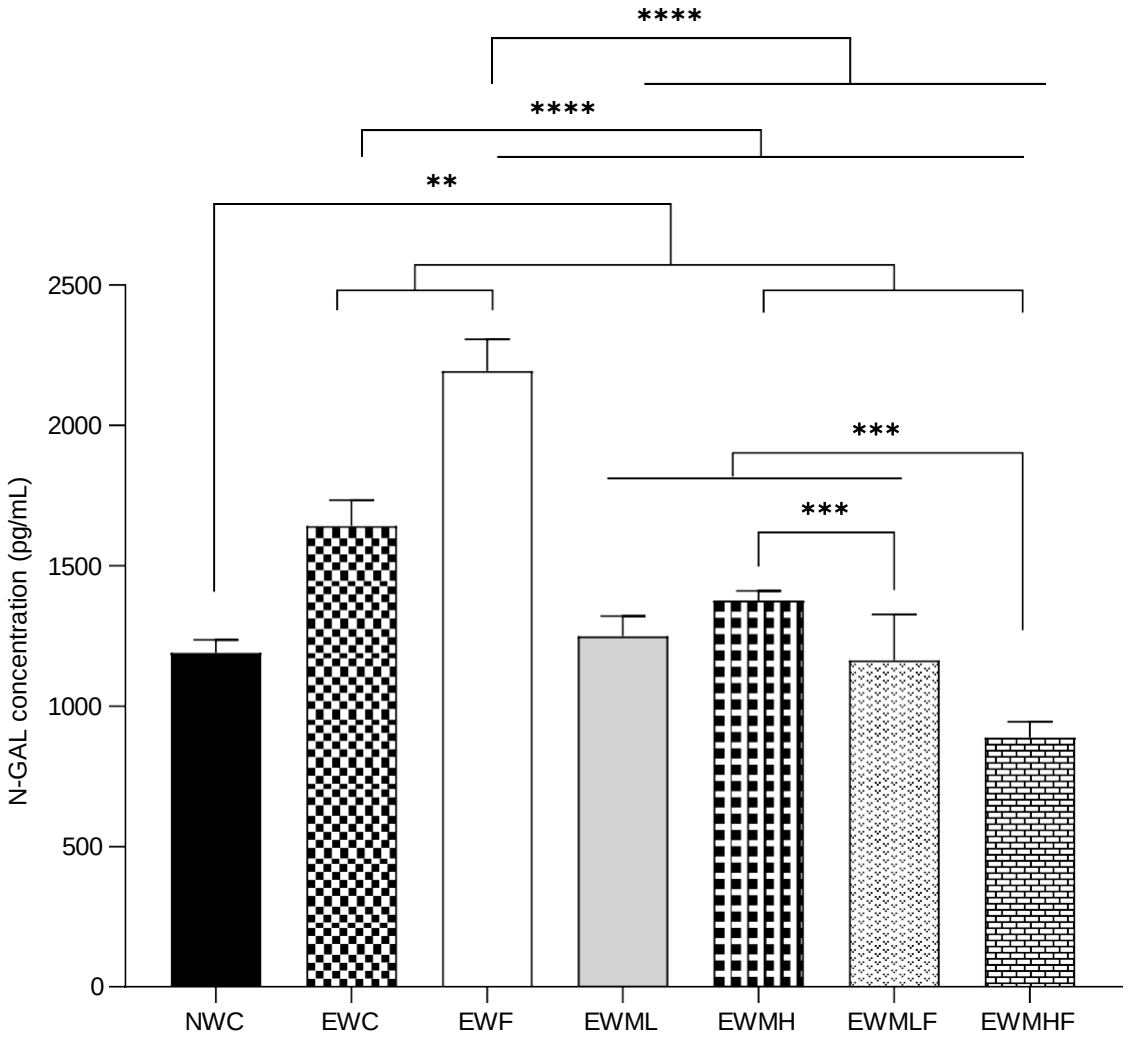


Figure 4. 3. The effect of Moringa leaf extracts and fructose consumption on serum NGAL concentration in early-weaned female Sprague-Dawley rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (** is $p < 0.01$, *** is $p < 0.001$ and **** is $p < 0.0001$) (n = 8 for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

4.3.4. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on serum creatinine, BUN concentrations and U: C ratio of early-weaned rats

Early weaning alone had no effect on serum creatinine concentrations compared to normal weaning ($p > 0.05$; Figure 4.4). However, early weaning with fructose significantly decreased serum creatinine concentration compared to all treatment groups ($p < 0.001$; Figure 4.4). The administration of both doses of Moringa for two weeks only post weaning prevented the fructose-induced decrease in serum creatinine concentrations compared to early weaning with fructose diet ($p < 0.001$; Figure 4.4).

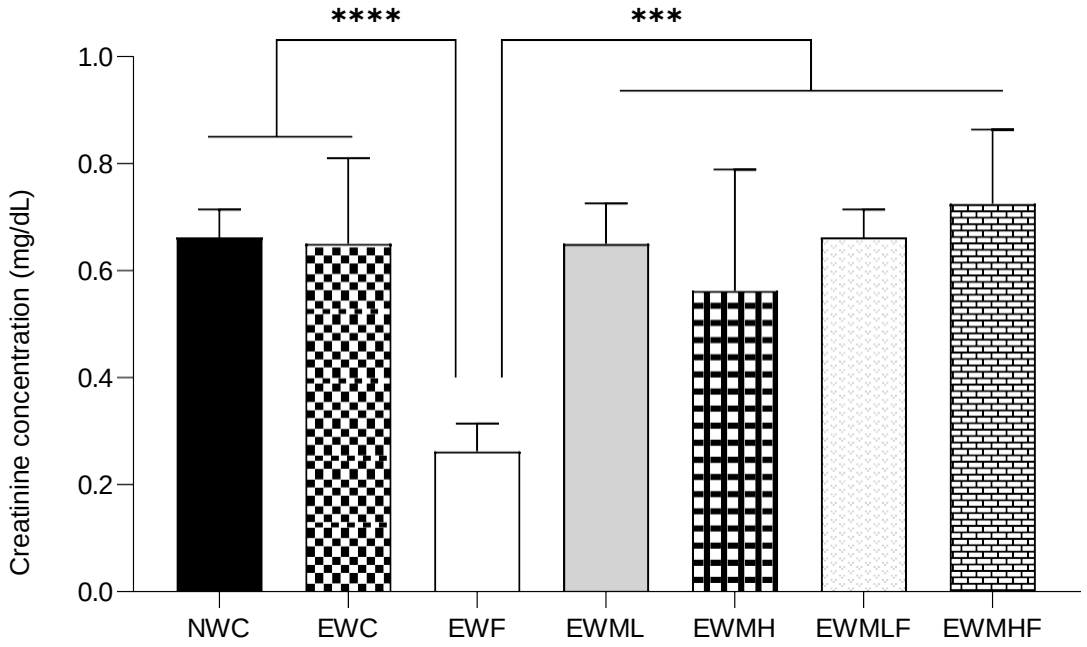


Figure 4. 4. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on creatinine concentration of early-weaned rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (*** is $p < 0.001$ and **** is $p < 0.0001$) (n = 8 for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

Early weaning did not have a significant effect on the serum concentration of BUN when compared to normal weaning ($p > 0.05$; Figure 4.5). However, early weaning with fructose significantly increased serum BUN concentration compared to all treatment groups ($p < 0.0001$; Figure 4.4). The administration of both doses of Moringa for only two weeks post weaning prevented the fructose-induced increase in BUN concentrations ($p < 0.0001$; Figure 4.5).

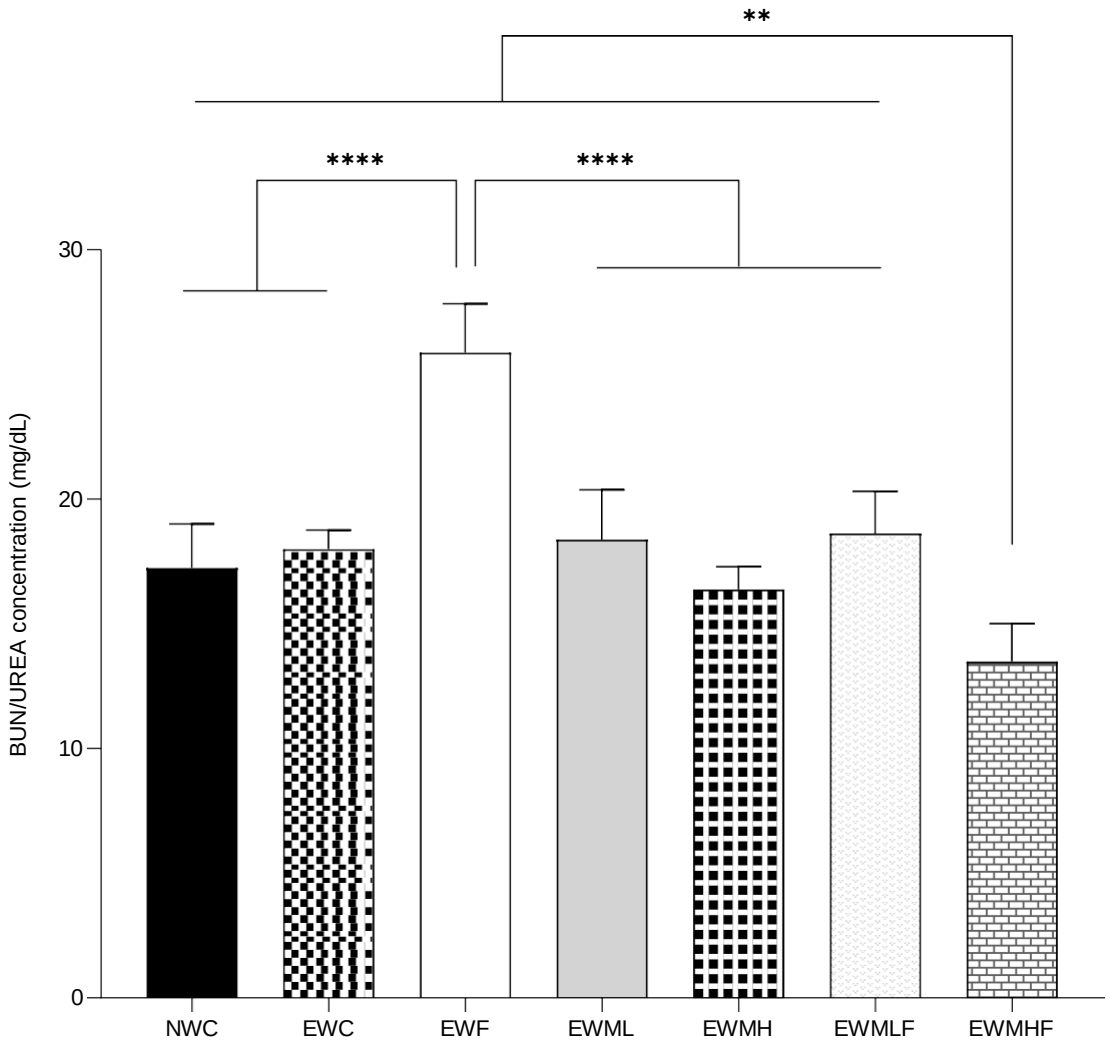


Figure 4. 5. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on BUN concentration of early-weaned rats at Termination, PD 112.

All data are presented as mean ± standard deviation. Significant difference (** is $p < 0.01$ and **** is $p < 0.0001$) (n = 8 for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

Early weaning alone had no effect on the U: C ratio compared to normal weaning ($p > 0.05$; Figure 4.6). Early weaning with fructose significantly increased the U: C ratio compared to both normal weaning and early weaning control groups (NWC and EWC) ($p < 0.0001$; Figure 4.6). However, the administration of both doses of Moringa for only two weeks post weaning prevented the fructose-induced high U: C ratio ($p < 0.0001$; Figure 4.6). The U: C ratios in the Moringa-treated groups (EWMLF and EWMHF) were not significantly different to the control groups (NWC and EWC) ($p > 0.05$; Figure 4.6).

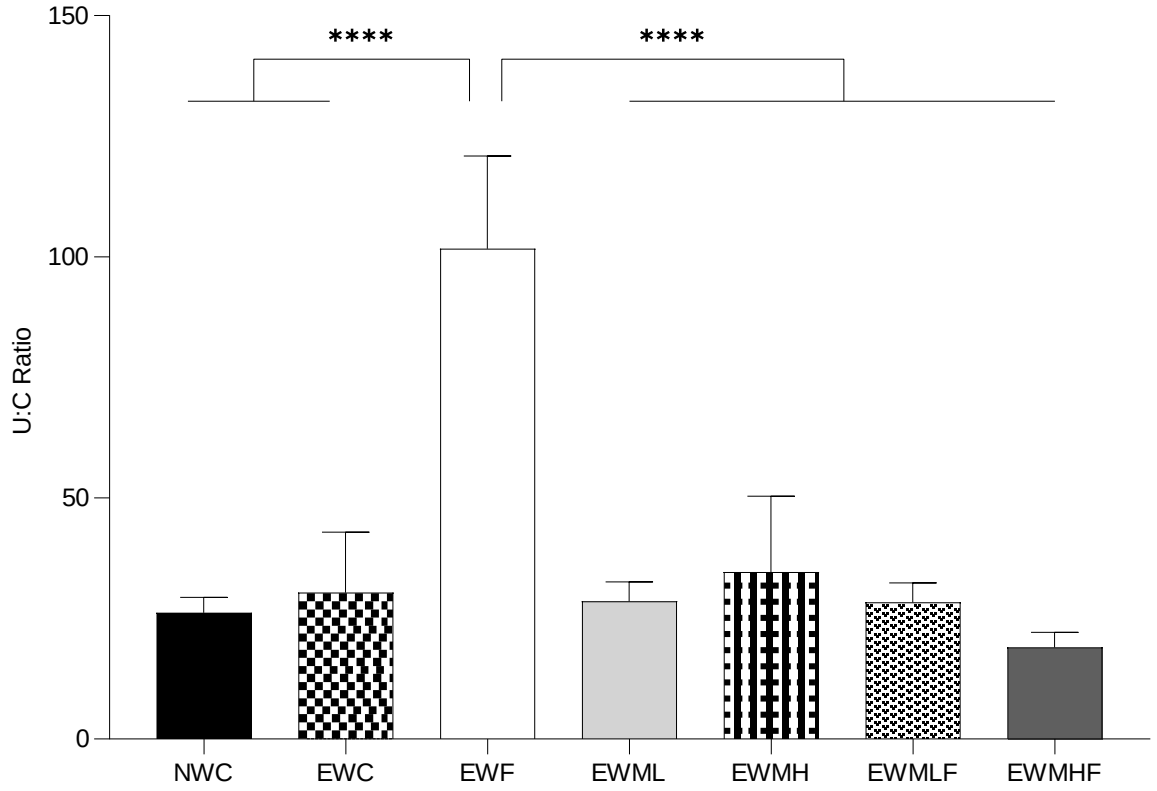
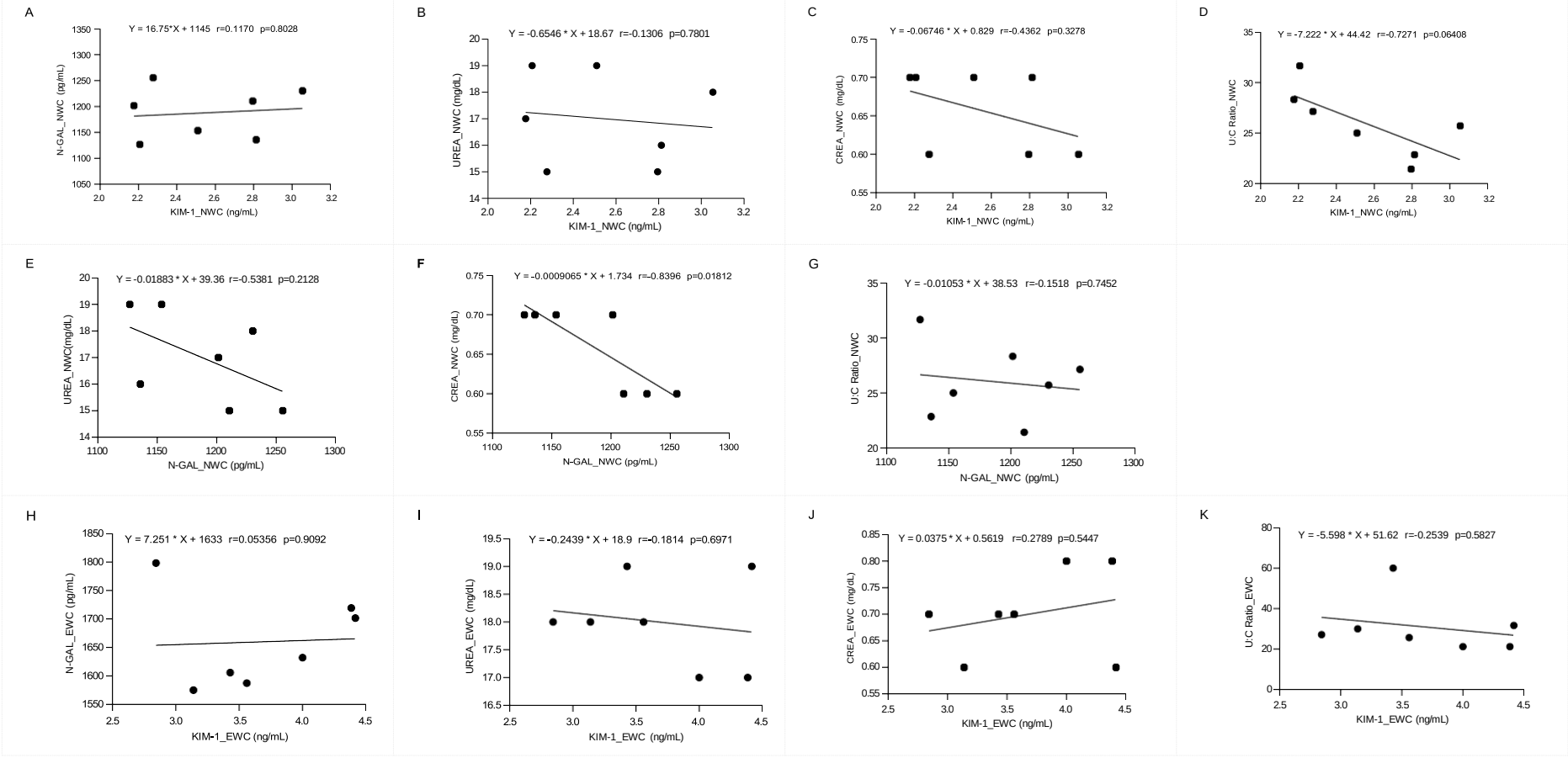


Figure 4. 6. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on the U: C ratio of early-weaned rats at Termination, PD 112.

