EFFICACY OF TWO MEDICINAL PLANT EXTRACTS AND METFORMIN IN THE PREVENTION OF DIET INDUCED FATTY LIVER

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2015

Efficacy of two medicinal plant extracts and metformin in the prevention of diet induced fatty liver

By

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Submitted in fulfilment of the requirements for the degree of Philosophiae Doctor in Biochemistry at the Nelson Mandela Metropolitan University

August 2012

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DECLARATION

I, *Shonisani Cathphonia Tshidino (206037511)*, hereby declare that the *thesis* for the *degree of Philosophiae Doctor in Biochemistry* is my own work and that it has not previously been submitted for assessment or completion of any postgraduate qualification to another University or for another qualification.

Shonisani Cathphonia Tshidino

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to:

- The almighty GOD for making all things possible for me.
- My promoter Prof Saartjie Roux for the interest she has shown in this study, inspiration, guidance, input into the study, invaluable help and sharing her knowledge on the study subject**.**
- My co-promoter Dr Trevor Koekemoer for the interest he has shown in this study, guidance and input into the study.
- The National Research Foundation (NRF) and NMMU/Vodacom postgraduate scholarship for the financial support.
- The Value Added Life (South Africa) for their generous gift of *Sutherlandia frutescens* used in the study.
- The Kenyan Medical Research Foundation (KMRF) and Dr Mathue for generous gift of *Prunus africana* extract used in the study.
- Prof Ryno Naude for his encouragement and financial support.
- My fellow postgraduate students for their support and encouragement.
- The staff members of the Department of Biochemistry and Microbiology for providing a working laboratory.
- My father Mr Ravhembelani Richard Tshidino and my mother Mrs Salminah Muofhe Mukovhi-Tshidino, for their prayers and financial support. I would not have completed this study if it was not for their love and encouragements.
- My friends and family for their generous help in time of needs.

DEDICATION

I dedicate this study to my father, Mr Ravhambelani Richard Tshidino and mother, Mrs Muofhe Salminah Mukovhi-Tshidino, who have been my inspirations in life. I also dedicate this study to my siblings, Hulisani Sister, Ndivhuweni Marvelous, Tshilidzi Marcia and Fhumulani Kipson Tshidino, for their moral support. I could not have finished this study if it was not through their love and encouragements.

ABSTRACT

Non-alcoholic fatty liver diseases (NAFLD) is manifested in the absent of alcohol abuse. This disease is the major cause of liver failure and death among adults and children worldwide, including South Africa. Its increasing prevalence urges the need of therapeutic intervention. The main objectives of this study were to investigate the following: (1) The effect of 38.9% high fat diet (HFD)-induced insulin resistance and fatty liver in male Wistar rats, (2) The efficacy of aqueous extracts from *Sutherlandia frutescens* leaves and *Prunus africana* bark and metformin in the treatment of HFD-induced insulin resistance and fatty liver. Male Wistar rats were fed on HFD (the HF group) or normal rat chow (the LF group) for 12 weeks. Even though the HFD-fed rats had developed insulin resistance by week 12, fatty liver developed by week 16. After week 12, the HF group was divided into four groups of 6-7 rats each and three of those groups were gavaged with either 0.125 mg *P. africana* extract/kg bwt/day (the HF+Pa group) or 50 mg *S. frutescens* extract kg bwt/day (the HF+Sf group) or 16 mg metformin/ kg bwt/day (HF+Met group), while kept on the same diet for an additional of 4 weeks, to investigate whether two medicinal plant extracts and metformin can prevent HFD to induce fatty liver or not.

After 16 weeks, the liver histological images revealed that the HF group developed fatty liver in the form of both microsteatosis and macrosteatosis. Fatty liver was confirmed by significant increased liver total lipid (TL) and activities of glucose-6-phosphate dehydrogenase (cG6PD) and xanthine oxidase (XO), mitochondrial NADH oxidase (mNOX) and by a decrease ($P<0.05$) in the activities of the homogenate superoxide dismutase (hSOD) and mitochondrial complex II in the HF group, when compared to the LF group. Since the activities of mCS and cACL enzymes were not changed in the HF group, hence increased cG6PD activity in the HF group indicates that there was increased NADPH demand for lipid accumulation from activated NEFAs taken up by the liver from circulation and for maintenance of the NADPH-dependent antioxidants and oxidants, respectively. The obtained data also show that mitochondria of the HFD-fed rats adapted to an increase in energy availability, thereby compensation through decreasing complex II activity, to allow electron flux from β-oxidation to respiratory chain in the HF group.

Liver TL content was significantly decreased in the rats treated with metformin and *P. africana* extract, but not in the rats treated with *S. frutescens* when compared to the HF group ($P < 0.05$). However, the TL content remained >5% per liver weight in all treated groups. The present study demonstrates that these two plant extracts and metformin have different glucogenic and lipogenic effects from that presented by HFD alone when compared to the LFD alone. In conclusion, metformin and *P. africana* extract can attenuate HFD-induced fatty liver without changing the dietary habits. Hence *S. frutescens* extract is less effective in the prevention of HFD-induced fatty liver. A change in the dietary habits is recommended to be considered during the use of these three remedies in the treatment of HFD-induced insulin resistance and fatty liver. All three treatments enhanced antioxidant capacity, and may improve insulin resistance and fatty liver mediated by the present HFD through different mechanism of actions in the liver.

Keywords: Fatty liver, high fat diet, metformin, *Prunus africana*, *Sutherlandia frutescens*

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ABRIVIATIONS

ACAT	Acyl-CoA: cholesterol acyl transferase
ACC	Acetyl-CoA carboxylase
ACL	Adenosine-5-triphosphate citrate lyase
ACS	Acetyl-CoA synthase
ADA	Adenosine deaminase
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AFLD	Alcoholic fatty liver disease
ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
AT	Angiotensinogen
ATP	Adenosine triphosphate
ATV	Atazanavir
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BCIP	5-bromo-4-chloro-3'-indolyphosphate
bFGF	Basic fibroblast growth factor
BHT	Butylyted hydoxytuluene or 2,6-ditert-butyl-4-methoxyphenol
BMI	Body mass index
BPH	Benign prostatic hyperplasia
c	Cytosolic
CDNB	1-Chloro-2,4dinitrobezene
ChoRE	ChREBP response element
ChREBP	Carbohydrate responsive element-binding protein
CREB	cAMP-responsive element-binding protein
CS	Citrate synthase
CT	Catalase
CTP	Citrate transport protein
CO_2	Carbon dioxide
CoA	Coenzyme A
con A	Concanavalin A

COX2	Cyclooxygenase-2
CPT-1	Carnitine palmitoyl-CoA transferase 1
CYP2E1	Cytochrome P450 2E1
D	Significantly decreased
ddH_2O	Deionized water
DGAT1	Diacylglycerol acyltransferases 1
DGAT2	Diacylglycerol acyltransferases 2
DHA	Decosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNPH	2,4-dinitrophenylhydrazine
DNSI	Insignificantly decreased
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Elovls	Long chain fatty acid elongases
EtOH	Ethanol
Em	Emission
ER	Endoplasmic reticulum
Ex	Excitation
F1,6P2	Fructose-1,6-diphosphate
F6P	Fructose-6-phosphate
FAs	Fatty acids
FAS	Fatty acid synthase
FFAs	Free fatty acids
FGF21	Fibroblast growth factor 21
FLD	Fatty liver disease
FLDs	Fatty liver diseases
Fox	Ferric xylenol orange
FOXO1	Forkhead box protein O1
g	Gram
$G3PO_4$	Glycerol-3-phosphate
G3P	Glyceraldehyde-3-phosphate

G3P	Glycerol-3-phosphate
G6P	Glucose-6-phosphate
G6Pae	Glucose-6-phosphatase
G6PD	Glucose-6-phosphate dehydrogenase
GABA	γ-Aminobutyric acid
GC	Guanylate cyclase
GK	Glucokinase
GLUT2	Glucose transporter 2
GSH	Reduced glutathione
GR	Glutathione reductase
GP	Glutathione peroxidase
GPAT	Glycerol-3-phosphate acyltransferase
GSSH	Oxidized glutathione
GST	Glutathione-S-transferase
γ-GT	γ-Glutamyltransferase
h	Homogenate
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HBV	Hepatitis B virus
HBVS-Tg	Hepatitis B virus surface antigen transgenic
HCD	High carbohydrate diet
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDL-C	High-density lipoprotein cholesterol
H&E	Haematoxylin and Eosin
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HF	HFD fed rats
HFD	High fat diet
HF+Met	High fat diet fed rats gavaged with 16 mg metformin/kg bwt per day
HF+Pa	High fat diet fed rats gavaged *P. africana* bark extract/kg bwt per day
HF+Sf	High fat diet fed rats gavaged *S. frutescens* leaves extract/kg bwt per day
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA

HNF4α	Hepatocyte nuclear factor 4α
HO	Heme oxygenase
HOC	Hypochlorous acid
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseredish peroxidase
I	significantly increase
IDH	Isocitrate dehydrogenase
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IMP	Inosine-5-monophosphate
iNOS	Inducible nitric oxide synthase
INT	Iodonitrotetrazolium chloride (INT)
K_2HPO_4	Dipotassium hydrogen phosphate
Kg	Kilogram
L	Liter
LCELovs	Long chain fatty acyl elongase
LCFAs	Long chain fatty acids
LDH	Lactate dehydrogenase
LDL-C	Low-density lipoprotein cholesterol
LF	LFD fed rats
LFD	Low fat diet
L-NMMA	NG-mono-methyl-L-arginine
LPK	L-pyruvate kinase
LPS	Lipopolysaccharide
LXR	Liver X receptor
m	Mitochondria
M	Molar
MCD	Methionine-choline deficient
MDA	Malondialdehydes
ME	Malic enzyme
mM	Millimolar
mg	Milligram

$MgCl_2$	Magnesium chloride
mmol	Millimole
mL	Milliliter
MRI	Magnetic resonance imagining
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
MTP	Microsomal triglyceride transfer protein
MUFA	Monounsaturated fatty acid
N_2	Nitrogen gas
NAC	N-Acetyl-Cysteine
NaN_3	Sodium azide
NaCl	Sodium chloride
NAD+	Oxidised nicotinamide adenine dinucleotide
NADP+	Oxidised nicotinamide adenine dinucleotide phosphate
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-Alcoholic Steatohepatitis
NBBS	N-butylbenzenesulfonamide
NC	Not changed
NEFAs	Non-esterified fatty acids
NF-kβ	Nuclear Factor-Kappaβ
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADH oxidases
NRF1	Nuclear respiratory factor 1
NRFs	Nuclear respiratory factors
NS	Not significantly increased or decreased
nSTZ	Neonatal streptozotocin
NTB	Nitro-blue tetrazolium
$O_2^{\bullet-}$	Superoxide
OAA	Oxaloacetate

$^{\bullet}OH$	Hydroxyl
$ONOO^{-}$	Peroxynitrite molecule
OXPHOS	Oxidative phosphorylation
6PGD	6-phosphogluconate dehydrogenase
pACC	Phospho-acetyl-CoA carboxylase
PC	Pyruvate carboxylase
PEP	Phosphoenol pyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	6-Phospho-1-fructokinase
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
Pi	Inorganic phosphate
PMCT	Plasma membrane citrate transporter
PMRS	Proton magnetic resonance spectroscopy
PNP	Purine nucleoside phosphorylase
PPARs	Peroxisome proliferator-activated receptors
PPAR-α	Peroxisome proliferator-activated receptor-α
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPAR-δ	Peroxisome proliferator-activated receptors-δ
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acids
qPCR	quantitative polymerase chain reaction
R5P	Ribose-5-Pphosphate
ROO	Peroxyl
ROS	Reactive oxygen species
RT	Room temperature
S1P	Site-1 protease
SCAP	SREBP cleavage-activating protein
SCAT	Succinyl-CoA acetoacetate transferase
SCD-1	Liver stearoyl-CoA desaturase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFA	Saturated fatty acid

SOD	Superoxide dismutase
SREBPs	Sterol regulatory element-binding proteins
T	Tween
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TBA	Thiobarbituric acid
TBS	Tris-buffer saline
TC	Total cholesterol
TCA	Trichloroacetic acid
TCA	Tricarboxylic acid
TGs	Triglycerides
TGF-b1	Transforming growth factor-b1
TLC	Thin layer chromatography
TLs	Total lipids
TNB	5-Thio-2-nitrobenzoic acid
TNF-α	Tumor necrosis factor-alpha
Tris	T*ris*(hydroxymethyl)aminomethane
UDCA	Ursodeoxycholic acid
US	United State
UTP	Uridine triphosphate
VDAC	Voltage-dependent anion channel
VLCFAs	Very-long-chain fatty acids
VLDL	Very-low-density lipoprotein
VLDL-Apo B	Very low density lipoprotein-apoprotein B
v/v	Volume per volume
WHO	World Health Organization
X5R	Xylupose-5-phosphate
xg	Times gravity
XO	Xanthine oxidase

CHAPTER 1: INTRODUCTION

Synopsis

This Chapter describes the fatty liver diseases (FLDs). The focus is granted to non-alcoholic fatty liver disease (NAFLD) as a core in this study. To understand NAFLD and its progression, two forms of vesicular steatosis and the NAFLD histological features are explained. The epidemiology of NAFLD and its associated risk factors per geographical area, pathogenesis, diagnosis and management are also depicted. In this Chapter, the two selected medicinal plants and metformin, and their known possible mechanisms of actions, together with the motivation, summary underlying the theme and aims, objectives, Justification and significance to the present study are also introduced. The effect of HFD, the participating pathways in liver glucose and lipid metabolism during induction of fatty liver and oxidative stress are introduced in ***Chapter 2****.*

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1. Introduction

1.1 Fatty liver disease

Liver diseases are currently a serious problem worldwide. Fatty liver is also known as fatty liver disease (FLD) or hepatic steatosis (Boyce *et al*., 2010). Its increasing prevalence urges the need of prevention and treatment as it remains a major public health problem worldwide (Erickson, 2009; reviewed in Zelber-Sagi *et al*., 2011).

There are two types of FLDs, namely; (i) alcoholic fatty liver disease (AFLD) which is manifested by alcohol abuse, and (ii) non-alcoholic fatty liver diseases (NAFLD) which is manifested in the absent of alcohol abuse (Reviewed in Obika and Noguchi, 2012). In

addition, there are two subtypes of fatty liver also known as hepatic steatosis, namely, (i) macrovesicular steatosis (large lipid droplets) and (ii) microvesicular steatosis (small lipid droplets) (Brunt, 2007). NAFLD can be induced by either malnutrition or overnutrition conditions or both, drug hepatotoxicity and autoimmune diseases. NAFLD has a serious health, economic and social consequences in both adult and children and the problem becomes bigger in developing countries and with urbanization (Pinidiyapathirage *et al*., 2011; Zelber-Sagi *et al*., 2011). However, NAFLD exact pathogenesis is still not well understood and renders further investigations. Therefore, recent studies focus increasingly on understanding the pathogenesis of NAFLD and to find a possible treatment to prevent NAFLD and its progression to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis.

Among other organs, including adipose tissue, skeletal muscle, heart, and kidney, liver plays a central role in lipid metabolism, importing serum free fatty acids (FFAs) and synthesizing, storing and exporting lipids and lipoproteins (Adams *et al*., 2005). The major dietary constituents, fatty acids (FAs) and carbohydrate participate in concert with several hormones including insulin and glucagon in the regulation of gene expression in response to food intake and qualitative nutritional changes. This regulation controls several metabolic pathways and involves mechanisms that control energy utilization in response to the availability of glucose and lipid, and that regulate the interconvertion, transport, storage, mobilization and the use of these nutrients and their metabolites. Altogether, these mechanisms ensure a healthy energy homeostasis, while alteration of this balance can results to the pathological conditions including metabolic syndrome and its associated risk factors (Cohen *et al*., 2011; Krey *et al*., 1997).

All features of metabolic syndrome are associated with non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), and its co-morbidities include obesity, T2DM, hypertension, and hyperlipidaemia in the form of elevated triglycerides (TG) levels (Yeh and Brunt, 2007). Recently, an increase in the prevalence of obesity and its related chronic NAFLD are observed in both adults and children (Frith *et al*., 2009; Patton *et al*., 2006; Stickel and Hellerbrand, 2010; Takahashi and Fukusato, 2010; Chavez-Tapia *et al*., 2009). NAFLD is an important cause of chronic liver diseases that include NASH, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In the most recent years, this disease has become more important worldwide (Takahashi and Fukusato, 2010). This phenomenal

recognition has been considered important for the hepatologists to be aware of the presence of NAFLD and to prevent or treat it adequately in order to benefit the current and future generations (Chavez-Tapia *et al*., 2009).

1.1.1 Non-alcoholic fatty liver disease (NAFLD)

In the most recent years, there is a growing interest in the elucidation of the mechanisms that are involved in the development and progression of the non-alcoholic fatty liver disease (NAFLD). NAFLD is a general term for the FLD induced by the environmental factors including HFD (Marczuk-Krynicka *et al*., 2009), drugs, HIV infection, and autoimmune disease including hepatitis induced by hepatitis B virus (HBV) and hepatitis C virus (HCV) (Konrinis and Dieterich, 2003; Thomas, 2006) without alcohol intake. Hence, NAFLD is defined as lipid accumulation that exceeds 5% to 10% by liver weight in the absence of other causes of fatty liver (Montecucco and Mach, 2008). NAFLD is the hepatic condition of the metabolic syndrome, a systemic condition mediated by elevated production of adipose tissue-derived and liver-derived factors that modulate energy substrate flux to coordinate liver catabolism and anabolism. These factors are produced by multiple types of cells in both tissues as well as the intermediary metabolism regulators which are potent regulators of immune system functions. These involve the immune regulators such as cytokines that modulate the production and biologic activity of the various metabolic factors. Normally, early stage of NAFLD is very common in individuals with the metabolic syndrome; however sustained exposure to the inflammatory mediators generally promotes the production of various profibrogenic factors that lead to progression of NAFLD from NASH to cirrhosis (Choi and Diehl, 2005).

Recently, lipid accumulation is noted to cause a direct damage to the hepatocytes, which is consequently followed by an inflammatory response, cytokine production, oxidative stress, abnormal cellular signalling and activation of stellate cells (Tessari *et al*., 2009). Even though NAFLD progression rate may be slower than that of other types of liver disease, its incidence is seen to be increasing at an alarming rate in parallel with obesity epidemic worldwide. Furthermore, there is no pharmacological licensed therapy for NAFLD (Basaranoglu and Neuschwander-Tetri, 2006). Thus, to date, the first line treatment of NAFLD is currently based on diet and lifestyle modifications (Mazza *et al*., 2012). NAFLD can be characterized quantitatively as mild, moderate or severe and quantitatively as macrovesicular or

microvesicular steatosis using various methods of hepatopathological examination (Fu *et al*., 2010a). Haematoxylin and eosin (H&E) staining can confirm two forms of vesicular steatosis that could be detected as either (a) microvesicular or (b) macrovesicular steatosis or as both types (Caldwell *et al*., 2010; Fu *et al*., 2010a).

(a) Microvesicular steatosis (small lipid droplets)

Microvesicular steatosis is uncommon form which is pathologically characterized by the variable of the enlarged hepatocytes with flocculent cytoplasm due to the numerous fine or small lipid droplets, while nuclei remain centrally located as shown in **Figure 1.1**. This type of fatty liver is manifested by a severe mitochondrial dysfunction which is commonly associated with significant elevated liver tests, without or with hepatic encephalopathy. This form of steatosis arises in very specific and typically situations such as an acute fatty liver of pregnancy, a certain drug hepatotoxicity, Reye syndrome, hormonal changes, cytokines and alcohol foamy degeneration (Fromenty and Pessayre, 1995; Fromenty and Pessayre, 1997; Yeh and Brunt, 2008). It is therefore diagnosed by the routing stains including Haematoxylin and Eosin as shown in **Figure 1.1** (H&E) (Yeh and Brunt, 2008).

Microvesicular steatosis may involve the entire liver lobule or may predominate in centrizonal, midzonal or periportal hepatocytes, while other liver lesions such as necrosis, cholestasis or fibrosis may be either absent or present depending on NAFLD stage. In addition, microvesicular steatosis is a serious condition which can be associated with the elevated serum transaminase activity, the prothrombin time and blood ammonia levels among others (Fromenty and Pessayre, 1995). It is documented that under severely altered mitochondrial function conditions in the liver, drugs can induce microvesicular steatosis which can be strongly associated with profound encephalopathy and hypoglycaemia, respectively. Moreover, this form of steatosis can also mediate hepatic apoptosis and/or necrosis inducing cytolytic hepatitis (NASH), which can lead to liver failure (Labbea *et al*, 2008).

(b) Macrovesicular steatosis (large lipid droplets)

Macrovesicular steatosis is a most common form in humans. As shown in **Figure 1.1**, macrovesicular steatosis can be visualized by H&E staining as clear, well-defined spaces

within the cytoplasm of normal sized or enlarged hepatocytes (**Figure 1.1**) (Yeh and Brunt, 2008).

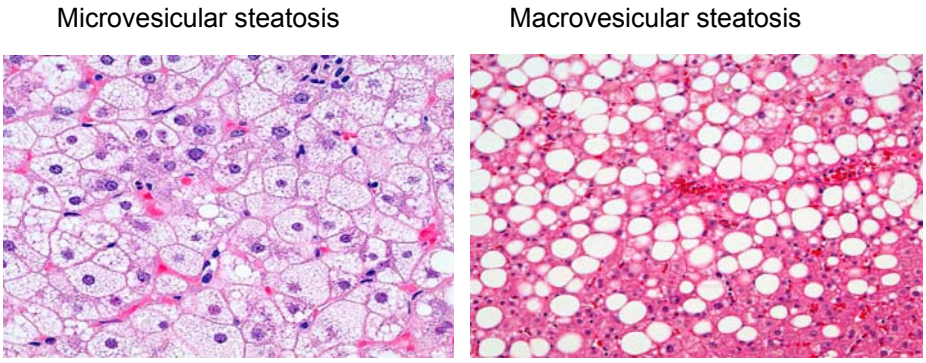


Figure 1.1 *Examples of two forms of steatoses (H&E) micrographs taken from Yeh and Brunt (2008).*

This type of fatty liver is characterized by the hepatocytes contain a single large, vacuole of lipid droplet (mainly TG), which fills up and rounds the hepatocyte, displacing the nucleus to the periphery of the cell or comprise several small clustered droplets, each of which can be well defined. Macrovesicular steatosis by itself in the absence of other liver lesions is a relatively benign condition, but only in the short term. Macrovesicular steatosis may also be strongly associated with a mild increase in serum transaminase, an enlarged liver on physical examination and a hyperreflective hepatomegaly on ultrasonography. Macrovesicular steatosis in humans is mostly caused by alcohol abuse, obesity, diabetes and dyslipidaemia (Fromenty and Pessayre, 1995; Yeh and Brunt, 2008). Moreover, a milder mitochondrial dysfunction together with an inhibition of TG secretion from the liver mediates macrovesicular steatosis in the drug-induced liver injury (Labbea *et al*, 2008).

1.1.1.1 NAFLD histological features

The NAFLD histological features have a wide spectrum ranging from a benign NAFLD to non-alcoholic steatohepatitis, cirrhosis, fibrosis and hepatocellular carcinoma (HCC). Suspected liver damage is accessed by digital diagnosis techniques such as ultrasound as well as the biochemical elevated liver tests. This procedure is important in helping patients who have mild and/or severe NAFLD to treat them in advance (Basaranoglu and Neuschwander-Tetri, 2006).

1.1.1.1.1 Non-alcoholic steatohepatitis (NASH)

A second NAFLD progressive stage is liver non-alcoholic steatohepatitis (NASH), which is an under diagnosed liver disease and it happens to be the main cause of elevated serum liver enzymes among the general population (Kabir *et al*., 2010). The presence of cellular ballooning in NAFLD is one of the principle histological findings used to identify the availability of significant and potentially progressive steatohepatitis (Caldwell *et al*., 2010). NASH can progress to cause an advanced fibrosis, cirrhosis, hepatocellular carcinoma (HCC), end-stage liver disease and liver-related death or transplantation (Basaranoglu and Neuschwander-Tetri, 2006). The characteristics of NASH are histologically elucidated including fatty infiltration with hepatocellular ballooning degeneration (Caldwell *et al*., 2010) and sinusoidal fibrosis, polymorphonuclear infiltration with or without Mallory hyaline (Kabir *et al*., 2010). Unlike other characteristics, hepatocellular ballooning is a key finding in NASH and it is well defined by haematoxylin and eosin (H&E) staining which reveals enlarged cells with rarefied cytoplasm and by changes in the cytoskeleton (Caldwell *et al*., 2010; Fu *et al*., 2010a).

In addition, the presence of high TG and cholesterol levels has been reported in the patients with NASH rather than in patients with NAFLD (Kruger *et al*., *2*010). It is well documented that NASH is most frequently associated with some metabolic disorders including hyperglycaemia, insulin resistance, obesity, T2DM, hyperlipidaemia, protein deficiency, and jejeunoileal bypass surgery (Brunt *et al*., 2003; Kabir *et al*., 2010).

1.1.1.1.2 Fibrosis

A third NAFLD progressive stage is liver fibrosis, which is strongly associated with the systemic hypertension and insulin resistance in obese subjects with NASH. In those obese patients, the high transforming growth factor-b1 (TGF-b1) and angiotensinogen (AT) are the risk factors of liver fibrosis (Dixon *et al*., 2003). In addition, Sakugawa *et al*. (2005) demonstrated that type 4 collagen 7S domain and hyaluronic acid are the useful biochemical markers to evaluate the degree of fibrosis and to discriminate patients with non-severe from those with severe fibrosis among patients with NASH. As shown in **Figure 1.2A**, the liver fibrotic tissues are presented with the lipid droplets and lipid degeneration, respectively (Cheng *et al*., 2004).

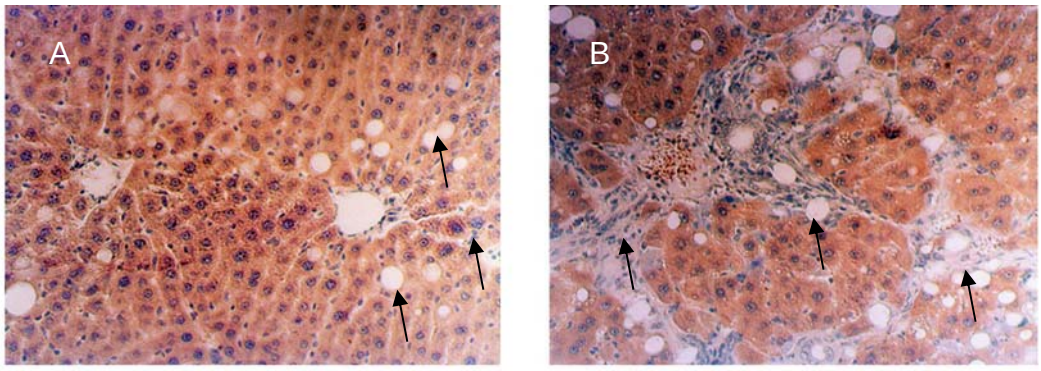


Figure 1.2 *Fibrosis and cirrhosis.* ***(A)*** *Liver fibrotic tissues, lipid drop accumulation, and lipid degeneration,* ***(B)*** *Liver widely fibrotic, lipid degeneration of hepatocytes and pseudo-lobuli as the typical expression of liver cirrhosis micrographs (H&E) taken from Cheng et al. (2004). Arrows indicate lipid droplets, lipid degeneration and fibrosis.*

1.1.1.1.3 Cirrhosis

A fourth NAFLD progressive stage is liver cirrhosis which is an irreversible form of chronic liver disease (Xu *et al*., 2004). Currently, liver cirrhosis is a worldwide health problem because it does not have effective therapy except liver transplantation (Horiguchi *et al*., 2009). This stage is one of the leading causes of morbidity and mortality in the United States in the persons with NAFLD (Heidelbaugh and Bruderly, 2006). In principle, as shown in **Figure 1.2B**, liver cirrhosis is defined as an architectural histological disorganization which is characterized by fibrosis and regenerative nodules This condition has a tendency to progress to portal hypertension, hepatocellular dysfunction and hepatocellular carcinoma (HCC) (Kudo *et al*., 2008). As a result, cirrhosis has been reported to have approximately 7 to 10 year liver related mortality (Farrell and Larter, 2006). In the last 2 decades, NASH patients who are older, obese, and have suffered from T2DM have been reported to be at highest risk of progressing to cirrhosis with its associated complications (Angulo *et al*., 1999).