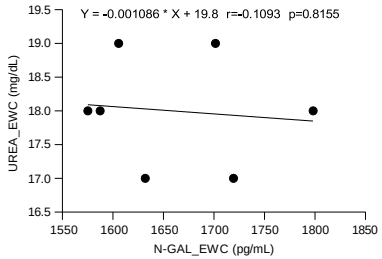
All data are presented as mean ± standard deviation. Significant difference (**** is $p < 0.0001$) ($n = 8$ for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

4.3.5. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on correlations of kidney biomarkers of health of early-weaned rats

Normal weaning with a normal diet resulted in a significant negative correlation between creatinine and NGAL concentrations ($r = -0.84$, $p < 0.05$; Figure 4.7F). However, there was no significant correlation between the other markers in the NWC group ($p > 0.05$; Figure 4.7 A to G). Early weaning alone or with fructose did not have any effect on the correlations of the kidney biomarkers of renal function ($p > 0.05$; Figure 4.7 H to V). Administration of Moringa low or high doses alone at early weaning did not have any significant effect on the correlation of kidney biomarkers of renal function ($p > 0.05$; Figure 4.7 W to JJ). Moreover, a two weeks post weaning Moringa low dose administration did not have any impact on the correlation of the kidney biomarkers ($p > 0.05$; Figure 4.7 KK to QQ). However, a two weeks post weaning administration of Moringa high dose with fructose resulted in a significant negative correlation between BUN and KIM-1 ($r = -0.96$, $p < 0.001$; Figure 4.7 SS), but did not affect the relations of the other markers ($p > 0.05$; Figure 4.7 RR to XX).


A
Y = 16.75*X + 1145 r=0.1170 p=0.8028
N-GAL_NWC (pg/mL)
KIM-1_NWC (ng/mL)
B
Y = -0.6546 * X + 18.67 r=-0.1306 p=0.7801
UREA_NWC (mg/dL)
KIM-1_NWC (ng/mL)
C
Y = -0.06746 * X + 0.829 r=-0.4362 p=0.3278
CREA_NWC (mg/dL)
KIM-1_NWC (ng/mL)
D
Y = -7.222 * X + 44.42 r=-0.7271 p=0.06408
U:C Ratio_NWC
KIM-1_NWC (ng/mL)
E
Y = -0.01883 * X + 39.36 r=-0.5381 p=0.2128
UREA_NWC(mg/dL)
N-GAL_NWC (pg/mL)
F
Y = -0.0009065 * X + 1.734 r=-0.8396 p=0.01812
CREA_NWC (mg/dL)
N-GAL_NWC (pg/mL)
G
Y = -0.01053 * X + 38.53 r=-0.1518 p=0.7452
U:C Ratio_NWC
N-GAL_NWC (pg/mL)
H
Y = 7.251 * X + 1633 r=0.05356 p=0.9092
N-GAL_EWC (pg/mL)
KIM-1_EWC (ng/mL)
I
Y = -0.2439 * X + 18.9 r=-0.1814 p=0.6971
UREA_EWC (mg/dL)
KIM-1_EWC (ng/mL)
J
Y = 0.0375 * X + 0.5619 r=0.2789 p=0.5447
CREA_EWC (mg/dL)
KIM-1_EWC (ng/mL)
K
Y = -5.598 * X + 51.62 r=-0.2539 p=0.5827
U:C Ratio_EWC
KIM-1_EWC (ng/mL)

L



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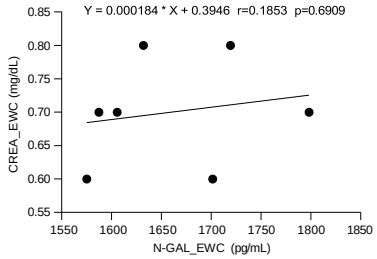

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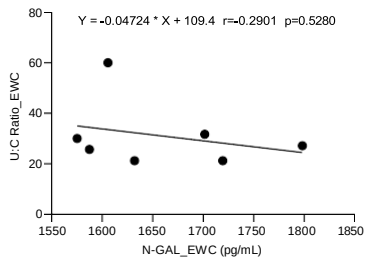

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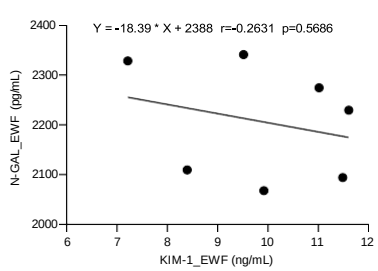

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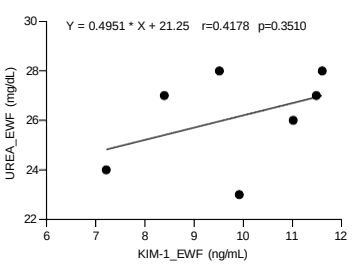

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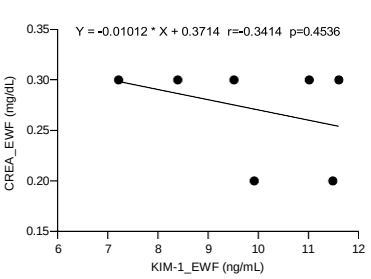

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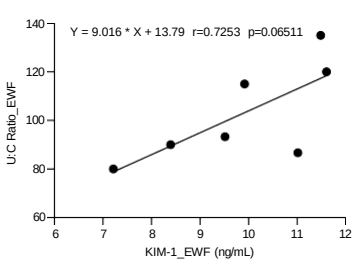

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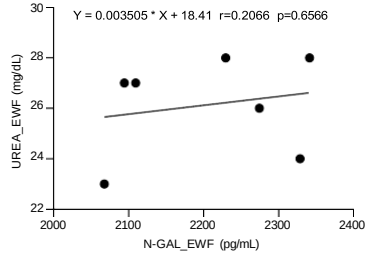

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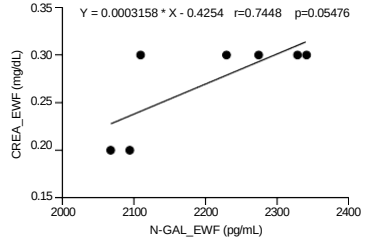

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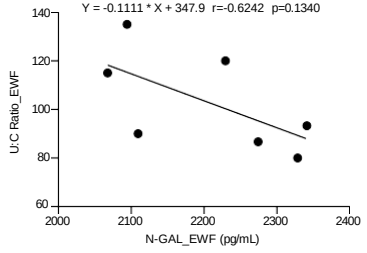
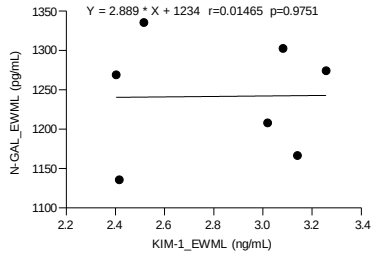


W



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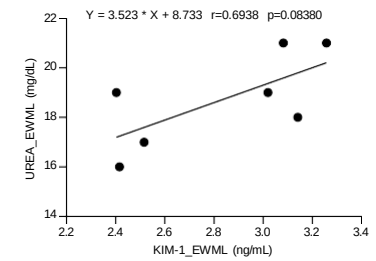

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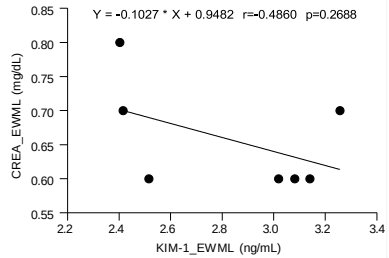

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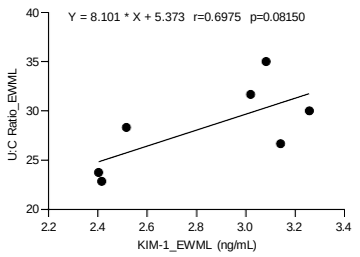

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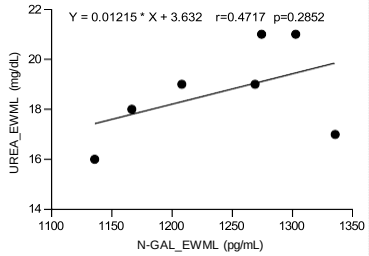

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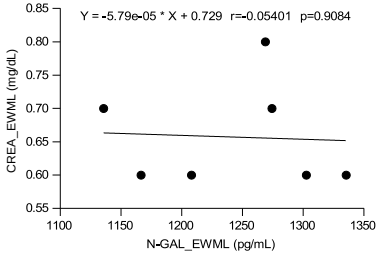

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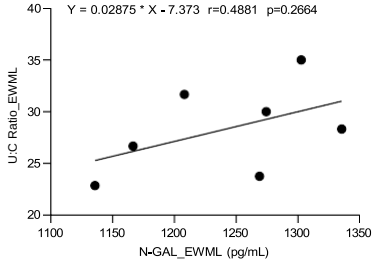

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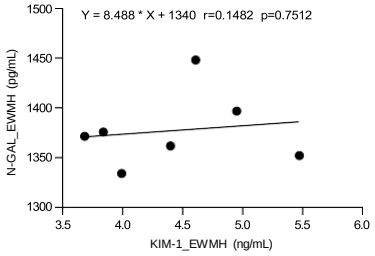

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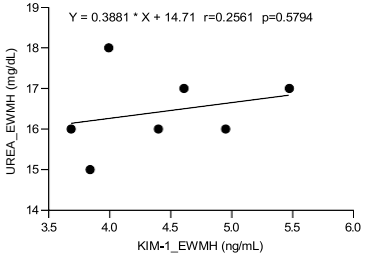

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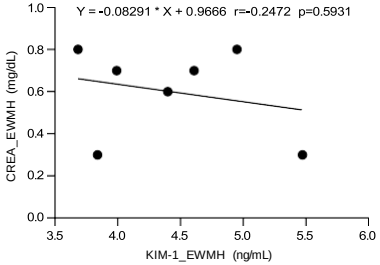

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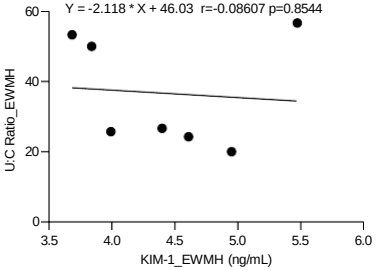

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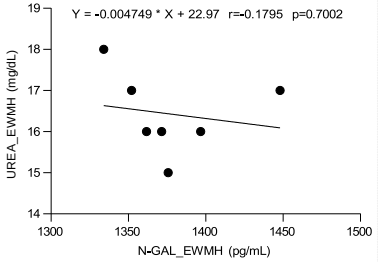

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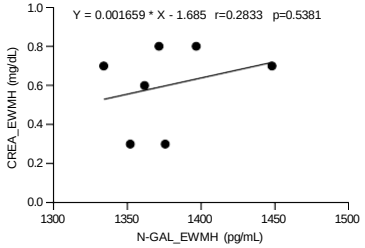

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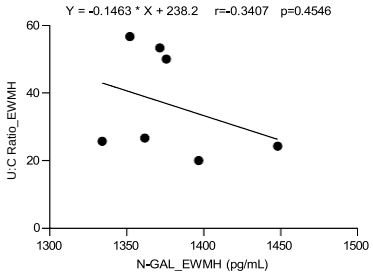

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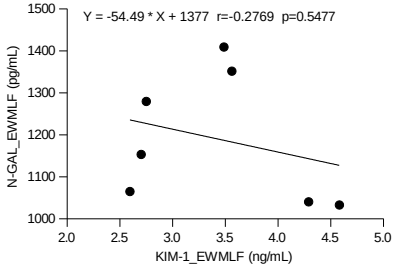

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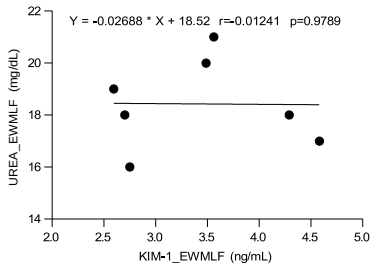

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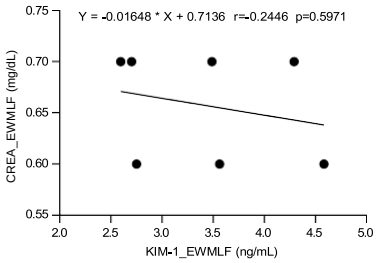

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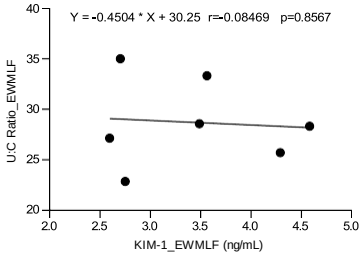

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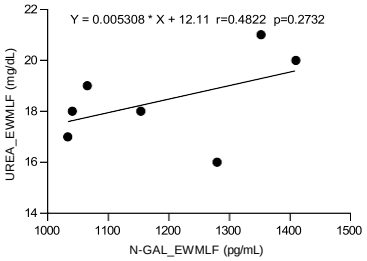

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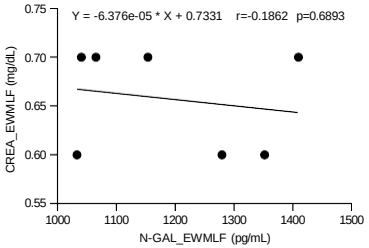

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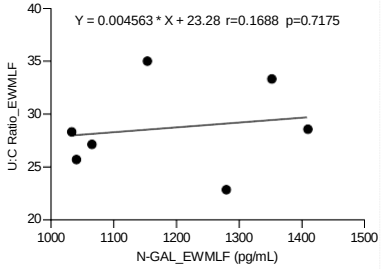

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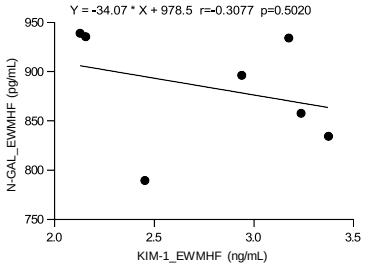

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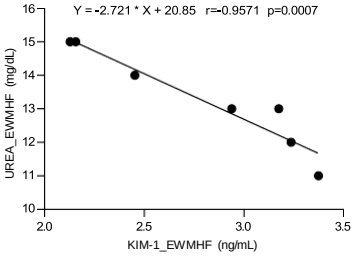

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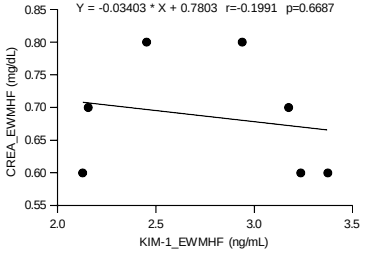

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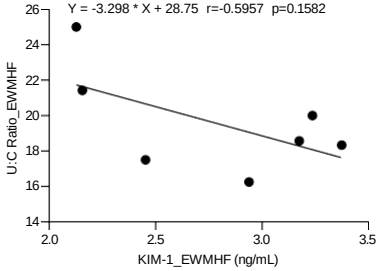

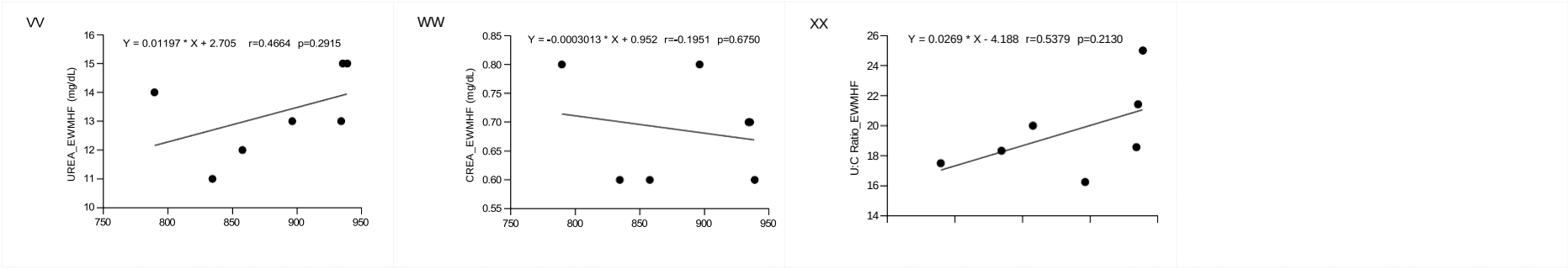

Figure 4. 7. The effect of early weaning, fructose consumption and Moringa administration on correlations of kidney biomarkers (KIM-1, NGAL, Creatinine, Urea, and U: C Ratio) across the experimental groups at Termination, PD 112.

All data are presented as mean ± standard deviation. Positive correlations ($r > 0$), negative correlations ($r < 0$), Significant difference ($p < 0.05$ and $p < 0.001$) ($n = 8$ for all groups). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.
CREA is creatinine. UREA is BUN.

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4.4. Discussion

The study investigated the potential protective effects of a strategic two week post weaning administration of Moringa hydroethanolic leaf extracts against fructose-induced renal dysfunction in early-weaned female rats. Specifically, the study assessed morphometrical changes in the kidneys, renal injury, and kidney function biomarkers.
The findings of this study indicate that neither early weaning alone or with fructose nor Moringa had any significant impact on kidney mass or relative mass. Administration of Moringa with fructose for two weeks post-weaning had no long-term effects on kidney mass or relative mass.

The study found that early weaning, combined with fructose, led to significant structural changes in the kidneys, including an increase in renal corpuscular area (RCA) and glomerular tuft area (GTA). Moringa administration with fructose, particularly at high doses, mitigated these changes. Early weaning alone did not significantly affect creatinine, BUN or KIM-1. However, early weaning with fructose significantly increased serum levels of BUN, NGAL and KIM-1 compared to normal weaning. Fructose-induced increases in NGAL and KIM-1 were most pronounced, indicating that fructose consumption at early weaning conditions exacerbated kidney injury. The administration of high-dose Moringa with fructose for only two weeks post-weaning successfully prevented the fructose-induced increase in these kidney injury biomarkers in the serum. The study found that normal weaning with a normal diet resulted in a significant negative correlation between creatinine and NGAL concentrations. However, early weaning, whether alone or with fructose, did not affect the correlations of BUN, creatinine, NGAL, and KIM-1. The two weeks post-weaning administration of Moringa with fructose at low and high doses also did not significantly alter these correlations. However, the administration of high-dose Moringa with fructose for two weeks post-weaning resulted in a significant negative correlation between BUN and KIM-1.

In toxicology studies, organ mass is often used as an indicator of potential toxicity or organ dysfunction, as changes in organ weight can reflect alterations in size, function, or metabolic processes due to exposure to various substances (Sellers *et al.*, 2007). However, changes in organ mass alone may not fully indicate early-stage toxic effects (Sellers *et al.*, 2007). Organ weight data is often supplemented with histological assessments and biomarkers of injury to

provide a more comprehensive evaluation of toxicity (Lazic, Semenova and Williams, 2020). In this study, the lack of change in kidney mass following the interventions of the study suggests that there were neither harmful effects nor developmental influences on the kidney, as measured by mass. This indicates that early weaning alone, or in combination with fructose or Moringa, did not cause significant kidney injury or developmental disturbances sufficient to alter kidney mass. In circumstances where harmful influences or developmental changes do affect kidney mass, they often do so through mechanisms such as inflammation, oxidative stress, or altered nephron development (Habib, 2018). A study by Nüsken *et al.*, (2020) examined the effects of early weaning on kidney function in rats. The study found that early weaning was associated with alterations in kidney function, including a reduced nephron count, endothelial dysfunction, oxidative stress, and dysregulation of the renin-angiotensin-aldosterone system (RAAS) (Nüsken *et al.*, 2020).

Another study revealed that high-fat or fructose diets can induce renal hypertrophy, primarily due to increased inflammation mediated by pro-inflammatory cytokines such as TNF-α and IL-1β, which promote kidney cell growth and increase kidney size (Akter *et al.*, 2021). Previous research have also shown that a high fructose diet significantly increases kidney mass (Nakayama *et al.*, 2010). Nakayama *et al.*, (2010) found that fructose significantly increased kidney mass of male Sprague Dawley rats weaned at PD28, even though the fructose concentration was not specified. In the current study, 20% fructose concentration was administered at PD18 in female Sprague Dawley rats, as such; the differences in comparison to the Nakayama study could be influenced by sex differences, weaning time or fructose concentration or duration of exposure. Overall, the above shows that dietary factor can lead to changes in kidney mass, either through compensatory hypertrophy or through atrophy, depending on the severity and duration of the weaning or dietary duration. Kidney mass alone is not a sufficient indicator of finer structural damage therefore, kidney microstructure and morphometry was investigated.

The kidney MT photomicrographs revealed significant structural changes induced by early weaning alone. These changes were worsened by fructose consumption. The quantitative histomorphometry analysis revealed that early weaning alone significantly enlarged the renal corpuscular area (RCA) and glomerular tuft area (GTA) compared to normal weaning ($p < 0.001$). However, this effect was not observed in glomerular/Bowman's capsule area (BCA) of rats early-weaned on a normal diet. These findings of enlarged RCA and BCA suggest that

early weaning alone can lead to imbalances of essential nutrients required for kidney development and maturation (Kotyk *et al.*, 2016). This then leads to significant glomerular hypertrophy and the compensatory response of the kidney to early dietary changes (Kotyk *et al.*, 2016; Abouhegazy *et al.*, 2019).

The glomerular capsule plays a crucial role in the kidney filtration processes, and abnormalities in BCA can impact filtration efficiency, leading to long-term structural damage such as tubulointerstitial fibrosis (Kotyk *et al.*, 2016). As such, early weaning alone can initiate renal injury and affect renal function. Whereas, fructose consumption at early weaning worsens the impairment of renal function. This is because of fructose metabolic effects of increasing oxidative stress and inflammatory cytokine production, resulting in the amplification of glomerular and tubular damage observed (Johnson, Sanchez-Lozada and Nakagawa, 2010; Nakayama *et al.*, 2010). The combined effect of early weaning and fructose highlights the kidney's vulnerability to dietary stress during developmental stages, resulting in chronic changes in kidney morphology and function. A study on albino male rats fed a 20% high fructose diet post-weaning for 8 weeks showed contradicting results where both the glomerular tuft area and corpuscular area in the kidneys were significantly reduced (Saleh, Merghani and Awadin, 2017). However, in this study, administering Moringa hydroethanolic leaf extract for only two weeks post-weaning, combined with long-term fructose consumption, was sufficient to mitigate kidney structural changes by reducing RCA, GTA, and BCA enlargement.

The renal corpuscular area (RCA), glomerular tuft area (GTA), and Bowman's/glomerular capsule area (BCA) are key structural parameters that reflect the filtration capacity of the kidney and overall health (Nakayama *et al.*, 2010; Habib, 2018; Nüsken *et al.*, 2020). RCA encompasses the glomerulus and glomerular/Bowman's capsule and it plays a crucial role in blood filtration to form urine (Habib, 2018). An increase in RCA can occur due to glomerular hypertrophy, often a compensatory response to elevated filtration pressures or kidney injury, such as those induced by hypertension, diabetes, or inflammation (Habib, 2018). Conversely, a decrease in RCA may result from reduced blood flow or nephron loss, indicative of kidney atrophy or compromised filtration capacity (Nüsken *et al.*, 2020). GTA is the size of the glomerular tuft and is directly involved in the filtration process through its capillaries (Nakayama *et al.*, 2010). Enlargement of the GTA is frequently observed in chronic kidney disease and diabetes, where compensatory hypertrophy occurs in response to increased

filtration demands (Nakayama *et al.*, 2010). A decrease in GTA, however, can result from glomerular injury, fibrosis, or glomerulosclerosis, impairing kidney function and leading to reduced glomerular filtration (Akter *et al.*, 2021). BCA surrounds the glomerular tuft within glomerular/Bowman's capsule and it facilitates the collection of filtrate (Arif and Nihalani, 2013). An increase in BCA, particularly when associated with an enlarged glomerular tuft, is often observed in kidney injury, inflammation, or fibrosis, typically associated with hypertension, obesity, or metabolic diseases (Johnson, Sanchez-Lozada and Nakagawa, 2010). On the other hand, a reduction in BCA may indicate glomerular atrophy or structural damage, resulting in impaired kidney filtration (Oraby *et al.*, 2019). These structural changes, assessed through histomorphometry, provide in depth status of the kidney functional and morphological health. Especially when combined with kidney biomarkers of health, together they help with understanding the underlying mechanisms of renal dysfunction in various pathophysiological states.

Blood urea nitrogen (BUN) and Creatinine (CREA) are commonly used markers of renal function, and their concentrations were also assessed in this study. Blood urea nitrogen (BUN) is a measure of the nitrogen component of urea, which is a waste product formed when proteins are broken down in the liver (Seki *et al.*, 2019). BUN is transported through the bloodstream to the kidneys, where it is filtered out and excreted in urine (Seki *et al.*, 2019). BUN originates primarily from the liver, where amino acids from proteins are deaminated (removal of an amino group (NH2) from an amino acid) (Wang, Ran and Jiang, 2014). The nitrogen from this process is converted into BUN, which is released into the bloodstream and carried to the kidneys (Wang, Ran and Jiang, 2014). Like creatinine, BUN levels reflect kidney function, as the kidneys are responsible for filtering urea (Liu *et al.*, 2024). Increased BUN levels may indicate renal dysfunction, dehydration, heart failure, or a high-protein diet (Seki *et al.*, 2019; Liu *et al.*, 2024). A high BUN concentration is commonly associated with a decreased glomerular filtration rate (GFR), which suggests that the kidneys are not functioning optimally (Kohl *et al.*, 2020). On the other hand, low BUN levels may occur in liver disease, malnutrition, or over hydration, as the liver is not producing enough urea or the kidneys are excreting it excessively (Seki *et al.*, 2019).

In this study, early weaning alone did not significantly affect serum BUN concentrations. This suggests that early weaning does not cause immediate renal dysfunction or compromise kidney filtration. However, the addition of fructose during early weaning led to a significant

increase in serum BUN concentrations. This indicates that fructose may impair kidney function, potentially leading to decreased renal filtration efficiency. However, the administration of both low and high doses of Moringa during the first two weeks post-weaning combined with a long-term fructose consumption effectively prevented this fructose-induced increase in BUN.

Since BUN was measured as an indicator of kidney function, it was important to also measure creatinine, as both are commonly used markers of renal health (Groothof *et al.*, 2022). BUN can be influenced by factors such as hydration status, diet, and liver function (Seki *et al.*, 2019). Creatinine is considered a more specific marker for kidney function, as it is produced consistently by muscle metabolism and primarily excreted by the kidneys (Groothof *et al.*, 2022). Therefore, creatinine levels provide a more reliable assessment of renal function, particularly in cases where BUN levels may be affected by non-renal factors (Seki *et al.*, 2019; Groothof *et al.*, 2022). Creatinine is produced as the end product of creatine and creatine phosphate metabolism (Kashani, Rosner and Ostermann, 2020). Creatine is a nitrogenous organic acid and is mainly synthesised in the liver and kidneys, using the amino acids glycine, arginine, and methionine (Kashani, Rosner and Ostermann, 2020). The formation of creatinine starts with a reaction between L-arginine and glycine, resulting in the production of guanidinoacetate, which is then converted into creatine in the liver (Kashani, Rosner and Ostermann, 2020). Creatine is transported to muscles where it is converted to creatine phosphate, a high-energy molecule used during muscle activity (Groothof *et al.*, 2022). Over time, creatine is spontaneously converted into creatinine through a non-enzymatic process (Kashani, Rosner and Ostermann, 2020). The kidneys filter creatinine from the blood and excrete it in urine (Groothof *et al.*, 2022). Serum creatinine levels are used as an important marker for assessing kidney function, as the kidneys are responsible for its elimination (Groothof *et al.*, 2022). Elevated serum creatinine levels are commonly associated with impaired kidney function, particularly with decreased glomerular filtration rate (GFR) (Groothof *et al.*, 2022). In healthy individuals, the serum creatinine concentration remains relatively stable, but it increases when kidney function declines, making it a useful indicator for diagnosing conditions like chronic kidney disease and acute kidney injury (Kashani, Rosner and Ostermann, 2020; Groothof *et al.*, 2022).

The results of this study revealed that early weaning alone did not affect serum concentrations of creatinine compared to normal weaning. However, when fructose was

added during early weaning, a significant decrease in serum creatinine levels was observed. This indicates potential renal dysfunction or reduced kidney filtration efficiency associated with fructose consumption. The administration of Moringa, both at low and high doses, for just two weeks post-weaning, combined with long-term fructose consumption, effectively prevented this decrease in serum creatinine levels. The ability of Moringa to regulate both serum BUN and creatinine concentrations suggests that Moringa has protective effects on renal health. The renal protective effects of Moringa may be attributed to its anti-inflammatory and anti-oxidative properties like vitamin C, flavonoids (such as quercetin, kaempferol, and rutin), phenolic compounds (like chlorogenic acid and caffeoylquinic acid), beta-carotene, and essential minerals like zinc and selenium (Dhakad *et al.*, 2019). The BUN results in relation to the creatinine results emphasise that while both markers are useful for assessing kidney function, they can be influenced by different factors. In this study, the fructose-induced increase in BUN, but not creatinine, indicates that BUN is more sensitive to changes in dietary factors. In contrast, the decrease in serum creatinine with fructose consumption suggests a more direct impairment of kidney function. The fact that Moringa supplementation prevented both the fructose-induced increase in BUN and the decrease in creatinine concentrations underscores its potential to protect against renal dysfunction.

In addition to its effects on kidney injury markers, Moringa has demonstrated a significant role in maintaining normal creatinine levels. Moringa extracts have been reported to restore creatinine concentrations to normal levels in high-fat, high-fructose diet-induced diabetes in male Wistar rats administered at 150 mg/kg – 200 mg/kg for 28 days (Putri *et al.*, 2023). Fresh leaf Moringa extracts also reduced creatinine levels back to normal in both male and female Sprague Dawley rats when administered for 28 days post-weaning (Zhang *et al.*, 2024). Additionally, studies in Wistar rats supports BUN results reported in this study where Moringa leaf extracts administered at 200 mg/kg for 7 days reduced elevated BUN and creatinine concentrations under dietary stress (Akinrinde *et al.*, 2020). The antioxidant, anti-inflammatory, and metabolic-stabilizing properties of Moringa helped counteract the oxidative stress associated with fructose-induced high-stress metabolic processes (Akter *et al.*, 2021). By reducing oxidative stress, Moringa protected renal cells from damage, particularly in the glomeruli and proximal tubules, which are susceptible to ROS-induced injury (Akinrinde *et al.*, 2020; Akter *et al.*, 2021).

Since both BUN and creatinine were measured as markers of kidney function in this study, it was important to also assess the urea-to-creatinine (U: C) ratio. The U: C ratio provides additional evaluation of renal function by reflecting the balance between urea and creatinine excretion (van der Slikke *et al.*, 2020). This ratio is valuable for differentiating prerenal causes of renal impairment, such as dehydration or diminished blood flow, from intrinsic renal injury, which may not be readily evident from BUN and creatinine assessments alone (van der Slikke *et al.*, 2020). A higher U: C ratio typically suggests prerenal causes of kidney dysfunction, where urea filtration is impaired but creatinine filtration remains relatively unaffected (van der Slikke *et al.*, 2020). In Contrast, a lower U: C ratio indicates impaired kidney function, such as a decreased glomerular filtration rate (GFR) or tubular dysfunction (Uchino, Bellomo and Goldsmith, 2012; Peng *et al.*, 2021). It is often associated with acute kidney injury (AKI) and intrinsic renal damage, which can occur within the glomeruli, tubules, interstitium, or blood vessels. This imbalance can also signal dehydration or early renal failure (Uchino, Bellomo and Goldsmith, 2012).

In the current study, the results indicate that early weaning alone does not significantly impact the U: C ratio, suggesting no immediate effect on kidney function. However, when fructose was introduced during early weaning, the U: C ratio significantly increased, pointing to potential kidney stress or impaired function associated with fructose consumption. The U: C ratio supported the BUN and creatinine findings, where early weaning with fructose resulted in kidney impairment. Additionally, the administration of both low and high doses of Moringa for just two weeks post-weaning combined with a long-term fructose consumption effectively prevented the fructose-induced increase in the U: C ratio. The U: C ratios in the Moringa-treated groups were comparable to the control groups. The U: C ratio also corroborates these findings seen in BUN and creatinine measures, reinforcing the potential of Moringa to mitigate the adverse effects of a high-fructose diet on kidney function.

It is important to note that early kidney injury often precedes significant changes in urea (BUN) and creatinine (CREA), which are traditional renal function markers. These markers typically become elevated only after a considerable loss of kidney function, often when approximately 40–50% of nephron function is compromised (Gounden, Bhatt and Jialal, 2024). This delay in elevation makes BUN and creatinine alone less reliable for detecting early-stage renal damage (Gounden, Bhatt and Jialal, 2024). To address this limitation, serum NGAL and KIM-1 concentrations were measured in this study. Both markers rise much

earlier in response to kidney injury, providing a more sensitive and specific indication of renal dysfunction (Zdziechowska *et al.*, 2020). NGAL is an early indicator of tubular damage and inflammation, while KIM-1 specifically reflects proximal tubular injury, a common early response to kidney stress (Zdziechowska *et al.*, 2020). By measuring these biomarkers, this study was able to detect kidney damage at an earlier stage, before the more traditional markers, thus offering a more accurate assessment of renal health.

Early weaning alone did not affect serum KIM-1 concentrations when compared to normal weaning. However, early weaning significantly increased serum NGAL concentrations. These findings coupled with the earlier findings on leptin, adiponectin, glucose and insulin concentrations (chapter 3 of this theses) suggests that premature dietary transition may initiate renal stress by disrupting the natural balance of metabolic and renal health markers (Abouhegazy *et al.*, 2019). This disruption increases the susceptibility of metabolic and renal regulatory systems to stress, initiating an elevated vulnerability to kidney injury and metabolic dysfunction under early dietary transition.

KIM-1 is highly specific to renal tubular cells, primarily indicating proximal tubule injury due to its low expression outside the kidney (Ostermann *et al.*, 2020). KIM-1 is a type 1 transmembrane protein, and its expression is significantly increased in the proximal tubule in the ischemic kidney, making it a critical factor in the diagnosis of acute kidney injury (Karmakova *et al.*, 2021). In contrast, NGAL, which can be elevated in non-renal tissues like the lungs and liver during inflammation, is less specific to kidney origin but still serves as a useful marker for tubular damage by reflecting active renal cellular stress (Coppolino *et al.*, 2020; Ostermann *et al.*, 2020). NGAL is a glycoprotein produced by neutrophils and epithelial cells of various organs, and it is released from the proximal nephron tubule cells into the bloodstream following ischemia and nephrotoxic damage (Coppolino *et al.*, 2020; Ostermann *et al.*, 2020). While NGAL levels in serum may indicate systemic inflammation or metabolic dysfunction, KIM-1 is a more specific marker of renal injury, especially in the proximal tubules, and has been identified as a predictive marker for acute kidney injury (Zdziechowska *et al.*, 2020; Karmakova *et al.*, 2021). Therefore, the high specificity of KIM-1 for proximal tubule injury makes it a reliable biomarker for detecting kidney damage in this study, while NGAL, although less specific to the kidneys, helps indicate renal stress and inflammation (Zdziechowska *et al.*, 2020). Together, these markers provide a comprehensive assessment of kidney injury, with KIM-1 offering targeted detection of kidney damage and

NGAL reflecting broader metabolic and inflammatory stress. Therefore, they enhance the understanding of renal health in response to early weaning alone or with fructose.

Early weaning combined with fructose had a marked impact on renal health, significantly increasing KIM-1 and NGAL levels. The elevated KIM-1 concentrations in the fructose group were consistent with the higher fasting glucose levels observed in these rats, indicating a link between fructose consumption, impaired glucose metabolism (reported in chapter 3), and kidney injury. High-fructose diets have been reported to induce kidney injury by elevating inflammatory markers, contributing to metabolic dysfunction such as increased blood glucose and kidney enlargement (Bier *et al.*, 2022). Furthermore, with the results reveal that when administered at early weaning, the high-fructose diet worsened renal injury, suggesting a compounding effect of early nutritional stress and fructose consumption that amplifies renal damage and increases the risk of long-term renal dysfunction.

Fructose metabolism bypasses the rate-limiting step of phosphofructokinase-1 (PFK-1), leading to rapid metabolism and the production of by-products such as uric acid and reactive oxygen species (ROS) (Vallon and Nakagawa, 2021). This can stimulate renal hyperfiltration, increasing glomerular filtration rate (GFR) and lowering creatinine concentrations due to more efficient renal clearance (Vallon and Nakagawa, 2021). High fructose intake has also been associated with reduced muscle mass, which may contribute to the observed decrease in creatinine levels, as creatinine is a by-product of muscle metabolism (Jang *et al.*, 2018; Gao and Gu, 2022).

The administration of Moringa for only two weeks post-weaning in combination with long-term fructose consumption provided renal function protection, as both low and high doses prevented fructose-induced increases in BUN, NGAL and KIM-1, with high doses demonstrating the strongest effects. This short-term Moringa administration with long-term fructose consumption also normalised CREA levels. In contrast, high doses of Moringa helped lower blood glucose without significantly affecting insulin or HOMA-IR (reported in chapter 3), suggesting a protective effect on glucose metabolism that could mitigate the induced metabolic dysfunction and kidney stress. Additionally, as reported in Chapter 3, early weaning with fructose significantly increased LEP and decreased ADP concentrations, which, combined with increased KIM-1 and insulin levels, further validated the presence of metabolic dysfunction and kidney injury. Research has shown that leptin and adiponectin

levels correlate with renal inflammation, with high leptin concentrations promoting inflammatory cytokine release, resulting in oxidative stress and kidney damage (Coimbra *et al.*, 2022). Furthermore, since Moringa has effects on the kidney biomarkers assessed in this study, it was essential to assess the correlations between the kidney biomarkers, in order to understanding the overall impact of early weaning with or without fructose and Moringa on renal health.

Normal weaning with a normal diet resulted in a significant negative correlation between creatinine and NGAL ($r = -0.84$, $p < 0.05$). This negative correlation suggests that as renal injury or stress (indicated by increased NGAL levels) occurs, creatinine levels decrease, possibly reflecting increased glomerular filtration rate (GFR) or altered renal clearance (Lei *et al.*, 2018). Based on these results, it makes this pair a potential indicator of renal health under normal conditions. Specifically, since NGAL is highly sensitive to renal tubular injury, the increased levels in this study show evidence of early signs of tubular stress or injury. This means that in relation to creatinine, NGAL serves as a useful marker to detect metabolic stress or adaptations occurring during early weaning, prior to creatinine showing significant impact. Our results are supported by a study in children, where urinary NGAL was found to be a better marker for early onset of kidney stress and injury compared to serum urea (Ostermann *et al.*, 2020). This is because NGAL is a glycoprotein produced by neutrophils and epithelial cells in different organs, where it is released into the bloodstream from proximal nephron tubule cells in response to ischemia and toxic kidney damage (Zdziechowska *et al.*, 2020).