1.1.1.1.4 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death. Only 30% of these cases are associated with obesity, T2DM and related metabolic diseases including insulin resistance and NASH. However, to date a direct relationship between these pathological conditions and HCC has not been convincingly established. In addition, HCC associated with NASH are thought to develop through a continuous transition of liver

pathologies, which begins with hepatic lipid accumulation and proceeds through hepatitis, fibrosis, cirrhosis, and ends with benign liver tumours and HCC, respectively (Hill-Baskin *et al*., 2009).

Amongst other factors, cirrhosis mediated by NASH is considered to be the major risk factor for HCC, which could be a complication of end-stage of NAFLD and other fibrosing liver diseases such as AFLD (Stickel and Hellerbrand, 2010; Ascha *et al*., 2010). Among HBV and HCV co-infected patients with cirrhosis the major risk factors for HCC development are male gender, but those who are older than 50 years of age (Chiaramonte *et al*., 1999). According to Mendez-Sanchez *et al*. (2008) this liver cancer disease will become a significant cause of adults and children morbidity and mortality worldwide in the near future. HCC has been observed to develop in a period of 27 years following the diagnosis of simple NAFLD in an old man case who had no history of alcohol intake or hepatitis virus markers neither (Nagaya *et al*., 2008).

Furthermore, HCC can occur in both obese diabetic and non-diabetic patients depending on the NAFLD progression rate (Chavez-Tapia *et al*., 2009). Recently, high saturated fatty acid (SFA) diet was recognized to be the most likely factor contributing to diet-induced metabolic disease and liver cancer (Hill-Baskin *et al*., 2009). In 1994, the DNA content of 41 South Africans and 47 Japanese diagnosed with HCC were analysed and compared in which the South African had higher DNA distribution pattern than that of the Japanese (Yoshida *et al*., 1994), indicating that there is high prevalence of liver diseases associated with liver cancer in South Africa. Therefore it is important to elucidate the effects of dietary compositions that play a central role in the aetiology of NAFLD and its associated liver diseases.

1.1.2 Epidemiology of NAFLD and associated risk factors per geographical area

The prevalence of contemporary life style which is characterized by increased consumption of high fat diet (HFD) and reduced physical activity leads to increase incidences of metabolic diseases such as NAFLD, hypertension, obesity, atherosclerosis and T2DM. It has been proposed that, by 2025, one in every three American children born in the year 2000 will carry a significant lifetime risk of T2DM and become prone to premature cardiovascular disease (Reviewed in Liu and Lin, 2011). Surprisingly, the prevalence of NAFLD is increasing at an alarming rate among both children and adults worldwide. NAFLD prevalence ranges from

10-24% in general population, hitting 28-55% in T2DM patients, 27-92% in hyperlipidemic patients and 60-95% in obese patients (Reviewed in Mazza *et al*., 2011). The factors associated with the higher prevalence of NAFLD are poor exercise, undernutrition of healthy and overnutrition of unhealthy diets, and low socioeconomic status. NAFLD is also associated with other risk factors and these might varies along the geographical distribution due to the lifestyle differences including dietary habits and physical activities e.g. exercise (Brunt, 2007). The international (other countries) and national (South Africa) prevalence of NAFLD and its associated chronic liver diseases including NASH, cirrhosis, HCC and hepatitis are summarized below. The international (other countries) and national (South Africa) prevalence of NAFLD and its associated chronic liver diseases including NASH, cirrhosis, HCC and hepatitis are summarized per country in **Table 1.1**.

Table 1.1 A summary of the international and national prevalence of NAFLD and its associated chronic liver diseases

Country	**Year**	**NAFLD (%)**	**NASH (%)**	**Cirrhosis (%)**	**HCC (%)**	**HBV (%)**	**HCV (%)**	**Study group**
United State[1]								
Hispanics	2004							
White		45	-	-	-	-	-	
Blacks		33	-	-	-	-	-	Ethnic adults
		24	-	-	-	-	-	
	2009							
		20	-	-	-	-	-	General
			-					population
Country	**Year**	**NAFLD (%)**	**NASH (%)**	**Cirrhosis (%)**	**HCC (%)**	**HBV (%)**	**HCV (%)**	**Study group**
United State[1]								Morbidity obese
	2009	75-92	-	-	-	-	-	Paediatric
		13-14	-	-	-	-	-	population
California[2]	2006	13.0	-	-	-	-	-	Obese children
China[3]								
South	2007	17.0	-	-	-	-	-	Adults
Central	2007	24.5	-	-	-	-	-	Adults
South-West	2009	12.4	-	-	-	-	-	Adults

Table 1.1 Continued

Italy[4]	2007 2008	65.4 23.0	- -	- -	- -	- -	- -	T2DM adults Obese children
Romania[5]	2008	20.0	-	-	-	-	-	Hospitalized patients
India[6]	2009	32.0	-	-	-	-	-	T2DM adults
Mexico[7]	2010	17.6	25	34.4	35.7	17.6	16.7	T2DM adults
Brazil[8]	2010	27.7 (obese) 3.4 (non-obese)	- -	- -	- -	- -	- -	Obese and non-obese students
South Africa[9]	2010	51	36	5	-	-	-	T2DM adults

1 *Browning et al., 2004; Erickson, 2009;* ***2*** *Schwimmer et al., 2006;* ***3*** *Zhou et al., 2007; Wang et al., 2007 and Li et al., 2009;* ***4*** *Targher et al., 2007 and Pozzato et al., 2008;* ***5*** *Radu et al., 2008;* ***6*** *Mohan et al., 2009;* ***7*** *Kobashi-Margain et al., 2010;* ***8*** *Lira et al., 2010;* ***9*** *Kruger et al., 2010. "-", indicates undetermined percentage.*

1.1.3 Pathogenesis

The etiology of NAFLD remains unclear; various lines of evidence have shown a pathogenetic role of insulin resistance (Reviewed in Mazza *et al*., 2012). There are two hits hypothesized to be involved in the pathogenesis of NAFLD:

First hit (1st): It is characterised by FFA and TG accumulation in liver (fatty liver). It is cause by insulin resistance via lipolysis and hyperinsulinemia, and obesity via leptin resistance which induces a chronic inflammation characterised by release of inflammatory cytokines and oxidative stress, both of which are responsible for 2nd hit.

Second hit (2nd): When there is an increased ratio of saturated to unsaturated FAs delivered to or stored within the liver may induce the progression from simple fatty liver to NASH. Validation of this concept has been rendered by recent data demonstrating that when TG precursors accumulate in the liver and the mechanism of liver detoxification are overwhelmed or inactivated, saturated fatty acids (SFAs) directly mediate liver insulin resistance and inflammation, both of which may lead to fatty liver progression towards more severe liver disease. It is vital to emphasize that insulin resistance remains a key player in NAFLD pathogenesis and that fatty liver may in turn enhance insulin resistance. It is also

documented that liver fat content affects insulin sensitivity in human more strongly than visceral fat, which may in part support a direct and vital role of fatty liver in the pathogenesis of insulin resistance (Reviewed in Mazza *et al*., 2012). Examples of the serial steps of NAFLD pathology from simple fatty liver to HCC are shown in **Figure 1.3**.

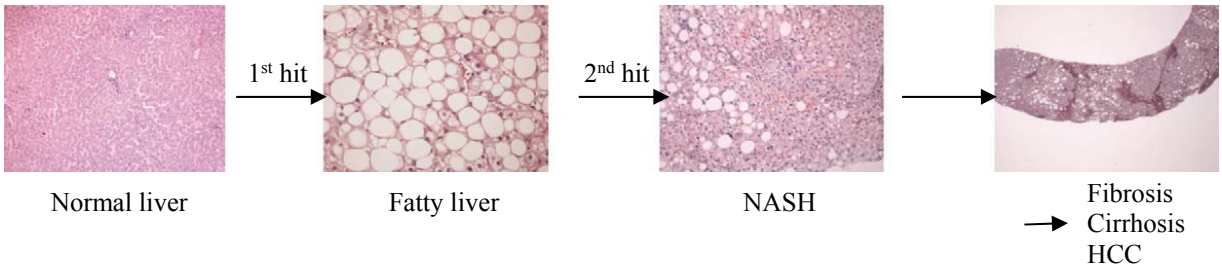


Figure 1.3 *Serial steps of NAFLD pathology representative images (left to right) of a normal liver, steatosis showing lipid filled hepatocytes, NASH showing inflammatory infiltrates and severe liver fibrosis adopted from Tailleux et al. (2012).*

Other studies have shown that the people with NAFLD have an increased risk of being infected with hepatitis B virus (HBV) or C virus (HCV) as depicted in **Figure 1.4**. These viruses are the most commonly involved in the NAFLD progression from simple fatty liver to NASH. HBV has been reported to have ability to interact with the hepatic microsomal triglyceride transfer protein (MTP), which might interfere with the TG secretion and storage (Fu *et al*., 2010a; Mirandola *et al*., 2010; Tsochatzis *et al*., 2007).

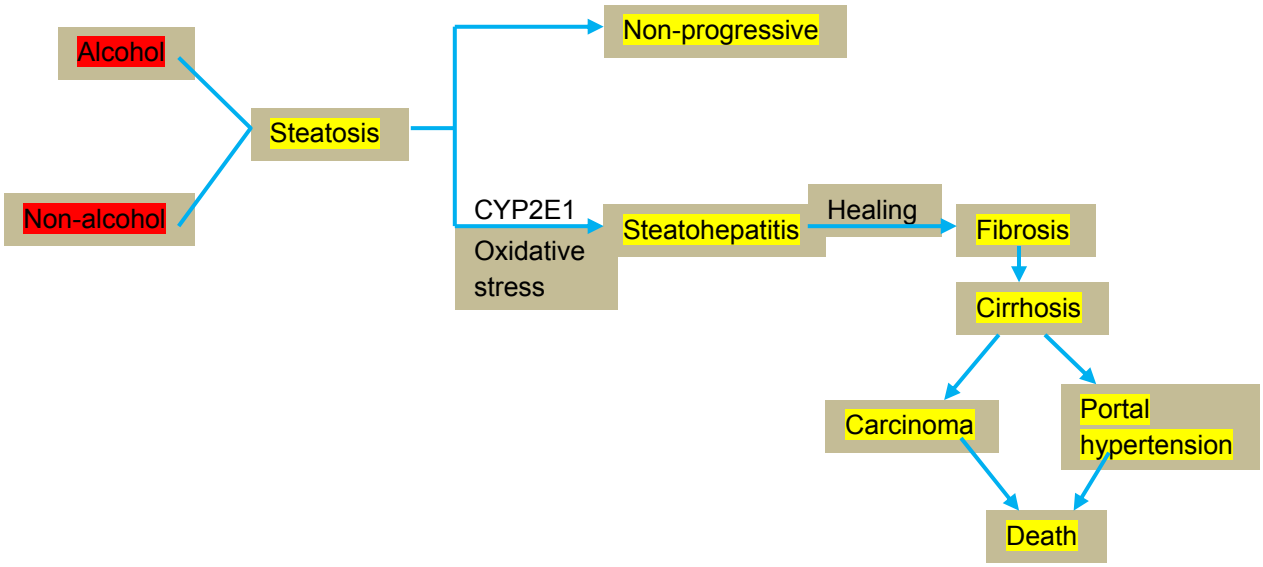


Figure 1.4*.The natural history of an NAFLD and its pathogenesis (Redrawn from Tolman and Dalpiaz, 2007).*

Although the NAFLD histological features have been elucidated, its aetiology and progression is complex and remains not well understood (Hijona *et al*., 2010). According to Kruger *et al.* (2010) among the South African overweight and obese adult patients with NAFLD and NASH, the presence of more marked obesity did not anticipate the present of more advanced disease, indicating that NAFLD progression could differ per geographical region depending on its cause. The effect mediated by accumulated TG and its metabolites (fatty acyl-CoA, ceramide and diacylglycerol) in the liver have been summarized by Tolman and Dalpiaz (2007) as follows:

i. Mitochondrial dysfunction
ii. Upregulation of apoptosis
iii. Induction of CYP2E1 produces ROS
iv. Induction of pro-inflammatory genes including TNF-α and cyclooxygenase-2 (COX2) that induce additional inflammatory mediators which are profibrotic
v. Indirectly upragulation of TNF-α which is pro-steatotic and proinflamatory, respectively.

1.1.4 Diagnosis

Non-invasive imaging techniques such as MRI and ultrasound are often used to diagnose lipid accumulation in the liver. However, neither approach can distinguish simple fatty liver from NASH, nor can they approximate inflammation severity, stage of disease or degree of fibrosis (Tailleux *et al*., 2012). Therefore, there is recognition that liver biopsy evaluation is the only way of diagnosis or exclusion of fatty liver disease (FLD) in clinical practice, followed by histological analysis (Obika and Noguchi, 2012). This recognition could be due to the fact that till now neither laboratory tests nor imaging studies can provide complete data related to amount of tissue lipid content, inflammation, liver cell injury, fibrosis, and structural changes. In addition, liver biopsy evaluation provides ways of grading and staging the lesions of FLD as well as detecting of clinically unsuspected progression. Moreover, liver biopsy evaluation serves as the primary end point in clinical trials of treatment (Brunt, 2007). In addition, the diagnosis of simple fatty liver is confirmed by the presence of macrovesicular steatosis without ballooned hepatocytes, Mallory hyaline, lobular inflammation, or perisinusoidal/perivenular fibrosis (Nagaya *et al*., 2008).

Due to the risks associated with invasive methods of diagnosis, these methods are limited in clinical situations. In addition, Nagaya *et al*. (2008) have demonstrated that a diagnosis of simple fatty liver does not guarantee non-progression to NASH, fibrosis, cirrhosis and HCC, therefore careful follow-up is needed in patients with simple fatty liver. In their case study, platelet counts and degree of fatty liver, as assessed by the periodic ultrasonography, were observed to gradually reduce with progression of fibrosis (Nagaya *et al*., 2008). According to Sunny *et al*., (2011), the exact cellular and/or metabolic event that accompanies NAFLD and threatens liver function remains unclear. Therefore, studies on the high fat diet (HFD) using animals are necessary in order to develop the NAFLD (fatty liver) and NASH models for the investigations of these pathological conditions, progression as well as their prevention and/or treatment.

1.1.5 Management

There is no licenced treatment for NAFLD (Mazza *et al*., 2012). Physical activity is one of the effective clinical management of NAFLD. Due to the relation of NAFLD to obesity, diabetes and metabolic syndrome the only accepted treatment for NAFLD, regardless of stage, is lifestyle modifications including weight loss by a combination of increased physical activity and decreased caloric intake (Erickson, 2009). The value of n3-PUFA in the diet has been described recently (Mazza *et al*., 2012).

1.1.5.1 Diet

To date, diet has been assigned as an effective treatment of NAFLD (Zelber-Sagi *et al*., 2011). The important factors in the diet therapy of the NAFLD and its associated diseases are composition, amount, distribution and time of feeding (Khan and Safdar, 2003). In addition, the beneficial diet should be high in complex carbohydrate such as starch, low in simple carbohydrates such as fructose, sucrose and glucose, low in fat such as saturated and *trans*-fatty acids, and high in mono-unsaturated fatty acid (MUFA) such as oleic acid and palmitoleic acid, and polyunsaturated fatty acids (PUFAs) such as α-linolenic acid (omega 3 fatty acid) (Khan and Safdar, 2003). Diet supplemented with antioxidants and essential FAs such as n-3 PUFA have been proposed to have potential in the treatment of NAFLD (Capanni *et al*., 2006).

1.1.5.1.1 n-3 PUFA

N-3 PUFA is a natural PPARα ligand that has been reported to induce hepatic adiponectin and PPARα expression, reduced hepatic TNF-α mRNA levels, reverse fatty liver and the degree of liver injury in the rats fed on 58% HFD enriched with n-6:n-3 ratio of 85.9. Furthermore, Svegliati-Baroni *et al*. (2006) found that increased dietary n-3 PUFA also reduces cholesterol and triglycerides (TG) deposition in the liver of their rat model (Svegliati-Baroni *et al*., 2006). In NAFLD patients who had depleted PUFA, enhanced oxidative stress and lipid accumulation in the liver were observed while dietary supplementation with n-3 PUFA has been found to restore the induced conditions. Capanni *et al*. (2006) have supported the efficacy of n-3 PUFA as a new therapeutic approach in the treatment of NAFLD patients. This support has arisen from their pilot study in which a 1 g n-3 PUFA capsule for 12 months reduced the serum fasting glucose, TG and inhibited liver injury as monitored by enzyme markers including plasma aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and γ-glutamyltransferase (γ-GT) (Capanni *et al*., 2006).

In **Figure 1.5** the interaction of n-3 PUFA with the glycolytic and lipogenic enzymes and genes including glucokinase (GK), glucose-6-phosphate dehydrogenase (G6PD) and carbohydrate responsive element-binding protein (ChREBP) is shown (Dentin *et al*., 2006a). Thus, under basal conditions of glucose and insulin concentrations, ChREBP is phosphorylated and localised in the cytosol of hepatocytes. Alternatively, under high glucose and insulin concentrations, ChREBP nuclear translocation is rapidly induced. This translocation is controlled by mechanism of phosphorylation and dephosphorylation (Dentin *et al*., 2005). An n-3PUFA has the ability to reverse fatty liver via enzymatic inhibition resulting in the reduction of glucose-6-phosphate (G6P), xylulose-5-phosphate (X5P) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Dentin *et al*., 2006a); the latter is a major co-factor for lipogenic enzymes (Horton, 2002a; 2002b). Furthermore, dephosphorylation of serine residue 196 allows ChREBP translocation into the nucleus, while dephosphorylation of threonine residue 666 increases DNA binding inhibition. In addition, X5P selectively activates protein phosphatase 2A (PP2A) is believed to be responsible for both nuclear and cytosolic dephosphorylation of ChREBP. Then ChREBP binds its response element (ChoRE) to activate lipogenic and glycolytic gene expression However, in the

presence of PUFA, ChREBP is retained in the cytosol via the specific inhibition of G6PD and GK activities, key enzymes of pentose phosphate pathway (PPP) and glycolysis, respectively (Dentin *et al*., 2005).

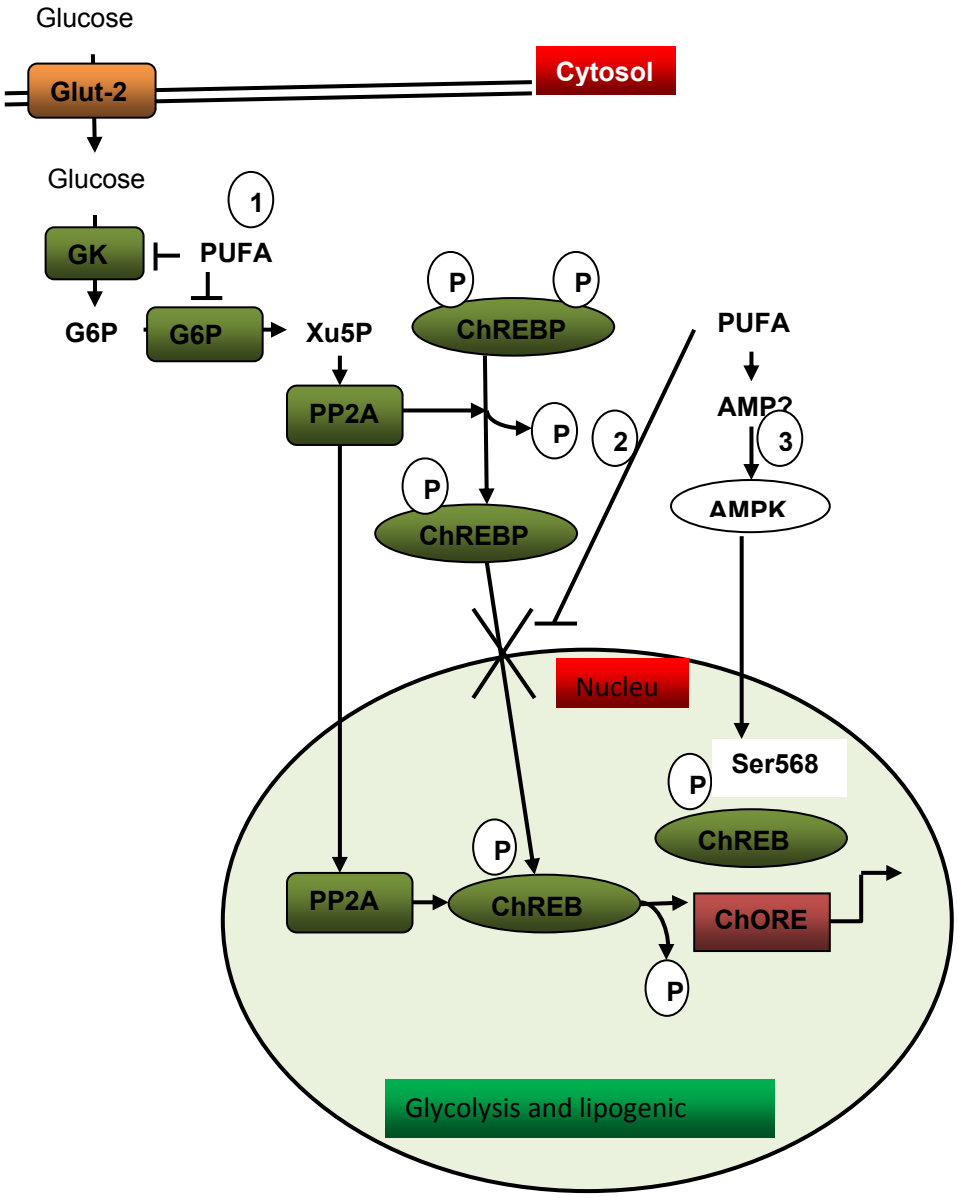


Figure 1.5 *Proposed PUFA mechanisms of actions in the liver diagram redrawn from Dentin et al. (2006a). PUFA inhibit ChREBP nuclear translocation in liver, while it pacifically inhibits the activities of two key enzymes of the glycolysis and pentose phosphate pathways, namely glucokinase (GK) and glucose-6-phosphate dehydrogenase (G6PD) (**1**), respectively by decreasing both glucose-6-phoaphate (G6P) and xylose-5-phosphate (X5P) concentrations retain ChREBP in the cytosol (**2**). AMPK was not involved in this mechanism of inhibition of ChREBP translocation. Although the ability of PUFA to activate AMPK remains controversial, the AMPK-mediated phosphorylation of ChREBP on Ser568 should not be excluded as having potential importance under specific physiological or physiopathology conditions (**3**).*

The ability of PUFA to directly activate AMPK remains controversial. However, AMPK-mediated phosphorylation of ChREBP on Serine 568 should not be excluded (Dentin *et al*., 2006a) Figure 1.5.

1.1.5.2 Insulin sensitizing agents

Metformin and rosiglitazone (also known as Avandia) are insulin sensitizing drugs that have been implemented in the treatment of NAFLD. Metformin is described in detail below under **Section 1.2**.

1.1.5.2.1 Rosiglitazone

According to Tiikkainen *et al*. (2004) fasting hyperinsulinemia is linked with an NAFLD due to impaired insulin clearance. In their study, increased insulin clearance was observed, while the hepatic lipid content decreased by rosiglitazone treatment of T2DM patients. Rosiglitazone is licensed for the treatment of T2DM (Tiikkainen *et al*., 2004). Recently, Omer *et al*. (2010) have reported that rosiglitazone treatment in the NAFLD patients with impaired glucose metabolism is more effective in metabolic control and histological improvement. However, most recently, this insulin sensitization drug has been found toxic to the liver (Holman *et al*., 2011). In 2011, this drug was withdrawn from the market in many countries, including South Africa, due to its side effects on cardiac tissue (Ye, 2011).

1.1.5.3 Other drugs and targets for the management of NAFLD

Other drugs and targets for the management of NAFD are shown in **Table 1.2**.

Table 1.2 Potential drugs and targets for NAFLD therapeutic management (Oneta and Dufour, 2002; Lam and Yonossi, 2009).

Target	Treatment
Obesity	Weight loss ▪ Surgical ▪ Pharmacologic - Sibutramine - Orlistat ▪ Diet with or without exercise

Table 1.2 Continued.

Oxidative stress	Antioxidants ▪ Vitamin C, E and others ▪ N-Acetyl-Cysteine (NAC) ▪ Betaine
Hyperlipidaemia	Lipid lowering agents ▪ Omega-3-fatty acids ▪ Statins
Hypertriglyceridemia	Triglyceride lowering agents ▪ Clofibrate ▪ Gemfibrozile ▪ Bezafibrate
Insulin resistance	Insulin sensitizing agents ▪ Metformin ▪ Theazadinediones ▪ Rosiglitazone (Withdrawn from market)
Pro-inflammatory cytokines	Anti-TNF-α agents ▪ Pentoxifyline
Apoptotic pathway	Cytoprotective agents ▪ Lecithin ▪ β-carotene ▪ Ursodeoxycholic acid (UDCA)
Increased endotoxin levels	Probiotics ▪ VSL#3 ▪ Oligofructose
Others	Novel treatment ▪ ACE inhibitors ▪ Second generation sulfonylureas ▪ Incretin analogues

1.2 Medicinal plants and metformin

African traditional medicinal plants have recently drawn scientific interest in elucidation of their efficacies and safety on the treatment and prevention of various diseases including hypertension, heart diseases, T2DM and NAFLD (Latha *et al*., 2011).

Karachi (2009) and Mackenzie (2009) reported the effectiveness of *Prunus africana* (bark) and *Sutherlandia frutescens* (leaves) aqueous extracts on the 38.9% HFD induced insulin resistance and body weight gain in the male Wistar rats. Therefore, due to relationship between insulin resistance and NAFLD as previously discussed above, the present study is designed to elucidate the pathogenesis of 38.9% HFD (see **Figure 3.1** for the dietary compositions) induced fatty liver rat model as well as the efficacy of two selected African traditional medicinal plant aqueous crude extracts, namely, *S. frutescens* (leaves) and *P. africana* (bark), and metformin on the liver metabolic changes during prevention of HFD-induced fatty liver and oxidative stress. These two medicinal plant extracts need to be investigated in the metabolic regulation of liver lipids, glucose, oxidants and antioxidant capacity during prevention of HFD-induced fatty liver and oxidative stress. On the other hand, metformin, which is a drug derived from medicinal plant namely, *Galleda officinalis* (Howlett and Bailey, 2007), is licensed for the treatment of T2DM due to its anti-hyperglycaemia and insulin sensitisation effects. Then metformin was also used as a control in the present study to compare its efficacy to that of the two selected medicinal plant extracts.

Prevention of HFD-induced fatty liver with unchanged dietary habits was implemented in the present study due to the experienced difficulties of the patients to change their dietary habits during treatment of fatty liver, obesity, T2DM and hypertension. Therefore, this study would serve as a trial to elucidate the risks as well as the benefits of unchanging the dietary habits during prevention of HFD-induced fatty liver with the selected crude aqueous plant extracts and metformin, independently. This study would also provide the effects of the HFD rich in palmitic acid in the presence of oleic acid, and the efficacy of two selected medicinal plant aqueous extracts, during prevention of dietary induced fatty liver rat model, as a preliminary test that could be later implemented to a human's pilot study, depending on the satisfactions of the required efficacy on the prevention of fatty liver disease as recommended by the World Health Organization (WHO). High fat diet (HFD) is high in calories that can induce NAFLD depending on the consumed fat type(s), content and time (Reviewed in Buerttner *et al*., 2007). Therefore, this phenomenon can be termed an HFD (or dietary) induced fatty liver to help in the description from those NAFLDs induced by other environmental factors such as toxins, drugs, malnutrition and autoimmune diseases. Thus in Chapter 2, NAFLD would be mentioned as fatty liver and/or HFD-induced fatty liver.

1.2.1 Two selected African traditional medicinal plants and metformin that can be used on the prevention of HFD-induced fatty liver.