The study findings also showed that early weaning alone or with fructose did not significantly affect the correlations among kidney function markers. This shows that early dietary stress from weaning alone does not significantly alter these renal biomarkers in isolation. However, weak correlations were observed between body mass and creatinine levels in the different experimental groups. Negative correlations in the early weaning with Moringa high-dose only (EWMH) group suggested increased filtration efficiency, and positive correlations in the early weaning with Moringa high dose and fructose (EWMHF) group potentially indicated a protective effect of Moringa on muscle mass or kidney stress. The urea-to-creatinine (U: C) ratio results showed that fructose consumption adds a considerable burden to kidney function under early weaning stress.

The study findings further indicate that when early weaning was combined with both fructose and Moringa at high doses, a significant negative correlation was observed between BUN and KIM-1 ($r = -0.96$, $p < 0.001$). This strong inverse relationship suggests that, in high-stress conditions, the antioxidant and anti-inflammatory properties of Moringa stabilize renal function by reducing protein catabolism and injury marker levels (Nüsken *et al.*, 2020). Studies have shown that BUN and KIM-1, in particular, are robust markers of renal stress and dysfunction in early developmental phases under metabolic stress, as they indicate how effectively the kidneys handle nitrogenous waste and respond to inflammatory signals (Akter *et al.*, 2021; Putri *et al.*, 2023). Overall, these results emphasize that in early developmental stages, markers such as BUN, creatinine, and KIM-1, when correlated, provide a clearer understanding of renal dysfunction under dietary stress. Moringa shows the potential to protect against long-term fructose-induced renal and metabolic dysfunction, even when administered for a short period post-weaning. This study revealed the critical role of nutritional interventions on kidney structure and function during early weaning.

4.5. Conclusion

Early weaning alone led to significant increases in NGAL concentrations and the enlargement of the renal corpuscular area and glomerular tuft area. When combined with fructose, the effects worsened, with significant increases in kidney injury markers (KIM-1, BUN, and NGAL) and exacerbated enlargement of RCA, GTA, and BCA. This suggests that fructose amplifies kidney damage under early weaning conditions, with a long-lasting impact on adulthood. Even when administered for only two weeks post-weaning, Moringa demonstrated long-term protective effects against fructose-induced renal stress and injury, particularly when administered at higher doses. Thus, the effects of Moringa hydroethanolic leaf extract were evident in rats at the terminal age (76 days old), even when administered for only two weeks post-weaning, providing significant protection against fructose-induced kidney damage. It most likely mitigated the renal stress and injury as evidenced by the reduced RCA, GTA, and BCA, serum creatinine levels, and KIM-1, BUN, and NGAL concentrations.

These protective effects could be attributed to the potential early life metabolic programming of renal antioxidant and anti-inflammatory processes. This suggests that Moringa has long-lasting effects on metabolism and could be used in weaning or early weaning diets.

Therefore, Moringa, is an accessible, low-cost prophylactic intervention for programming metabolism and supporting renal health. The findings of this study are significant for resource-poor communities, particularly where access to expensive pharmaceuticals for renal dysfunctions treatments is limited. In the current study, the urea: creatinine (U:C) ratio was measured as a preliminary indicator of kidney function. We acknowledge that while the U:C ratio can suggest impaired renal function, it does not provide detailed information on electrolyte handling or glomerular filtration rate (GFR), and it is not a direct measure of GFR. Electrolytes and GFR measurements were not included due to resource limitations and the study design, which primarily focused on metabolic and endocrine outcomes rather than full renal physiology assessment. Furthermore, monitoring GFR and blood pressure from weaning into adulthood would have provided additional information into the extent to which early weaning and fructose intake affect kidney function and long-term cardiovascular risk. Another limitation is the lack of direct red blood cell count measurements, which limited the ability to fully assess haematopoietic responses. This reduced the scope for evaluating potential hormonal influences such as erythropoietin activity, which may have provided additional clarity into the haematological and renal implications of fructose intake and Moringa supplementation.

Additionally, identifying the specific bioactive compounds in Moringa responsible for its protective effects and understanding their mechanisms of action could offer more targeted therapeutic strategies for preventing metabolic dysfunction induced renal stress and injury. This would further solidify Moringa as a viable intervention in diet-induced kidney stress and injuries.

Building on the analyses of liver and kidney, this chapter presents supplementary findings on the effects of Moringa leaf extract and fructose consumption on feed intake, body and organ morphometry, pancreatic structure, and haematological parameters in early-weaned female rats. Investigating these parameters provides a broader understanding of how early weaning and high-fructose diets impact overall metabolic health and organ function. Since liver and kidney dysfunction can influence, and can be influenced by, systemic metabolic status, including energy intake, pancreatic insulin production, and blood cell health, these additional assessments offer a more holistic view of the physiological consequences of the interventions. Collectively, they help contextualise the hepato-renal findings within a wider framework of metabolic programming, allowing for a more comprehensive evaluation of the protective effects of Moringa supplementation.

CHAPTER 5: THE EFFECT OF HYDROETHANOLIC MORINGA LEAF EXTRACT ADMINISTRATION FOR THE FIRST TWO WEEKS POST-WEANING AND LONG-TERM FRUCTOSE CONSUMPTION ON FEED INTAKE, PANCREATIC MORPHOMETRY, AND HAEMATOLOGICAL PARAMETERS IN EARLY-WEANED FEMALE RATS.

5.1. Introduction

In the earlier chapters of this thesis, measures of growth were restricted to body mass gain and visceral fat accretion. However, other measurements such as body length, waist circumference, body mass index (BMI), and waist-to-body length ratio (WtBLR) are crucial to quantify in detail the physical condition and body composition of the rats (Leopoldo *et al.*, 2016). Body length measurement assesses linear growth and overall body development (Bell *et al.*, 2017).

Body mass index (BMI) is a standardised efficient metric used to assess body condition in relation to size in obesity studies (Rabiu *et al.*, 2017). BMI is calculated using body mass and length, and provides information on whether rats maintain a healthy mass or are more likely to be underweight or overweight (Rabiu *et al.*, 2017).
Finally, the waist-to-body length ratio (WtBLR) provides useful information on the ratio of waist size to body length, helping to clarify the distribution of fat and classification of degree of adiposity (Leopoldo *et al.*, 2016). Therefore, these morphometric and physiological measures offer important additional metabolic function associated data.
The pancreas is a key organ in the regulation of metabolism via its exocrine and endocrine functions. Like other organs, pancreas mass is regulated by genetic factors and environmental influences such as food intake (Williams, 2017). High-energy diets have been shown to significantly increase pancreas mass by 14% (Rospond *et al.*, 2022). High-sugar diets also affects insulin production and signalling and pancreatic general function, where pancreatic β cells are inactivated and therefore results in impaired insulin secretion (Rad *et al.*, 2022). Hence, the mass of the pancreata of the study animals were assessed in the current study as supplementary information on the earlier reported findings on insulin secretion (chapter 3).

Impaired metabolic function can also have an impact on erythrocytes (Misiti, 2024). Erythrocytes play a crucial role not only in oxygen transport but also in regulating redox balance and vascular tone (Misiti, 2024). Under oxidative stress, erythrocytes undergo structural changes, such as alterations in the cell membrane and a reduction in membrane elasticity (Massaccesi, Galliera and Corsi Romanelli, 2020). These changes are critical indicators of various diseases, including neurodegenerative disorders, cardiovascular diseases, and chronic kidney disease (CKD) (Pretorius *et al.*, 2016; Misiti, 2024). These

alterations are linked to the increased release of reactive oxygen species (ROS), which exacerbate cellular damage and contribute to disease progression (Massaccesi, Galliera and Corsi Romanelli, 2020). Additionally, erythrocytes play an essential role in regulating vascular tone by synthesising and releasing nitric oxide (NO), which is a key vasodilator (Massaccesi, Galliera and Corsi Romanelli, 2020). Changes in erythrocyte function, such as impaired membrane fluidity and deformability, are common in conditions like type 2 diabetes, preeclampsia, and COVID-19, and indicate underlying metabolic and inflammatory imbalances (Pretorius *et al.*, 2016). Furthermore, RBC aggregation, which is influenced by factors such as oxidative stress, hyperglycaemia, and inflammatory cytokines, is a critical parameter in assessing circulatory health and disease progression (Misiti, 2024). Given their sensitivity to changes in the systemic environment, erythrocytes are important biomarkers for early disease detection and monitoring (Misiti, 2024). This makes them an essential tool in precision medicine and therapeutic interventions in oxidative stress-related pathologies.

Indeed, erythrocyte indices, including haemoglobin concentration (Hb), haematocrit (Hct), and mean corpuscular haemoglobin concentration (MCHC), have been linked to metabolic dysfunction, particularly central obesity, hypertension, dyslipidaemia and glucose intolerance (Monteomo, Kamagate and Yapo, 2018). Huang *et al.*, (2018) found that red blood cell count, haematocrit, and haemoglobin, were positively associated with insulin resistance and chronic low-grade inflammation in men and women, suggesting their potential role as predictors for metabolic dysfunction. Haematocrit has also been positively associated with insulin resistance in both male and female Wistar rats, further suggesting that erythocytes indices could indeed be potential predictors for the risk of metabolic dysfunction (Monteomo, Kamagate and Yapo, 2018). This association highlights the broader relevance of erythocytes indices in monitoring systemic health, as variations in these markers may reflect underlying metabolic stress or dysfunction beyond insulin resistance alone.

In particular, the mean corpuscular haemoglobin concentration (MCHC) plays a key role serving as an indicator of alterations in erythrocyte integrity and oxygen-carrying capacity (C. Yan *et al.*, 2023). Therefore, the MCHC is crucial for diagnosing and characterising different types of anaemia (C. Yan *et al.*, 2023). This suggests that erythocytes indices serve as important indicators of potential risks associated with development of metabolic dysfunction.

Food consumption patterns during early weaning are strongly influenced by the composition and energy density of the diet (Clawson *et al.*, 2019). High-fructose diets can significantly affect the regulatory mechanisms of appetite (Hadzhibozheva *et al.*, 2023). Studies have shown that the introduction of fructose during weaning can lead to an increase in preference for sweet foods and an overall increase in caloric intake (Softic, Cohen and Kahn, 2016). This preference can persist into adulthood, contributing to long-term changes in eating behaviour and metabolic health (Softic, Cohen and Kahn, 2016). Furthermore, high-fructose diets, especially when consumed in liquid form, have been linked to reduced intake of solid food (Lindqvist, Baelemans and Erlanson-Albertsson, 2008). This may be due to changes in the regulation of hunger and satiety hormones, such as cholecystokinin (CCK), which is essential for the feeling of fullness (Lindqvist, Baelemans and Erlanson-Albertsson, 2008).

Fluid consumption also plays a key role during early weaning, as it is closely linked to overall hydration status and nutritional intake (Suzan *et al.*, 2023). High-fructose diets can not only affect food intake but also alter fluid preferences, potentially leading to an increase in the consumption of sugary beverages (Mennella, Bobowski and Author, 2015; Hadzhibozheva *et al.*, 2023). This can exacerbate the effects of the diet by promoting further calorie intake and altering hydration patterns (Hadzhibozheva *et al.*, 2023). Early weaning onto a high-fructose diet may create a preference for sweet liquids, influencing fluid balance and contributing to metabolic dysregulation.

Both the nutritional content of the diet and the timing of the dietary transitions influence metabolic programming during early weaning (Joshi, Rathore and Deshpande, 2019). Research suggests that early exposure to high-calorie diets can induce long-lasting changes in metabolic pathways, affecting energy regulation and fat storage (Heyman and Abrams, 2017). These changes may predispose individuals to metabolic diseases such as obesity, diabetes, and cardiovascular disease in later life (Heyman and Abrams, 2017). As such, food and fluid consumption during early weaning play a crucial role in shaping long-term health outcomes, with the potential for both positive and negative impacts depending on the dietary interventions used.

The aim of this chapter was to present how the above parameters (morphometric measures of growth, pancreas mass and erythrocyte indices) were influenced by early weaning, Moringa

administration in the first two weeks post weaning and a high fructose diet. The objectives of this chapter were;

1. To assess the effect of early weaning, Moringa supplementation, and long-term fructose consumption on physical morphometric measurements, including body length, waist circumference, body mass index (BMI), and waist-to-body length ratio (WtBLR).
2. To evaluate the influence of early weaning and dietary interventions on pancreas mass and organ weight relative to body mass.
3. To examine the impact of Moringa supplementation and fructose consumption on erythrocyte indices, including haemoglobin concentration (Hb), haematocrit (Hct), and mean corpuscular haemoglobin concentration (MCHC).

5.2. Methods

5.2.1. Study setting

The study setting is as described in chapter 3, section 3.2.1.

5.2.2. Study animals and husbandry

The study animals and husbandry methods are described in chapter 3, section 3.2.4.

5.2.3. Feed and fluid intake measurement

The methods followed are described in chapter 3, section 3.2.5, and illustrated in Figure 3.1. In summary, from PD 36 to the end of the experiment at PD111, the pups were housed in pairs, with two female rats from the same treatment group per cage. This arrangement, based on comparable body weights, was implemented to ensure socialization and promote the welfare of the rats throughout the study. Feed intake and the 20% fructose solution and plain water consumption were monitored and recorded twice a week.

Feed intake of 20% fructose solution and plain water consumption were measured once a day and twice a week. SRC Feed was provided at a maximum of 400 g fluid was provided at 500 ml (fructose and water) and consumptions were calculated using the following formulas;

1. Consumed Feed (g) = Initial Feed Provided (g) – Previous Remaining Feed (g)
   For Stage 2: Consumed Food (g) = 400 g − Previous Remaining Feed (g)

2. Fluid Consumed (ml) = Initial Fluid Provided (ml) – Previous Remaining Fluid (ml)
   For Stage 2: Fluid Consumed (ml) = 500 ml – Previous Remaining Fluid (ml)

5.2.4. Terminal procedure

The termination methods are as described in chapter 3, section 3.2.7.

5.2.5. Body mass measurements

5.2.5.1. Body length measurement

After the rats were anesthetised, as described in chapter 3, they were placed on a flat surface and gently stretched to ensure they were in full length. A flexible measuring tape was used to measure length from the tip of the nose to the base of the tail, excluding the tail itself. This measurement was recorded in centimetres (cm) (Timotius *et al.*, 2019).

5.2.5.2. Waist circumference measurement

While the anesthetised rats were placed on a flat surface, the narrowest point around the abdomen was identified behind the rib cage. A flexible tape measure was then wrapped around this point. The waist circumference was recorded in centimetres (cm) (Novelli *et al.*, 2007).

5.2.5.3. Body mass index (BMI) computation

In order to determine the BMI of the rats, the rats were weighed. Mass were recorded in grams (g). With the body length already measured (from the nose-tip to tail-base), the following formula (Novelli *et al.*, 2007) was then used;

$$BMI = \frac{Body\ mass\ (g)}{Body\ length\ (cm^2)}$$

5.2.5.4. Waist-to-body length ratio (WtBLR) measurement

For calculating the WtBLR, both the waist circumference and body length measurements obtained earlier were utilised. The below formula was then applied to derive the ratio (Yan *et al.*, 2007).

$$WtBLR = \frac{Waist\ circumference\ (cm)}{Body\ length\ (cm)}$$

This ratio was used to describe the proportion of the rat's waist size to its overall body length, which was useful for assessing its body fat distribution.

5.2.5.5. Pancreas mass measurement

The rats pancreata were dissected out and weighed on an electronic balance (Precisa Instruments AG, Dietikon, Switzerland). The pancreas mass relative to body mass was calculated by expressing the absolute pancreas mass as a percentage of the terminal body mass of each rat using the below formula (National Toxicology Program, 2014);

$$Pancreas\ mass\ (\%TBM) = (\frac{Pancreas\ mass\ (g)}{Terminal\ body\ mass\ (g)})\ x\ 100$$

5.2.5.6. Haematological parameters measurements

In Chapter 3, blood collection, preparation and storage is explained in section 3.2.7. Haemoglobin concentration and haematocrit were measured using an Insight HCT meter (EKF Diagnostics Holdings plc, Penarth, Cardiff, United Kingdom). Mean corpuscular haemoglobin concentration (MCHC) was calculated using the below formula (Wahed and Dasgupta, 2015);

$$MCHC(g/dL) = haemoglobin\ \div\ haematocrit$$

5.3. Results

5.3.1. The effect of hydroethanolic Moringa leaf extracts and fructose on feed and fluid consumption of early-weaned female rats

From week 5, rats fed a high fructose diet (in the EWF group) exhibited a significantly lower consumption of SRC compared to those in the early weaning control group (EWC), the Moringa low dose only group (EWML), the Moringa high dose only group (EWMH), and the Moringa low dose with fructose group (EWMLF) ($p < 0.05$, $p < 0.01$; Figure 5.1). In week 7, SRC consumption in the EWF group was significantly reduced when compared with that in both the EWC and the normal weaning group (NWC) ($p < 0.05$, $p < 0.01$; Figure 5.1). Furthermore, from weeks 10 through 13, the EWF group consistently recorded significantly lower SRC consumption than all other experimental groups. ($p < 0.01$; Figure 5.1).

In terms of fluid intake, there was no significant differences observed in both plain water and fructose solution intake throughout the 13 weeks of phase two of the experiment among the groups ($p > 0.05$; Figure 5.2).