It is well announced that Africa and the African traditional healers are greatly blessed with the vegetation and its biodiversity resources (Atawodi, 2005; Elujoba *et al.*, 2005; Hoareau and DaSilva, 1999). Medicinal plants are of great importance to the health of communities and individuals. The medicinal value of these plants lies in some chemical substances that produce a distinct physiological action on the human body (Edeoga *et al*., 2005; Adongo *et al*., 2012). Traditionally, plants are selected for the preparation of herbal medicine as a result of the healers' guidance by their ancestors as an assurance of the plant safety (Elujoba *et al.,* 2005; Okpuzor *et al*., 2008). WHO (1978) defined the traditional medicine as the sum of all the knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. According to WHO (1978), the traditional medicine has to be considered as a solid amalgamation of dynamic medicine and ancestral experience. African traditional medicines are used by over 80% of the populations of the developing world, as the primary health care due to the socioeconomic and socio-cultural heritage, respectively (Elujoba *et al*., 2005). In South Africa, 60 to 80% of people consult the traditional healers before attending the primary health care facilities (Setswe *et al*., 1999).

Alternatively, biomedicine also known as the Western medicine is usually counterpointed with the approach taken by traditional medicine practitioners. The biomedical is often associated with diseases of the physical body only, and is dependent on the principles of science, technology, knowledge and clinical analysis developed in Northern America and Western Europe. Furthermore, most people in South Africa associate traditional medicine with the herbs, remedies (muti or mushonga) and advice imparted by sangomas or izinyangas (vhomaine) traditional healers from African indigenous groups and with strong spiritual components (Richter, 2003). According to Truter (2007) the modern allopathic medicine has its cores in ancient traditions of healing, and some views of those traditions are still relevant and complementary to modern medical practice. Hence biomedical literature refers to the use of traditional medicines as phytotherapy (Richter, 2003). Levin and Das (2000) speculated that each individual of phytotherapeutic agents must be considered differently even from the

same plant source because of differences in extraction technique, preparation of product, composition, clinical and biological activities. This objection could be helpful in the investigation of new active compounds from the same part of plant which was already researched using different solvent. It is also imported for the researchers to mention the solvent used for the medicinal plant preparation to avoid contradictions to the new researchers who would be interested on further investigation of the reported plant extracts.

The active extract from dried medicinal plant known as Milk thistle (*Silybum marianum*) seeds, namely, silymarin is a flavanolignans complex mixture of isosilibin, silidianin, silibinin and silichristin has been used exclusively for the treatment of NAFLD. Amongst others, silybin is the most active and commonly used compound (Pradhan and Girish, 2006). However, all these active components of silymarin have been reported to have protective actions against hepatotoxic effects of drugs used in the chemotherapy of tuberculosis in animal models (Eminzade *et al*., 2008). The silymarin efficacy, safety and future uses in the treatment of liver diseases have been reviewed by Parmar and Gandhi (2008). These include its mechanisms of actions via anti-inflammatory, anti-fibrotic, antioxidative, anti-lipid peroxidative, immunomodulatory, membrane stabilizing and liver regenerating activities. In addition, the silymarin clinical application has been attributed to the treatment of AFL, liver cirrhosis, Amanita mushroom poisoning, viral hepatitis, toxic drug induced liver diseases and diabetes, respectively (Parmar and Gandhi, 2008; Abenavoli *et al*., 2010).

In the present study, the biomedical approach was employed, wherein two African medicinal plants, namely, *Sutherlandia frutescens* (leaves) and *Prunus africana* (bark) were selected to study efficacy of their aqueous extracts on the prevention of HFD-induced fatty liver. It is vital to investigate the medicinal plant extract(s) that can be implemented on the prevention of a HFD-induced fatty liver. However, various *S. frutescens* leaves and *P. africana* bark extracts as well as a biguanidine driven from a medicinal plant, namely metformin have been used in the *in vitro* and *in vivo* studies as depicted in **Sections 1.2.1.1**, **1.2.1.2** and **1.2.1.3**, respectively. Thus, the efficacy of *S. frutescens* leaves and *P. africana* bark aqueous extracts and metformin on the prevention of HFD high in 40.9% palmitic acid in the presence 29.8% oleic acid (**Table 3.1**) induced fatty liver associated with oxidative stress and mitochondrial respiratory chain dysfunction has not yet been studied. The efficacy of metformin on the

treatment of fatty liver in the diabetic patients remains controversial with the recent study reporting that its treatment does not reduce liver lipids (**see Section 1.2.1.3**).

1.2.1.1 *Sutherlandia frutescens* (*Lessertia frutescens*)

Figure 1.6 *Sutherlandia frutescens leaves, wigs, flowers and pods images taken from van Wyk and Albrecht (2008).*

Sutherlandia frutescens is the South African traditional medicinal plant, recently known as *Lessertia frutescens* (**Figure 1.6**) (Johnson *et al*., 2007). The *S. frutescens* is a member of the family Fabaceae and it is traditionally used as a remedy for internal cancers, stomach problems, various inflammatory conditions and diabetes (Avula *et al*., 2010). *S. frutescens* whole dried plant material produced by Phyto Nova in Cape Town is on the market to fight muscle wasting and general infirmity in HIV patients (Mulholland and Drewes, 2004).

In vitro studies have been conducted to elucidate anti-HIV (Bessong *et al*., 2005; Harnett *et al*., 2005), anti-proliferation (Stander *et al*., 2007, 2009; Tai *et al*., 2004), anti-inflammatory (Kundu *et al*., 2005), anti-cancer (Chinkwo, 2005; Steenkamp and Gouws, 2006), anti-oxidant (Fernandes *et al*., 2004, Prevoo *et al*., 2008), antithrombiotic (Low Ah Kee *et al*., 2008) and toxicity (Phulukdaree *et al*., 2010) from various extracts of *S. frutescens* leaves. The *S. frutescens* leaves aqueous extract has been reported to exhibit anti-HIV activity (Hamett *et al*., 2005), while the other studies did not detect its anti-HIV activity (Bessong *et al*., 2005); therefore these different observations were attributed to different experimental assays used per study. Hence the recent study used Phyto Nova *S. frutescens* tablet's ethanol extract reported conflict with preliminary clinical evidence which has suggested that *S.*

frutescens extracts are possibly beneficial in the treatment of HIV infection (Korb *et al.* 2010). In addition, according to Korb *et al.* (2010) despite a lack of knowledge of *S. frutescens* extract potential immune toxicity or mechanism of action, its extracts have been recommended as an adjuvant in the HIV/AIDS treatment by the South African Ministry of Health following its immune-enhancing potential recognition. Moreover, both *S. frutescens* aqueous and methanol extracts have been reported to possess the potential to alter the absorption of atazanavir (ATV, the protease inhibitor which has been included in the South African clinical guidelines for the management of HIV/AIDs in adults and adolescents patients who experienced intolerable gastrointestinal problems, hyperglycaemia and/or hyperlipidaemia) via drug to drug interaction mechanism (Muller *et al*., 2012).

Phulukdaree *et al*. (2010) have reported that Phyto Nova *S. frutescens* tablet's aqueous extract at high concentrations seem to induced an increase in cultured renal proximal (LLC-PK) and distal tubule (MDBK) epithelial cells oxidative stress via decreasing the GSH and increase lipid peroxidation, and to change mitochondrial integrity as observed by increase in caspase3/7 activity and to promote apoptosis in renal tubule epithelial. Most recently, Vorster *et al*. (2012) reported that the carcinogenic MCF-7 cell line are more susceptible to the cytotoxic and cytostatic effects of *Sutherlandia frutescens* aqueous extract, when compared to the non-tumorigenic MCF-12A cell line. Steenkamp and Gouws (2006) provided evidence that different cell lines exhibit different sensitivity towards this plant extract. These differences were due to an observed variations resulted from the oestrogen dependent cancer cell lines, which their growth was inhibited by *S. frutescens* aqueous extract, while stimulating growth of the MCF-12A and MDA-MB231, respectively (Steenkamp and Gouws, 2006).

In addition, *S. frutescens* aqueous extract possess antioxidant activity, which has been attributed to its neutrophil superoxide and H_2O_2 scavenging at concentration 10 μg/mL (Fernades *et al*., 2004). This plant aqueous extract also possess a greater inhibition towards steroidogenic P450 enzymes that are the microsomal CYP17 and CYP21 resulting in reduce glucocorticoid biosynthesis (Prevoo *et al*., 2008). Topical application of the *S. frutescens* leaves methanol extract on the mice skin demonstrated anti-inflammatory activity, which is attributed to its inhibitory action towards ATP induced COX-2 and activation of activator protein 1 (AP-1), while it could not affect ATP induced activation of NFκB (Kundu *et al*.,

2005). On the other hand, *S. frutescens* ethanol extract demonstrated anti-proliferation and induced morphological hallmarks of apoptosis in tumorigenic MCF-7 cells (Stander *et al*., 2007).

In contrast, *S. frutescens* leaves aqueous extract also possess anti-proliferative activity against tumorigenic MCF-7 and non-tumorigenic MCF-12A cell lines through its ability to increase apoptosis at concentrations 5 mg/ml and 10 mg/ml, respectively (Stander *et al*., 2009). A dose dependent from 2 to 10 mg/ml *S. frutescens* extract can mediate a decrease in malignant cell numbers, human breast adenocarcinoma and human-non-tumorigenic epithelial mammary gland cell (Stander *et al*., 2009). *S. frutescens* whole plant aqueous extract induced-cytotoxicity in cervical carcinoma and Chines hamster ovary cell lines showing its apoptotic activity compared to other apoptosis inducer including ceramide and staurosporine (Chinkwo, 2005). Anti-proliferation of *S. frutescens* leaves ethanol extract activity has been reported, while same extract did not possess antioxidant activity against several human tumour cell lines (Tai *et al*., 2004). In addition, this plant extract cannot significantly reduce lipopolysaccharides (LPS) induced NO production in murine/monocyte macrophages (RAW 264.7) cells as well as not significantly inhibits TNF-α and IL-1β mRNA expression in those cells (Tai *et al*., 2004). However, this plant ethanol extract exhibited 50% anti-proliferative activity against several tumor cells including MCF7, MDA-MB-468, Jurkat and HL60 cells at 200, 150 and 250 times dilutions (Tai *et al*., 2004). Recently, Shaik *et al*. (2010) described a protocol for rapid and efficient *in vitro* micropropagation of *S. frutescens*, which can facilitate the easier and faster plantation of this plant.

On the other hand, few *in vivo* studies on *S. frutescens* extract activities have been reported in humans (Johnson *et al*., 2007), mice (Ojewole, 2008) and rats (Chadwick *et al*., 2007, Mackenzie, 2009; Ojewole, 2004). Healthy individuals given 800 mg *S. frutescens* leaves powder/day for three months had presented with a significantly lower respiratory rate, protein, haemoglobin and albumin levels as compared to the placebo group (Johnson *et al*., 2007). In their study, the scientific information on the safety of *S. frutescens* in the South African healthy adults that lead to grounding strict and ethical procedures for directing clinical trials on indigenous medicinal plant treatments has been described (Johnson *et al*., 2007). *S. frutescens* shoot aqueous extract (50-800 mg/kg bwt) has been reported to possess analgesic effects against pain stimuli in mice as well as anti-inflammatory and anti-

hyperglycaemic effects in diabetic rats (Ojewole, 2004). Ojewole (2008) demonstrated that *S. frutescens* shoot aqueous extract possess anticonvulsant activity in mice, which can contribute to the treatment of childhood convulsion and epilepsy, respectively.

In our laboratory, Chadwick *et al*. (2007) demonstrated that in the male Wistar rat fed on 40% HFD induced obese/insulin resistant model, *S. frutescens* leaves aqueous extract normalized blood insulin levels and increased peripheral glucose uptake, while inhibiting its absorption by the intestines. However, HFD used in the present study is different from that used by Chadwick *et al*. (2007). Mackenzie (2009) reported that *S. frutescens* leaves aqueous extract possess anti-insulin resistant activity in the male Wistar rats fed on 38.9% HFD induced insulin resistance ,with no change in the blood glucose levels. It can be suggested that this HFD induced insulin resistance was accompanied by compensatory hyperinsulinemia (Stefanovski *et al*., 2011). It is also well established that enhanced nocturnal FFA, but not glucose, may be responsible for development of insulin resistance and fasting hyperinsulinemia in the HFD-fed model (Kim *et al*., 2007). Thus *S. frutescens* leaves aqueous extract mechanism of actions on the prevention of 38.9% HFD-induced fatty liver and oxidative stress are still unknown. The present study addresses the role of *S. frutescens* leaves aqueous extract in the metabolic regulation of liver lipids, glucose, oxidants and antioxidant capacity during the prevention of HFD-induced fatty liver and modest oxidative stress, thereby elucidating the metabolic pathways that are preserved and/or altered by this plant extract in this HFD rat model.

1.2.1.1.1 The *S. frutescens* leaves identified compounds and their mechanism of actions

Several compounds have been identified from the *S. frutescens* leaves extract including non-protein amino acids, free amino acids, flavonoids, pinitol, triterpenoid (saponins) and other components as depicted below.

i. Non-protein amino acid.

   a) L-canavanine

L-canavanine is a non-protein amino acid reported to be found in *S. frutescens* leaves at a concentrations range from 0.42 to 14.5 mg/g in different populations of this plant and 1.3 to

3.1 mg/g in a commercially available extract (Tai *et al*., 2004; Van Wyk and Albrecht, 2008). Canavanine, a natural analogue of arginine is well known as a specific inhibitor of iNOS and mediator of apoptosis by activating caspase-3 (Suzuki *et al*., 2002). Canavanine is also a substrate of arginyl-RNA synthase and it can replace arginine, thereby incorporating to protein due to its structural similarity to that of arginine, resulting in reduce arginine utilization for protein synthesis, disruption of enzyme activity and rapid protein degradation (Reviewed in Akaogi *et al*., 2006). In addition, the rat liver arginyl-RNA synthase has been reported by Sivaram and Deutscher (1990).

b) γ-Aminobutyric acid (GABA)

γ-Aminobutyric acid (GABA) is an amino acid that functions as an inhibitor for neurotransmitter. GABA content is 0.4 mg/g *S. frutescens* ethanol extract (Tai *et al*., 2004). GABA and glutamate AMPK/Kainate receptors have been reported to negatively regulate DNA synthesis in embryonic cortex mostly via a depolarization-based mechanism (LoTurco, 1995).

ii. Free amino acids

Commercial available *S. frutescens* leaves extract called Phyto Nova SU-1 type contains higher amounts of free amino acids (Tai *et al*., 2004; Van Wyk and Albrecht, 2008) including Proline (0.7-7.5 mg/g), asparagine (1.6-35.0 mg/g), L-arginine (0.5-6.7 mg/g), alanine, aspartate, tryptophan, isoleucine or leucine and phenylalanine. Phenylalanine is an antagonist of L-canavanine that attenuates its anti-proliferative activity (Van Wyk and Albrecht, 2008). Asparagine can be converted to oxaloacetate, whereas proline and arginine can be converted to α-ketoglutarate (Owen *et al*., 2002) that can be used as the sources for the acetyl-CoA synthesis via the TCA cycle (Resendis-Antonio *et al*., 2010). Proline is also known to be used in collagen synthesis, while hyperprolinemia has been proposed as a marker of fibrogenesis (Mukherjee *et al*., 2010).

In human, rats and other mammals, arginine is synthesised from other amino acids, which include proline, glutamine and glutamate through the intestinal-renal axis (Wu *et al*., 2009). Proline is also contained in this plant aqueous extract as indicated above. Arginine degradation occurs through multiple pathways that are catalysed by all three NOS isoforms,

arginase, argininеglycine amidinotransferase, and arginine decarboxylase. These metabolic pathways produce polyamines, glutamate, agmatine and creatine with each having tremendous biological greatness (Wu *et al*., 2009). Amongst other arginine metabolites, creatine has been reported to possess anti-oxidant, anti-inflammatory and anti-hyperglycaemic effects in human (Fang *et al*., 2002; Gualano *et al*., 2008). In the liver, arginine (L-arg) mainly serves as an iNOS and arginase substrate, enzymes that produce NO, L-citrulline and L-ornithine, respectively (**Figure 1.7**) (Tang *et al*., 2009a; Taylor *et al*., 1998).

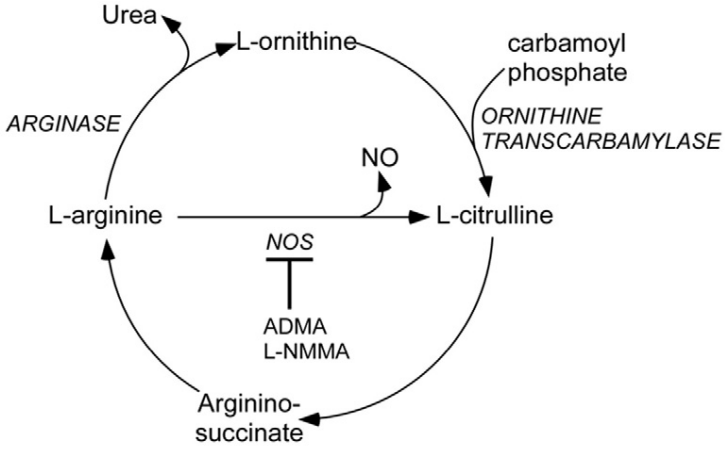


Figure 1.7 *Pathways for nitric oxide production and arginine metabolism (Taken from Tang et al., 2009a). ADMA, asymmetric dimethylarginine; L-NMMA, NG-mono-methyl-L-arginine (NOS inhibitor); NO, nitric oxide; NOS, nitric oxide synthases (Tang et al., 2009a).*

The produced NO may result in an increased NO levels leading to NO-induced cytotoxicity via oxidative injury accompanied with cellular and organ dysfunctions (reviewed by Sia, 2004). In the liver and other tissues, during *de novo* biosynthetic pathway for arginine, L-citrulline is recycled to L-arginine by two enzymes, namely, argininosuccinate synthase and argininosuccinate lyase, generating the essential arginine for NO generation via NOS catalyses (**Figure 1.7**). It has been documented that the induction of iNOS is always accompanied by the enhancement of argininosuccinate synthase expression for NOS synthesis. Therefore, argininosuccinate synthase catalyses condensation of L-citrulline and aspartate to produces argininosuccinate. Then argininosuccinate lyase cleaves argininosuccinate to release arginine and fumarate (Reviewed in Haines *et al*., 2011).

Arginine is also a good substrate for the TCA cycle because it may serve as precursor of α-ketoglutarate, which is the most important precursor of oxaloacetate. In the liver, arginase activities strongly correlate with arginine concentrations, and arginases inhibition uniquely increases the activity of the cytokine-inducible isoform of NOS, namely iNOS, which then activates inflammation and cyclo-oxygenase-2. Thus increased arginase activity or expression has a serious impact on NOS ratio. For example, arginase activity is increased in T2DM patients with impaired NOS activity, and such impairment correlates with the degree of hyperglycaemia and is reversed by insulin (Reviewed in Dioguardi, 2011). According to Sia (2004) L-arginine may be harmful or beneficial to the body, but the question of its safety may be related to the dose administered. Therefore, it can be proposed that *S. frutescens* leaves aqueous extract may play a critical role on the L-citrulline-NO cycle and TCA cycle due to its aspartate and high arginine content.

iii. Pinitol (3-O-methyl-chiroinositol)

Pinitol is a sugar found in many types of leguminous plant. Pinitol acts as an insulin-like thereby reduces the blood glucose levels in hypoinsulinaemic STZ-diabetic mice. In contrast, non-diabetic and severely insulin resistant obese/obese mice 100 mg/kg n-pinitol was found not to affect blood insulin or glucose level (Bates *et al*., 2000). Sia (2004) has documented that pinitol has been described to be possessing anti-acute adema activity and it reduces the production of pro-inflammatory cytokines including IL-1β and TNF-α from the macrophagic cells. It has also been documented that *S. frutescence* aqueous extract and pinitol have similar metabolic effects (Muller *et al*., 2012).

iv. Flavonoids

The novel flavonoids were elucidated from soluble portion of methanol extract prepared from the *S. frutescens* leaves n-butanol extract, including Sutherlandins A, Sutherlandins B, Sutherlandins C and Sutherlandins D. These flavonoids were elucidated from soluble portion of methanol extract prepared form the *S. frutescens* leaves n-butanol extract (Fu *et al.,* 2010b). Avula *et al*. (2010) also elucidated similar four flavonoids from methanol extract to that reported by Fu *et al.* (2010). However, their mechanism of action in the treatment of fatty liver disease and oxidative stress remains elusive.

v. Triterpenoids (saponins)

Triterpenoids including SU1, SU2, SU3, sutherlandioside A, sutherlandioside B, sutherlandioside C and sutherlandioside D. Triterpenoid (cycloartane glycoside), SU3 is an oxonocycloartane diglucoside which was isolated from *S. frutescens* leaves methanol extract as well as the SU1 and SU2 (Olivier *et al*., 2009). The *S. frutescens* leaves-stem methanol extract contains 4 triterpenoids, namely sutherlandiosides A-D in which sutherlandiosides B is dominant triterpenoid compound (Avula *et al*., 2010). Flavonol glycoside and triterpenoid fractions containing the sutherlandiosides and sutherlandins were found to be present in both aqueous and methanolic extracts of *S. frutescens* (Muller *et al*., 2012).

vi. Other compounds (Sia, 2004; Tai *et al*., 2004; Van Wyk and Albrecht, 2008) include γ-sitosterol-stigma-4-en-one, palmitic acid (Hexadecanoic acid), methyl parabens, propyl parabenes, several long chain alcohols, normally found in plants, three long chain fatty acids and 35% polysaccharides/*S. frutescens* aqueous extract dry weight. On the other hand, *S. frutescens* leaves aqueous extract contains approximately 35% polysaccharides/dry weight, however those polysaccharides still need to be identified (Reviewed in Van Wyk and Albrecht, 2008). Bai *et al*. (2009) demonstrated that the crude polysaccharides from plant extract possess liver insulin sensitivity and hypoglycaemic activities, thereby inhibiting G6Pase and activating GK activities, resulting in an increase glycogen synthesis in the liver. Hence it can be proposed that *S. frutescens* leaves aqueous extracts may have an effect in the liver glucose metabolism in the present rat model, due to its reported high content of polysaccharides.

1.2.1.1.2 The *S. frutescens* toxicity

Though the safety of *S. frutescen* extract is based on its long usage in South Africa, there are known side effects such as dry mouth, mild diuresis in cachectic patients, occasional mild diarrhoea and L-canavanine induced autoimmunity (reviewed in Mills *et al*., 2005). L-canavanine, a non-protein amino acid, which can incorporate into protein in place of arginine (Arg) may result in autoimmunity, after its usage for long time (Mills *et al*., 2005). Phulukdaree *et al.* (2010) reported the effect of *S. frutescens* water extract at various concentration using *in vitro* studies in which cultured LLC-PK and MDBK cells were used. Higher concentrations of *S. frutescens* aqueous extract (12 and 24 mg/ml) have resulted in

toxicity to the LLC-PK and MDBK cell lines, thereby inducing an increase in lipid peroxidation, thus MDA, a marker for oxidative stress, to alter mitochondrial integrity and to promote apoptosis, respectively (Phulukdaree *et al*., 2010).

1.2.1.2 *Prunus africana*

Figure 1.8 *Prunus africana's images taken from the http://www.plantzafrica.com/plantnop/prunusafri.htm*

Prunus africana (African cherry) formally known as *Pygeum africanum* (**Figure 1.8**) is the African traditional medicinal plant, which is a member of family Rosaceae (WHO, 2002; Stewart, 2003a). The *P. africana* bark aqueous extract has been traditionally used for many years in the treatment of tuberculosis, fever, chest pain, malaria, sexual dysfunction, malaria and diabetes (Bii *et al*., 2010; Park *et al*., 2008; Stewart, 2009; WHO, 2002). Gathumbi *et al*. (2000) reported the biochemical and haematological responses in healthy Sprague Dawley rats administered oral doses at 10, 100 and 1000 *P. africana* stem-bark aqueous extract mg/kg bwt/day for 8 weeks. Only 1000 mg/kg bwt/day demonstrated to have mild toxicity in the liver and heart of the rats (Gathumbi *et al*., 2000). This plant root and stem bark aqueous extracts at a concentration 80 µg/ml have been reported to possess anti-viral activity against human cytomegalovirus *in vitro* (Tolo *et al*., 2007). Bii *et al*. (2010) demonstrated the potential use of *P. africana* bark methanol extract for the control, management and treatment of common bacterial and fungal infections (Bii *et al*., 2010). However, this plant bark aqueous extract efficacy has not yet being scientifically studied on the prevention of HFD-induced fatty liver and oxidative stress. The *P. africana* bark chloroform extract has been used worldwide over the past 40 years, in the treatment of benign prostatic hyperplasia (BPH)

and it is sold in a tablet form under the trade name Tadenan of the French Dabat laboratories among other various names (Andro and Riffaud, 1995; Yablonsky *et al*., 1997).

1.2.1.2.1 Tadenan's compounds and their mechanisms of action

P. africana bark chloroform extract FFAs composition has been identified by Ganzera *et al*. (1999) as depicted in **Table 1.3**.

Table 1.3 *P. africana* bark chloroform extract FFAs measured as trimethylsilyl derivatives (Ganzera *et al*., 1999)

Fatty acid	Content (%)
Caproic	-
Caprylic	-
Capric	-
Lauric	0.38
Myristic	0.94
Palmitic	28.30
Linoleic	30.60
Linolenic	4.30
Oleic	24.90
Stearic	10.58
Total	100.00

A standardized *P. africana* bark chloroform extract (Tadenan) has been found to possess rat prostatic fibroblast anti-proliferation activity via inhibition of the 5-lipoxygenase activity, the basal growth of human cultured prostatic fibroblast and myofibroblast of the stimulatory effect of the growth factors, such as insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (Boulbes *et al*., 2006; Yablonsky *et al*., 1997).

A. Tadenan (chloroform extract) contains active compounds including

i. Phytosterols include 3-β-sitosterol, 3-β-sitostenone and 3-β-sitosterol-glucoside
ii. Pentacyclic triterpenes include oleanolic and ursolic acids

iii. Long chain fatty acids (LCFAs) include unsaturated and saturated fatty acids from C_{12} to C_{22}, namely -myristic, oleic, linoleic, steric, arachidic, behenic, lignoceric of which palmitic acid is most dominant saturated fatty acid (SFA) while linoleic acid is a dominant unsaturated fatty acid (MUFA) (**Table 1.3**)

iv. Alkanes namely, nonacosane and hentriacontane (Founeau *et al*., 1996; Ganzera *et al*., 1999; Longo and Tira, 1981; Quiles *et al*., 2010; WHO, 2002).

B. Dichloroform and ethanol extracts contain compounds including atratic acid, benzoic acid, N-butylbenzenesulfonamide (NBBS) (Schleich *et al*., 2006a; Schleich *et al*., 2006b; Papaioannou *et al*., 2009)

Recently, Schleich *et al*. (2006a) selectively elucidated atraric acid from dichloromethane extract, which has effective anti-androgenic activity, as determined using the androgenic receptor reporter gene assay for drug discovery. This atratic acid was elucidated next to benzoic acid from *P. africana* for the first time; however, benzoic acid did not show anti-androgenic activity. The atratic acid derivative esters namely, n-propyl and n-butyl have shown higher anti-androgenic activities (Schleich *et al*., 2006a).

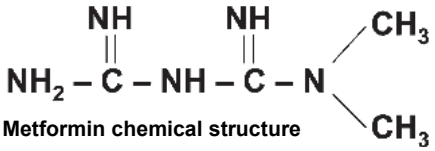
The *P. africana* bark ethanol extract also shown a very strong anti-androgenic activity, while its methanol extract did not show a significant effect, while aqueous extract had no effect on the androgenic activity (Schleich *et al*., 2006b). N-butylbenzenesulfonamide (NBBS) has been elucidated from *P. africana* bark ethanol extract and then latter from dichloromethane extract (Schleich *et al*., 2006b), indicating the presence of the atraric acid and NBBS with anti-androgenic activity on the *P. africana* bark dichloromethane extract, respectively (Schleich *et al*., 2006a; 2006b). NBBS has been further synthesized into 14 derivatives (S1-14) that can serve as novel drugs for treatment of prostate cancer, BPH and prostatitis (Papaioannou *et al*., 2009). Johnson *et al*. (2008) have demonstrated that *P. africana* bark ethanol/acetone/water acetic acid mixture extract contains large amount of total phenols like garlic acid compared to other selected medicinal plants on their study, using gallic acid as control. Tadenan dissolved in peanut oil has shown to suppress oxidative stress in early treatment of the diabetic bladder (Wang *et al*., 2009b). Alternatively, Tadenan dissolved in DMSO has been reported to possess the antiproliferative and apoptotic effects in the cultured prostate stromal cells from patients with BPH (Quiles *et al*., 2010). The Tadenan active compounds, namely, ferulic acid esters are the potent prostatic anti-hypercholesterolemia

(Cristoni *et al*., 2000; Stewart, 2003b). To date there are very few studies on the activity of other *P. africana* bark extracts and no other long term studies on the efficacy of Tadenan has been conducted (Lowe, 2001; Lowe and Fagelman 1996, 1999; Madersbacher *et al*., 2007).

In addition, this plant stem-bark methanol extract has been reported to possess anti-HIV-1 reverse transcriptase activity *in vitro*, at a concentration 100 μg/ml (Rukunga *et al*., 2002). Most recently, *P. africana* ethyl acetate extract's yield and composition from barks collected from the selected Kenyan provenances, namely Eburu, Kakamega, Kinale, Kobujoi and Timboroa forests were compared. From their study, Kinale provenance has been found to contain high number of compounds, while that from Eburu contains high contents of β-sitosterol than that obtained from other provenances (Gachie *et al*., 2012). Furthermore, the amounts of zinc, iron and magnesium have been reported in the *P. africana* leaves and stem bark, revealing that this plant contains some essential minerals (Adongo *et al*., 2012). These minerals were at the recommended levels and are needed for the functioning of body. Thus iron is an essential component of hundreds of proteins and enzymes. Moreover, zinc facilitates the pancreas with its digestive functions; aids metabolize fat, protein and carbohydrate, release vitamin A from storage in the liver and scavenge of damaging ROS. Magnesium is involved in more than 300 essential metabolic reactions (Adongo *et al*., 2012). However it also remains important to study the efficacy of *P. africana* bark aqueous extract on the prevention of HFD-induced fatty liver. Therefore, the present study would report the efficacy of *P. africana* bark aqueous extract on the prevention of HFD-induced fatty liver.

1.2.1.3 Metformin

Metformin (dimethyl guanidine) is an antihyperglycemic drug, derived from a guanidine, which is naturally synthesized in the traditional medicinal plant *Galleda officinalis* (**Figure 1.9**) (Goat's rue, French lilac) (Hadden, 2005; Howlett and Bailey, 2007). This guanidine was discovered in 1918 as an active plant compound possessing low anti-diabetic activity. However, it was found to be too toxic for the clinical use that led to the development of its two derivatives in 1950s, namely, phenformin and metformin (Patel, 2003). Metformin is licensed for the treatment of polygenic disease which in characterised by high glucose levels in the blood namely, type 2 diabetes mellitus (T2DM, insulin dependent diabetes). This is due to metformin's effectiveness in the increase of insulin sensitivity, but is only effective in the presence of insulin (Patel, 2003).



(A) **(B)**

Figure 1.9 *Metformin origin and structure. (**A**) Galleda officinalis also known as Professor Weed (Goat's rue, French lilac) image, and (**B**) metformin chemical structure taken from Howlett and Bailey (2007).*

Most recently, the role metformin on the management of fatty liver has been reviewed in Mazza *et al*. (2012), but the precise conclusion on its treatment guideline could not be reached due to differences in variety of studies (Mazza *et al*., 2012). Hundal *et al*. (2000) demonstrated that patients with uncontrolled T2DM have increase rates of endogenous glucose production, which is related to an increased rate of gluconeogenesis.

On the hand, Leverve *et al.* (2003), metformin demonstrated to possess a unique efficacy in the treatment of hyperglycaemia or insulin resistance related complication including its mild inhibitory of the mitochondrial respiratory chain complex I (NADH dehydrogenase), activation of AMPK in several models and activation of G6PD in the HFD group related insulin resistant animal model. In contracts to their study, Pradhan *et al*. (2009) treatment with insulin causes weight gain while its combination with metformin resulted in a severe hypoglycaemia among patients with T2DM. Furthermore, metformin and/or insulin treatment in T2DM patients improved glucose control, but did not reduced inflammatory biomarker levels (Pradhan *et al*., 2009). Peripheral insulin resistance state accompanied with increase lipogenic activity and lipid storage, but associated with low blood glucose levels is known to be allowed by a low G6P hydrolysis. This protects the animals from the potentially negative effects of insulin resistance by the low blood glucose concentrations (Koceir *et al*., 2003). Lipid oversupply in the male Wistar rats has been demonstrated to diminish the AMPK pathway in the liver that led to induction of metabolic defects, including decrease in phospho-

acetyl-CoA carboxylase (pACC) and PPAR-α mRNA levels, while this pathway was unchanged in the adipose tissue and activated in skeletal muscle (Anavi *et al*., 2010).

Metformin has been demonstrated to induce an increase in the liver GK and G6PD activities, which were significantly decreased in the neonatal streptozotocin (nSTZ) non-insulin-dependent diabetic rats (Chakrabarti *et al*., 2003; Natarajan and Pari, 2005). Metformin and other known hypoglycaemic plant extracts and drugs are known to induce an increase in liver glycogen storage that can lead to hyperuracemia under uncontrolled treatment (Chakrabarti *et al*., 2003; Natarajan and Pari, 2005). Mithieux *et al*. (2002) demonstrated that metformin treatment on the liver of rat fed on HFD had led to the activation of G6PD activity and reduced liver glucose production, indicating its hypoglycaemic effects on the HFD induced insulin resistance and hyperglycaemia rat model with decreased G6PD activity. In the last decade, injection of 120 mg metformin/kg body eight (bwt) unexpectedly stimulated food intake in both rats fed on HFD and HCD, without affecting the blood glucose levels. Alternatively, injection of 200 mg metformin/kg bwt mediated coma and convulsion in five rats, the events hypothesised to be due to metformin induced hypoglycaemia or had unspecific toxic effect at this dose (Prete *et al*., 1999).

Metformin has been suggested to decrease glucose production via short term which is metabolic and long term which is genetic effects (Fulgencio *et al*., 2001), but by then metformin has no well-defined cellular mechanism of action (Zhau *et al*., 2001). Recently, metformin demonstrated to reduce lipolysis in primary rat adipocytes stimulated by TNF-α or isoproterenol, respectively. This antilipolytic action of metformin in the adipocytes can be the mechanism by which its cellular action reduces systemic FFAs concentration and thus improve insulin sensitivity in obese patients and the hyperglycaemia states in T2DM (Ren *et al*., 2006). The antihyperglycemic effects of metformin thereby increasing insulin sensitivity in peripheral tissues, suppressing liver glucose output and increase glucose transport and utilization are well documented (Bailey, 1992). Metformin significantly reduced body weight, but it could not reduce the significantly raised liver fat in T2DM patients (Gupta *et al*., 2010).

Mice fed HFD (rich in lard fat, HF group) containing 2-3 g/kg metformin for 8 weeks had normal plasma fasting TG, low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), but liver TG and TC were significantly lower than that in the HF group, while

significantly higher than that in the LF control. In addition to their study, metformin also prevented body and weight gain induced by HFD alone in their mice model and metformin action was suggested to be via activation of AMPK and suppression of FAS and SREBP-1c expression, respectively (Kim *et al*., 2010). In addition, AMPK is known to increase FA oxidation and to prevent TG synthesis in the liver, thereby leading to decreased lipogenesis and liver glucose production. However, AMPK function in the liver is also enhanced by adipose-derived adiponectin (Reviewed in Gruzman *et al*., 2009). Suppression of G6Pase expression via inhibition of complex I independently from AMPK activation has been reported in the rat H4IIE cells (Ota *et al*., 2009). Foretz *et al.* (2010) demonstrated that metformin inhibits gluconeogenesis independently of AMPK and LKB1 actions, but via decrease expression of the gene encoding the catalytic subunit of G6Pase without affecting cytosolic PEPCK gene expression in wild-type, AMPK-deficient, and LKB1-deficient hepatocytes, respectively.

Metformin decreased liver glucose output in the insulin resistant rats fed HFD (Leverve *et al*., 2003; Mithieux *et al*., 2002) via inhibition of liver glucose-6-phosphatase (G6Pase) activity, promoting either glycogen sparing or the diversion of G6P into PPP, inhibition of liver lactate uptake from plasma and/or activation of liver G6PD activity. A treatment of the male Wistar rats with 631 mg metformin/kg bwt/day has been reported to results in the following changes in three muscles such as increased hexokinase activity in rat white gastrocnemius, increased mitochondrial citrate synthase (mCS) activity in rat soleus, red and white gastrocnemius muscles as well as increased β-hydroxyacyl-CoA dehydrogenase activity in rat soleus (Suwa *et al*., 2006).

Despite the above mentioned changes mediated by metformin in the muscles, GLUT 4 content was unchanged in these rats skeletal muscle that led to a suggesting that metformin can enhance the PPARα-co-activator 1α (PGC1α) expression and mitochondrial biogenesis, via AMPK-phosphorylation partly in the skeletal muscle (Suwa *et al*., 2006). Recently, adult male Wistar rats administered with 100 mg metformin/kg body weight per day for 3 weeks exhibited no notable effects on the significantly raised blood TG, TL and LDL-C levels and in the reduced HDL-C levels, respectively. In addition metformin neither reduce the pancreatic MDA levels nor activates the SOD and GP activities in the diabetic rats. However, this metformin dose did significantly reduce the elevated blood glucose levels and the

pancreatic injury score in these diabetic rats, while significantly increased the serum NO levels (Tang *et al.,* 2009b). The latter effect indicates that metformin may serve as NOS activator. Therefore, metformin effects on the liver iNOS protein expression in the HFD-induced fatty liver and oxidative stress in rats would be reported in present study.

Recently, metformin attracted the researchers to study its effects in the treatment of fatty liver (Cone *et al*., 2010; Forcheron *et al*., 2009; Haukeland *et al*., 2009; Nobili *et al*., 2008). However, in the last decade, metformin has been recommended not to be taken by patients with history of the following disorders: hepatic insufficiency, chronic metabolic acidosis, alcohol abuse, renal insufficiency and severe cardiac or respiratory disease (Brown and Brillon, 1999b). Four months of metformin treatment in the obese insulin resistant patients increased liver insulin sensitivity and decreased blood FFAs without changing the liver lipid contents (Yki-Jarvinen and Westerbacka, 2005). Recently, 1700 mg metformin/day for a year, improved all the liver tests with no change in the elevated blood fasting insulin and glucose levels in the fatty liver patients, with impaired glucose metabolism for 6 month before they start the treatment. In addition, insulin resistance was also not improved by this dose of metformin (Omer *et al* 2010). A long term study on the effects of metformin combined with lifestyle modification suggested being more effective than lifestyle change alone in the treatment of fatty liver (Tock *et al*., 2010). Even lifestyle modification in the form of optimization of diet and physical activity is a primary mode of intervention in the treatment of fatty liver (Yu *et al*., 2009).

1.2.2.3.1 Metformin mechanism of actions

Metformin mechanisms of action are as follows:

i. To attenuate hyperinsulinaemia.
ii. To improve hyperglycaemia via AMPK activation and liver G6Pase inhibition.
iii. To attenuate blood and liver hypertriglyceridemia.
iv. Antioxidant activity via its liver insulin sensitivity action, liver G6PD and GK activation and its mild inhibition of the skeletal muscle mitochondrial complex I activity (Gad *et al*., 2010; Srividhya and Anuradha, 2002, Yki-Jarvinen and Westerbacka, 2005).

1.2.1.3.2 Metformin toxicity

Metformin therapy-induced hepatotoxicity cases are documented (Aksay *et al.,* 2007; Kutoh, 2005), in which the liver biopsy presented severe hepatitis with pericentral necrosis, portal and parenchymal inflammation (Babich *et al*., 1998).

1.3 Motivation to the present study

Since people from different countries have different eating habits, it is critical to investigate the metabolic effects of an HFD prepared following their indigenous-Westernized eating pattern. The amount(s) and composition(s) of a particular dietary fat can play both physiological and pathophysiological functions (Callow *et al*., 2002). SFAs including palmitic and stearic acids, and non-essential MUFA including oleic acid are mainly found in plant and animal products (Erdei *et al*., 2006). SFAs are attributed to mediate body weight gain (obesity), elevate blood fasting glucose and insulin levels and to induce lipid accumulation in the tissues, depending on the amount(s) and type(s) of the consumed FA compositions (Callow *et al*., 2002). HFD rich in either *cis*-MUFAs (Neat *et al*., 1980; Ros, 2003) or (PUFAs) are essential for the mammalian body, due to their ability to improve insulin action and to reduce lipid accumulation in the tissues in both non-diabetic and diabetic subjects, respectively (Clandinin *et al*, 1993; Montoya *et al*., 2002; Sohal *et al*., 1992).

In the year 2002, health survey study was conducted in South Africa and their findings demonstrated that overnutrition and obesity are increase with age, and that higher levels of obesity were in the urban African women. Overnutrition was observed to be strongly associated with age, level of education, ethnicity, and area of residence (Puoane *et al*., 2002). Chronic intake of high calorie is a risk factor for development of obesity, whereas HFD promotes lipid accumulation more than high carbohydrate diet (HCD), because of high energy density and other characteristics (Goedecke *et al*., 2005). It is generally accepted that naturally oxidation of palmitic acid yields 38.9 kJ/g, while that of glucose yields 15.64 kJ/g. Theoretically, the carbons in fat are known to be highly reduced than that in carbohydrate. This is because glucose has more oxygen that does palmitic acid. Therefore, many reducing equivalents are needed to complete palmitic acid reduction for the FAs, TG and cholesterol esters biosynthesis. Thus, NADPH is the major source of electrons for reductive biosynthesis (Mathews and Van Holde, 1996).

On the other hand, increase urbanization is strongly associated with the adoption of the Western diet, which is rich in fat and has less carbohydrate and fiber than the traditional diet (Goedecke *et al*., 2005). In South Africa, a higher prevalence of obesity and hypertension among poor groups has been recently reported, suggesting that this dimensional change might lead to unmanageable chronic diseases epidemic with future socioeconomic development in this country (Schneider *et al*., 2009). Furthermore, Kruger *et al*. (2010) revealed a higher prevalence of fatty liver (87%) among South African obese diabetic patients who are > 45 years of age from the Western Cape Province. Analysis of liver biopsy from these patients confirmed that 51% had fatty liver, 36% had NASH, and 5% had cirrhosis without fatty liver. Their study shows that there is a higher prevalence of both fatty liver (51%) and NASH (36%) among South African adults, which is strongly associated with insulin resistance, dyslipidaemia and obesity, respective (Kruger *et al*., *2*010). Liver remains an organ to be studied in the present, in order to elucidate its metabolic pathways involvement in the development of fatty liver induced by HFD. The efficacy of *S. frutescens* and *P. africana* extracts, and metformin in the metabolic regulation of liver lipid, glucose, oxidants and antioxidant capacity during prevention of HFD-induced fatty liver and oxidative stress would also be reported in the present study under **Chapters 5**, **6**, **7** and **8**.

Liver is a vulnerable organ to toxicity in the body due to its involvement in the lipid, glucose, proteins, amino acids and drug metabolism. Therefore, it is of important to study the effects of HFD rich in palmitic acid in the metabolic pathways during induction of fatty liver and oxidative stress. In the present study, HFD's mechanism of action in the liver as well as to elucidate novel therapeutic targets for prevention of HFD-induced fatty liver with minimal or no side effects that can be used without change in the dietary habits would be established. Mitochondrial would be studied because they play a critical role in liver metabolism. Mitochondria are the primary site for FA oxidation, oxidative phosphorylation for electron transport chain to generate energy and for glucose metabolism via TCA cycle (Review in Wei *et al*., 2008). As previously mentioned, peroxisomes do not have electron transport chain for oxidative phosphorylation, thus $FADH_2$ generated by β-oxidation in peroxisomes is not available for energy production. Thus these differences announce that peroxisomal β-oxidation is less efficient than the mitochondrial pathway (Clark-Taylor and Clark-Taylor, 2004). Indeed, fatty liver has been reported to be closely associated with mitochondrial abnormalities designated ultrastructural lesions, decreased activity of respiratory chain

(electron transport) complexes, depletion of mitochondrial DNA (mtDNA), and impaired mitochondrial β-oxidation (Review in Wei *et al*., 2008). In the present study, the elucidated HFD high in palmitic acid mechanism of action in the etiology of fatty liver *in vivo* will serve as a reference to the future studies.

1.4 Summary of the pathways underlying the theme of the present study

The PPP oxidative branch is a link between carbohydrate, FA, antioxidant, purine and pyrimidine nucleotide metabolism (**Figure 1.10**) and the overview of these metabolic pathways are depicted in **Chapter 2**.

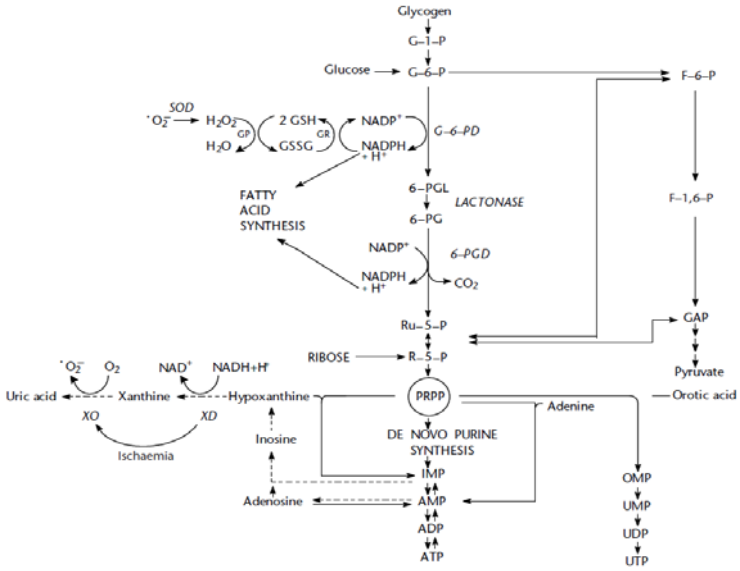


Figure 1.10 *Pathways underlying theme of the present study illustrative diagram taken from Zimmer, (2001). The oxidative PPP (centre), its connections to glycolysis (non-oxidative branch, right-hand side) via the transaldolase and transketolase reactions (arrows), to synthesis of purine and pyrimidine nucleotide via the de novo synthesis and the salvage pathways, and the routes of degradation of ATP (broken arrows) Abbreviations: G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose-6-phosphate; F1,6-P, Fructose-1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; 6-PGL, 6-phosphoglucono-d-lactone; 6-PG, 6-phosphogluconate; Ru-5-P, ribulose 5-phosphate; R-5-P, ribose 5-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; NADP1, nicotinamide adenine dinucleotide phosphate; IMP, inosinemonophosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; OMP, orotidine monophosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; G-6-PD, glucose-6-phosphate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase; GP, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; XD (XDR), xanthine dehydrogenase; XO, xanthine oxidase (Taken from Zimmer, 2001)* .

These pathways include glycolysis via glucokinase (GK), oxidative branch of PPP via glucose-6-phosphate dehydrogenase (G6PD), gluconeogenesis via lactate dehydrogenase (LDH) and glucose-6-phosphotase (G6Pase), purine (hypoxanthine) degradation via xanthine oxidase (XO), antioxidant defence via glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GP), glutathione reductase (GR), catalase (CT) and glutathione-S-transferase (GST) and acetyl-CoA oxidation and *de novo* FA synthesis via mitochondrial citrate synthase (CS) and cytosolic ATP citrate synthase (ACL) as illustrated in **Figure 1.10**.

The liver histology of HFD rich in palmitic acid (40.9%) in the presence of oleic acid (29.8%) induced fatty liver remains elusive. The efficacy of 4 weeks of *S. frutescens* leaves and *P. africana* bark aqueous extract and metformin on the liver histology and metabolic changes during their prevention of HFD-induced fatty liver render investigations. Under inflammatory and apoptotic conditions, iNOS and caspase-3 protein expressions are increased and these mechanisms are involved in the progression from simple fatty liver to NASH. These conditions are mainly enhanced under severe oxidative stress due to decrease in SOD activity, GSH depletion and decrease in its dependents enzymes including GP, GST and GP. Depressed antioxidant enzymes lead to increase accumulation of ROS under severe oxidative stress. ROS may then mediate lipid peroxidation that lead to inflammation and cell death if sufficient ROS are generated by oxidative stress (Koteish and Diehl, 2002). XO and mitochondria NADH oxidase (mNOX) among other oxidases remain the enzymes responsible for generating H_2O_2 and superoxides in the liver. Hence, the effect of HFD on the male Wistar rat liver's pro-oxidants enzyme namely XO and antioxidants, namely GSH, total SOD, CT, GP, GST, GR activities still need to be investigated. Efficacy of 4 weeks of *S. frutescens* leaves and *P. africana* bark aqueous extracts and metformin on these parameters during prevention of HFD-induced fatty liver also remain to be investigated.

The liver cytosolic G6PD is a lipogenic enzyme, which also fuels 6PDH; both are the PPP enzymes, responsible for NADPH generation, when its demand is increased in the tissue. The mitochondrial CS is a lipogenic enzyme, which is a rate limiting in the TCA cycle. This enzyme is responsible for condensing the acetyl-CoA synthesised from either pyruvate or FA oxidation with oxaloacetate to form citrate. The cytosolic lipogenic enzyme, namely, ACL then catalyses citrate from mitochondrial back to oxaloacetate and acetyl-CoA, the latter is a key substrate for the *de novo* FA synthesis via ACC-1 and FAS enzymes. Hence the effect of

HFD-induced fatty liver on the liver cG6PD, mCS and cACL activities remain elusive. The efficacy of 4 weeks of *S. frutescens* leaves and *P. africana* bark aqueous extracts and metformin on these enzyme activities during prevention of HFD-induced fatty liver also remains to be investigated in the present study.

Amongst other organelles, mitochondria dysfunction is strongly associated with fatty liver (Maza *et al*., 2012). Mitochondria play a significant role in the liver during fasting, thereby providing the liver with energy. These organelles have been valued due to their special pathways, including TCA cycle, FA β-oxidation and electron transport chain (Sunny *et al*., 2011). Thus, mitochondria metabolic enzymes remain to be investigated in the present study at levels of mCS activity for the TCA cycle, oxidative phosphorylation complexes I, II and III for electron transport chain, pro-oxidative and anti-oxidative defence mXO, mNOX, mSOD, mGP, mGST and mGP. Complexes I and II play an important roles in the acceptance of NADH and succinate from mitochondrial FA oxidation and acetyl-CoA oxidation through the TCA cycle and pass them to complex III in the mitochondria for ATP synthesis via complexes IV and V. ATP is a major source of energy in the liver because it is also used for gluconeogenesis during fasting. Therefore, the effect of HFD on the liver histology and lipid, glucose, oxidants and antioxidants metabolic enzymes in the male Wistar rat during the induction of fatty liver in week 16 are investigated in the present study. The liver lipid profiles are investigated in weeks 12 and 16 to confirm the onset of fatty liver induced by HFD, before and after prevention studies. The role of 4 weeks of *S. frutescens* leaves and *P. africana* bark aqueous extract and metformin in the metabolic regulation of liver lipids, glucose, oxidants and antioxidants capacity during prevention of HFD-induced fatty liver are also investigated in the present study.

1.5 Aims of the study

This study is aimed at investigating the liver metabolic effect of a 38.9% HFD-induced fatty liver associated with oxidative stress and decreased mitochondrial complex II activity as well as the prevention of HFD-induced fatty liver with metformin and two selected medicinal plant extracts in the male Wistar rats. These were achieved by extracting the liver total lipid (TL) and by measuring the TG and TC levels from those TL. Furthermore, the selected fatty liver's lipid, glucose, oxidative stress and anti-oxidative stress metabolic enzyme markers and the inflammatory and apoptotic protein markers were analysed using the frozen liver samples,

obtained from the overnight fasted rats. Moreover, the study aims were also achieved by conducting the microscopic assessment of the liver histology, considering the metabolic changes induced by HFD and their improvement or worsen by gavage with S. *frutescens* extract or *P. africana* extract or metformin using the liver samples obtained from the lean rats as a control for every experiment.

1.6 Objective of the study

The objectives of this study are as follows:

- To investigate the effect chronic feeding the male Wistar rats on HFD rich in 40.9% palmitic acid (SFA) in the presence of oleic acid (29.8%) in the liver glucose, lipid, oxidants, antioxidants and mitochondrial respiratory chain metabolic changes, which are involved during induction of fatty liver in 16 weeks.
- To investigate the rat liver's histological features mediated by HFD and/or HFD plus 4 weeks of gavage with either *S. frutescens* leaves extract (HF+Sf) or *P. africana* bark extract (HF+Pa) or metformin (HF+Met) per kg body weight per day.
- To elucidate the liver metabolic effects of gavaging the rats with *S. frutescens* leaves aqueous extract (HF+Sf) from week 12 to 16 to prevent HFD-induced fatty liver and oxidative stress.
- To elucidate the liver metabolic effects of gavaging the rats with *P. africana* bark aqueous extract (HF+Pa) from week 12 to 16 to prevent HFD-induced fatty liver and oxidative stress
- To elucidate the liver metabolic effects of gavaging the rats with metformin (HF+Met) from week 12 to 16 to prevent HFD-induced fatty liver and oxidative stress.

1.7 Justification and significance of the study

Exposure to chronic feeding on diet high in saturated fatty acids (SFAs) is attributed to various health risks to human and animals including fatty liver, obesity, insulin resistance, liver dysfunction, heart failure, atherosclerosis and cancer of various body organs. This may affect children and adults of all age groups who reside at the industrialized areas. Given that morbidity of fatty liver includes long-term developmental and liver failure complications are

real and well documented. The knowledge on how the HFD high in SFAs, mainly palmitic acid in the presence of oleic acid during induction of fatty liver remains to be fully investigated. This can be done by targeting some of the liver metabolic enzymes that are involved in the liver lipid and glucose synthesis, pro-oxidants generation and antioxidant defence systems and quantification of lipid contents. There is no information on the efficacy of *S. frutescens* and *P. africana* aqueous extracts and metformin on those metabolic enzymes and antioxidant capacity during the prevention of HFD-induced fatty liver in the rat model used in the present study. Thus, availability of data and information about the HFD rich in palmitic acid and oleic acid induced fatty liver in the male Wistar rats will be helpful in understanding the roles of this diet in the etiology of fatty liver disease.

In addition, elucidation of the roles of *S. frutescens* and *P. africana* aqueous extracts in the metabolic regulation of liver lipids, glucose, oxidants and antioxidant capacity during prevention of HFD rich in palmitic acid induced fatty liver would provide a novel knowledge on these plants safety and/or side effects on this pandemic liver disease. Hence, little is known about rat model used in the present study that was fed on 38.9% HFD high in palmitic acid for 16 weeks (**See Chapter 3**, **Tables 3.2, 3.3** and **3.4** and **Figure 3.1**). The *S. frutescens* leaves aqueous extract and metformin have been reported to exhibit some health improvements in the treatment of insulin resistance, cancer, HIV and diabetes in both *in vitro* and *in vivo* studies as depicted above. In contrast, *P. africana* bark chloroform extract has been attributed to improve prostate cancer and other urinary tract infections. Altogether, given that 16 weeks of chronic feeding on 38.9% HFD induced insulin resistance accompanied with hyperinsulinemia, elevated blood FFAs, TG and TC in the presence of normal blood fasting glucose, HDL-C, LDL-C and TNF-α levels in this rat model (Karachi 2009; Mackenzie, 2009, **see Chapter 3**), mechanism by which 16 weeks of feeding the rats on this HFD-induced fatty liver remains to be fully elucidated and further studies are necessary to investigate the participating body organs during these metabolic changes as well as to study their prevention using either *S. frutescens* leaves or *P. africana* bark aqueous extracts or metformin**.**

The present study is a follow-up to the research studies conducted in our laboratory by Karachi (2009) and Mackenzie (2009). The frozen liver samples obtained from the overnight fasted rats are used in the present study. Since the liver exerts the most important metabolic

functions controlling triglycerides (TG), cholesterol and glucose biosynthesis and uptake, and drug metabolism, it is contributing to maintenance of the body energy balance during the modulation between fed and fasted states. Therefore, the present study would elucidate the liver metabolic changes that are involved during the development of HFD-induced fatty liver associated with oxidative stress, as well as investigating the efficacy of two medicinal plant (*S. frutescens* and *P. africana*) aqueous extracts and metformin on the prevention of this fatty liver condition as mentioned above. The present study's key targets were the lipid, glucose, oxidants and antioxidants metabolic pathways. Influence of gavage with either plant extract or metformin on these selected metabolic pathways would provide new knowledge underlying these plant extracts usage on the prevention of fatty liver without changing the dietary habits. Hence, depending on the required efficacy, data and information from the present study would serve as the guidelines for usage or prohibition of usage of these remedies on the prevention of fatty liver in the presence of chronic feeding on HFD mainly rich in palmitic acid in the presence of oleic acid.