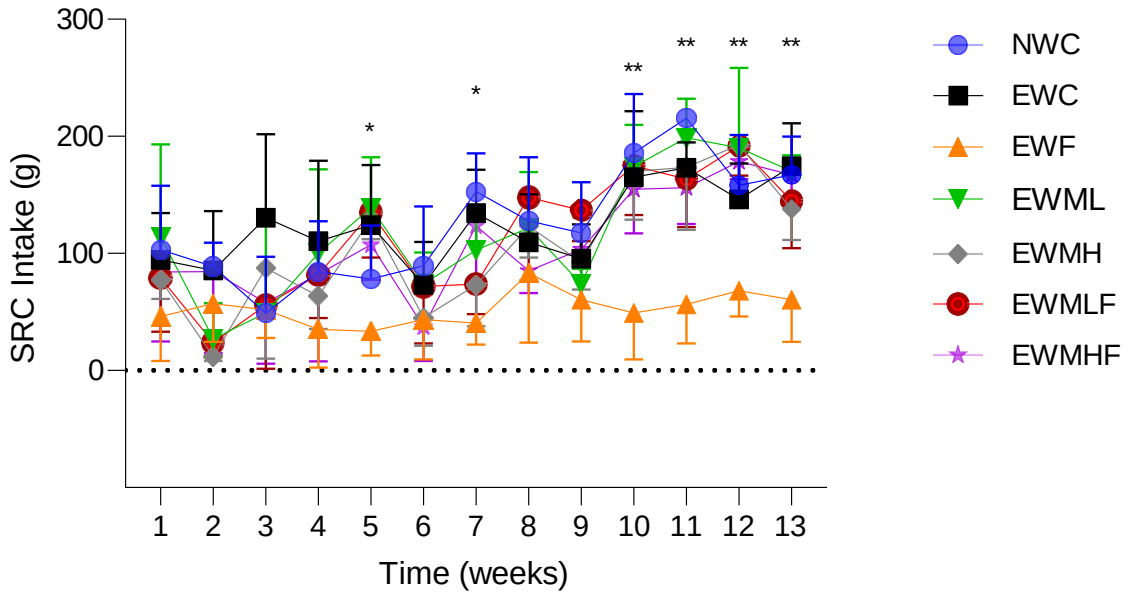

Figure 5. 1. Weekly consumption of standard rat chow (SRC) by female rats during phase 2 of the study from week one until at termination, PD 112.

n = 4 pairs for all groups. All data are presented as mean ± standard deviation. (* is $p < 0.05$ and ** is $p < 0.01$). **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water until PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

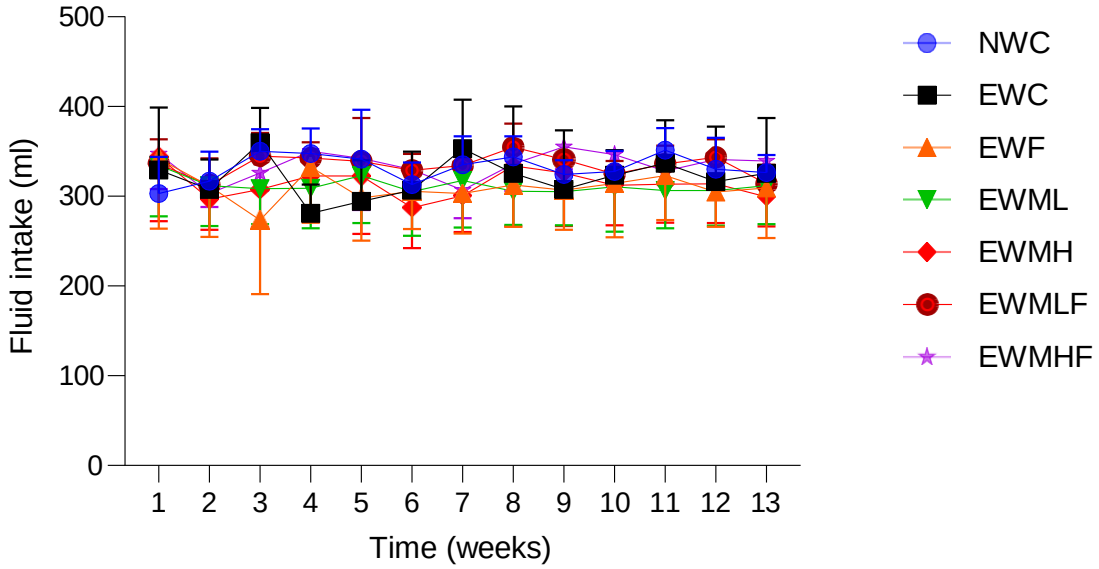

Figure 5. 2. Weekly consumption of fluid (Fructose solution (FS)/plain drinking water (PDW)) by female rats during phase 2 of the study from week one until at termination, PD 112.

n = 4 pairs for all groups. All data are presented as mean ± standard deviation. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution PD35. **EWML** = (early weaning hydroethanolic Moringa low dose only) received Moringa low dose (50 mg/kg) administered in gelatine cubes and plain drinking water until PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low dose and fructose) received Moringa low dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

5.3.2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on physical morphometric measurements of early-weaned female rats

There were no significant differences in body length, waist circumference, body mass index and waist to body length ratio of rats across the treatment groups ($p > 0.05$; Table 5.1). Terminal body mass of rats early-weaned on a Moringa high dose only diet was significantly higher when compared to those in the early weaning with fructose group ($p < 0.01$; Table 5.1).

Table 5. 1. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on body length, waist circumference, body mass index (BMI) and waist to body length ratio (WtBLR) of early-weaned female rats at Termination, PD 112.

Group	Body length (cm)	WC (cm)	Terminal body mass (g)	BMI (g/cm^2)	WtBLR
NWC	23.88 ± 1.73^{a}	16.00 ± 1.69^{a}	268.75 ± 16.52^{ab}	11.28 ± 0.64^{a}	0.67 ± 0.03^{a}
EWC	22.88 ± 0.64^{a}	15.25 ± 1.04^{a}	269.88 ± 9.42^{a}	11.81 ± 0.70^{a}	0.67 ± 0.05^{a}
EWF	22.88 ± 0.35^{a}	15.25 ± 1.04^{a}	269.88 ± 10.77^{ab}	11.18 ± 0.42^{a}	0.64 ± 0.05^{a}
EWML	23.75 ± 0.89^{a}	15.38 ± 0.52^{a}	274.13 ± 13.07^{ab}	11.56 ± 0.67^{a}	0.65 ± 0.02^{a}
EWMH	23.75 ± 1.16^{a}	15.50 ± 0.76^{a}	282.63 ± 22.62^{b}	11.91 ± 0.88^{a}	0.65 ± 0.04^{a}
EWMLF	23.38 ± 0.92^{a}	1588 ± 083^{a}	276.25 ± 13.47^{ab}	11.85 ± 1.01^{a}	0.68 ± 0.06^{a}
EWMHF	23.25 ± 1.16^{a}	1563 ± 0.74^{a}	275.88 ± 14.04^{ab}	11.87 ± 0.47^{a}	0.67 ± 0.06^{a}

All data are presented as mean ± standard deviation. ^{a,b,} Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. Termination. All data are presented as mean ± standard deviation. n = 8 per group. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low-dose only) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35**. EWMLF** = (early weaning hydroethanolic Moringa low-dose and fructose) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low-dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. WC = waist circumference, BMI = body mass index and WtBLR = waist to body length ratio.

5.3.3. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on the mass of pancreata of early-weaned rats

There were no significant differences in the absolute and relative masses of the pancreata of the rats across the treatment groups of the study ($p > 0.05$; Table 5.2).

Table 5. 2. The effect of hydroethanolic Moringa leaf extracts and fructose consumption on absolute mass and mass relative to body mass of the pancreata at Termination, PD 112.

Treatment groups	Pancreas (g)	Pancreas (%TBM)
NWC	0.93 ± 0.27[a]	0.35 ± 0.10[a]
EWC	0.94 ± 0.22[a]	0.35 ± 0.08[a]
EWF	0.98 ± 0.16[a]	0.38 ± 0.06[a]
EWML	0.93 ± 0.20[a]	0.34 ± 0.06[a]
EWMH	1.28 ± 0.33[a]	0.45 ± 0.12[a]
EWMLF	1.16 ± 0.15[a]	0.42 ± 0.05[a]
EWMHF	1.16 ± 0.32[a]	0.42 ± 0.10[a]

n=8 per group. All data are presented as mean ± standard deviation. [a] Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. **NB:** %TBM = organ mass relative to terminal body mass. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low-dose only) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMLF** = (early weaning hydroethanolic Moringa low-dose and fructose) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low-dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

5.3.4. The effect of hydroethanolic Moringa leaf extract and fructose consumption on erythrocyte indices in early-weaned rats

There were no significant differences in haemoglobin concentration (Hb), haematocrit and MCHC among the treatment groups ($p > 0.05$; Table 5.3)

Table 5. 3. The effect of hydroethanolic Moringa leaf extract and fructose consumption on haematological parameters early-weaned rats at Termination, PD 112.

Group	Hb concentration (g/dL)	Haematocrit (%)	MCHC (g/dL)
NWC	19.96 ± 0.99[a]	59.88 ± 3.00[a]	33.33 ± 33.00[a]
EWC	19.26 ± 1.67[a]	57.88 ± 4.97[a]	34.49 ± 33.60[a]
EWF	18.93 ± 1.75[a]	57.00 ± 5.26[a]	33.21±33.26[a]
EWML	19.24 ± 1.58[a]	57.88 ± 4.94[a]	33.24 ± 31.98[a]
EWMH	19.19 ± 2.06[a]	57.50 ± 6.26[a]	33.37 ± 32.91[a]
EWMLF	18.10 ± 0.72[a]	54.50 ± 2.20[a]	33.21 ± 32.73[a]
EWMHF	18.20 ± 0.55[a]	54.63 ± 1.69[a]	33.32 ± 32.54[a]

n = 8 per group. All data are presented as mean ± standard deviation. MCHC is mean corpuscular haemoglobin concentration, Hb is haemoglobin. [a] Significant difference represented by groups with different letters and non-significance is represented by at least one similar letter among the groups in the same column. **NWC** (PD21) = (normal weaning control, weaned at postnatal day 21) received plain gelatine cubes and plain drinking water daily until PD35. **EWC** (PD18) = (early weaning control, weaned at postnatal day 18) received plain gelatine cubes and plain drinking water daily until PD35. **EWF** = (early weaning fructose only) received plain gelatine cubes and 20% fructose solution from PD18 to PD35. **EWML** = (early weaning hydroethanolic Moringa low-dose only) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMH** = (early weaning hydroethanolic Moringa high dose only) received Moringa high dose (500 mg/kg) administered in gelatine cubes and plain drinking water from PD18 to PD35. **EWMLF** = (early weaning hydroethanolic Moringa low-dose and fructose) received Moringa low-dose (50 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35. **EWMHF** = (early weaning hydroethanolic Moringa high dose and fructose) received Moringa low-dose (500 mg/kg) administered in gelatine cubes and 20% fructose solution from PD18 to PD35.

5.4. Discussion

The impact of a two-week administration of hydroethanolic Moringa leaf extracts and long-term fructose consumption on standard rat chow (SRC), fluid (including fructose solution and plain water), body length, waist circumference, body mass index (BMI), waist-to-body length ratio (WtBLR), the absolute and relative mass of the pancreas in relation to body mass, haematological parameters, including haemoglobin concentration (Hb), haematocrit (Hct), and mean corpuscular haemoglobin concentration (MCHC) in early-weaned female rats, including feed and fluid intake, morphometric measurements, and pancreas mass and erythrocyte indices. It was found that early weaning had a significant effect on feed intake, as rats in the early weaning control group (EWC) consumed more standard rat chow (SRC) compared to those in the early weaning fructose (EWF) group, which exhibited a significantly lower consumption of SRC throughout the study in phase 2 of the study. Early weaning with fructose (EWF) and continued fructose solution as drinking fluid led to a decrease in SRC intake, particularly in the last six weeks of the experimental study. However, there was no significant difference in fructose solution consumption of rats early-weaned on fructose only, or Moringa with fructose (EWMLF and EWMHF). Additionally, the early weaning with Moringa high dose only (EWMH) increased body mass compared to early weaning with fructose only group. Overall, there were no significant changes noted in the other morphometric parameters (BMI and waist-to-body length ratio), irrespective of diet. Furthermore, neither early weaning alone nor any of the dietary interventions (fructose or Moringa) significantly affected pancreatic mass or erythrocyte indices (Haemoglobin concentration, haematocrit, and mean corpuscular haemoglobin concentration (MCHC)).

The observed decreases in weekly SRC intake from Weeks 5 to 13 from the high fructose diet group highlights the potential affect of a high-fructose diet on appetite regulation.
Rats are able to regulate their appetite based on the dietary calorific content as well as the state of the consumed calories (solid vs liquid diet) (Hadzhibozheva *et al.*, 2023). Previous research suggests that when fructose is administered in liquid form, it significantly affects solid food intake by modulating appetite-regulating hormones and metabolic signals (Lindqvist, Baelemans and Erlanson-Albertsson, 2008; Hadzhibozheva *et al.*, 2023). Hadzhibozheva *et al.*, (2023) showed similar findings that liquid fructose reduces the intake of solid food in Wistar male and female rats. This is likely due to its rapid absorption and

distinct effects on hypothalamic appetite-regulation signals, where it decreases cholecystokinin (CCK), which is an essential satiety hormone produced in the gastrointestinal tract but impacts hypothalamic satiety centres (Lindqvist, Baelemans and Erlanson-Albertsson, 2008; Hadzhibozheva *et al.*, 2023). In contrast to the results of this study, when fructose is incorporated into solid feed, it can enhance palatability and increase overall caloric intake by adding sweetness, making the food more appealing for consumption (Colley and Castonguay, 2015).

Research also shows sexually dimorphic metabolic impacts of fructose such that fructose consumption affects male and female rats differently (Hadzhibozheva *et al.*, 2023). Research also indicate that in females, there are notable variations between pregnant and non-pregnant females (Murphy *et al.*, 2018). When fructose was administered as liquid to pregnant female Wistar rats, it resulted in elevated calorie intake, elevated levels of insulin, leptin, and ghrelin (Vickers *et al.*, 2011). However, in studies were non-pregnant females were compared to males, fructose resulted in pronounced elevated plasma levels of insulin, leptin, and ghrelin (Lindqvist, Baelemans and Erlanson-Albertsson, 2008; Hadzhibozheva *et al.*, 2023). These sex-specific differences suggest that hormonal factors significantly influence the impact of fructose on feeding and metabolism, with pregnant females experiencing an amplified metabolic response to fructose due to increased energy demands than non-pregnant females and males. However, exploring this further will be the subject of future research on Moringa. The groups supplemented with Moringa for two weeks post-weaning, did not show the long-term fructose-induced reduction in SRC consumption. This indicates that in this study, Moringa, when administered for two weeks only post-weaning seems to have blunted the appetite-modulating effects of fructose . Studies have shown that proteins (globulins and albumins), which are the predominant proteins in Moringa (reference), offer stronger appetite-regulatory effects than carbohydrates like fructose and glucose (Murphy *et al.*, 2018; Gehring, Gaudichon and Even, 2020). The early life consumption of Moringa seems to have programmed against appetite regulating impact of fructose.

Indeed studies in male and female mice indicate that dietary protein (globulins and albumins) in Moringa increases satiety and reduce overall food intake , as proteins engage more substantial feedback mechanisms, including increased cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) (Gehring, Gaudichon and Even, 2020). This important finding on

early life consumption of Moringa programming for long-term appetite regulation needs further exploration.

Irrespective of dietary components, it is important to note that energy intake plays a crucial role in the regulation of appetite in rats, with caloric needs influencing food consumption patterns depending on physiological states, such as growth, pregnancy, or lactation (Menaker and Navia, 1973; Vickers *et al.*, 2011). Research has revealed that rats have the ability to adjust their food intake based on the caloric content of their diet, which shows a high capacity for energy regulation to meet their metabolic demands (Beheshti *et al.*, 2018). In non-pregnant female rats, food intake is primarily regulated by caloric needs, but during energy-intensive conditions like pregnancy or lactation, appetite adjusts to meet higher demands, even if dietary protein is limited (Menaker and Navia, 1973; Vickers *et al.*, 2011; Hadzhibozheva *et al.*, 2023). The ability of rats to regulate food intake based on caloric content reveals their broader metabolic flexibility. This flexibility extends to fluid consumption as well.

Early weaning control with plain drinking water (EWC) or with fructose (EWF) did not significantly affect fluid consumption patterns. These findings are consistent with a study by (Díaz, de Souza and de Sousa, 2022), which reported that early weaning did not impact water consumption in female Wistar rats. However, the results for early weaning with fructose contradict findings from another study, where early-weaned Wistar rats exhibited significantly higher fructose consumption compared to the control group, which was provided with water. This suggests that while early weaning with water may not affect baseline fluid intake, early weaning with fructose appears to significantly influence subsequent fluid consumption. These findings imply that fructose may act as a potent factor in altering fluid intake patterns in early-weaned rats. Fructose is often considered palatable, which may contribute to increased intake, especially during early life (Softic, Cohen and Kahn, 2016). Infants and young children have innate preferences for sweet tastes, which may lead them to consume foods and beverages containing fructose willingly (Weiss, Bremer and Lustig, 2013). Hence, early weaning onto high fructose diets could prime them for longer term preference for sweetened foods and beverages.

Interestingly, the two week post-weaning administration of Moringa at both low and high doses combined with a long-term fructose consumption significantly reduced the fructose-

induced increase in fluid consumption. Other studies have shown that phytochemicals in plants can block sugar preference and potentially reduce the desire for sweet foods by altering taste perception and sugar absorption (Turner *et al.*, 2020). These findings suggest that while Moringa supplementation may not directly influence fluid consumption or adiposity measures, its impact on nutrient absorption and metabolism could contribute to changes in body mass.

The indirect measures of increased adiposity revealed no significant differences in body mass index (BMI) and waist-to-body length ratio (WtBLR) across treatment groups, except for body mass, where early-weaned rats on a high Moringa dose exhibited a notable increase compared to those weaned early on SRC. These findings are consistent with the results on visceral fat and body mass presented in Chapter 3. These findings validate BMI and WtBLR as alternative measures of adiposity. Fructose consumption did not significantly alter BMI or WtBLR. High-dose Moringa supplementation may promote muscle gain, possibly through improved nutrient absorption or the metabolic effects of Moringa components, such as polyphenols and flavonoids (Dhakad *et al.*, 2019). However, the mass gain could be attributed to lean body mass, which would explain the insignificant differences in measures of obesity observed between the rat groups in the current study. While changes in body mass were observed with high-dose Moringa supplementation, it was necessary to also assess organ mass relative to body mass to determine if the observed increases in body mass were associated with changes in organ size, such as the pancreas.

The justification for measuring organ mass relative to body mass lies in understanding whether the observed increases in body mass are due to changes in overall body composition or if corresponding changes in organ size, such as the pancreas, accompany them. When organ mass measurements are expressed relative to body mass, they reveal changes in diet, metabolism, and treatment can impact not only body composition but also organ health and function (Lazic, Semenova and Williams, 2020). Studies have revealed that changes in organ mass, particularly in response to chronic metabolic disturbances or dietary modifications, are indicative of long-term physiological adaptations (Furman *et al.*, 2019; Kivimäki, Bartolomucci and Kawachi, 2023). For instance, obesity or metabolic stress could lead to either organ hypertrophy (enlargement) or atrophy (Kivimäki, Bartolomucci and Kawachi, 2023). Therefore, assessing organ mass relative to body mass is crucial for understanding the broader effects of Moringa supplementation on both metabolic health and the structural

integrity of vital organs, such as the pancreas. Because the pancreas can be influenced by changes in metabolic function and nutrient absorption.

The absence of significant differences in pancreata mass across treatment groups indicates that neither early weaning, fructose consumption, nor Moringa supplementation had a significant effect on pancreatic mass. Previous studies have shown that high-sugar diets can increase organ mass, including the pancreas (Rospond *et al.*, 2022), but this was not observed in the current study. Neither were there any changes in the liver or kidney masses, as presented earlier in both Chapter 3 and 4.

Research has shown that modulation of cytokines associated with obesity, insulin resistance and dyslipidaemia could result in pancreatic inflammation, β cells damage, hypertrophy, apoptosis and eventually an increase or decrease of the mass of the pancreas (Rebours *et al.*, 2018). This study has shown no impact of treatments regarding the pancreatic mass. This is despite the insulin resistance, increased and decreased obesity associated cytokines (LEP and ADP) and dyslipidaemia (increased LDL, TG and TC and decreased HDL) observed from the early weaning with or without fructose results presented in Chapter 3. This is most likely due to the fact that for organ mass changes to occur, there is need for severe or sustained long-term insults (Furman *et al.*, 2019). Organ mass changes, particularly in response to chronic metabolic disturbances or stress, can be valuable in predicting long-term complications, but such changes often require prolonged or significant physiological insults (Kivimäki, Bartolomucci and Kawachi, 2023). Therefore, to assess the effects of early weaning with different diets on pancreatic health, crucial biomarkers such as histology of the pancreas and related cells (alpha, beta, delta, pancreatic polypeptide cells, and epsilon cells) will be considered in our future studies on Moringa.