CHAPTER 2: LITERATURE REVIEW

High fat diets (HFDs) and participating metabolic pathways during induction of fatty liver.

Synopsis

This Chapter describes the harmful and beneficial effects of chronic feeding on a high fat diet (HFD) on the liver parameters as well as the glucose, lipid, pro-oxidant and antioxidant metabolic pathways that are involved in the pathogenesis of fatty liver and oxidative stress. Background of two selected African traditional medicinal plants namely, Sutherlandia frutescens and Prunus africana, and metformin as well as their possible mechanism of actions are also described herein. Moreover, the motivation, summery of theme and aims, objectives, Justification and significance to the present study are also depicted in this chapter. Introduction to the present study and its background are described in ***Chapter 3****.*

OUTLINE

2. Literature review
    - 2.1 Harmful and beneficial effects of chronic feeding on a high fat diet (HFD)
    - 2.2. Liver glucose and lipid metabolism
        - 2.2.1 Metabolic pathways
            - 2.2.1.1 Glycolysis
            - 2.2.1.2 Gluconeogenesis and glycogenolysis
            - 2.2.1.3 Pentose phosphate pathway (PPP)
            - 2.2.1.4 Tricarboxylic acid (TCA) cycle in the mitochondrial
                - 2.2.1.4.1 Citrate cleavage pathway in the cytosol
                - 2.2.1.4.2 Acetate as source of fuel for the TCA cycle
                - 2.2.1.4.3 Gluconeogenic or ketogenic precursors for the TCA cycle
                    - 2.2.1.4.3.1 Gluconeogenic anions disposal to the TCA cycle
            - 2.2.1.5 *De novo* lipid synthesis
    - 2.3. Sources of the free fatty acids (FAs) in fatty liver
    - 2.4 Mechanisms of HFD-induced fatty liver

2. Literature review

The liver can be described as the main metabolic organ of the body, but also renders other crucial functions in the body. This organ serves as a principal site for the synthesis of proteins, lipids, lipoproteins, ketone bodies and releases most of the plasma proteins and coagulation factors. The liver remains a central to the metabolism of drugs and nutrients and is one of the major storage places for macro- and micronutrients. The liver also plays a key role in the unspecific immune system by maintaining Kupffer cells that are the bulk of all macrophages. It is well documented that the liver is a key organ in the maintenance of systemic glucose homeostasis in mammals. It keeps blood glucose levels nearly constant under various nutritional conditions and provides a crucial source of fuel for the function of many tissues and organs under conditions of fasting (food deprivation) (Lin *et al*., 2003). The liver is the only organ capable of removing elevated TG and FFAs from the circulation when the adipose tissues are reduced or saturated (Iozzo *et al*., 2004). Together, these functions of the liver must be coordinated and regulated in response to metabolic changes and minor or major injuries (Andus and Holstege, 1994; Dentin *et al*., 2006a). A typical westernized high fat diet (HFD), which is dense in energy, places a heavy burden on the metabolic role of liver function (Manco *et al*., 2004; Puoane *et al*., 2002). Liver's specific lipid metabolism is affected differently by various types of saturated fatty acids (SFAs) (Kien *et al*., 2005; Oosterveer *et al*., 2009a).

As shown in **Table 2.1,** blood and liver parameters, which are altered in the animals fed on an HFD during the fed and fasted states are summarised. High fat diet can mediate metabolic disorders and body weight gain in rodents that resemble human metabolic syndrome (Reviewed in Buerttner *et al*., 2007). In the last decade, HFDs mainly rich in SFAs (Asai and Mayazawa, 2001; Erdei *et al*., 2006) and *trans*-unsaturated FAs (Araya *et al*., 2004; Aro *et al*., 2006; Han *et al*., 2002; Machado *et al*., 2010; Oosterveer *et al*., 2009a) have been reported to mediate body weight gain (obesity), elevate fasting blood glucose and insulin levels (hyperglycaemia and hyperinsulinemia), tissues lipid accumulation and inflammation. On the other hand, HFD rich in either *cis*-monounsaturated fatty acids (MUFAs) (Neat *et al*., 1980; Ros, 2003) or polyunsaturated fatty acids (PUFAs) are essential to the mammalian body, to improve insulin action and to reduce tissues lipid accumulation in both healthy and

diabetic subjects (Clandinin *et al*, 1993; Field *et al*. 1990; Montoya *et al*., 2002; Sohal *et al*., 1992).

Mice fed on 60% HFD [rich in palmitic acid (45%) and oleic acid (40%)] for 16 weeks have been suggested that might have had insulin resistance, due to increase in the fasting blood insulin levels, but they showed less glycaemic control compared to other six HFDs containing less palmitic acid, high oleic and linoleic acids contents (Ikemoto *et al*., 1996, **Table 2.1**). Ikemoto *et al*. (1996) demonstrated in mice that the fatty acid compositions of a HFD influence fasting blood insulin levels that vary amongst fat types, since HFD rich in lard and vegetable oil mediated insulin resistance with no change in the fasting blood insulin levels (Ikemoto *et al*., 1996).

It is also well established that when the peripheral tissues become resistant to insulin, long-term glucose concentrations do not necessarily rise in a pathological characteristic, because the pancreas secrets additional insulin. This maintenance of normal blood glucose via elevated plasma insulin concentrations is termed compensatory hyperinsulinemia. The onset of type 2 diabetes mellitus (T2DM) or impaired glucose tolerance (IGT) indicates a failure of the pancreas to maintain state of compensatory hyperinsulinemia (Cordani *et al*., 2003). Recently, Stefanovsk *et al*., (2011) contended that in dogs, early in the development of HFD induced insulin resistance, a change in plasma FFAs may directly, via signalling at the levels of beta-cells or indirectly, by decreasing liver insulin clearance, result in the observed compensatory hyperinsulinemia. The metabolic effects of three HFDs composed of various fat types have been compared in the male Wistar rats. High fat diet enriched with lard fat (comparative quantities of oleic acid and palmitic acid) demonstrated to induce fatty liver and obesity associated with insulin resistance. Lard fat has been then recommended as one of the standard fats to be used for the propagation of a valid rat model for the metabolic changes associated with obesity. Intake of an HFD enriched with olive oil (monounsaturated fatty acid, MUFA) has been demonstrated not to be protective against metabolic changes.

2.1 Harmful and beneficial effects of chronic feeding on a high fat diet (HFD)

The harmful and beneficial effects induced by chronic feeding on HFD(s) enriched with different FAs are depicted in **Table 2.1**

Table 2.1 Summary of different HFDs and their effects on the liver and blood parameters (**14 pages incessantly**)

HFD	Liver parameters	Plasma parameters	Body weight gain	Feeding time (weeks)	Fasting time (hrs)	Reference
60% HFD enriched with corn oil (palmitic acid: 6.3-18.2%, stearic acid: 0.9-4.5%, oleic acid: 18.5-46.1%, linoleic acid: 36.6-66.8%, linolenic cid: 0-2%, arachidonic acid: 0-1.4%)	iNOS protein expression (I) Caspase-3 expression and activity (I) NO (I) MDA levels (I) SOD activity (D) CT activity (D) GSH level (D)	Bilirubin (I) Glucose(I) Cholesterol (I) TG (I) VLDL (I) LDL-C (I) HDL-C (D)		4	Fasted, time not indicated	**Karmakar *et al.*(2011)** [HFD induced NASH and oxidative stress in the male and female Wistar rats]
60% HFD	TG (I)	Insulin (NC) Glucose(NC) TG (I)	Body weight (NC)	4	12 hrs	**Kosone *et al.* (2007)** [HFD-induced fatty liver in the Tg and WT mice]
45% HFD rich in Palm oil (43% palmitic acid and 41% oleic acid)	TG (I)	Glucose (NC) Glucose clearance (NS)	Body weight (I)	8	Postprandial phase	**Timmers *et al.* (2011)** [HFD palm oil induced insulin resistance in mice]

45% HFD rich in cocoa butter (CB, 25% Palmitic acid, 34% stearic acid and 33% oleic acid)	TG (NC)	Glucose (NC) Glucose clearance (NS)	Body weight (D)	8	Postprandial phase	**Timmers *et al*. (2011)** [HFD cocoa butter in mice]
45% HFD rich in olive oil (OO) (73% oleic acid)	TG (I)	Glucose (NC) Glucose clearance (NS)	Body weight (D)	8	Postprandial phase	**Timmers *et al*. (2011)** [HFD olive oil in mice]
45% HFD rich in safflower oil (SO, 78% linoleic acid)	TG (NC)	Glucose (NC) Glucose clearance (NS)	Body weight (I)	8	Postprandial phase	**Timmers *et al*. (2011)** 8[HFD sunflower oil]
HFD (31.6% saturated fat and 3.2% unsaturated fat)	TG (I) Cholesterol (I) Oxidative stress (I)	Glucose (NC) Insulin (I) HOMA-IR (I)	Body weight (I)	18	Overnight	**Bravo *et al*. (2011)** [HFD feeding induced fatty liver and oxidative stress in rats]
42% HFD consisted of 80 % lard (41 % saturated fat, 44 % MUFA and 9 % PUFA; g fatty acids/100 g of lard)	TG (NS) Glycogen (D)	Glucose (I) Insulin (I) TG (I) NEFA pool (I) Glycerol (I)	Body weight (I)	16	2-3 hrs (Postprandial phase)	**Gauthier *et al*. (2006)** [HFD induced body weight gain in Female Sprague - Dawley strain rats]
42% HFD consisted of 20 % corn oil (13 % saturated fat, 25 % MUFA and 58 % PUFA; g fatty acids/100 g of corn oil).	TG (I) Glycogen (D)	Glucose (I) Insulin (I) Leptin (I) TG (I) NEFA pool (I) Glycerol (I)	Body weight (I)	16	2-3 hrs (Postprandial phase)	**Gauthier *et al*. (2006)** **[**HFD-induced fatty liver in Female Sprague-Dawley rats]

59% HFD rich in Sunflower oil (26% carbohydrate/59% fat/15% protein)	TG (I) Free fatty acyl-CoA (I) Glycogen synthase activity (D) Hepatic insulin resistance (I)	Glucose (NC) FFA (NC)	Body weight (NC)	0.4	12 hrs	**Samuel *et al*. (2004)** [HFD-induced fatty liver in male Sprague-Dawley rats]
66.4% HFD rich in lard fat (15.5% carbohydrate, 66.4% fat, 18.1% protein)	TG (I) ACC-1 (I) CPT-1 expression (D) Glucose infusion rate (D)	FFA (NC)	Body weight (I)	8	8-10 hrs	**Bai *et al*. (2010)** [HFD-induced fatty liver in male Sprague-Dawley rats]
66.4% HFD rich in lard fat (15.5% carbohydrate, 66.4% fat, 18.1% protein)	TG (I) Glucose infusion rate (D)	FFA (NC)	Body weight (I)	12	8-10 hrs	**Bai *et al*. (2010)** [HFD-induced fatty liver in male Sprague-Dawley rats]
66.4% HFD rich in lard fat (15.5% carbohydrate, 66.4% fat, 18.1% protein)	TG (I) ACC-1 (I) CPT-1 expression (NC) Glucose infusion	FFA (I)	Body weight (I)	20	8-10 hrs	**Bai *et al*. (2010)** [HFD-induced fatty liver in male Sprague-Dawley rats]

	rate (D)					
HFD rich in 400 kg/g Beef tallow fat	TG (I) TC (NS) GSH (D) GP activity (D) GR activity (D) GST activity (D)	Insulin (I) Leptin (I) Glucose (NC) TG (I) TC (I) Phospholipids (I) LDL-C (I) HDL-C (NC) Uric acid (NC) Creatinine (NC)	Body weight (I)	10	overnight	**Hsu and Yen (2007)** [HFD-induced fatty liver and oxidative stress in Male Wistar rats]
67% HFD coconut (86% SFA especially lauric acid)	TG (NC)		Body weight (NC)	14	Not fasted	**Romestaing *et al*. (2007)** [Male Wistar rats resistance to HFD-induced fatty liver]
67% HFD milk butter (51% SFA, the most abundant being palmitic acid)	TG (NS)		Body weight (NC)	14	Not fasted	**Romestaing *et al*. (2007)** [Male Wistar rats resistance to HFD-induced fatty liver]
MCD	TG (I)		Body weight (D)	14	Not fasted	**Romestaing *et al*. (2007)** [MCD-induced fatty liver and NASH in Male Wistar rats)
52.6% HFD rich in palm oil (PO, predominantly contains SFA, nearly 50% palmitic and 5% stearic acids)	TG (I) Cholesterol (I) GK expression (I) SREBP-1c expression (I) ACL expression	Glucose (NS) TG (NS) Cholesterol (I)	Body weight (I)	8	3 hrs	**Nisikata *et al*. (2011)** [HFD-induced fatty liver in male mice (C57B6)]

	(NS) ACC expression (NS) FAS expression (D) SCD-1 expression (NS) Elovl-6 expression (NS) DNL (I) Non-DNL FAs (I, palmitic, oleic and linoleic acids)					
52.6% HFD rich in oleic sufflower oil (FO, predominantly contains MUFA, over 80% oleic acid)	Cholesterol (I) TG (I) GK expression (NC) SREBP-1c expression (I) ACL expression (NS) ACC expression (NS) FAS expression (D) SCD-1 expression	Glucose (NS) TG (NS) Cholesterol (I)	Body weight (NC)	8	3 hrs	**Nisikata *et al*. (2011)** [HFD-induced fatty liver in male mice (C57B6)]

	(D) Elovl-6 expression (NS) DNL (I) Non-DNL FAs (I, palmitic, oleic and linoleic acids)					
52.6% HFD rich in soy oil (SO, predominantly contains PUFA, 52% linoleic and 6% linoneic acids)	TG (NC) Cholesterol (NC) GK expression (NC) SREBP-1c expression (I) ACL expression (NS) ACC expression (NS) FAS expression (D) SCD-1 expression (D) Elovl-6 expression (NS) DNL (D) Non-DNL FAs (I,	Glucose (I) TG (NS) Cholesterol (I)	Body weight (I)	8	3 hrs	**Nisikata *et al*. (2011)** [HFD prevented fatty liver in male mice (C57B6)]

	palmitic, oleic and linoleic acids)					
40% HFD rich *Trans*-FA (elaidic acid, 18:1-trans; source (100% hydrogenated soy bean oil). ***Trans*-FA versus PUFA and SFA diets**	TG (I, higher) TC (I, higher) PPAR-γ (greatly I) SREBP-1c (greatly I) PPAR-α (same) CPT-1 (same) MTP (D)	Insulin (I) Glucose (I) HOMA-IR (I, greater) TG (I, higher) TC (I, higher) VLDL (I) LDL-C (I) HDL-C (NC)	Body weight (I. lesser)	16	12 hrs	**Machado *et al.* (2010)** [HFD-induced fatty liver and NASH with mitochondrial dysfunction (inflammatory NASH-like lesions, and impaired glucose tolerance) in male mice (C57BL/6J)]
40% HFD rich in PUFA (Linoleic acid, C18:2 enriched; source 54% sunflower oil, 46% canola oil). **PUFA versus *Trans*-FA diet**	TG (I, lesser) TC (I, lesser) PPAR-γ (D) SREBP-1c (D) PPAR-α (same) CPT-1 (same) MTP (I)	Insulin (I) Glucose (D) HOMA-IR (I, lesser) TG (I, lesser) TC (I, lesser) VLDL (I) LDL-C (I) HDL-C (NC)	Body weight (I, greater)	16	12 hrs	**Machado *et al.* (2010)** [HFD induced mild fatty liver with normal mitochondrial β-oxidation and phosphorylation in male mice (C57BL/6J)]
40% HFD rich in SFA (Palmitic acid, C16:0 enriched; source 11% olive oil, 5% soy bean oil, 84% palm oil). **SFA versus *Trans*-FA diet**	TG (I, lesser) TC (I, lesser) PPAR-γ (D) SREBP-1c (D) PPAR-α (same)	Insulin (I) Glucose (I) HOMA-IR (I, lesser) TG (I, lesser) TC (I, lesser) VLDL (I) LDL-C (I) HDL-C (NC)	Body weight (I, in between that of the PUFA and Trans-FA diets)	16	12 hrs	**Machado *et al.* (2010)** [HFD-induced fatty liver in male mice (C57BL/6J)]

	CPT-1 (same) MTP (D)					
46% HFD rich in 25.5% corn oil and 20.5% beef tallow , 24% carbohydrates [corn starch 6% and sucrose 18%], 20.3% proteins [casein 20% and DL-Methionine 3%], 5% fiber, 3.7% salt mixture and 1% vitamin mixture	MDA (I) Protein carbonyl (I) GSH (D) GP activity (D) GST activity (D) CAT activity (NC)	TG (I) TC (I) LDL-C (I) VLDL-C (I) HDL-C (D)	Body weight (I)	16	Not indicated	**Noeman *et al*. (2011)** [HFD induced obesity and liver oxidative stress in males and females albino rats]
71% HFD	CYP2E1 (I) caspase-3 (I) TNF-α (I) MDA (I) 4-HNE (I) BAX (I) Apoptosis (I) Lipid droplets (I)			6	Not indicated	**Wang *et al*. (2008)** [HFD induced NASH and apoptosis in male Sprague-Dawley rats]
HFD, which contained whole wheat (50.0 g), yellow corn (50.0 g), barley (25.0 g), anik spray (37.5 g), bone meal (2.5 g), calcium chloride (2.5 g), salt (2.5 g),	TL (I) TG (I) TC (I) Free cholesterol (I) Ester cholesterol	VLDL-C (I) LDL-C (I) HDL-C (D) TG/HDL-C (I) LDL-C/HDL-C (I)	Body weight (I)	8	Overnight	**Vijaimohan *et al*. (2006)** [HFD-induced fatty liver in rats]

oil (25.0 g), butter (25.0 g) and vitamin B12 1 tablet	FFAs (I) Phospholipids (I)					
59.2% HFD components were pate, bacon, chips, cookies, chocolate, and chow with proportions 2:1:1:1:1:1	MDA (I)	Glucose (I) Insulin (I) Leptine (I) HOMA-1R (I) TG (NC)	Body weight (I)	8	Overnight	**Milagro *et al*. (2006)** [HFD induced body weight gain and liver oxidative stress]
60% HFD rich in palm oil (45% palmitic acid, 16:0 and 40% oleic acid, 18:1)		Insulin (I) TG (DNS) TC (I)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** [HFD induced hyperglycaemia and obesity in mice]
60% HFD rich in lard (24% palmitic and 44% oleic acid)		Insulin (NS) TG (D) TC (I)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** [HFD induced hyperglycaemia and obesity in mice]
60% HFD rich in rapeseed oil (59% oleic and 20% linoleic Acid, 18:2)		Insulin (NS) TG (D) TC (D)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** [HFD induced hyperglycaemia and obesity in mice]
60% HFD rich in soybean oil (24% oleic and 54% linoleic acid)		Insulin (NS) TG (D) TC (NC)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** HFD induced hyperglycaemia and obesity in mice

60% HFD rich in safflower oil (76% linoleic acid)		Insulin (NS) TG (D) TC (NC)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** [HFD induced hyperglycaemia and obesity in mice]
60% HFD rich in perilla oil (58% α-linolenic acid)		Insulin (NS) TG (D) TC (NC)	Body weight (I)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** HFD induced hyperglycaemia and obesity in mice
60% HFD rich in fish oil (7% eicosapentaenoic acid and 23% docosahexaenoic acid)		Glucose (I) Insulin (DNS) TG (D) TC (NC)	Body weight (NC)	16, 18 and 19 for different parameters	Overnight	**Ikemoto *et al*. (1996)** [HFD prevented obesity in mice]
42% HFD rich in lard fat	TG (I) SREBP-1c (I) PPAR-γ (NC) ACL expression (I) ME expression (I) FAS expression (I) PK (I) FA elongase 1 (I) FA elongase 2 (NC) FA desaturase 2 (I)	Glucose (NC) Insulin (I) HOMA-IR (I) Glucagon (I) TG (NS) FFAs (NS) Adiponectin (D)	Body weight (I)	12	Overnight	**Buettner *et al.* (2006)** [Defining HFD rat models]

	SCD (I) CPT-1 (D) Glycogen synthase (D) GK expression (I) G6Pase catalytic (NC)					
42% HFD rich in olive oil	TG (I) SREBP-1c (I) PPAR-γ (NC) PPAR-α (NC) ACL expression (I) ME expression (I) FAS expression (I) PK (I) FA elongase 1 (I) FA elongase 2 (NC) FA desaturase 2 (I) SCD (I) CPT-1 (D) Glycogen synthase (D) GK expression (I) G6Pase catalytic	Glucose (I) Insulin (NS) HOMA-IR (NS) Glucagon (NS) TG (NS) FFAs (NS) Adiponectin (D) Creatinine (NC)	Body weight (I)	12	Overnight	**Buettner *et al.* (2006)** [Defining HFD rat models]

	(NC)					
42% HFD rich in coconut fat	TG (I) SREBP-1c (I) PPAR-γ (NC) PPAR-α ACL expression (I) ME expression (I) FAS expression (I) PK (I) FA elongase 1 (I) FA elongase 2 (I) FA desaturase 2 (I) SCD (I) Glycogen synthase (D) GK expression (I) G6Pase catalytic (NC)	Glucose (NC) Insulin (NS) HOMA-IR (NS) Glucagon (NS) TG (I) FFAs (NS) Adiponectin (DNS) Creatinine (NC)	Body weight (I)	12	Overnight	**Buettner *et al.* (2006)** [Defining HFD rat models]
42% HFD rich in fish oil	TG (NC) PPAR-γ (NC) PPAR-α (I) SREBP-1c (I) ACL expression	Glucose (NC) Insulin (DNS) HOMA-IR (NC) Glucagon (NS) TG (NC) FFAs (NC) Adiponectin (DNS) Creatinine (NC)	Body weight (D)	12	Overnight	**Buettner *et al.* (2006)** [Defining HFD rat models]

	(NC) ME expression (I) FAS expression (NC) PK (NC) FA elongase 1 (I) FA elongase 2 (NC) FA desaturase 2 (I) SCD (I) CPT-1 (NC) Glycogen synthase (NC) GK expression (NC) G6Pase catalytic (NC)					
60% HFD rich in sunflower oil	Glycogen (ND) G6Pase activity (I) GK activity (D) G6PD activity (D)	Glucose (I) Insulin (NC) TG (NC)	Body gain (NC)	6	5 hrs	**Gad *et al*. (2010)** [Two HFD rat models]

D, significantly decreased; **I**, significantly increased; **NS**, insignificantly increased, **DNS**, insignificantly decreased; **NC**, not changed

In addition, intake of an HFD enriched with coconut fat (SFA) has demonstrated to be less harmful based on obesity and insulin resistance, but it was associated with prominent fatty liver and hypertriglyceridemia, via upregulation of Sterol regulatory element-binding protein-1c (SREBP1c) and lipid synthetic genes. High fat diet enriched with fish oil (PUFA) has demonstrated to sustained insulin sensitivity and lean conditions, which were attributed to PPARα induced predominant fat oxidation gene clusters. From their study, it is believed that dissociation between the peripheral and/or liver resistance to the metabolic and sustained sensitivity to the transcriptional insulin effects is a major aspect in the pathogenesis of obesity related metabolic disorders (Buettner *et al*., 2006).

High fat diet (25.5% corn oil and 20.5% beef tallow) significantly induced body weight gain and liver oxidative stress after 16 weeks in the albino rats (Noeman *et al*., 2011; **Table 2.1**). Ten weeks of feeding male Wistar rats an HFD, enriched with beef tallow (400 g/kg dietary weight), induced macrosvesicular fatty liver associated with oxidative stress and increased blood insulin levels without affecting glucose levels (Hsu and Yen, 2007). A 66.4% HFD enriched with lard fat mediated fatty liver associated with insulin resistance and decreased mitochondrial β-oxidation in the male Sprague-Dawley rat model in 20 weeks (Bai *et al*., 2010). Overall, various HFDs mediate different effects on blood and liver parameters including insulin, TG, FFAs and others, possibly depending on their fatty acids and other dietary compositions as shown in **Table 2.1**.

Stearic, palmitic, linoleic, oleic, arachidonic, eicosapentaenoic and docosahexaenoic acids each has been shown to stimulate nitric oxide (NO) production from inducible nitric oxide synthase (iNOS) *in vitro* at low doses using the microphages J774 cells (de Lima *et al*., 2006). Ricchi *et al.* (2009) demonstrated that in hepatocyte cell cultures, oleic acid is more steatogenic, but less damaging than palmitic acid. Kien *et al*. (2005) showed that high amounts of palmitic acid in diet decreases daily energy utilization and fat oxidation, while increases oleic acid and low amounts of palmitic acid have opposite effects in healthy men. Thus, increased dietary palmitic acid may elevate the risk of insulin resistance and obesity (Kien *et al*., 2005). Moreover, *in vitro* study showed that co-supplementation of palmitic acid with oleic acid partition palmitic acid towards TG accumulation in the non-adipose tissue cell cultures, implying that TG accumulation protects against FA induced apoptosis and lipotoxicity in the tissues (Listenberger *et al*., 2002).

According to Schaffer (2003) there is accumulated evidence from both animal and human studies, suggesting that lipid accumulation in the liver and other non-adipose tissue organs play a critical role in the pathogenesis of obesity, diabetes and heart failure. Thus, excess of nonessential FFAs may impair normal cell signalling, causing cellular dysfunction (Schaffer, 2003). However, HFD mechanism of actions on the induction of fatty liver remains complex and not yet fully understood, because it depends on the duration, amount and type of FA(s) consumed as mentioned above. It remains vital to fully investigate animal model of interest in order to understand its altered pathways mediated by a given HFD-induced fatty liver. Hence, HFD enriched with palmitic acid in the presence of oleic acid can mediate fatty liver, depending on other compositions of diet and the duration of its intake, but its mechanism of actions on the liver glucose and lipid metabolic enzymes and their associated parameters remain complex and not yet fully elucidated. The present study investigates some of the lipids, glucose, oxidants and antioxidants metabolic changes caused by this HFD in the liver as well as their prevention by two selected medicinal plant extracts and metformin.

2.2. Liver glucose and lipid metabolism

Regulation of liver glucose and lipid metabolism plays a vital role in whole body energy-balance. This organ is a major location of glucose metabolism including glycolysis, pentose phosphate pathway (PPP), glycogenesis, gluconeogenesis and glycogenolysis, TCA cycle as well as lipid metabolism including FA oxidation, respiratory chain and biosynthesis of TG, cholesterol, phospholipids and fatty acids (FAs) (Dentin *et al*., 2005; Dentin *et al*., 2006a). According to Dentin *et al*. (2006a), metabolic balance in the liver leads to "partitioning" of FAs away from TG synthesis toward β-oxidation. Lipogenesis is regulated via the acute control of key enzyme activities by allosteric and covalent modifications and by the synthesis of most glycolytic enzymes (Dentin *et al*., 2005). Glucose response occurs in huge part via transcriptional regulation of genes encoding of both glycolytic and lipogenic enzymes and it can be metabolized in the liver to provide substrates like glucose-6-phosphate (G6P) and pyruvate (Kahn, 1997) and acetyl-CoA from pyruvate oxidation for *de novo* FA synthesis (Dentin *et al*., 2005) through mitochondrial TCA cycle and cytosolic PPP **(Figure 2.1)** (Horton, 2002; Resendis-Antonio *et al*., 2010).

After overnight fasting, the liver is the exclusive organ releasing glucose into the systemic circulation, while taking up lactate and glycerol from endogenous and/or circulation for

energy supply and/or storage (Tappy *et al*., 2000). Under these conditions, sources of circulating glucose are derived mainly from liver *de novo* glucose processes including glycogenolysis and gluconeogenesis (the synthesis of glucose primarily from lactate, amino acids or glycerol). Mitochondrial FA oxidation remains the major contributor of acetyl-CoA for energy production in the fasting state (Postic *et al*., 2004). In the liver, acetyl-CoA is also a substrate for biosynthesis of both FAs and ketone bodies. Fatty acids are then incorporated into TG in the cytoplasmic site of the liver for a long term energy reservoir or can result in fatty liver (Dentin *et al*., 2006a). Fatty liver is a core feature for the metabolic syndrome diseases including T2DM and obesity that can lead to liver insulin resistance (Savage *et al*., 2005). Liver insulin resistance is strongly related to intrahepatic lipid contents in human (Sunny *et al*., 2011). Lipids play a role in both metabolic and inflammatory effects during fatty liver, but their effects depend on multiple factors such as dietary compositions and hormonal imbalance. An increase in glucose metabolism is attributed to rise in mitochondrial production of reactive oxygen species (ROS) in obese objects and enhances activation of the inflammatory pathways (Wellen and Hotamisligil, 2005). Metabolic pathways that are involved in cholesterol, phospholipids and TG synthesis from glucose and/or FAs and that of the oxidants and antioxidant are described in **Section 2.2.1**.

2.2.1 Metabolic pathways

A constraint-based analysis of a metabolic network cooperated by a core of the metabolic pathways are recently presented including TCA cycle, glycolysis, PPP and oxidative phosphorylation (Resendis-Antonio *et al*., 2010) (**Figure 2.1**). The liver has a central role in controlling energy homeostasis through glucose, protein and lipid metabolism (Fritsche *et al*., 2008). Metabolic and nutritional aberrations are common to various liver diseases such as fatty liver (Fong *et al*., 2000). Glucose metabolism in the liver takes place from the fed to the fasted state and back. Tight control of blood glucose levels is crucial, since a fall in blood glucose can cause hypoglycaemia, coma, death, seizures, metabolic and brain dysfunctions. A persistent increase in blood glucose levels can cause directly or indirectly glucose toxicity through hyperglycaemia induced tissue damage by increasing mitochondrial superoxide production, which contributes to β-cell dysfunction and other complications of diabetes (Reviewed in Peeters and Baes, 2010).

In order to normalize blood glucose levels, the liver glucose metabolism undergoes a shift from glucose storage through glucose uptake and glycogenesis during feeding and towards glucose output via gluconeogenesis and glycogenolysis during fasting. These metabolic changes are reflected in the expression and/or activity of liver enzymes (Reviewed in Peeters and Baes, 2010).

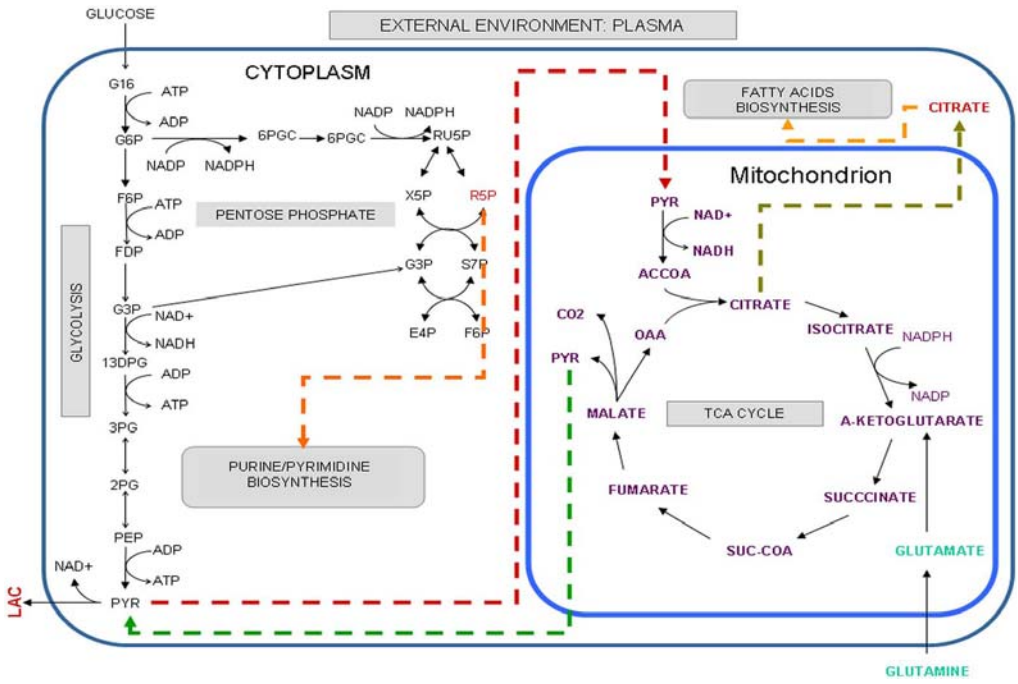


Figure 2.1 *Metabolic pathways (Taken from Resendis-Antonio et al., 2010). Orange, red and green dashed lines indicate metabolites that participate in other biosynthetic pathways, metabolites that can be transported from mitochondrion to cytoplasm and metabolites that can be transported from cytoplasm to mitochondrion. Compartment information has been denoted by external environment, cytoplasm (cytosol) and mitochondria. LAC, lactate; OAA, oxaloacetate; G6P, glucose-6-phosphate (Resendis-Antonio et al., 2010).*

2.2.1.1 Glycolysis

Glycolysis serves as a small generator of ATP and the principal supplier of pyruvate, which can be further metabolized to acetyl-CoA or oxaloacetate. Crucial players in glycolysis include transporters for glucose entry, glucokinase (GK) and the key glycolytic enzymes pyruvate kinase (PK) and phosphofructokinase (PFK). In the fed state, when blood glucose levels are high, glucose is taken up by the liver and rapidly phosphorylated to G6P by GK. Liver GK expression levels and glycolytic flux are mainly regulated in response to the feeding status. Therefore, GK transcription is induced by insulin through sterol regulatory

SREBP-1c and is repressed by glucagon (Ferre *et al*., 2003; Reviewed in Peeters and Baes, 2010; Shonk *et al*., 1965).

During diabetic state, the activity and expression of GK are relatively low as a result of either liver insulin resistance (T2DM) or a lack of insulin (type 1 diabetes mellitus, T1DM). Under these conditions, the liver is ineffective to utilize glucose as a source of energy (Iynedjian *et al*., 1988; Magnuson *et al*., 1989). Alternatively, overexpression of liver GK in the diabetic mice results in improved glucose homeostasis (Ferre *et al*., 1996). Long-term overexpression of liver GK increases *de novo* lipids synthesis and circulating lipids that result in insulin resistance (Ferre *et al*., 2003).

Under the aerobic glycolysis conditions, acetyl-CoA produced from pyruvate oxidation by pyruvate dehydrogenase complex and/or from FA oxidation in the mitochondria enters the TCA cycle to produce the electron donors, namely, NADH and $FADH_2$. Under the anaerobic glycolysis conditions, pyruvate is converted to lactate by muscle lactate dehydrogenase (LDH) which is transported to the liver (Resendis-Antonio *et al*., 2010; Yeluri *et al*., 2009). In the fasting state, liver LDH converts lactate to pyruvate for gluconeogenesis (Peeters and Baes, 2010).

2.2.1.2 Gluconeogenesis and glycogenolysis

Prolonged fasting induces a decrease of the maximal activity of the glycolytic enzyme GK and stimulates the expression of the gluconeogenic gene phosphoenolpyruvate carboxykinase (PEPCK). Simultaneously, the lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC) are suppressed and the activity of mitochondrial *β*-oxidation is enhanced. As a result, the liver carbon flux is directed towards gluconeogenesis and glucose output, rather than glucose uptake and glycolysis, and towards FA oxidation and ketogenesis rather than *de novo* FA synthesis (Reviewed in Peeters and Baes, 2010). Thus, under these conditions, blood glucose for consumption by the red blood cells, kidney, medulla and brain are entirely maintained by gluconeogenesis, which primarily occurs in the liver. The main precursors for liver gluconeogenesis are amino acids, lactate, pyruvate and glycerol. The main portion of lactate is produced in a process known as the Cori cycle, in which lactate produced by glycolysis in exercising muscle is transported to the liver, where it is converted back to glucose. Also in adipose tissue, glucose is metabolized to lactate, which

can subsequently be shuttled to the liver. Glucose can also be transported back to muscle in the glucose-alanine cycle (Reviewed in Peeters and Baes, 2010; Postic *et al*., 2004)

Glucose-6-phosphatase (G6Pase) is a multicomponent complex enzyme located in the endoplasmic reticulum (ER). Hydrolysis of G6P involves the coupled functions of different membrane-spanning translocases that mediate penetration of G6P and efflux of glucose and inorganic phosphate (Pi) (Van Schaftingen and Gerin 2002; Salgado *et al*., 2004). The G6Pase system consists of a putative translocator that transports G6P from the cytosol into the ER lumen and a catalytic subunit that converts G6P to glucose and Pi in the inner ER membrane (Brandsma *et al*., 2001). An increase in glycogenolysis may result in hyperglycaemic conditions. However, insulin inhibits gluconeogenesis by suppressing the expression of key enzymes including G6Pase and PEPCK (Salgado *et al*., 2004). Gluconeogenesis can also be influenced by increased glycerol flux in the liver, a condition which cannot be controlled by insulin in this tissue, since PEPCK is not involved in G6P formation under these conditions (Postic *et al*., 2004)**.**

2.2.1.3 Pentose phosphate pathway (PPP)

Pentose phosphate pathway (PPP) is also known as pentose shunt, hexose monophosphate shunt or phosphogluconate oxidative pathway. This pathway is useful in maintaining the integrity of red blood cell membranes, in biosynthesis of lipid, steroid, antioxidants and in hydroxylation and other anabolic reactions. It is notably active in the liver, lactating mammary glands, and adipose tissue (Bouche *et al*., 2004). The pathway occurs in the cytosol, where it forms a link between glycolysis, gluconeogenesis, glycogenolysis, FA and nucleotides metabolism (Postic *et al*., 2004; Reviewed in Zimmer, 2001). However, PPP also plays a qualitative role as an alternative to TCA cycle in the oxidation of carbohydrate (**Figure 2.1**). This pathway is stimulated by the presence of glucose, which is taken up from blood and phosphorylated to G6P (Zimmer, 2001) and/or by G6P generated from glycogenolysis and glycogenolysis pathways (Postic *et al*., 2002). G6P can then be degraded to a reduced nicotinamide adenine dinucleotide phosphate (NADPH), ribose-5-phosphate (R5P) and glyceride-3-phosphate (G3P) via PPP (Mycielska *et al*., 2009). There are three principal monosaccharides that are resulted from the digestive process, namely fructose, galactose and glucose, in which both fructose and galactose are readily converted to glucose in the liver; thus, for all these simple sugars, glucose metabolism becomes a critical factor in

determining their fates (Bouche *et al*., 2004). G6PD is a rate-limiting enzyme in the PPP (Frederiks *et al*., 2007; Tian *et al*., 1999). This pathway is needed for the following two primary functions in the tissues (Tian *et al*., 1999; reviewed in Zimmer, 2001) namely, to produce ribose-5-phosphate (R5P) and NADPH (**Figure 2.15**). According to Mathews and van Holde (1996) the PPP meets varying metabolic needs via different ways including when:

i. The primary need in the cell is for reducing power i.e. NADPH; the fructose-6-phospates (F6P) from the non-oxidative branch are reconverted to glucose-6-phosphate (G6P) for re-oxidation in the oxidation phase of operation.
ii. The primary need in the cell is for nucleotide biosynthesis, the primary product is R5P
iii. Only moderate quantities of NADPH and pentose phosphates are needed, the pathway can be used to supply energy with the reaction products oxidized via glycolysis and citric acid cycle (TCA cycle).

Pentose phosphate pathway consists of two main branches that are oxidative branch (1st step) and non-oxidative branch (2nd step) **Figure 2.1**. The generated NADPH is cytosolic that serves as a coenzyme for redox processes different from reactions involving NADH. It is now known that the actual entry of glucose into the PPP is controlled by the consumption of NADPH within the cell. Upon utilization of NADPH in the tissues, G6P oxidation increases significantly in the PPP, at which the maximal flux is determined by the cellular activity of G6PD (Spolarics, 1998; Xu *et al*., 2010). Tepperman and Tepperman (1965) suggested that increased G6PD activity in rats fed diets enriched with SFA is secondary to essential fatty acid-deficiency, in which NADPH demand is increased for chain lengthening and desaturation of SFAs.

2.2.1.4 Tricarboxylic acid (TCA) cycle

The sources and fates for the acetyl-CoA that is used for the TCA cycle and synthesis of FAs, sterols and ketone bodies are depicted in **Figures 2.2**. The major source of acetyl-CoA for lipids synthesis is the pyruvate dehydrogenase (PD), enzyme located in the mitochondrial matrix, in contrast to FA oxidation that occurs in the same compartment (Elliott and Elliott, 2001). For acetyl-CoA production, pyruvate becomes a dominant producer in the feeding state, whereas FA oxidation is a dominant producer in the fasting state. The resultant acetyl-CoA cannot cross mitochondrial membrane to cytosol, thus it is converted to citrate by citrate

synthase (CS), an enzyme which is a rate limiting in the TCA cycle (Delvin, 2006). Citrate is an important anion essential for both cation chelation and cellular metabolism (Mycielska *et al*., 2009)

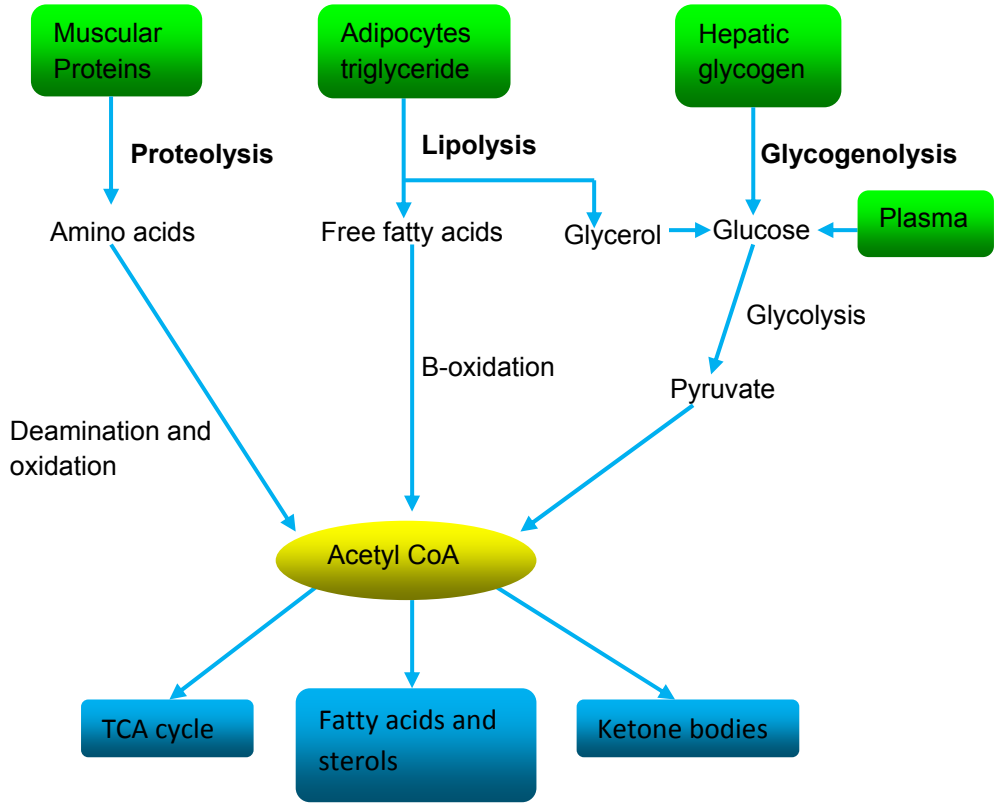


Figure 2.2 *Illustrative diagram of sources and fates of acetyl-Coenzyme A (acetyl-CoA) redrawn from Delvin (2006). Carbohydrates, lipids and proteins are broken down to produce acetyl-CoA that is used for the TCA cycle and synthesis of FAs, sterols and ketone bodies (Delvin, 2006).*

2.2.1.4.1 Citrate cleavage pathway in cytosol

Citrate is then transported from mitochondria by the mitochondria membrane system (**Figure 2.3**) to the cytosol, where it is cleaved to acetyl-coA and oxaloacetate by a reaction catalysed by ATP citrate lyase (ACL) also known as citrate cleavage enzyme (Owen *et al*., 2002; Sun *et al*., 2010; Mycielska *et al*., 2009). Citrate is implicated in the activation of acetyl-CoA carboxylase (ACC); enzyme that produces malonyl-CoA from acetyl-CoA. Malonyl-CoA is an inhibitor of carnitine palmitoyl-CoA transferase 1 (CPT-1), an enzyme involved in the transport of FAs into mitochondria for their oxidation. CPT-1 inhibition eventually raises the level of long-chain (LC)-acyl-CoAs in the cytosol, which can be utilized for lipid synthesis

(MacDonald *et al*., 2005; Wei *et al*., 2008). For every acetyl-CoA produced in the cytosol from citrate generates one NADPH molecule, while each cycle of *de novo* FA synthesis process requires two NADPH molecules. Indeed, other mechanisms for producing extra NADPH is required (Elliott and Elliot, 2001), which is the PPP (**Figure 2.1**) (Zimmer, 2001). In the last 4 decades, citrate has been considered the major carrier in the transfer of acetyl groups from the mitochondrial matrix space to the cytosol during lipid synthesis in the rat liver (Watson *et al*., 1970). This is because citrate cleavage provides both acetyl-CoA and NADPH molecules for *de novo* lipid synthesis in the tissues (Devlin, 2006). It remains important to determine the mitochondrial CS and cytosolic ACL activities to elucidate the role of acetyl-CoA in the *de novo* palmitic acid synthesis in the livers of an HFD-induced fatty liver rat model.

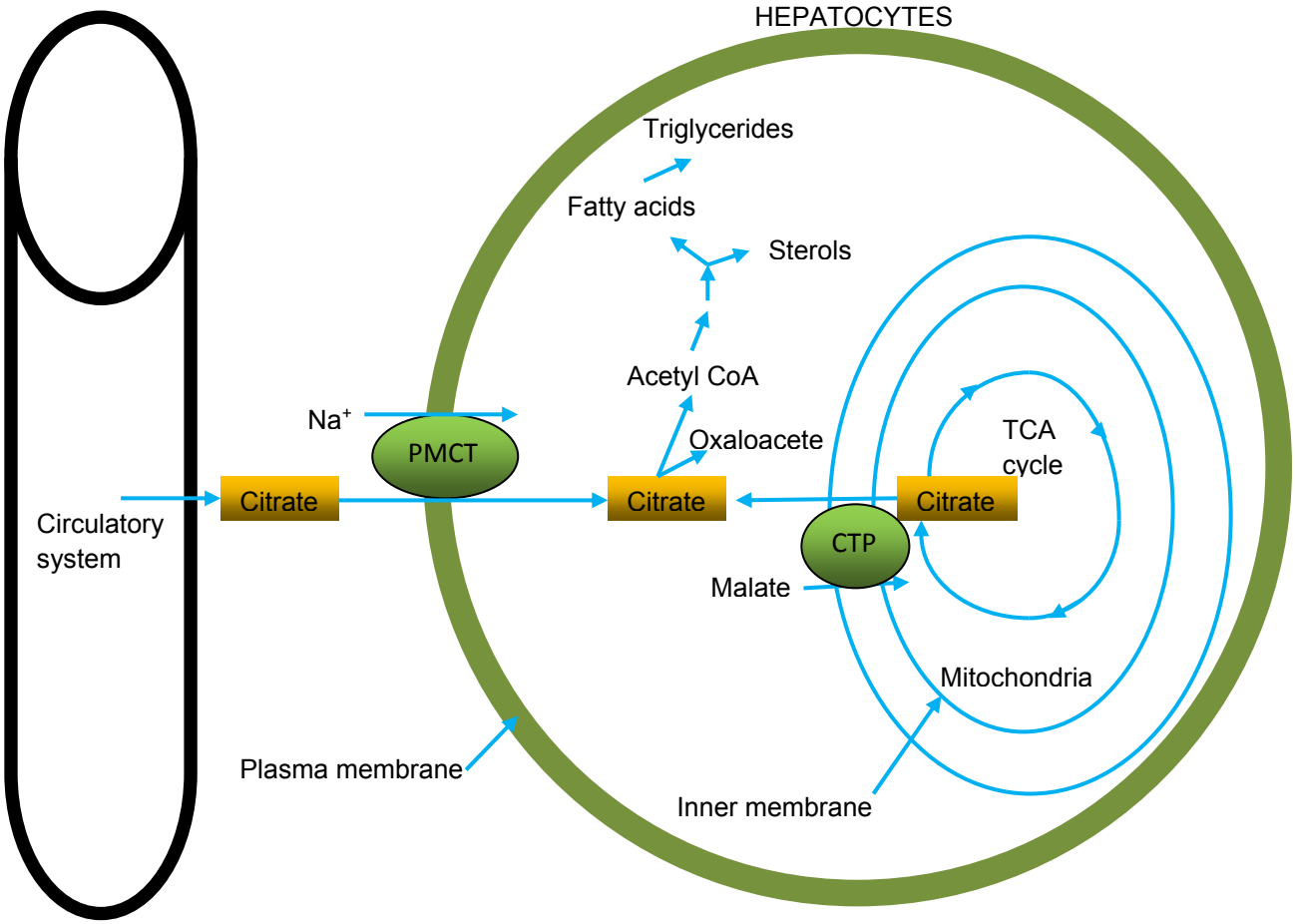


Figure 2.3 *Illustrative diagram of the roles of the PMCT and the CTP in supplying citrate to fuel liver fatty acids (FAs), triglycerides (TG), and sterol biosynthesis redrawn from Sun et al. (2010). Citrate can be transported from the blood across the hepatocyte plasma membrane into the cytoplasm on the PMCT, or it can be released from the mitochondrial matrix across the mitochondrial inner membrane on the CTP. Cytoplasmic*

citrate derived from either source is then broken down to acetyl-CoA by ATP citrate lyase (ACL) and the resulting acetyl-CoA provides the entire carbon precursor to fuel the FAs, TG, and sterol biosynthetic pathways (Sun et al. 2010).

2.2.1.4.2 Acetate as a source of fuel for the TCA cycle

Acetate group that serves as a source of fuel for the TCA cycle is produced from the major-generating pathways in the cells (Delvin, 2006), including oxidation of ethanol and certain amino acids, β-oxidation of long chain FAs such as palmitic (C16:0) and stearic acids (C18:0), breakdown of stored or ingested carbohydrates by glycolysis. These metabolic pathways all produce acetyl-CoA, in which is completely oxidized in the TCA cycle to CO_2 (Delvin, 2006). Thus mitochondria are the primary location of enzymes for the TCA cycle (Wei *et al*., 2008).

2.2.1.4.3 Gluconeogenic or ketogenic precursors for the TCA cycle

The gluconeogenic or ketogenic precursors for the TCA cycle that are produced from catabolism of amino acids (Owen *et al*., 2002) are depicted as follows:

i. Alanine, serine, glycine, threonine, cysteine and tryptophan are converted to pyruvate.
ii. Aspartate and asparagine are converted to oxaloacetate.
iii. Glutamate, glutamine, proline, histidine, arginine are converted to α-ketoglutarate.
iv. Phenylalanine and tyrosine are converted to furmarate.
v. Methionine, isoleucine and valine are converted to succinyl-CoA.
vi. Leucine, isoleucine, lysine, phenylalanine, tyrosine, tryptophan and threonine are converted to acetyl-CoA.

The only known pathway for the terminal oxidation of leucine is via acetoacetate to acetyl-CoA, and subsequent oxidation in the TCA cycle. Other amino acids also have disposal alternate ketogenic pathways for their terminal oxidation. The ketogenic amino acids from proteolysis can be terminally oxidized in muscle, whereas the gluconeogenic amino acids dependent upon the anaplerosis and cataplerosis for their conversion to glucose in the liver and kidney, prior oxidation to CO_2 and H_2O (Owen *et al*., 2002) as described below.

2.2.1.4.3.1 Gluconeogenic anions (negatively charged ions) disposal to the TCA cycle

Theoretically, the disposal of gluconeogenic anions for their terminal oxidation in the TCA cycle involves two pathways, namely anaplerotic and cataplerosis. Anaplerosis is an obligatory during both gluconeogenesis and lipogenesis, when malate (gluconeogenesis) or citrate (lipogenesis) leaves the mitochondria for further metabolism to produce glucose or FAs. Normally, if intermediates can be added to the TCA cycle, it is equally important to remove them to avoid the accumulation of anions in the mitochondrial matrix. Cataplerosis describes reactions involved in the disposal of the TCA cycle intermediates (Owen *et al*., 2002). Therefore, mitochondria remain primary organelles involved in the lipogenesis due to lack of the TCA cycle in the peroxisomes (Mehta *et al*., 2002; Yamashina *et al*., 2009).

2.2.1.5 *De novo* lipid synthetic pathways

The major metabolic intermediates in the *de novo* lipid synthesis (lipogenesis) are depicted in **Figure 2.4**. Cleavage of citrate into acetyl-CoA and oxaloacetate by the cytosolic enzyme namely, ACL remains a key step in the *de novo* lipid synthesis (Leonhardt and Langhans, 2002). Savage and co-workers (2005) suggested that pharmacological inhibition of this lipogenic enzyme, ACC1 may be a novel approach in the treatment of fatty liver and hepatic insulin resistance. Wang *et al*. (2009a) also reported that ACL deletion in the liver improves insulin sensitivity, hyperglycaemia and fatty liver in mice. NADPH is produced by four enzymes, namely G6PD and 6PGD (the first two enzymes in the PPP), malic enzyme (ME) and isocitrate dehydrogenase (IDH, cytosolic). Among other NADPH-producing enzymes, G6PD is the rate-limiting enzyme of PPP, which is controlled by both nutritional and hormonal signals, including high-carbohydrate diet, PUFA, insulin, glucagon, thyroid, and glucocorticoids (Jain *et al*., 2003; Park *et al*., 2005). Second enzyme in the PPP, namely, 6PGD produces as much NADPH as G6PD; but the NADPH produced by 6PGD is entirely dependent on the G6PD activity (Xu *et al*., 2010).

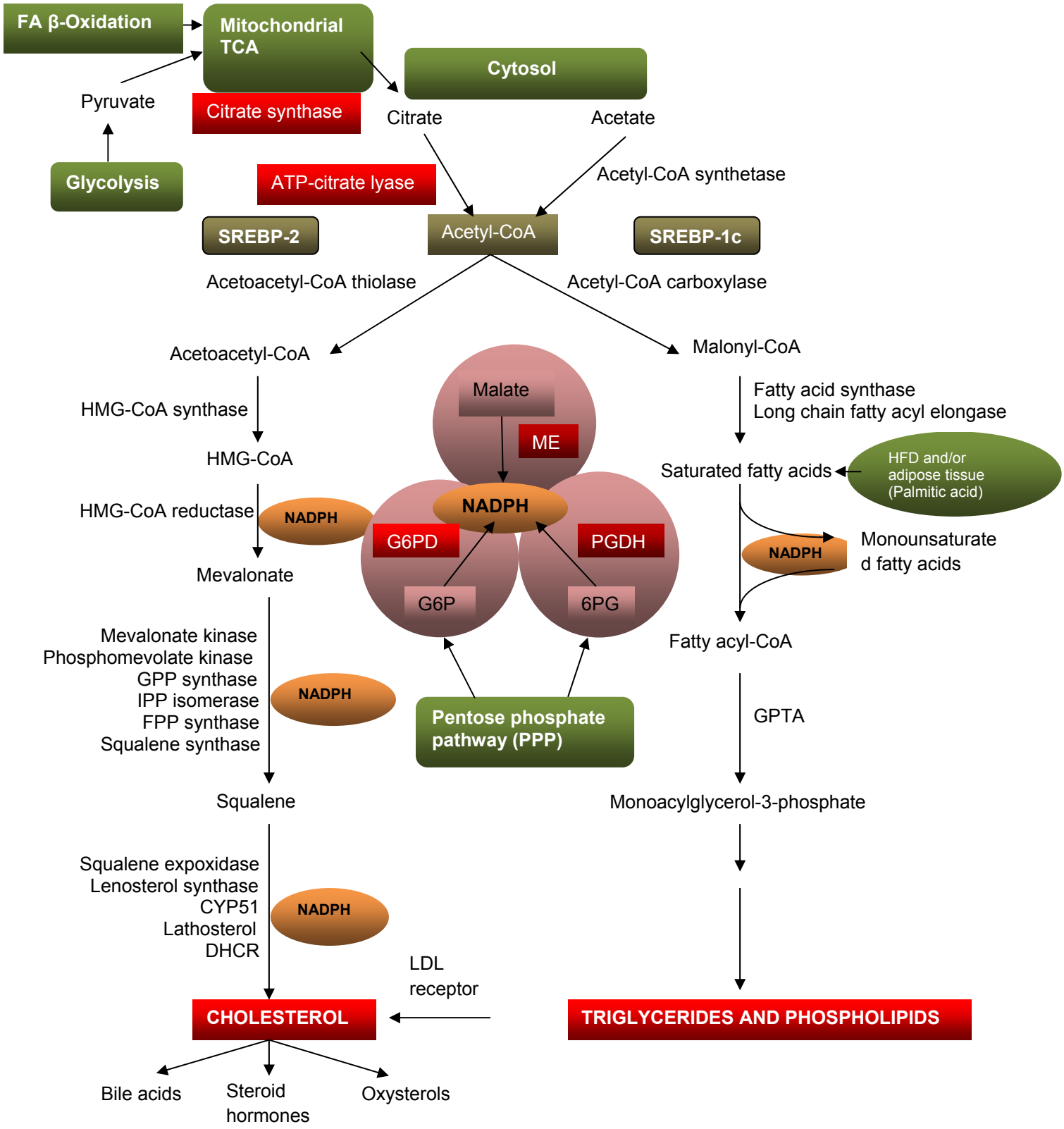


Figure 2.4 *Major metabolic intermediates in the de novo synthesis pathways for TG, FAs, phospholipids and cholesterol illustrative diagram redrawn from Horton, 2002; Dentin et al., 2005; Webber et al., 2004; Block et*

al., 2011) with minor modifications. The measured metabolic fates and the end products in this study are highlighted in red.