Analysis of the erythrocyte indices revealed no significant differences in haemoglobin concentration (Hb), haematocrit (Hct), and mean corpuscular haemoglobin concentration (MCHC) among the treatment groups. However, the results contrast with others that have linked high sucrose/fructose intake to altered haematological profiles. In rats with metabolic dysfunction, haematocrit, MCHC and haemoglobin concentration were significantly decreased and haemoglobin was increased (Monteomo, Kamagate and Yapo, 2018). These changes reflect the response of the body to the metabolic stresses imposed on the rats (Monteomo, Kamagate and Yapo, 2018). In compensation, the body increased haemoglobin

production in the remaining red blood cells, even when their numbers were reduced (Monteomo, Kamagate and Yapo, 2018; Qiu *et al.*, 2022). Studies in Sprague-Dawley female rats fed a fructose-rich diet, found elevated haemoglobin, which may be attributed to increase due to oxidative stress, low-grade inflammation, dehydration, and hormonal changes, all of which drive adaptive erythropoiesis to support oxygen delivery and counteract metabolic stress (Tran, Yuen and McNeill, 2009).

The lack of significant impact on erythocytes indices suggests that the interventions, including early weaning with or without fructose and the two weeks post-weaning Moringa supplementation, may not have been severe enough to induce changes in red blood cell health. This indicates that the degree of metabolic stress or dysfunction induced by these interventions was likely mild, or the duration of the intervention was too short to cause detectable alterations in erythrocyte indices. Therefore, while the interventions may have had some metabolic effects, they did not appear to cause substantial alterations in erythropoiesis or overall red blood cell function. This could point to the need for more prolonged or intensified interventions to observe changes in erythrocyte health and function. Furthermore, the absence of red blood cell count measurements limited the ability to comprehensively assess haematopoietic responses and potential hormonal influences such as erythropoietin activity, which could have provided clarity into the haematological and renal implications of fructose intake and Moringa supplementation.

5.5. Conclusion

This chapter aimed to assess the effect of early weaning and Moringa supplementation on various physiological parameters, including body composition, pancreas mass, erythrocyte indices, and fluid intake, with a specific focus on the potential protective effects of Moringa in mitigating fructose-induced metabolic disturbances. The findings suggest that while early weaning with a high fructose diet led to changes in body mass, the supplementation of Moringa at a high dose during the first two weeks post-weaning effectively prevented the adverse effects of fructose on body mass. The study highlighted the potential of Moringa to regulate appetite, as evidenced by the reduced impact of fructose on food and fluid consumption in Moringa-supplemented groups. Despite these findings, linear growth, as indicated by body length and other morphometric measures, was not significantly affected by the interventions, suggesting that the impact of early weaning or Moringa supplementation

did not impair overall growth. Furthermore, the dietary interventions did not influence erythrocyte indices, which are important indicators of systemic health, indicating that the metabolic stress induced by early weaning and fructose consumption was not severe enough to impact red blood cell health. The study suggests that Moringa supplementation may offer some protective benefits against fructose-induced metabolic stress, particularly regarding appetite regulation and body mass. However, more extended supplementation periods are needed to observe more profound, long-term effects on metabolic health.

The results of this study are important for resource-poor communities because they suggest that Moringa supplementation, particularly at high doses, may have potential benefits for improving body mass and possibly enhancing nutrient absorption. This could be beneficial in regions where malnutrition and nutrient deficiencies are prevalent.

The following chapter is a concluding chapter. The chapter summarises the key findings of the research, reaffirming the long-term potential protective effects of *Moringa oleifera* when administered for only two weeks post-weaning against metabolic, MAFLD and renal dysfunctions induced by early weaning and high-fructose diets. The chapter further discusses the broader implications of the results and the relevance to human health. It also addresses the limitations of the study and suggests areas for future research.

CHAPTER SIX: CONCLUSIONS, LIMITATIONS AND DIRECTIONS FOR FUTURE STUDIES

6.1. Conclusion

The main aim of this study was to assess the metabolic outcomes of early weaning in a multipronged approach focusing on long-term metabolic impact of:
i. early weaning onto a normal diet,
ii. early weaning onto a normal diet with supplementary hydroethanolic leaf extracts of Moringa in the first two weeks post-weaning,
iii. Early weaning onto a high fructose diet until termination and
iv. Early weaning onto a high fructose diet with supplementary hydroethanolic leaf extracts of Moringa in the first two weeks post-weaning.

Hence the metabolic programming potential of a strategic two weeks administration of Moringa hydroethanolic leaf extract post-weaning against long-term fructose-induced metabolic dysfunction was explored. The focus areas were general metabolic dysfunction and target organs responsible for significant morbidities and mortalities presenting as metabolic-associated fatty liver disease (MAFLD) and renal dysfunction, using an early-weaned (PD 18) rat model.

i. Long-term metabolic impact of early weaning onto a normal diet

Early weaning did not significantly affect body mass or growth performance. There were no significant differences in measures of body composition and fat distribution (Waist circumference, body mass index (BMI), waist-to-body length ratio (WtBLR) and visceral fat mass). However, early weaning had significant impact on the hormonal profile of the rats. Early weaning increased serum leptin concentrations and reduced adiponectin concentrations. These hormonal disruptions are critical as they play a major role in regulating appetite and insulin sensitivity, energy expenditure, and inflammation. Therefore, by increasing serum leptin and decreasing adiponectin concentrations, early weaning resulted in leptin resistance, promotion of inflammation and decreased insulin sensitivity. Early weaning also resulted in decreased serum HDL, which is a marker of dyslipidaemia. All this consequently resulted in insulin resistance, and metabolic dysfunction. However, since early weaning did not affect body mass and visceral adiposity, it only caused lean metabolic dysfunction and not obesity.

The impact of early weaning on liver function and structure was significant, predisposing the rats to the development of MAFLD. Histological analysis of the liver revealed steatosis, hypertrophy, and inflammation. The quantification of serum lipid content further supported

these findings, as early-weaned rats showed reduced levels of HDL. Moreover, there was an increase in serum ALT concentration. These changes suggest that early weaning disrupts normal liver metabolism, making the liver more susceptible to fat accumulation and subsequent MAFLD.

The study further explored molecular mechanisms and showed that early weaning altered the expression of key genes involved in lipid metabolism, which were *SREBP-1c*, *ACC-1*, *CPT-1*, *PPAR-α*, and *FAS*. These alterations disrupted the balance between lipid synthesis and degradation, further contributing to hepatic injury, steatosis and MAFLD.

Early weaning alone did not significantly affect kidney mass, but renal histology revealed an enlargement of the renal corpuscular area (RCA) and glomerular tuft area (GTA), indicating early kidney dysfunction. There was a significant increase in NGAL concentrations, suggesting early renal stress. These findings suggest that early weaning alone results in early structural changes and stress within the kidney, suggesting the onset of renal dysfunction and potential impairment of kidney function.

ii. Long-term impact of early weaning onto a high fructose diet until termination

Early weaning combined with a high fructose diet worsened the development of metabolic dysfunction more severely than early weaning alone. The inclusion of fructose led to a pronounced increase in blood glucose levels, indicating a deterioration in glucose metabolism. Insulin resistance was significantly worsened in the fructose-fed group, as indicated by elevated insulin levels and disrupted hormonal profiles, including a further increase in serum leptin and a decrease in adiponectin. The high-fructose diet had a substantial impact on the development of MAFLD in early-weaned rats. Hepatic steatosis was significantly more extensive, with large fat droplets present in hepatocytes, suggesting a more severe form of fatty liver disease. This was accompanied by markedly further elevated levels of ALT, indicating a more severe form of fatty liver disease. Additionally, circulating serum LDL, triglycerides and total cholesterol were elevated in the early-weaned fructose fed rats, with a further decrease in HDL concentrations. At the molecular level, there was a significant upregulation of m-RNA expressions of lipogenic genes as indicated by the elevated expressions of *SREBP-1c* and *FAS*. Concurrently, m-RNA expressions of genes involved in fatty acid oxidation, such as *CPT-1* and *PPAR-α*, were downregulated, indicating a disruption in lipid metabolism and an enhancement of lipogenesis. Therefore, early weaning with fructose resulted in the development of severe metabolic dysfunction,

including impaired glucose metabolism, insulin resistance, exacerbated hepatic steatosis, and dyslipidaemia, alongside molecular disruptions in lipid metabolism, leading to enhanced lipogenesis and reduced fatty acid oxidation.

In terms of renal function and structure, early weaning with fructose also worsened renal dysfunction observed from early weaning alone. Early weaning with fructose caused further significant increases in KIM-1, NGAL, and BUN levels, while creatinine levels were decreased. Renal histology showed further enlargement and deformation in structure of renal anatomical structures (BCA, RCA and GTA). Early weaning with fructose significantly increased the urea-to-creatinine (U: C) ratio. Both early weaning and early weaning with fructose did not significantly affect pancreatic mass or haematological parameters, although they resulted in lean metabolic dysfunction, MAFLD and renal structural changes.

iii. Long-term metabolic impact of early weaning onto a normal diet with supplementary hydroethanolic leaf extracts of Moringa in the first two weeks post-weaning

One of the notable findings was the increase in body weight in the high-dose Moringa diet group compared to the early-weaned control group. However, this increase was not linked to an increase in fat mass, as visceral fat remained unaffected by the short-term Moringa supplementation alone. These results suggest that post-weaning sort-term Moringa supplementation may promote muscle or lean mass gain, potentially through its bioactive compounds such as flavonoids and polyphenols, which are known for their anti-inflammatory and antioxidant properties. The absence of significant changes in visceral fat, which is an adiposity marker, is important, as it indicates that the weight gain was not due to fat accumulation, but likely due to lean tissue growth or muscle mass development. This is consistent with findings from studies on the potential of Moringa to enhance nutrient absorption and metabolic processes. In terms of metabolic markers, no significant differences were observed in glucose, insulin, or HOMA-IR, indicating that Moringa supplementation did not significantly alter insulin sensitivity or glucose metabolism compared to normal weaning with the control under the conditions of this study. Additionally, there were no significant changes in lipid metabolism markers such as LDL, HDL, TG, or TC, further suggesting that Moringa supplementation did not significantly alter lipid profiles in the short term.

Liver structure and function, as assessed by liver weight and relative mass, showed no significant changes with Moringa supplementation. This suggests that Moringa did not induce liver hypertrophy or alter liver function through liver enlargement or deduction in size. Similarly, the study found no significant changes in kidney function or structure, with creatinine, BUN, NGAL, KIM-1 and the U: C ratio remaining largely unaffected. These results suggest that short-term post-weaning Moringa supplementation did not have a harmful effect on renal function or structure later in life, nor did it exacerbate kidney stress or injury induced by early weaning. Furthermore, the two weeks only low-dose of Moringa supplementation post-weaning reduced the severity of hepatic steatosis.

The lack of significant changes in haematological indices, including haemoglobin, haematocrit, and MCHC, suggests that short-term Moringa supplementation post-weaning did not alter red blood cell health or oxygen-carrying capacity, which aligns with previous research highlighting the nutritional benefits of Moringa without significant effects on erythropoiesis under non-anaemic conditions.

iv. Long-term metabolic impact of early weaning onto a high fructose diet with supplementary hydroethanolic leaf extracts of Moringa in the first two weeks post-weaning

Early weaning combined with a low-dose of Moringa hydroethanolic leaf extract and long-term 20% fructose consumption demonstrated a protective effect against the development of lean metabolic dysfunction, MAFLD, and renal dysfunction in the female early-weaned rat model.

The low-dose of Moringa with fructose, even when administered for two-weeks only post-weaning managed to partially mitigate the adverse effects of early weaning and high fructose consumption. It improved insulin sensitivity, as evidenced by lower serum insulin concentrations and a more balanced hormonal profile, including higher serum adiponectin and lower leptin concentrations. Serum glucose concentrations were better regulated compared to the early-weaned and fructose-only groups, indicating an improvement in glucose metabolism.

Histological analysis revealed fewer and smaller fat droplets in the hepatocytes compared to the fructose-fed group without Moringa. Serum ALT concentrations were significantly lower, suggesting reduced liver injury. At the molecular level, there was a downregulation of m-RNA expressions of lipogenic genes such as *SREBP-1c* and *FAS*, alongside an upregulation

of m-RNA expressions of fatty acid oxidation genes like *CPT-1* and *PPAR-α*, indicating a shift towards improved lipid metabolism and reduced lipogenesis.

Renal function also showed improvement two weeks post-weaning with low-dose Moringa supplementation. Biomarkers of kidney injury, including CREA, BUN, KIM-1, and NGAL, were all lower compared to the fructose-only group. Histological examinations revealed reduced structural damage, accompanied by less glomerular hypertrophy, indicating the protective effects of Moringa on renal structural integrity. Additionally, oxidative stress and inflammatory markers in the kidneys were reduced, indicating a long-term protective effect against renal dysfunction of administering Moringa at high doses for a short period post-weaning.

High-dose Moringa supplementation with fructose provided even more significant protective effects against metabolic dysfunction, MAFLD, and renal dysfunction. The two weeks post-weaning administration of a high dose of Moringa, combined with long-term fructose consumption, significantly enhanced insulin sensitivity, as reflected by normalised insulin levels and improved hormonal balance, including further increased serum adiponectin and decreased leptin concentrations. Blood glucose levels were maintained closer to normal, showing a robust improvement in glucose metabolism. For liver health, the administration of a high dose of Moringa for only two weeks post-weaning combined with a long-term high fructose diet consumption showed a pronounced reduction in hepatic steatosis. Liver histology revealed minimal fat accumulation in hepatocytes, and serum ALT levels were comparable to those of the control, suggesting minimal liver injury. At the molecular level, there was a significant downregulation of m-RNA expression of *SREBP-1c* and *FAS*, and a marked upregulation of m-RNA expression of *CPT-1* and *PPAR-α*. This indicates that Moringa when administered for a short period post-weaning results in substantial improvement in lipid metabolism and reduced lipogenesis, protecting against development of MAFLD later in life.

The administration of Moringa high dose for two weeks only post-weaning combined with a long-term high fructose diet consumption also showed protective effects against renal dysfunction. Serum concentrations of renal injury biomarkers (CREA, BUN, KIM-1, and NGAL) were significantly lower than those in the fructose-fed group and did not differ significantly from the concentrations seen in the healthy, normally weaned (PD 21) control

group. Histological analysis revealed that the short-term administration of Moringa at a high dose post-weaning resulted in minimal structural damage in the kidneys, with normal glomerular and Bowman's capsule sizes and minimal fibrosis. Oxidative stress and inflammation markers serum concentrations (KIM-1 and NGAL) were also significantly reduced by Moringa high dose with fructose diet. This indicates that when administered for a short-term at post-weaning, Moringa strongly protects against renal dysfunction and structural damages later in life.

The administration of both low and high doses of Moringa for two weeks post-weaning, in combination with a long-term high-fructose diet, did not significantly affect body mass index, waist-to-body length ratio, and pancreatic mass, or haematological parameters. This indicates that the short-term administration of Moringa did not have a substantial impact on body composition, pancreatic mass, or blood cell health, suggesting that its effects may be more focused on metabolic or organ-specific functions rather than broad physiological changes such as adiposity, organ size, or erythrocyte function.

In comparison of the two doses used in this study, the effects of low-dose Moringa supplementation were mainly comparable to those observed in the normal weaning group, suggesting that the low dose did not significantly alter the metabolic or organ health outcomes compared to the normal weaning control group. This indicates that the low dose may not have been potent enough to induce substantial physiological changes. Alternatively, because its effects were consistent with those of the control, it may mean that the low dose did not produce any adverse effects and maintained a metabolic balance similar to that of normal weaning conditions. On the other hand, the high-dose Moringa supplementation was more effective in counteracting or preventing the negative effects of early weaning and a high-fructose diet. This suggests that the higher dose provided more substantial protective effects against the development of metabolic dysfunction, liver damage, and renal stress associated with early weaning and fructose consumption.

The null hypothesis for this study was that early weaning, a high fructose diet, and a two weeks administration of hydroethanolic Moringa leaf extract post-weaning would not significantly affect growth performance, metabolic dysfunction, liver function, or kidney function in female Sprague-Dawley rats. The alternative hypothesis suggested that early weaning, a high fructose diet, and a two weeks administration of hydroethanolic Moringa leaf

extract would significantly affect the growth performance, metabolic function, liver function, and kidney function in female Sprague-Dawley rats. The findings of this study support this alternative hypothesis, as early weaning and high fructose diets resulted in significant metabolic dysfunction, liver injury, and renal damage, while a two weeks administration of Moringa supplementation, especially at high dose provided protective effects, mitigating some of the adverse outcomes. Moringa achieved this through its anti-inflammatory and antioxidant properties. These results suggest that Moringa may offer a short-term post-weaning therapeutic intervention for programming long-term metabolic, hepatic and renal good health if early weaning cannot be avoided.

The results of this study indicate that Moringa may offer a viable short-term post-weaning therapeutic intervention for promoting long-term metabolic, hepatic, and renal health in populations affected by early weaning and high fructose diet. The finding that Moringa supplementation produced lasting effects even after only two weeks of administration post-weaning reinforces the concept of metabolic programming, where the metabolic systems of the body adapt during critical developmental stages, such as the weaning period. Even brief exposure to bioactive compounds found in Moringa, including polyphenols, flavonoids, and essential minerals, appears to programme the body for better metabolic health later in life. This highlights the potential for early interventions, even if short-term, to prevent chronic metabolic diseases such as metabolic-associated fatty liver disease (MAFLD), metabolic-induced renal dysfunction, and other related conditions like type 2 diabetes, cardiovascular disease, hypertension, dyslipidaemia, and obesity.

From an economic perspective, short-term interventions using Moringa hydroethanolic leaf extract as a dietary supplement post-weaning could offer a cost-effective strategy to prevent long-term health complications, which often require expensive medical interventions. If Moringa supplementation can effectively reduce the onset of metabolic diseases like MAFLD and chronic kidney disease (CKD), the long-term healthcare costs associated with treating these conditions could be substantially reduced. In resource-poor communities, where malnutrition and metabolic diseases are becoming increasingly prevalent, the use of Moringa as a low-cost therapeutic dietary supplement could offer a practical solution to alleviate healthcare burdens. Notably, short-term supplementation can offer affordable and scalable benefits compared to long-term pharmaceutical treatments, thereby promoting health equity in areas with limited access to healthcare resources.

The uniqueness of this study lies in its demonstration that Moringa supplementation during the critical post-weaning period can programme long-term health benefits, especially in terms of metabolic and organ health. This is particularly significant as early weaning practices, especially in developing countries, are unlikely to end in the near future. According to the World Health Organisation (WHO) and United Nations International Children's Emergency Fund (UNICEF), early weaning remains a common practice due to factors like socio-economic pressures, limited access to maternal milk, and public health policies encouraging the introduction of solid foods at an early age. Studies indicate that the rate of early weaning is rising globally, particularly in regions like South Africa, where urbanization and socio-economic challenges increasingly influence infant feeding practices. Given the growing prevalence of early weaning, the results of this study suggest that Moringa supplementation can serve as a viable intervention to counteract the long-term adverse effects of early weaning, offering a long-term solution to mitigate metabolic dysfunction, hepatic damage, and renal impairment in early-weaned individuals.