2.3. Sources of free fatty acids (FAs) in fatty liver

Non-adipose tissue such as liver has a limited capacity for TG storage (Videla *et al*., 2006). Fatty liver is characterized histologically, by TG accumulation within the cytoplasm of hepatocytes among other lipid types and relates to lipid accumulation in the liver exceeding 5% to 10% by liver weight (Reviewed in Obika and Noguchi, 2012). Fatty liver referred to pathological conditions, characterized by the excess accumulation of lipids within hepatocytes, even though various classes of lipids may accumulate in the liver secondary to chronic HFD consumption, hepatotoxic drug reaction and/or inherited metabolic disorders, cholesterol and TG are the lipids most often involved in fatty liver development (Fong *et al*., 2000). The TG are major lipids component that renders high levels of SFAs, which can aid tissue dysfunction or cell death (Videla *et al*., 2006). Accumulation of lipids in the liver results from imbalance between supply, formation, consumption and liver FA oxidation or disposal of TG. Fatty liver is also caused by a decrease in FA oxidation or their increase synthesis in the liver and release of VLDL from the liver (Trustwell, 1974; Videla, 2009). General sources of the storage and influx of the FFAs to the liver that mediate fatty liver thereby increasing TG accumulation within the hepatocytes cytoplasm are well documented, (Donnelly, 2005; Fong *et al*., 2000; Mantena *et al*., 2008; Wei *et al*., 2008) including:

i. Peripheral adipocytes TG e.g. TG in the adipose tissue can be enzymatically hydrolysed by hormone-sensitive lipase to release glycerol-3-phosphate and FFAs.
ii. Dietary TG including HFD (enriched with SFAs) in the form of chylomicrons, which are enzymatically hydrolysed by lipoprotein lipase.
iii. Endogenous (*de novo*) FAs synthesis in the liver.
iv. Diminished export of lipids from the liver.
v. Reduced FAs oxidation.

Possible sources of lipids that contribute to fatty liver also include plasma NEFA pool from adipose tissue, newly made FAs within the liver via *de novo* lipogenesis and dietary FAs that can enter the liver by two means, through:

i. Overflow into the plasma NEFA pool.

ii. The uptake of intestinally derived chylomicron remnants.

Nearly 60-80% of liver TG is derived from circulating FFAs. About 25% of liver TG are derived from increased *de novo* FA synthesis. *De novo* lipogenesis indicates new palmitic acid synthesis from non-fat precursors such as dietary carbohydrate and it is mediated by enzymes under the transcriptional regulation of SREBP-1c, which is upregulated by insulin and is likely to be activated by hyperinsulinaemia. Imbalance in these pathways is implicated in the pathogenesis of fatty liver. Dietary fat intake is responsible for 15% of FFA supplied to the liver (Donnelly *et al*., 2005; Reviewed in Paschos and Paletas, 2009).

2.4 Mechanisms of HFD-induced fatty liver

Fatty liver is confirmed to be caused by the availability and mobilization of FFAs from diet and/or adipose tissue to the liver, thereby increasing *de novo* FA synthesis, esterification of FFAs into TG and/or decrease in TG export from the liver. These metabolic defects can lead to accumulation of lipids in the liver, serving as an initial insult of the liver by chronic intake of HFD (Lira *et al*., 2010; Liu *et al*., 2010).

In the liver, insulin regulates *de novo* TG, very low density lipoprotein-apoprotein B (VLDL-Apo B) synthesis and their secretion (Basaranoglu and Neuschwander-Tetri, 2006; Tessari *et al*., 2009). The VLDL-Apo B plays an important role in the transportation of TG and FAs from the liver to the blood. The hepatitis B virus surface antigen transgenic mice (HBVS-Tg mice) fed HFD-methionine-choline deficient (MCD) for 20 days developed severe macrovesicular lipid droplets earlier, which was coupled with a significant lost in body weight by 34% and hepatocyte proliferation that may result in increased susceptibility to steatohepatitis. Methionine-choline deficient was confirmed to induce hepatic liver injury by causing an elevation of serum ALT and a decline of serum TG (Fu *et al*., 2010a). Decline in the serum TG may result from impaired VLDL secretion from the liver. Methionine and choline are known as the principal precursors of phosphatidycholine (Fu *et al*., 2010a). Methionine is also an essential amino acid involved in reduced glutathione (GSH) synthesis and DNA methylation (Oz *et al*., 2008). Even though low-density lipoprotein (LDL) cholesterol concentrations maybe unchanged, cholesterol metabolism in NAFLD is characterized by increased synthesis and diminished absorption of cholesterol. Together,

these changes are associated with increased lipid content in the liver, independently from body weight gain (Simonen *et al*., 2011).

2.4.1. Palmitic acid, elongation, desaturation and esterification

Pathway for synthesis of palmitioleic and oleic acids from palmitic and stearatic acids, and their incorporation into TG, phospholipids, and cholesterol esters is shown in **Figure 2.5**. Palmitic acid is the most common SFA in plants and animal lipids (Nishina *et al*., 1993). Among other FA types, oxidation of palmitic acid yields 38.9 kJ/g, while that of simple carbohydrate, namely glucose yields 15.64 kJ/g. Theoretically carbons in lipid are known to be highly reduced relative to that in carbohydrate. This is because glucose has more oxygen than does palmitic acid. Many reducing equivalents are required to completely reduce palmitic acid for biosynthesis of FAs, TG and cholesterol esters. A reducing agent, NADPH is the major source of electrons for biosynthesis (Mathews and Van Holde, 1996). Fujimoto *et al*. (2006) demonstrated that long chain fatty acids (LCFAs) induce lipid droplets formation in a cultured human hepatocyte in a manner dependent of acetyl-CoA synthase (ACS). *In vitro*, 0.3 mM palmitic acid leads to intracellular lipid accumulation in hepatocytes (Joshi-Barve *et al*., 2007). Altogether, these effects indicate that HFD enriched with palmitic acid can induce fatty liver *in vivo* as well as previously depicted in **Table 2.1**.

Palmitic acid is a principal saturated LCFA containing 16 carbons designated as C16:0 and is specifically produced by FAS in the rodents. A high proportion of palmitic acid is converted to stearic acid (18:0) by an elongation process localized in the ER. It has been observed that FAs that accumulate in the liver of SREBP transgenic mice are 18 carbons, rather than 16 carbons in length, indicating that enzymes required for the elongation of palmitic acid to stearic acid may be activated (Moon *et al*., 2001). In the eukaryotic cell, FAs are either synthesized in the cytosol by an enzyme FAS or derived from diet or taken up from circulation can be further desaturated and/or elongated into long-chain (e.g. C16, C18) and very-long-chain fatty acids (VLCFAs) (≥C20) by specific membrane-bound enzymes localized in the endoplasmic reticulum (ER) (Guillou *et al*., 2010). Fatty acid elongases and desaturases play a vital role in whole body and liver lipid compositions (Wang *et al.*, 2006b). Several elongases are governed by stage of development, hormones, dietary factors, pharmacologic compounds and tissue specific factors. Changes in the activity of FA elongases (Elovls) affect cellular FA composition, whilst changes in cellular FA contents

disturb cell function via transcriptional and posttranscriptional regulatory mechanisms (Tripathy *et al*., 2010).

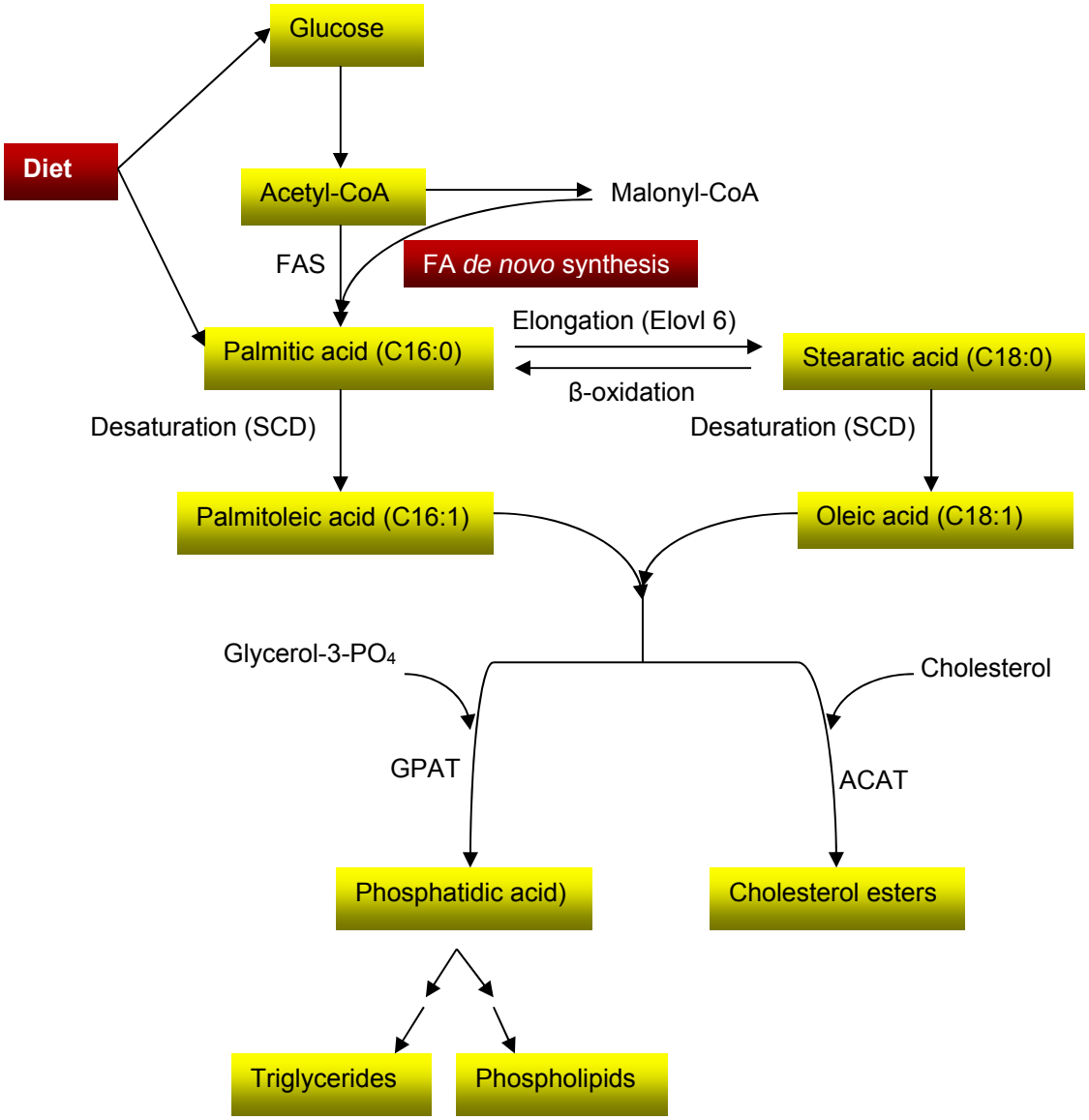


Figure 2.5 *Pathway for synthesis of palmitioleic and oleic acids from palmitic and stearatic acids and their incorporation into triglycerides (TG), phospholipids and cholesterol esters diagram redrawn from Ntambi and Kim (2001) and Jump (2011) with minor modifications. SCD, stearoyl-CoA denaturise; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; ACAT, acyl-CoA: cholesterol acyl treansferase; G-3-PO4, glycerol-3-phosphate (Ntambi and Kim, 2001).*

Palmitic acid (C16:0) is also synthesized endogenously in the body from carbohydrates and other fatty acids containing C12 and less., a mechanism which involves enzymes such as ATP citrate lyase (ACL), acetyl-CoA carboxylase 1 (ACC1), and FAS amongst others. Similarly, subsequent palmitic acid is desaturated and/or elongated by stearoyl-CoA

desaturase (SCD) and long-chain fatty acyl elongase enzymes to yield palmitoleic acid (C16:1, non-essential MUFA), stearic acid (C18:0, nonessential SFA), and oleic acid (C18:1, non-essential MUFA) (Ntambi and Kim, 2001; Postic and Girar, 2008; Jump, 2011). Esterification of FAs to yield TG and eventual packaging into VLDL molecules involve several enzymes, however this process is not yet completely elucidated (Postic and Girar, 2008).

2.4.1.1 Fatty acid elongation (Fatty acid activation step)

After entry of LCFAs into the cells through FA transporters, the long chain acyl-CoA synthetases (LCACS) catalyse the first step in lipid metabolism (**Figure 2.6)** (Li and Wurtman, 1999; Postic and Girard, 2008), which is the formation of fatty acyl-CoA from the fatty acid, ATP and CoA. This activation process of FAs is an important step in their utilization and degradation via β-oxidation and the anabolic pathway for the synthesis of TG, cholesterol esters and phospholipids (Cao *et al*., 2010). Livers of human, mouse and adult rat express four FA elongase (Elovls) subtypes, namely, Elovl-5; Elovl-1; Elovl-2 and Elovl-6 (Jump, 2011; Moon *et al*., 2001; Tripathy *et al*., 2010).

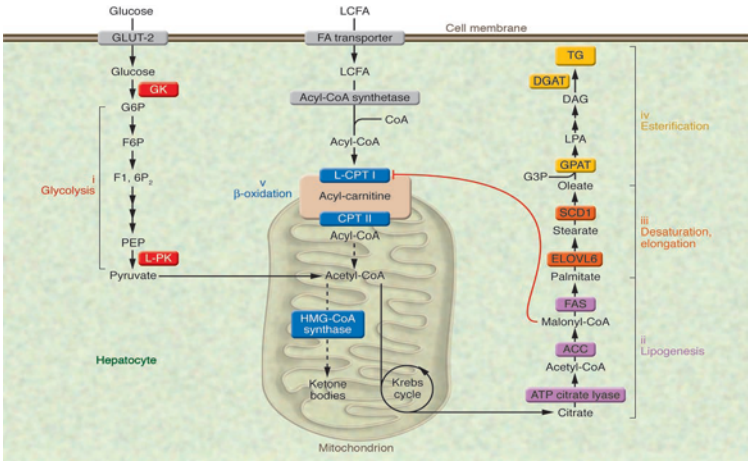


Figure 2.6 *Illustrative diagrams of metabolic pathways that lead to the TG synthesis in the liver (Taken from Postic and Girar, 2008). The TG synthesis in the liver is also nutritionally regulated. The enzymes involved in key metabolic pathways are as follows (i) glucokinase (GK) and L-PK for glycolysis; (ii) ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) for lipogenesis; (iii) ELOVL6 and SCD1 for fatty acid (FA) elongation and desaturation steps; and finally (iv) GPAT and DGAT for TG synthesis. The elevation in malonyl-CoA concentrations, the product of the lipogenic enzyme ACC, inhibits L–CPT-1, the rate-limiting enzyme of β-oxidation (v), which regulates the transfer of long-chain acyl-CoAs from the cytosol into the mitochondria, thus resulting in a shift from an oxidative (production of ketone bodies) to an esterification*

pathway (TG synthesis). F6P, fructose-6-phosphate; F1, 6P2, fructose-1, 6-diphosphate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphatase; PEP, phosphoenol pyruvate; LCFA, long-chain fatty acids; CPT II, carnitine palmitoyltransferase II (Postic and Girar, 2008)

A dietary induced change in elongase activity correlates with Elovl-5 and Elovl-6 mRNA abundance (Wang *et al*., 2005). Both Elovl 1 and Elovl 6 play a role in the elongation of SFA and MUFA, whilst both Elovl 2 and Elovl 5 play a role in the synthesis of n3 and n6 PUFA (Tripathy *et al*., 2010; Jump, 2011). Both Elovl 2 and Elovl 5 elongate 20 carbons (C20) PUFA to produce 22C PUFA. In addition, Elovl 2 exclusively elongates 22 carbons PUFA to form 24 carbons PUFA, a precursor for decosahexaenoic acid (DHA, 22:6 n-3) synthesis (Tripathy *et al*., 2010; Jump, 2011). The HFDs that induce hyperglycaemia are reported to suppress the Elovl 5 activity, while increasing Elovl 6 activity in the liver, by a recombinant adenoviral approach restore blood glucose, insulin and glucose tolerance and suppress the G6Pase and PEPCK activities and increase Elovol 5 products that are 20:3 (n-3) and 22:4 (n-6) PUFAs (Tripathy *et al*., 2010). Thus, Elovl-6 preferred substrates are C12-16 saturated and unsaturated fatty acids, respectively (Wang *et al*., 2005). Elovl-6 is regulated by multiple factors, including insulin and LXR agonist that increases SREBP-1 nuclear abundance, which leads to its expression. Elovl-6 is regulated during postnatal development, its expression declines at birth and is induced at weaning. Elovl-6 expression during early postnatal development is parallels SREBP-1 nuclear abundance (Wang *et al.,* 2005).

2.4.1.2 Fatty acid (FA) desaturation

After FAs activation step, a key enzyme involve in the *de novo* synthesis of monounsaturated fatty acids (MUFAs) from dietary or endogenously synthesized SFAs in the liver is stearoyl-CoA desaturase-1 (SCD-1) also known as delta-9-desaturase (Dobrzyn *et al*., 2004; Liu *et al* 2010). SCD-1 is a rate limiting enzyme that catalysis the synthesis of MUFAs mainly oleic (18:1) and palmitoyl (16:1) acids from the activated stearic and palmitic acids (stearyl-CoA and palmitoyl-CoA) (Ntambi and Kim, 2001). The SCD-1 preferred substrates are stearoyl-CoA and palmitoyl-CoA, which are converted to oleoyl-CoA and palmitoleoyl-CoA. These products are the most abundant MUFAs of TG, cholesterol esters, phospholipids, and wax esters formation. Expression of SCD-1 can influence membrane fluidity, lipid metabolism, and adiposity. An increase in SCD-1 activity and imbalance between MUFAs and SFAs are involved in diseases such as obesity, fatty liver, cancer, diabetes, and atherosclerosis

(Dobrzyn *et al*., 2004; Ntambi and Miyazaki, 2004). This enzyme is an important metabolic control designated in body weight regulation. Inhibition of SCD-1 can lead to decrease in HFD induced obesity and increases insulin sensitivity and metabolic rate (Dobrzyn and Ntambi, 2004). Both Elovl-6 and SCD-1 are induced along with L-PK and FAS, indicating that these enzymes also play a role in the liver response to excess carbohydrate consumption (**Figures 2.7**) (Reviewed in Wang *et al*., 2006a). Insulin-induced glucose metabolism increases ChREBP nuclear content and the ChREBP/MLX, a heterodimer modulates glucose regulated genes such as L-PK, ACC, FAS, and SCD. Elovl-6 is among glucose-regulated genes and it is also activated by the PPAR-α (Wang *et al*., 2006a).

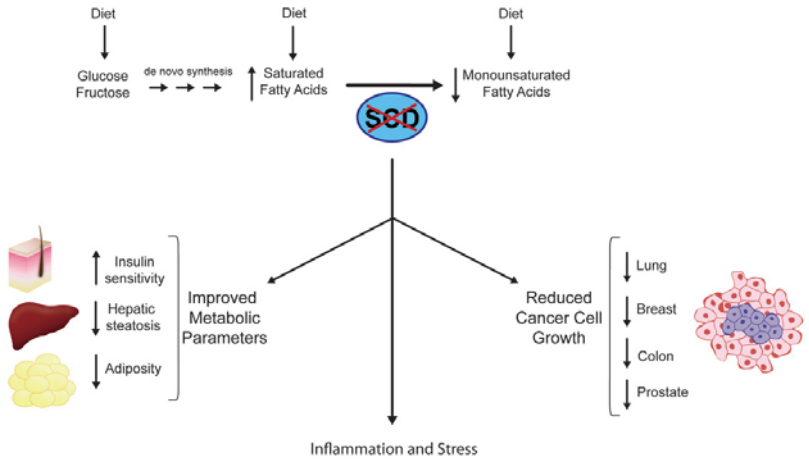


Figure 2.7 *Illustrative diagram of the role of stearoyl-CoA desaturase (SCD) in pathological processes (Taken from Liu et al., 2011). The SCD1 mediates the synthesis of MUFA from dietary or endogenously synthesized saturated fatty acids (SFA). Loss of SCD1 results in a favourable metabolic profile, including an increase in insulin sensitivity and a decrease in hepatic steatosis and adiposity. Inhibition of SCD-1 is also associated with a reduction in cancer cell growth. Additionally, suppression of SCD-1 alters cellular function by modulating inflammation and stress in a number of cell types and tissues, such as adipocytes, liver, macrophages, aorta, skin, myocytes, b-cell and endothelial cell (Liu et al., 2011).*

FAs such as sterculic acid and conjugated linoleic acid are known to inhibit liver enzymes involved in the oleic acid synthesis, result in a decrease in TG synthesis, whereas linoleic and palmitic acids increase the oleic acid synthesis for the TG production. This is due to the fact that in the liver, there is a correlation between VLDL and SCD-1 activity in response to different FAs. Fatty liver can also result from certain FAs that inhibit secretion of TG from the liver to serum and by altering apo-B production. As a consequence, disruption of lipoprotein transport causes FAs to accumulate in the liver, instead of being secreted. This

imbalance in the TG secretion can result a decrease in the blood TG levels. It has been suggested that if the blood TG decreases, it does not mean that FAs are being oxidized and not accumulated in the liver (Aydin, 2005). Inhibition SCD-1 activity is a key target in the treatment of fatty liver and other metabolic parameters (**Figure 2.7**). In supports to their ideologies, it is important to investigate the lipid profiles in the liver and blood in parallel to elucidate the cause of lipid accumulation in this organ (Liu *et al*., 2011). According to Postic and Girard (2008) a decrease in TG synthesis in liver is a potential and key target for the treatment of fatty liver.

2.4.1.3 Esterification

Esterification of FAs to yield TG and eventual packaging into VLDL molecules involve several enzymes, but this process is not yet completely elucidated. Diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2) are important enzymes in the esterification of diacylglycerol to yield TG. It is believed that one of the two enzymes is cytosolic and plays a role in the esterification of FAs to yield TG stored in the liver. Second enzyme is present in the ER lumen, where it re-esterifies FAs released from TG pool to incorporate them into the TG-rich particles that form the mature VLDL molecules (Morral *et al*., 2007). Chylomicrons and VLDLs are TG-rich, apo B-containing lipoprotein particles that are secreted by the liver and intestines. Both are structurally spherical particles consist of a phospholipid monolayer surface, interlarded with proteins and free cholesterol, and a core filled with neutral lipids that are TG and cholesteryl esters. The VLDLs size and composition varies depending on the nutritional state of the animal. Hence, the diameter of VLDL ranges from 30-70 nm, whereas chylomicrons are 70-600 nm in diameter. The average compositions of VLDL are: 60% TG, 15% phospholipids, 15% cholesterol and 10% protein, whilst that for chylomicrons is: 90% TG, 5% phospholipids, 4% cholesterol and 1% protein, respectively (Reviewed in Gordon *et al*., 1995).

Microsomal triglyceride transfer protein (MTP) is required for transferring the bulk of TG into the lumen of the ER for VLDL assembly and secretion of apo B-100 from the liver (Raabe *et al*., 1999). The microarray analysis revealed that gene expression pattern induced by glucose metabolism favours FA storage in the liver, rather than secretion into the circulation (Morral *et al*., 2007). Choi *et al.* (2007) demonstrated that DGAT1 and DGAT2 (two isoforms), catalyse a final step in TG synthesis and their data indicate that knocking

down DGAT2 protects against HFD induced liver insulin resistance, by paradoxically lowering liver diacylglycerol content and protein kinase C activation through decreasing SREBP1c-mediated lipogenesis and increased liver FA oxidation, simultaneously (Choi *et al*., 2007). It has been recommended that diets for the treatment of hypercholesterolemia should contain low myristic and palmitic acids. These were recommended after treating healthy men and women with dietary myristic acid or palmitic acid (10% total energy) for 3 weeks and these fats both had increased LDL-C and apo-B levels and decreased HDL-C/LDL-C ratios (Zock *et al*., 1994). However, their livers lipid contents were not studied. Moreover, the effect of deletion of SCD-1 on lipid metabolism and energy storage has also been outlined by Paton and Ntambi, (2009) as follows:

i. In the presence of SCD-1, liver SFA are converted to MUFAs, which increases SREBP-1c maturation, TG and cholesterol esters synthesis.
ii. In the absence of SCD-1 (SCD-1–/–), the inability to desaturate liver SFA leads to an inability to upregulate *de novo* lipogenesis via SREBP-1c and storage as TG. Hence, β-oxidation and uncoupling increase to decrease TG and VLDL.

2.5 β-oxidation

β-Oxidation is known to partition FFAs away from lipogenesis. Imbalance in β-oxidation may results in increased lipogenesis in the liver, due to a decrease in FA oxidation or increase in ROS production from the mitochondria and peroxisomes as a result of increased FAs oxidation (Purohit *et al.* 2009). Within the liver, hepatocytes play a central role in lipid metabolism. Fatty acids taken up by the hepatocytes are then oxidized by the peroxisomes, mitochondria, and microsomes. Both LCFAs and VLCFAs are oxidised by microsomal, resulting in the production of dicarboxylic acids that are further degraded by peroxisomes (Mehta *et al*., 2002). Peroxisomes also oxidise VLCFAs, showing their important role in lipid disposal from the tissues and they may provide an intracellular therapeutic target for the treatment of the lipid accumulation related diseases such as fatty liver (Noland *et al*., 2007). Purohit *et al.* (2009) demonstrated that inactivation and oxidative modifications of the enzymes involved in mitochondrial and/or peroxisomal β-oxidation may contribute to lipid accumulation in the liver. However, the exact mechanism of action of the enzymes involved in fatty liver development remains elusive (Purohit *et al.,* 2009) and need to be determined. Mitochondria also play a key role in cell death, cellular energy homeostasis and amino acid

metabolism in the tissues (Wolin *et al*., 2005). Mitochondrial β-oxidation is upregulated 10-fold to accommodate large differentials in lipid flux and insulin action during fasting (**Figure 2.8**) (Sunny *et al*., 2011).