By integrating a short-term intervention, such as Moringa supplementation, during the critical post-weaning period, the health of individuals can be "programmed" for better metabolic outcomes in the long run. This underscores the importance of implementing Moringa as part of a strategic dietary approach to health management, particularly for populations affected by early weaning practices. Additionally, the ability of Moringa to offer protective effects across multiple organs, including the liver and kidneys, positions it as a valuable candidate for addressing widespread public health concerns, such as MAFLD, renal dysfunction, and insulin resistance.

In conclusion, while long-term consumption of Moringa may offer sustained benefits, the results of this study highlight the potential of short-term Moringa supplementation as a cost-effective, accessible, and sustainable intervention that can have significant long-term health benefits. The findings present a promising avenue for future interventions in regions where early weaning is prevalent, offering a simple and practical solution to prevent the onset of chronic metabolic diseases in individuals who may otherwise be at risk due to early weaning.

6.2. Limitations

Despite the significant findings of this study, several limitations should be acknowledged.

The study focused exclusively on female rats, which limits the generalisation of the results to both sexes. While female sex hormones, particularly oestrogen, can confer protection against insulin resistance and related metabolic complications, the study design age-matched pups within groups, ensured that any hormonal surges during adolescence would have been similar across all experimental and control groups. Significant differences observed between dietary intervention groups, such as Moringa-supplemented versus fructose-only rats, are therefore more likely attributable to the interventions rather than endogenous hormonal effects. Nonetheless, sex-specific differences in metabolic susceptibility, influenced by hormones, genetic factors, and gut microbiota composition, remain an important consideration. Future studies including male rats would provide a broader understanding of sex-dependent responses and the potential differential effects of dietary interventions.

The study utilised a crude hydroethanolic extract of Moringa leaves, which may not fully represent the bioactive components present in whole Moringa leaf powder or other forms of Moringa supplementation. While hydroethanolic extraction can capture a wide range of compounds due to the presence of both polar and non-polar solvents, it may not efficiently extract highly non-polar molecules found in Moringa leaves. Therefore, certain bioactive constituents, particularly those with strong non-polar characteristics, may have been underrepresented in the extract used in this study. Additionally, the exact concentration and nature of active ingredients in the hydroethanolic extract was not quantified, making it difficult to assess the relationship between the dose and the observed protective effects.

Furthermore, key markers of oxidative stress (such as malondialdehyde or superoxide dismutase) or inflammatory cytokines (such as TNF-α, IL-1β, and IL-6), which are often implicated in metabolic dysfunction and organ damage, were not measured. These markers could provide additional insights into the protective effects of Moringa, particularly in relation to the observed renal and hepatic improvements. Future studies should incorporate these markers to assess the role of oxidative stress and inflammation in mediating the effects of Moringa.

Additionally, while renal function was assessed using serum biomarkers and histological evaluations, the study did not measure glomerular filtration rate (GFR), a critical indicator of kidney function. Urine analysis of GFR would have provided a more direct and comprehensive measure of renal health, particularly in relation to kidney damage and the

potential nephroprotective effects of Moringa. Including GFR measurements would help better understand the extent of renal dysfunction and the protective effects of Moringa supplementation.

Furthermore, muscle mass was not measured, which could influence creatinine concentrations due to its dependence on muscle metabolism.
Moreover, oxidative stress markers such as malondialdehyde (MDA) and superoxide dismutase (SOD) were not measured in this study, due to technical limitations. Nevertheless, such information could have provided some information the balance between oxidative damage and antioxidant defences in the kidney tissue.

The study also did not include pancreatic histology, surrogate markers of pancreatic function and genes. While pancreatic mass was measured, no histological assessments of pancreatic cells (such as alpha, beta, delta, and epsilon cells) were performed. Given that the pancreas central role in regulating metabolism and insulin secretion, histological analysis would have provided additional information into how early weaning and high fructose diet affect pancreatic structure and function. This would complement the findings related to metabolic dysfunction and offer a more complete understanding of how Moringa may influence pancreatic health.

Additionally, the study did not quantify lipid profiles in detail. While lipid levels were assessed, a more in-depth lipidomic analysis of phospholipids, triglycerides, cholesterol esters, sphingolipids, and fatty acids would have provided additional information into the molecular mechanisms behind the effects of early weaning, high fructose diet and Moringa on lipid metabolism and its role in reducing the risk of conditions like dyslipidaemia, MAFLD and cardiovascular disease.

Another limitation of this study is that epigenetic mechanisms were not investigated. Epigenetic modifications, such as DNA methylation, histone modifications, and microRNA expression, play a critical role in mediating long-term metabolic programming during periods of developmental plasticity, such as the immediate post-weaning phase. Assessing these changes could provide mechanistic insights into how a brief two-week administration of hydroethanolic Moringa leaf extract post-weaning conferred lasting benefits on metabolic, hepatic, and renal health. For instance, Moringa bioactive compounds may influence gene

expression through epigenetic regulation, thereby promoting insulin sensitivity, reducing hepatic lipogenesis, and protecting renal structures. Future studies examining these epigenetic pathways could help elucidate the molecular basis of the long-term protective effects observed in this study.

6.3. Directions for future studies

Future studies should address the limitations identified in this study. First, future research should include male and female rats to account for potential sex-specific differences in metabolic responses to dietary interventions. Additionally, future studies should focus on quantifying the active ingredients in Moringa extracts and comparing different extraction methods. A focus could be on either whole-leaf powder or water extracts, which would be more accessible to beneficiaries without the need for complex processing associated with hydroethanolic extraction. They would also be cheaper, and there would be less chance of toxic residues from alcohol, etc.

Furthermore, future research should measure key markers of oxidative stress (such as malondialdehyde and superoxide dismutase) and inflammatory cytokines (such as TNF-α, IL-1β, and IL-6) to better assess the role of oxidative stress and inflammation in mediating the effects of early weaning, high fructose diet and Moringa. In addition, incorporating glomerular filtration rate (GFR) analysis would provide a more direct and comprehensive measure of kidney function. Pancreatic histology should also be included in future studies to understand better the effects of early weaning, high fructose diet, and Moringa on pancreatic structure and function. Lastly, a detailed lipidomic analysis should be conducted to profile and quantify lipid profiles. Addressing these limitations will provide a more holistic understanding of the effects of early weaning and a high fructose diet on broader metabolism function and the therapeutic potential of Moringa and its mechanisms of action in metabolic and organ-specific diseases.

It could clarify how Moringa influences gene expression related to energy metabolism and fat storage. For instance, examining DNA methylation and histone modifications in key metabolic genes, such as Peroxisome Proliferator-Activated Receptor Gamma (*PPARγ*) in adipose tissue and AMP-activated Protein Kinase (*AMPK*) in muscle, could shed light on how Moringa impacts metabolic pathways at the epigenetic level. Future studies could also

incorporate transcription factor (TF) binding predictions using databases such as JASPAR and targeted DNA methylation analyses to further elucidate the precise molecular mechanisms and regulatory pathways influenced by dietary interventions.

Additionally, detailed dietary assessments would allow for a more accurate understanding of the nutritional intake and how it might interact with Moringa supplementation to influence metabolic outcomes.

This could involve tracking the intake of macronutrients, micronutrients, and phytochemicals to determine their contribution to observed metabolic changes. Their absence in this study limited the comprehensive understanding of the further effects of early weaning on pancreatic health. Additionally, future studies should include immunohistochemistry of pro-inflammatory cytokines like IL-1, IL-6, and TNF-α, as well as related regulatory genes such as Pancreatic and Duodenal Homeobox 1 and Forkhead Box O1. This approach to assessing pancreatic health using both cellular and molecular markers will help clarify the effects of early weaning and diet on metabolic function, which may also extend to haematological changes observed in metabolic stress.

CHAPTER SEVEN: REFERENCES

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APPENDICES

APPENDIX 1: Study Ethical Clearance

1. **Ethical Clearance Certificate**

AN IMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2020/08/04/B

APPLICANT: Ms M Mosana
School: School of Physiology; Department: N/A; Location: CAS

PROJECT TITLE: The potential prophylactic effect **of Moringa (Moringa oleifera** Lam.) leaf extracts against **fructose- induced metabolic dysfunction in early weaned female Sprague-Dawley** rats;
Category: B; **Species and Numbers involved:** *48X 16 day old, female Sprague Dawley (Rattus Norvegicus)*

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 25 Aug 2020. This approval remains valid until 29 Sep 2022 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: __01/10/2020 _____
(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).

CC: Student supervisor: «Title1» «Initials1» «Supervisor surname»
Director Central Animals Service: Dr Kim Jardine

Signed: _____ _____ Date: _2/10/2020_____

(Registered Veterinarian)

2. Modifications and Extensions to Experiments 1

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: **Mmahiine Mosana**

b. Department: Physiology

c. Experiment to be modified / extended AESC NO

Original AESC number	**2020**	**08**	04B
Other M&Es :			1

d. Project Title: **The potential prophylactic effect of Moringa (Moringo *a/ei/era* torn.) leaf extracts against fructose-induced metabolic dysfunction in early weaned female Sprague-**Dawley rats.

		No.	Species
e.	Number and species of animals originally approved:	48	*Rattus novergicus*
f.	Number of additional animals previously allocated on M&Es:	8	*Rattus novergicus*
g.	Total number of animals allocated to the experiment to date:	56	*Rattus novergicus*
h.	Number of animals used to date:	*0*	*Rattus novergicus*

i. Specific modification / extension requested: I would like to add the following co-workers to my ethics, namely:

Dr Pilani Nkomozepi — staff member at the University of Johannesburg (assistance with histological samples analyses)

Dr Craig Grobbelaar — staff member at the University of Pretoria (technical assistance with SEM of blood samples and brain samples)

Reveshini Moodley — student at the University of Johannesburg (Student number: 201114158) — collection of intestinal samples far the assessment of gut micrabiota

Carrina Claire Marcus — student at the University of Johannesburg (Student number: 221053909) — collection of heart and aorta samples for histochemical analyses.

j. Motivation for modification / extension:

The above mentioned additional co-workers will assist with technical and animal work as well as sample collection/analysis in this project.

Date: 21/01/2021 Signature:

RECOMMENDATIONS

Approval of the additional co-workers (Dr P. Nkomozepi, Dr C. Grobbelaar, R. Moodley (UJ Student

number 201114158) and C. Marcus (UJ Student number 221053909) to the study as stipulated. Students registered with the University of Johannesburg have to obtain the necessary ethical clearance from their institution prior to the commencement of their research component. Co-workers directly involved with the animal handling aspect of the project are requested to attend the WRAF orientation course. Please contact Lorraine Matjila lorraine.matjila@wits.ac.za for further details.

Date:22 January 2021 Signature:

Deputy Chair, AESC

3. Modifications and Extensions to Experiments 2

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: **Mmahiine Mosana**

b. Department: **Physiology**

c. Experiment to be modified / extended AESC NO

Original AESC number	**2020**	**08**	04B
Other M&Es :			2

d. Project Title: **The potential prophylactic effect of Moringa (Moringo *a/ei/era* torn.) leaf extracts against fructose-induced metabolic dysfunction in early weaned female Sprague-**Dawley rats.

		No.	Species
e.	Number and species of animals originally approved:	48	*Rattus novergicus*
f.	Number of additional animals previously allocated an M&Es:	8	*Rattus novergicus*
g.	Total number of animals allocated to the experiment to date:	56	*Rattus novergicus*
h.	Number of animals used to date:	56	*Rattus novergicus*

Specific modification / extension requested:

- to add 2 more female rat pups to my study

-to include co-worker, namely Miss Chigoziem Ohaegbulam — student at the University of the Witwatersrand (Student number: 1475622)

j. Motivation far modification / extension:

-The above mentioned additional animals will replace animals that are going to be removed from the study due to poor performance (weight lag of *15% relative to the peersj. Therefore two additional rats will be required to ensure that the affected groups have the correct sample size for statistical power.

-Miss Ohaegbulam will assist with feeding and weighing as well as data collection in this project.

Date: 03/06/2021 Signature:

RECOMMENDATIONS

Approval for the addition of the two rat pups as requested and the additional of co-worker C. Ohaegbulam to the study. Please ensure that the new co-worker attends the WRAF orientation course prior to initiating any of the animal work. Please contact Dr Jardine for details (Kim Jardine@wits ac za).

Date:07 June 2021 Signature:

Otehp ty ir AESC

APPENDIX 2: Project Manuscript

1. Proof of manuscript submission

Mmahiine Mosana

From:	em.sajb.0.91ea8c.c960c7d4@editorialmanager.com on behalf of South African Journal of Botany <em@editorialmanager.com>
Sent:	Saturday, 08 March 2025 11:55
To:	Mmahiine Mosana
Subject:	Confirming submission to South African Journal of Botany

CAUTION: External email - verify sender before opening links or attachments. Report issues to ithelp@wits.ac.za.

This is an automated message.

Strategic administration of Moringa oleifera Lam. hydroethanolic leaf extracts in the first two weeks post-weaning protects against subsequent development of fructose-induced metabolic fatty liver disease (MAFLD) in early weaned female Sprague-Dawley rats

Dear Mrs Mosana,

We have received the above referenced manuscript you submitted to South African Journal of Botany.

To track the status of your manuscript, please log in as an author at https://www.editorialmanager.com/sajb/, and navigate to the "Submissions Being Processed" folder.

Thank you for submitting your work to this journal.

Kind regards,
South African Journal of Botany

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South African Journal of Botany

Strategic administration of Moringa oleifera Lam. hydroethanolic leaf extracts in the first two weeks post-weaning protects against subsequent development of fructose-induced metabolic fatty liver disease (MAFLD) in early weaned female Sprague-Dawley rats

--Manuscript Draft--

Manuscript Number:	SAJB-D-25-00886
Article Type:	Research Paper
Section/Category:	Ethnobotany
Keywords:	Early weaning; fructose; Moringa; metabolic programming; metabolic fatty liver disease (MAFLD); gene expression
Corresponding Author:	Mmahine Christina Mosana, MSc University of the Witwatersrand Johannesburg, Gauteng SOUTH AFRICA
First Author:	Mmahine Christina Mosana, MSc
Order of Authors:	Mmahine Christina Mosana, MSc
	Ashwell Ndhlala, PhD
	Abe Kasonga, PhD
	Trevor Tapiwa Nyakudya, PhD
	Kennedy Honey Erlwanger, PhD
Abstract:	Early weaning, often onto high fructose diets, is commonly practiced for socioeconomic and health related reasons. However, early weaning and high fructose diets can cause metabolic disorders including metabolic-associated fatty liver disease (MAFLD) later in life. Moringa oleifera (Moringa) possess numerous beneficial bioactivities. We investigated whether a two weeks administration of hydroethanolic Moringa leaf extract could protect against poor metabolic health in high fructose-fed early-weaned female rats. Female Sprague Dawley rat pups were weaned early (18 days old) onto either normal rat feed, a fructose-rich diet, a normal diet with Moringa extracts or a combination of high fructose with Moringa extracts for 18 days. The control group was weaned at 21 days old. They were then fed a high-fructose diet or a normal diet for 111 days. The rats were terminated on the 112th day. Body mass, liver histology, hormone and lipid profile analyses, and metabolic gene expression were determined to evaluate the metabolic health of the rats. Early weaning with fructose significantly induced ($p<0.05$, ANOVA) elevated concentrations of serum leptin, low density lipoprotein (LDL), triglyceride, total cholesterol and ala-nine transaminase. It also resulted in significantly increased ($p<0.05$) hepatic steatosis, hypertrophy, inflammation, area fraction of the collagen, peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element-binding protein-1c (SREBP-1c) gene expression. Early weaning with fructose also induced decreased ($p<0.05$) concentrations of serum insulin, adiponectin, high density lipoprotein (HDL) and carnitine palmitoyltransferase-1 (CPT-1) gene expression. The two weeks only administration of Moringa at early weaning pre-vented fructose induced metabolic impairments in adulthood. Specifi-cally, reduced hepatic steatosis scores and modulated gene expression related to lipogenesis and fatty acid oxidation ($p<0.05$). Moringa leaf extracts showed potential as a natural intervention for preventing MAFLD and associated metabolic disorders, even when administered for a short period post weaning. This suggests a need for further exploration of use of Moringa in weaning diets.

APPENDIX 3: Protocols

1. **Moringa supplementation dose calculations for Phase 1 intervention (PD18 – PD38)**

Table 1: Doses for Moringa low-dose intervention (50mg/kg)

Rat weight (g)	Moringa (mg) in Gelatine cubes (amount (1ml)) per rat	Moringa (mg) in Gelatine cubes (amount (10ml)) per rat	Moringa (mg) in Gelatine cubes (amount (100ml)) per rat
10	0,5	5	50
20	1	10	100
30	1,5	15	150
40	2	20	200
50	2,5	25	250
60	3	30	300
70	3,5	35	350
80	4	40	400
90	4,5	45	450
100	5	50	500
TOTAL AMMOUNT OF MOR needed (mg)	**27,5**	**275**	**2750**

Table 2: Doses for Moringa high dose intervention (500mg/kg)

Rat weight (g)	Moringa (mg) in Gelatine cubes (amount (1ml)) per rat	Moringa (mg) in Gelatine cubes (amount (10ml)) per rat	Moringa (mg) in Gelatine cubes (amount (100ml)) per rat
10	5	50	500
20	10	100	1000
30	15	150	1500
40	20	200	2000
50	25	250	2500
60	30	300	3000
70	35	350	3500
80	40	400	4000
90	45	450	4500
100	50	500	5000
TOTAL AMMOUNT OF MOR needed (mg)	**275**	**2750**	**27500**

NB: PD18 Weight (g) range (30 – 40)

PD21 Weight (g) range (50 – 60)

2. Lipids and Hormones ELISA Protocols

Total Cholesterol (TC) Colorimetric Assay Kit

(Single Reagent, COD-PAP Method)

Catalog No: E-BC-K109-S

Method: Colorimetric method

Specification: 100 Assays (Can detect 96 samples without duplication)

Instrument: Spectrophotometer

Sensitivity: 0.09 mmol/L

Detection range: 0.09-25.85 mmol/L

Elabscience®

Assay protocol

▲Operating steps

1. Blank tube: Add 10 μL of double distilled water into a 2 mL EP tube.
   Standard tube: Add 10 μL of reagent 2 into a 2 mL EP tube.
   Sample tube: Add 10 μL of sample into a 2 mL EP tube.
2. Add 1000 μL of reagent 1 and mix fully.
3. Incubate at 37℃ for 10 min. Set the spectrophotometerto zero with double distilled water and measure the OD values of each tube at 550 nm with 0.5 cm diameter cuvette.

▲Operation table

	Blank tube	Standard tube	Sample tube
Double distilled water (μL)	10		
Standard (μL)		10	
Sample (μL)			10
Reagent 1 (μL)	1000	1000	1000
Mix thoroughly and incubate at 37°C for 10 min. Set the spectrophotometer to zero with double distilled water and measure the OD values of each tube at 510 nm with 0.5 cm optical path quartz cuvette.			

Triglyceride (TG) Colorimetric Assay Kit

(Single Reagent, GPO-PAP Method)

Catalog No: E-BC-K238

Method: Colorimetric method

Specification: 96T (Can detect 92 samples without duplication)

Instrument: Microplate reader, biochemical analyzer

Sensitivity: 0.14 mmol/L

Detection range: 0.14-9.5 mmol/L

Assay protocol	
Ambient temperature	25-30℃
Optimum detection wavelength	510 nm

Instructions for the use of transferpettor:

(1) Equilibrate the pipette tip in that reagent before pipetting each reagent.

(2) Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Assay protocol

▲Plate set up

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85
B	B	B	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86
C	S1	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87
D	S2	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88
E	S3	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
F	S4	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
G	S5	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S91
H	S6	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S92

Note: A, blank wells; B, standard wells; S1-S92, sample wells.

▲Operation table

Operate with microplate reader			
	Blank well	Standard well	Sample well
Double distilled water (μL)	2.5		
Standard (μL)		2.5	
Sample (μL)			2.5
Working solution (μL)	250	250	250
Mix thoroughly, incubate at 37℃ for 10 min, measure the OD value at 510 nm with microplate reader.			

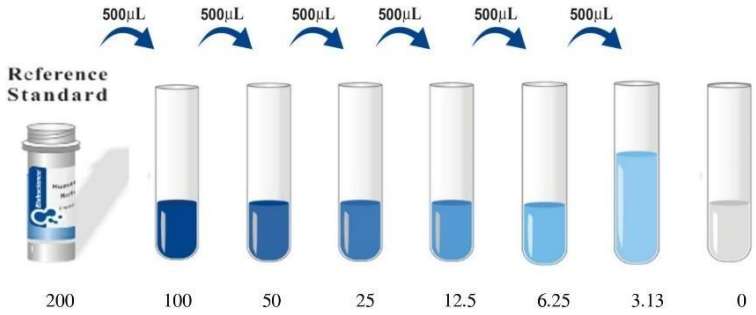
Operate with automatic biochemical analyzer	
Sample volume/ Double distilled water (μL)	2.5
Working solution (μL)	250
Incubate at 37℃for 10 min, set zero with distilled water + working solution, measure the absorbance value A at 510 nm.	
Main wavelength (nm)	510
Reaction type	Endpoint method
Reaction direction	(+)

Assay protocol for HDL, LDL, LEP, NGAL, ADP, KIM-1 and INS

NB: Each has its own specific standard working solutions, buffers and concentrations.

Reagent preparation

1. Bring all reagents to room temperature (18~25℃) before use. Follow the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.
2. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved
3. **Standard working solution:** Centrifuge the standard at 10,000×g for 1 min. Add 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 200 ng/mL. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 200, 100, 50, 25, 12.5, 6.25, 3.13, 0 ng/mL.
   Dilution method: Take 7 EP tubes, add 500uL of Reference Standard & Sample Diluent to each tube. Pipette 500uL of the 200 ng/mL working solution to the first tube and mix up to produce a 100 ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to these steps. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



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

5th Edition, revised in May, 2020

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

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

3. Aurum™ total RNA mini extraction kit Protocol

Animal and Plant Tissue

Follow steps D1—DS, then continue with step 1 of “All Starting Sample Types" on page 13.

DI. Cut the tissue into small pieces (<5 mm long) and grind it into a tne powder with a monar and pestle containing liquid nitrogen. Make sure that the tissue does not tnaw by periodically adding liquid nitrogen to the molar.

D2. Transfer up to 20 mg nard animal tissue, up to 40 mg sob animal tissue, or up to 60 mg plant tissue into an RNase-free 2.0 ml caDped microcentrifuge tube (provided).

Note: Plant tissue requires the Aurum lysis solution to be supplementea with 2% (w/v) polyvinylpyrrolidone-40 (PVP). For each column processed, add 14 ul PVP to every 700 II of lysis solution before proceeding to step 3.

D3. Add 700 pl of lysis solution to tne tube. Resuspend tne sample by pipetting up and down. Use a rotor-stator homogenizer for 30—60 sec or otner equivalent method to disrupt the sample.

D4. Centrifuge the lysate for 3 min and transfer the supernatant into a new 2.0 ml capped microcentrifuge tube (provided).

D5. Add 700 pl of 70% ethanol for plant tissue or 60% ethanol for animal tissue to the supernatant. Mix thoroughly by pipetting up and down or by using a rotor-stator homogenizer. Make sure tnat no bilayer is visible.

All Starting Sample 3gpes:

1. Attach an Aurum total RNA binding column to a luer fitting of the column adaptor plate on the Aurum vacuum manifold or to a compatible vacuum manifold. Refer to Figure 2 for setup. The vacuum source should be turned off and tne vacuum regulator should be completely open.

2. Decant or pipet the homogenized lysate into the RNA binding column. Turn tne vacuum on and adjust to —J 7 to —23 inHg by closing the vacuum regulator. Continue to apply vacuum until all tne lysate has passed tnrough the column. Open the vacuum regulator until the gauge indicates 0 inHg.

3. The low stringency wasn solution is provided as a 5x concentrate. Add 4 volumes (80 ml) of 95-100% ethanol to the low stringency wash solution concentrate before initial use.

4. Add 700 II of low stringency wash solution to the RNA binding column and close tne vacuum regulator dial until the gauge indicates —17 to 23 inHg. Continue to apply the vacuum until the low stringency wash solution has passed tnrough the column. Open tne vacuum regulator until the gauge indicates 0 inHg.

5. The RNase-free DNase I is provided as a lyophilized powder. Reconstitute the DNase I by adding 250 pl 10 mM Tris, pH 7.5 (not provided) to the vial and pipetting up and down briefly to mix.

6. For eacn column processed, mix 5 pl of reconstituted DNase I with 75 ul of DNase dilution solution in a 1.5 ml microcentrifuge tube (not provided). Scale up proportionally if processing more than one column. Add 80 II of diluted DNase I to the membrane stack at the bottom of each column. Allow the digest to incubate at room temperature for 15 min (25 min for animal tissue).

7. Add 700 II of hign stringency wasn solution to the RNA binding column and close tne vacuum regulator dial until the gauge indicates —17 to —23 inHg. Continue to apply the vacuum until the hign stringency wasn solution has passed tnrough the column. Open tne vacuum regulator until the gauge indicates 0 inHg.

8. Add 700 pl of low stringency wash solution to the RNA binding column and close tne vacuum regulator dial until the pauge indicates —17 to —23 inHg.

9. Transfer the RNA binding column to a 2 ml capless tube (provided). Centrifuge for an additional 2 min to remove residual wash solution.

10. Transfer the RNA binding column to a 1.5 ml capped microcentrifuge tube (provided). Pipet 80 II (or 40 uI)t of the elution solution onto the membrane stack at the bottom of the RNA binding column and allow 1 min for the solution to saturate the membranes. Centrifuge for 2 min to elute the total RNA.

    tNote: Pipet 40 pl when isolating total RNA from small amounts ol starting material (<10 mg of tissue or 500,000 cells).

    The eluted total RNA samples can be used immediately in downstream applications. Alternatively, the total RNA can be aliquoted stored at —20°C or at —80°G for later use.

Spin Protocol

Important: Please read Section 5, “Before Using the Aurum™ Total RNA Mini Kit,” before proceeding.

The Aurum total RNA mini kit can be used with any commercially available microcentrifuge that can accommodate 1.5 and 2.0 ml tubes. All centrifugation steps are performed at maximum speed (>12,000 x g) at room temperature.

Note: Except for the first few steps that are specific for the starting sample types (A. for cultured cell lines, B. for bacteria, C. for yeast D. for animal and plant tissue), the remaining procedures within "All Starting Sample Types" share a common protocol.

Animal and Plant Tissue

Follow steps D1–D5, then continue with step 1 of "All Starting Sample Types" on page 17.

D1. Cut the tissue into small pieces (<5 mm long) and grind it into a fine powder with a mortar and pestle containing liquid nitrogen. Make sure that the tissue does not thaw by periodically adding liquid nitrogen to the mortar.

D2. Transfer up to 20 mg hard animal tissue, up to 40 mg soft animal tissue, or up to 60 mg plant tissue into an RNase-free 2.0 ml capped microcentrifuge tube (provided). Let the tissue thaw before adding the lysis solution or precipitation may occur.

Note: Plant tissue requires the Aurum lysis solution to be supplemented with 2% (w/v) polyvinylpyrrolidone-40 (PVP). For each column processed, add 14 µl PVP to every 700 µl of lysis solution before proceeding to step 3.

D3. Add 700 µl of lysis solution to the tube. Disrupt the sample by pipetting up and down. Use a rotor-stator homogenizer for 30–60 sec or other equivalent method to disrupt the sample.

D4. Centrifuge the lysate for 3 min and transfer the supernatant into a new 2.0 ml capped microcentrifuge tube (provided).

D5. Add 700 µl of 70% ethanol for plant tissue or 60% ethanol for animal tissue to the supernatant. Mix thoroughly by pipetting up and down or by using a rotor-stator homogenizer. Make sure that no bilayer is visible.

All Starting Sample Types

1. Insert an RNA binding column into a 2 ml capless wash tube (provided).
2. Decant or pipet the homogenized lysate into the RNA binding column. Centrifuge for 30 sec (for cultured, bacterial, or yeast cells) or 60 sec (for animal or plant tissue). Remove the RNA binding column from the wash tube, discard the filtrate from the wash tube, and replace the column into the same wash tube.
3. The low stringency wash solution is provided as a 5x concentrate. Add **4 volumes (80 ml) of 95–100% ethanol to the low stringency wash solution concentrate before initial use.**
4. Add 700 µl of low stringency wash solution to the RNA binding column. Centrifuge for 30 sec. Discard the low stringency wash solution from the wash tube and replace the column into the same wash tube.
5. The RNase-free DNase I is provided as a lyophilized powder. Reconstitute the DNase I by adding 250 µl 10 mM Tris, pH 7.5 (not provided) to the vial and pipetting up and down briefly to mix.

6. For each column processed, mix 5 µl of reconstituted DNase I with 75 µl of DNase dilution solution in a 1.5 ml microcentrifuge tube (not provided). Scale up proportionally if processing more than one column. Add 80 µl of diluted DNase I to the membrane stack at the bottom of each column. Allow the digest to incubate at room temperature for 15 min (25 min for animal tissue).

7. Add 700 µl of high stringency wash solution to the RNA binding column. Centrifuge for 30 sec. Discard the high stringency wash solution from the wash tube and replace the column in the same wash tube.

8. Add 700 µl of low stringency wash solution to the RNA binding column. Centrifuge for 1 min (for cultured, bacterial, or yeast cells) or 30 sec (for animal or plant tissue). Discard the low stringency wash solution from the wash tube and replace the column in the same wash tube.

9. Centrifuge for an additional 2 min to remove residual wash solution.

10. Transfer the RNA binding column to a 1.5 ml capped microcentrifuge tube (provided). Pipette 80 µl (or 40 µl)† of the elution solution onto the membrane stack at the bottom of the RNA binding column and allow 1 min for the solution to saturate the membranes. Centrifuge for 2 min to elute the total RNA.

    †Note: Pipet 40 µl when isolating total RNA from small amounts of starting material (<10 mg of tissue or 500,000 cells).

    The eluted total RNA samples can be used immediately in downstream applications. Alternatively, the total RNA can be stored at –20°C or at –80°C for later use.

4. Protocol for LunaScript® RT SuperMix Kit (E3010)

1. Mix components briefly and spin down if necessary.
2. Prepare cDNA synthesis reaction as described below:

COMPONENTS	20 µl REACTION	FINAL CONCENTRATION
LunaScript RT SuperMix (5x)	4 µl	1x
RNA Sample	variable	(up to 1 µg)*
Nuclease-free Water	to 20 µl	

3. For no-RT control reaction, mix the following components:

COMPONENTS	20 µl REACTION	FINAL CONCENTRATION
No-RT Control Mix (5x)	4 µl	1x
RNA Sample	variable	(up to 1 µg)*
Nuclease-free Water	to 20 µl	

4. *Up to 1 µg total RNA, 1 µg mRNA or 100 ng specific RNA can be used in a 20 µl reaction. However, the cDNA input for downstream qPCR detection should typically contain < 10^9 copies of the target to ensure that quantitation remains linear. To accommodate larger amounts of input RNA (> 1 µg), the reaction should be scaled up to ensure optimum cDNA synthesis.

   For no template controls, mix the following components:

COMPONENTS	20 µl REACTION	FINAL CONCENTRATION
LunaScript RT SuperMix (5x)	4 µl	1x
Nuclease-free Water	to 20 µl	

5. Incubate reactions in a thermocycler with the following steps:

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	10 minutes	
Heat Inactivation	95°C	1 minute	

Protocol accessed from https://www.neb.com/en/protocols/2018/01/31/protocol-for-lunascript-rt-supermix-kit-e3010

5. SensiMix SYBER No-ROX kit Protocol

Storage and Stability:
SensiMix SYBR® No-ROX Kit is shipped on dry/blue ice. All kit components should be stored at -20°C upon receipt. Excessive freeze/thawing is not recommended.

Expiry:
When stored under the recommended conditions and handled correctly, full activity of the kit is retained until the expiry date on the outer box label.

Quality Control:
SensiMix SYBR® No-ROX Kit and its components are extensively tested for activity, processivity, efficiency, heat activation, sensitivity, absence of nuclease contamination and absence of nucleic acid contamination prior to release.

Safety Precautions:
Please refer to the material safety data sheet for further information.

Notes:
For research or further manufacturing use only.

Trademarks:
SensiMix and SensiFAST (Bioline Reagents Ltd), SYBR (Molecular Probes), ROX, iCycler MyiQ5, Opticon, Chromo4, Miniopticon, (Bio-Rad), LightCycler (Roche), StepOne (ABI), SmartCycler (CEPheid), RotorGene (Qiagen), RealPlex (Eppendorf), Quantica (Techne), MX4000 (Stratagene).

SensiMix™ SYBR® No-ROX Kit

Shipping: On Dry/Blue Ice Catalog Numbers

Batch No.: See vial

Concentration: See vial QT650-05: 500 x 50 µL reactions: 10 x 1.25 mL

Store at –20 °C

meridian BIOSCIENCE™

Description

The SensiMix™ SYBR® No-ROX Kit is a high-performance reagent designed for superior sensitivity and specificity on various real-time instruments, in which a passive reference signal is not required. The SensiMix SYBR® No-ROX Kit employs a hot-start DNA polymerase, for high PCR specificity and sensitivity. SensiMix is inactivated and possesses no polymerase activity during the reaction set-up, preventing non-specific amplification including primer-dimer formation.

For ease-of-use and added convenience, SensiMix SYBR® No-ROX is provided as a 2x master mix containing all the components necessary for real-time PCR (qPCR), including the SYBR® Green I dye, dNTPs, stabilisers and enhancers. As a ready-to-use premix, only primers and template need to be added.

Kit components

Reagent	500 x 50 µL reactions
SensiMix™ SYBR® No-ROX (2x)	10 x 1.25 mL(12.5 mL)

Kit compatibility

The SensiMix SYBR® No-ROX Kit contains premixed SYBR® Green I dye for compatibility with real-time instruments that do not need a passive reference signal for normalization of the data. The SensiMix SYBR® No-ROX Kit is optimized for use on the real-time instruments listed in the following compatibility table.

Manufacturer	Model
Bio-Rad	Opticon™, Opticon2™, MiniOpticon, Chromo4™, CFX96, CFX384
Cepheid	SmartCycler™
Qiagen	Rotor-Gene™ 3000 & 6000
Eppendorf	Mastercycler ep Realplex, ep Reaplex 2S
Roche	LightCycler® 480
Techne	Quantica®
BMS	Mic
Takara	Thermal Cycler Dice® TP800

General considerations

To help prevent any carry-over DNA contamination we recommend that separate areas be maintained for PCR set-up, PCR amplification and any post-PCR gel analysis. It is essential that any amplified PCR product should not be opened in the PCR set-up area.

Primers: the sequence and concentration of primer as well as the amplicon length can be critical for specific amplification, yield and overall efficiency of any qPCR. We strongly recommend taking the following into consideration when designing and running your PCR reaction:

- use primer-design software, such as Primer3 or visual OMP™ (http://frodo.wi.mit.edu/primer3/ and DNA Software, Inc ; http://dnasoftware.com/ respectively). Primers should have a melting temperature (Tm) of approximately 60 °C
- optimal amplicon length should be 50-150 bp
- a final primer concentration of 250 nM is suitable for most PCR conditions, however to determine the optimal concentration we recommend a primer titration in the range of 0.1–1 µM
- use equimolar primer concentrations
- when amplifying from cDNA use gene-specific primers. If possible use intron-spanning primers to avoid amplification from genomic DNA

Template: it is important that the DNA template is suitable for use in PCR in terms of purity and concentration. Also, the template needs to be devoid of any contaminating PCR inhibitors (e.g. EDTA). The recommended amount of template for PCR is dependent upon the type of DNA used. The following should be considered when using genomic DNA and cDNA templates:

- **Genomic DNA:** use up to 1 µg of complex (e.g. eukaryotic) genomic DNA in a single PCR. We recommend using the ISOLATE II Genomic DNA Mini Kit (BIO-52066) for high yield and purity from both prokaryotic and eukaryotic sources
- **cDNA:** the optimal amount of cDNA to use in a single PCR is dependent upon the copy number of the target gene. We suggest using 100 ng cDNA per reaction, however it may be necessary to vary this amount. To perform a two-step RT-PCR, we recommend using the SensiFAST cDNA Synthesis Kit (BIO-65053) for reverse transcription of the purified RNA. For high yield and purity of RNA, use the ISOLATE II RNA Mini Kit (BIO-52072)

$MgCl_2$: The $MgCl_2$ concentration in the 1x reaction mix is 3 mM, which is optimal for SensiMix in the majority of qPCR conditions. If necessary, we suggest titrating $MgCl_2$ to a maximum of 5 mM.

PCR Controls: It is important to detect contamination by DNA that may affect the reliability of the data. Always include a no-template control (NTC), replacing the template with PCR-grade water. When performing a two-step RT-qPCR, set-up a no-RT control as the NTC for the PCR.

Procedure

Reaction mix composition: Prepare a PCR master mix. The volumes given below are based on a standard 50µl final reaction mix and can be scaled accordingly.

Reagent	Volume	Final concentration
2x SensiMix™ SYBR® No-ROX	25 µL	1x
25 µM Forward Primer	0.5 µL	250 nM
25 µM Reverse Primer	0.5 µL	250 nM
H_20	Up to 45 µL	-
Template	5 µL	
	50 µL Final volume	

Suggested thermal cycling conditions

The PCR conditions described below are suitable for SensiMix SYBR® No-ROX Kit for the majority of amplicons and qPCR instruments. However, the cycling conditions can be varied to suit customer or machine-specific protocols. The critical step of the PCR is the 10 minute initial activation at 95 °C. The detection channel on the real-time instrument should be set to (SYBR®) Green or FAM.

Cycles	Temperature	Time	Notes
1	*95 °C	*10 min	Polymerase activation
40	95 °C 55-60 °C 72 °C	15 s 15 s 15 s	Temp. depends on the Tm of primers Acquire at end of step

***Non-variable parameter**

Optional analysis:
After the reaction has reached completion refer to the instrument instructions for the option of melt-profile analysis.