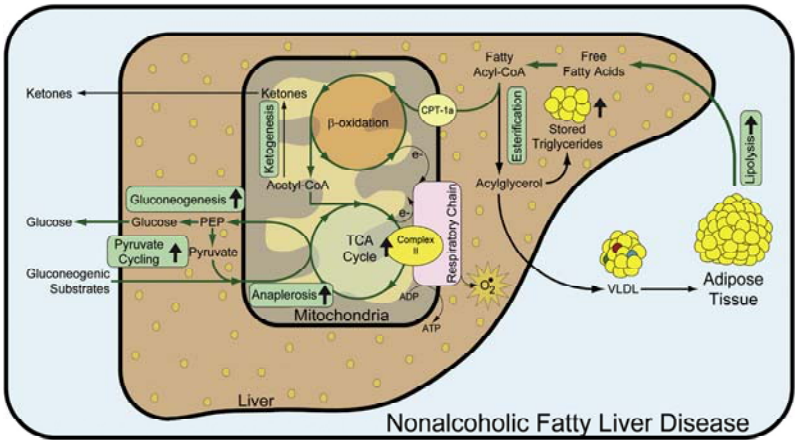


Figure 2.8 *Illustrative diagram of liver mitochondrial metabolisms in subjects with NAFLD (fatty liver) (Taken from Sunny et al., 2011). Subjects with increased liver TG had elevated adipose tissue lipolysis, which contributes to increase lipid delivery to liver (Sunny et al., 2011).*

Induction of lipid oxidation is needed for the endogenic steps of gluconeogenesis and ureagenesis pathways, which are partially localized in liver mitochondria and constitutively upregulated during insulin resistance. Unlike the skeletal muscle, fatty liver and insulin resistance may activate oxidative metabolism (**Figure 2.8**) (Reviewed in Sunny *et al*., 2011). Thus, oxidation of short-, medium-, and long-chain FAs occurs in mitochondria. After the VLCFAs and LCFAs are shortened by the peroxisomal and microsomal oxidation, the mitochondrial β-oxidation system completes the process. This is why mitochondria play a dominant role in FA oxidation and are responsible for majority of disturbances occurring in the lipid metabolism (Mehta *et al*., 2002; Yamashina *et al*., 2009).

Mitochondrial β-oxidation represents a physiological response to tissue energy depletion such as during fasting. β-Oxidation provides approximately 80% of energy for the liver functions (Reviewed in Schmidt and Mandrup, 2011). In addition, liver initiates the synthesis of ketone bodies from acetyl-CoA resulted from increased FA oxidation that spares the use of glucose in other tissues. Adaptation of liver to fasting is mediated by several transcription factors, such as hepatocyte nuclear factor 4 α (HNF4 α), forkhead box protein O1 (FOXO1), cAMP-responsive element-binding protein (CREB), nuclear respiratory factor 1 (NRF1), and

peroxisome proliferatoractivated receptor α (PPAR-α), which activate specific, but overlapping sets of target genes. HNF4α, FOXO1 and CREB are particularly critical for the induction of gluconeogenesis, while PPAR-α is important for induction of genes involved in peroxisomal and mitochondrial β-oxidation and ketogenesis (Reviewed in Schmidt and Mandrup, 2011). Therefore, β-oxidation within mitochondria and FA synthesis in cytoplasm are tightly governed so that they do not occur simultaneously. These two metabolic processes are mainly co-ordinately regulated by malonyl-CoA, a substrate for FAS and an inhibitor of carnitine palmitoyl-CoA transferase-1 (CPT-1), an enzyme involved in the transfer of LCFAs into mitochondria for their oxidation. High levels of malonyl-CoA block FA β-oxidation, whilst promoting FA synthesis (reviewed in Mycielska *et al*., 2009). These alterations may promote fatty liver development. Major enzymes and a key regulator for FA oxidation in the liver include CPT-1, enoyl-coA hydratase and PPAR-α (Buettner *et al*., 2006).

In comparison to mitochondria, peroxisomes do not have the TCA cycle and electron transport chain for oxidative phosphorylation (OXPHOS). These deficiencies render peroxisomal β-oxidation to be incomplete (Noland *et al*., 2007). Thus $FADH_2$ generated by peroxisomes β-oxidation is not available for energy production. These differences announce that peroxisomal β-oxidation is less efficient than the mitochondrial pathway (Clark-Taylor and Clark-Taylor, 2004). It has been documented that inhibition of mitochondrial β-oxidation in the rat liver mediates an increase in TG and cholesterol synthesis (Yamamoto *et al*., 1997). Fatty liver has been reported to be closely associated with mitochondrial abnormalities designated ultrastructural lesions, decreased activity of respiratory (electron transport) chain complexes, mCS activity, depletion of mitochondrial DNA (mtDNA), and impaired mitochondrial β-oxidation in fatty-insulin resistant Zucke rat, whereas CO_2 production and malonyl-CoA levels were significantly increased. Thus mitochondria play a critical role in liver metabolism as they are the primary site for oxidation of FAs, respiratory chain and OXPHOS to generate energy (Noland *et al*., 2007; Reviewed in Wei *et al*., 2008). Therefore, in the present study, mitochondria remain a target to be investigated for their participation in the HFD-induced fatty liver and during its prevention with metformin and two selected medicinal plant extracts.

2.5.1 Mitochondrial respiratory chain reactions

Mitochondria are sites for OXPHOS as mentioned above and they produce most of ATP and ROS via the TCA cycle in animal cells. The mitochondrial inner membrane contains five large enzyme complexes of the respiratory chain (Althoff *et al*., 2011; Murphy, 2009) as depicted in **Figure 2.9**.

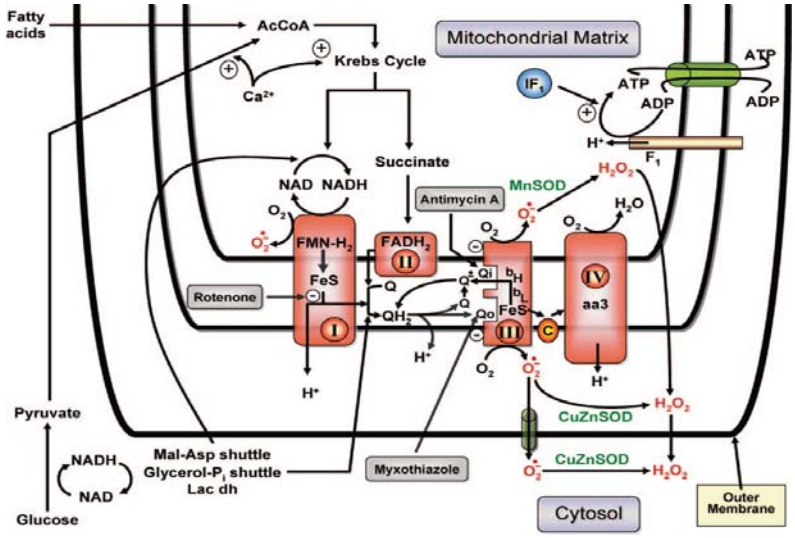


Figure 2.9 *Illustrative diagrams of mitochondrial matrix's respiratory chain pathways (Taken from Wolin et al., 2005).*

Mitochondria are also essential for the cytoplasmic processes of glycolysis and lipolysis products to generate energy in the form of ATP. To the electron transport chain, NADH donates electrons to complex I, whilst $FADH_2$ donates electrons to complex II. In the mitochondrial oxidative phosphorylation, oxygen acts as the final electron acceptor. Mitochondrial energy metabolism modulates cell proliferation and its biogenesis. Cell death pathways are also regulated in the mitochondrial through production of ROS from complexes I and III (**Figure 2.10**) (Wolin *et al*., 2005; Yamashina *et al*., 2009). Breakdown of cellular homeostasis mediated by mitochondrial dysfunction develops chronic liver diseases including fatty liver, cirrhosis, and HCC. According to Yamashina *et al*. (2009), elucidation of mitochondrial energy metabolism and transcriptional mechanisms that regulate mitochondrial function and biogenesis can offer insights into possible therapeutic interventions in the treatment of liver diseases. It is well recognized that in mammals, food-derived and TCA cycle-driven $FADH_2$ or NADH is the electron donor and transfers electron to an O_2 molecule

through a number of redox components in complex I via IV in the mitochondrial respiratory chain (**Figure 2.10**) (Wolin *et al* 2005; Yamashina *et al*., 2009).

Glycolysis and TCA cycle and generate less energy as ATP than that generated from mitochondrial FA oxidation and respiratory chain. Interestingly, six dehydrogenation processes, in which one takes place in glycolysis, another in the pyruvate dehydrogenase reaction and four in the TCA cycle collectively, reduce 10 moles of NAD^+ to NADH and 2 moles of FAD to $FADH_2$ per molecule of glucose. These reduced products are then re-oxidized by electron transport proteins that are bound in the inner mitochondrial membrane (Mathews and van Holde, 1996). The protein carries primarily cytochromes constitutes the respiratory chain that are assembled in the form of five large enzyme complexes (**Figures 2.9** and **2.10**) (Althoff *et al*., 2011; Yamashina *et al*., 2009), assigned as

- Complex I also known as NADH dehydrogenase.
- Complex II also known as succinate dehydrogenase.
- Complex III also known as cytochrome c reductase.
- Complex IV also known as cytochrome c oxidase.
- Complex V also known as ATP synthase.

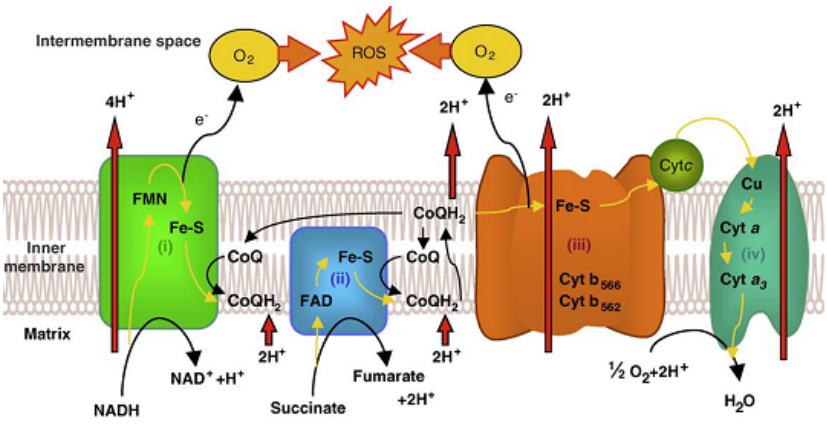


Figure 2.10 *Illustrative diagram of the mitochondrial electron transport chain (Taken from Yamashina et al., 2009).*

Electrons are transported between large electron transport complexes by small, lipid-soluble electron carrier ubiquinol and by cytochrome c, a soluble electron carrier protein. Amongst others, complexes I, III, and IV use the energy released in electron transfer reactions to pump protons out of the matrix across the inner membrane. The resulting proton gradient powers

the synthesis of ATP by complex V (ATP synthase) (Weiss *et al*., 1991; Althoff *et al*., 2011). Thus complexes I and II receive electrons namely, NADH and $FADH_2$ that from both TCA cycle and FA β-oxidation and then pass them to a lipid electron carrier, which is coenzyme Q (**Figures 2.9** and **2.10**) (Cole *et al*., 2011; Wolin *et al*., 2005; Yamashina *et al*., 2009). Cytochrome Q moves freely via the membrane. Complex III is a dimer (Castellani *et al*., 2010), which then oxidizes the reduced form of cytochrome Q and in turn cytochrome c, which is a protein electron carrier that is also mobile in the inner mitochondrial membrane (Labbea *et al*., 2008; Althoff *et al*., 2011). It has also been shown that complex III monomer proximal to complex I is the one that binds cytochrome c (Althoff *et al*., 2011). Finally, complex IV couples the oxidation of cytochrome c to the reduction of O_2 to H_2O.

Complexes I and III can directly react with O_2 to produce superoxide anion, which is dismutased by the mitochondrial manganese superoxide dismutase (MnSOD or mSOD) to hydrogen peroxide (H_2O_2) (**Figure 2.10**). The resultant H_2O_2 is detoxified to H_2O by the mitochondrial glutathione peroxidase (mGP). Under normal conditions, most of the ROS generated by the mitochondrial respiratory chain are detoxified by the mitochondrial antioxidant defence system including glutathione peroxidase (mGP), glutathione reductase (mGR) and reduced glutathione (GSH) (Labbea *et al*., 2008). Mitochondrial complex I proximal is inhibited by rotenone, while complex III is inhibited by antimycin A to inhibit electron transport (**Figure 2.10**), resulting in a decrease ROS production (Wolin *et al* 2005). Leakage of electrons in the mitochondrial respiratory pathway induces the production of ROS involved in the DNA damage and cellular aging. The advancement of therapeutic approaches to protect mitochondria acclaims a new strategy to apprehend progressive liver diseases and cancer (Yamashina *et al*., 2009). According to Esposti *et al*. (2012) better knowledge of the mechanisms and pathways involved in mitochondria homeostasis may improve preventive and therapeutic strategies for liver diseases. Therefore, it can be proposed that complex II might also be involved in the aetiology of dietary-induced fatty liver associated with modest oxidative stress.

2.5.2 Peroxisome proliferator-activated receptors (PPARs)

There are three 'lipid-sensing' peroxisome proliferator-activated receptors **(**PPARs) that represent connection of energy balance and insulin signalling, governing diverse aspects of glucose and lipid metabolism, and serving as *bona fide* therapeutic targets, namely PPAR-α,

PPAR-γ and PPAR-δ (Evans *et al*., 2004). Both PPARα and PPARγ can increase steaoryl-CoA desaturase-1 activity, which is required for very-low-density lipoproteins (VLDLs) secretion. Therefore, PPARα and PPARγ are important pharmacological targets for the treatment of insulin resistance, fatty liver, obesity and atherosclerosis (Kallwitz *et al*., 2008). The PPAR-δ is ubiquitously expressed and also facilitates energy combustion, but it is less studied (Lopez-Velazquez *et al*., 2012; Reddy and Rao, 2006).

The PPAR-α is highly expressed in the liver, skeletal muscle, heart, brown adipose tissue, kidney, and at lower levels in other organs (Lopez-Velazquez *et al*., 2012). It is a nuclear receptor that plays a central role in regulating gluconeogenesis in the liver and FAs oxidation in both liver and skeletal muscle, particularly at the fasted state or following feeding on HFD (Reviewed in Ament *et al*., 2012). It also regulates many of the genes encoding enzymes that are involved in the peroxisomal and mitochondrial β-oxidation pathways. The PPAR-α activity reduces accumulation of lipid in the liver by elevating β-oxidation (Kallwitz *et al*., 2008). The PPARγ is highly expressed in the adipose tissues and is present in the lymphoid organs and colon (Reviewed in Lopez-Velazquez *et al*., 2012). It is found in two isoforms, PPAR-γ1 and PPAR-γ2 that differ at their N termini. The shorter PPAR-γ1 has a relatively broad expression pattern including in the gut, brain, vascular cells, and immune and inflammatory cells, while PPAR-γ2 is expressed at high levels mainly in the adipose tissues (Wahli and Machilik, 2012). Although PPAR-γ expressions are relatively low in healthy liver, this receptor is important for the development of fatty liver (Lopez-Velazquez *et al*., 2012). Thus, PPAR-γ increases insulin sensitivity of the adipose tissue, while decreasing FA flux to the liver (Kallwitz *et al*., 2008).

The PGC-1 comprised of PGC-1α and PGC-1β, interact with PPAR-γ, allow interaction with multiple proteins, which are involved in the regulation of cellular metabolism such as nuclear respiratory factors (NRFs) and cAMP-response-element-binding protein (CREB) (Ament *et al*., 2012). This PGC-1 family is emerging as a centre connecting hormonal and nutritional signals and energy metabolism (Liu and Lin, 2011). It also plays a key role in regulatory network that transcriptionally controls mitochondrial biogenesis and respiratory function (Tailleux *et al*., 2012).

Under normal fed conditions, PGC-1α is expressed at very low levels in the liver. Hence, fasting induces liver PGC-1α expression in response to glucagon, a pancreatic hormone that

induces cAMP and CREB. Increased PGC-1α expression can lead to induction of several gluconeogenic enzymes such as G6Pase and PEPCK through its association with several transcription factors. In addition, p38 MAPK activation in the liver by the fasting-glucagon-cAMP-PKA axis increases PGC-1α transcription and induces gluconeogenesis. In addition, p38 MAPK is also necessary for FFA-mediated activation of PGC-1α expression in the liver, which in turn, enhances gluconeogenic genes. Together, PGC-1α stimulates both gluconeogenesis and FA oxidative during fasting in the liver (Liang and Ward, 2006; Fernandez-Marcos and Auwerx, 2011). Expression of PGC-1β is increased in response to dietary intake of fats, resulting in hyperlipidaemia through activation of VLDLs secretion and liver lipogenesis. Several factors are involved in mediating the effects of PGC-1β on plasma TG, FFA and cholesterol metabolism, including SREBP, liver X (LXR), and Foxa2 (Reviewed in Liu and Lin, 2011).

2.6 Oxidants metabolism

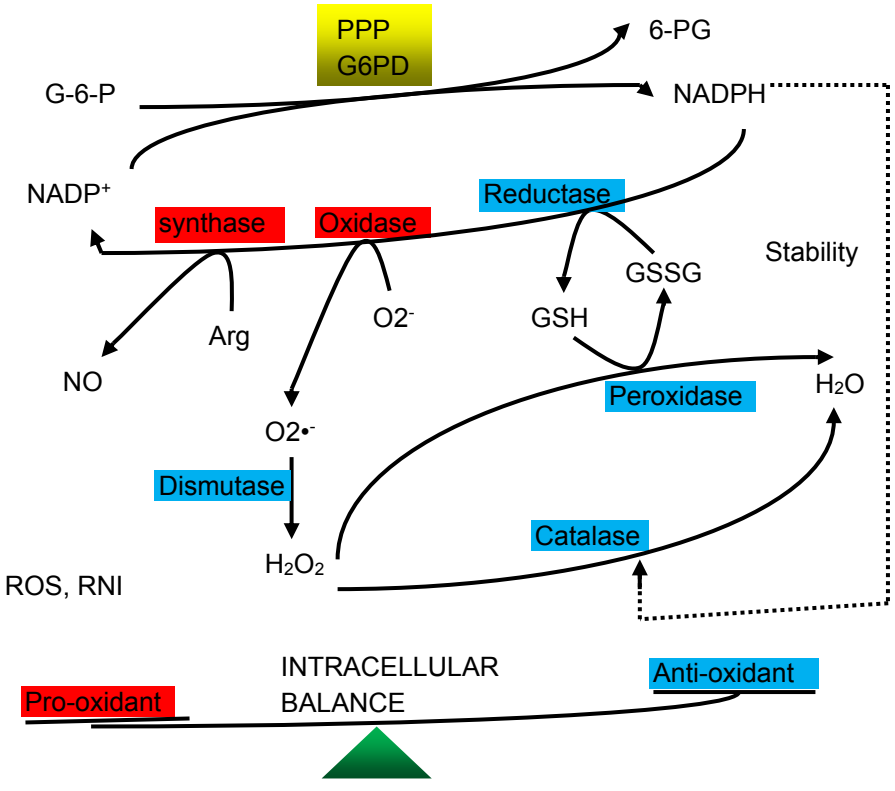


Figure 2.11 *Illustrative diagram of the dual functional of PPP (HMS) in the intracellular oxidant balance redrawn from Spolarics (1998). Pro-oxidants measured in the present study are highlighted in red, whereas the measured anti-oxidants are highlighted in blue. ROS, Reactive oxygen species, RNI, reactive nitrogen intermediates (Spolarics, 1998).*

A dual functional of PPP in the intracellular oxidant balance is illustrated in **Figure 2.11**. In phagocytes, NADPH generated from the PPP is also used by NOS and NADPH oxidase for the production of NO and superoxide anion. Superoxide anion and NO are converted to peroxynitrite and H_2O_2, by interaction with superoxide anions or by enzymatic or spontaneous dismutation by superoxide dismutase (SOD) enzyme. Subsequent elimination of peroxides is also dependent on NADPH. In this manner, antioxidant enzymes including glutathione peroxidase (GP) converts H_2O_2 to H_2O, via the use of reduced glutathione (GSH) and glutathione reductase (GR) reduces oxidized glutathione (GSSH) to GSH during the oxidation of NADPH to $NADP^+$, which is then used in the PPP. Catalase (CT), which is other major H_2O_2 eliminating enzyme also utilizes NADPH to protect protein from inactivation by its substrate i.e. H_2O_2 (**Figure 2.11**) (Spolarics, 1998).

2.6.1 Oxidant producing enzymes

Oxidase enzymes and NOSs are the main contributors for superoxide, H_2O_2 and NO in the body. Amongst other oxidases and NOSs, herein xanthine oxidase (XO), NADH oxidase (NOX) and inducible nitric oxide synthase (iNOS) are described as the main contributors of ROS and RNS, under pathophysiological conditions.

2.6.1.1 Xanthine oxidase (XO)

Purine nucleotides are synthesized preferentially by the salvage pathway as long as hypoxanthine, the most essential source of purine salvage, can be utilized. The preferential usage of the salvage pathway results in saving the energy expenditure required for *de novo* biosynthesis. The regulatory capacity of the *de novo* biosynthesis (approximately 200%) is larger than that of the salvage pathway (approximately 20%) with constant hypoxanthine phosphoribosyltransferase (HPRT) activity **Figure 2.12** (Yamaoka *et al*., 1997). In the eukaryotic cells, regulation of pentose phosphate production by nucleotide catabolism is affected by developmental and physiological factors on enzymatic levels. Ribose phosphates are either synthesized through the oxidative branch of the PPP or are supplied by nucleotide phosphorylases. The two main pentose phosphates, namely, R5P and ribose-1-phosphate (R1P) are readily interconverted by the action of R5P is a direct precursor of 5-phosphoribosyl-1-pyrophosphate, for both *de novo* and salvage synthesis of nucleotides (Tozzi *et al*., 2006).

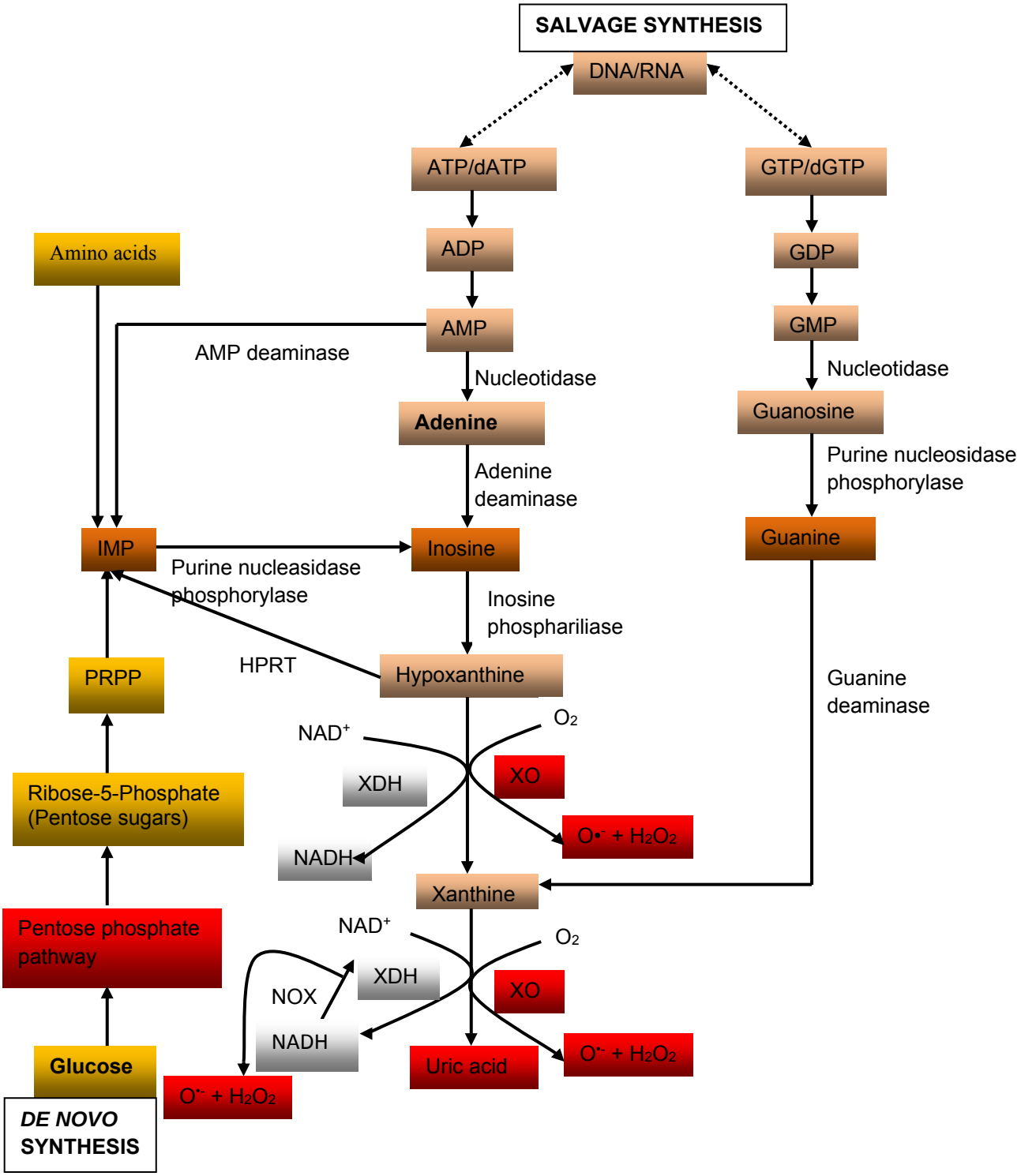


Figure 2.12 *The purine nucleotide synthesis and degradation pathway illustrative diagram redrawn from Boueiz et al. (2008); Mathews and van Holde (1996) and Pacher et al. (2006) with minor modifications. The enzymatic activities and the products highlighted in red were measured in this study (**see Chapter 4**). ATP, adenosine triphosphate; ADP, adenosine diphopsphate, AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; PRPP, 5-phospho-α-D-riboxy-1-pyrophosphate; IMP, inosine monophosphate; XO, xanthine oxidase; XDH, xanthine dehydrogenase; NOX, NADH oxidase, H_2O_2, hydrogen peroxide, HPRT, hypoxanthine phosphoribosyltransferase (Mathews and van Holde, 1996). It should be noted that a fully reduced XO contains 6 electrons and its re-oxidation involves*

electrons transfer to oxygen molecule which generates 2 H_2O_2 and 2 superoxides ($O^{\bullet -}$) species, respectively (Gearge and Struthers, 2009).

Purine nucleotide degradation is accelerated to produce inosine-5-monophosphate (IMP), hypoxanthine (HX), xanthine and uric acid (Kinugawa *et al*., 2001). Xanthine dehydrogenase (XDH) is the enzyme that utilizes NAD^+ as electron acceptor is converted to the XO form, which uses O_2. Both superoxide radical and H_2O_2 are produced from XO reaction (Doctor and Mandel, 1991). Both oxidative and non-oxidative branches of PPP generate R5Ps, which can be further utilized for purine and pyridine *de novo* synthesis (Resendis-Antonio *et al*., 2010; Tozzi *et al*., 2006). The rat liver XO has been found to be the only oxidizing enzyme available for purine substrates (Boueiz *et al*., 2008).

2.6.1.2 NADH oxidase

In the rat liver, both XO and XDH have NOX activity, thereby catalysing oxidation of NADH to generate ROS that induce peroxidation of liposomes. Under pathological conditions, where an increase in NADH levels occurs, its oxidation catalysed by XDH may constitute an important pathway for ROS mediated tissue damages (Maia *et al*., 2005).

2.6.1.3 Nitric oxide synthase

There are three NOS isoforms, which differ by their site of expression and catalytic activation in the tissues (Reviewed in Nathan and Xie, 1994; Stuehr, 1997), namely:

i. Inducible nitric oxide synthase (iNOS).
ii. Endothelial nitric oxide synthase (eNOS).
iii. Neuron nitric oxide synthase (nNOS).

In mammals, these enzymes catalyse the generation of NO through NADPH-dependent oxidation of L-arginine (L-Arg) to NO and L-citrulline, respectively (Panda *et al*., 2005). Herein, iNOS is described as it is the main contributor for NO production in the tissues under pathological conditions.

2.6.1.3.1 Inducible nitric oxide synthase and nitric oxide

The NO biosynthetic pathways are shown in **Figure 2.13**. Liver hepatocytes, macrophages (Kupffer cells) and endothelial cells play a vital role in the clearance of endotoxin from the portal circulation (Laskin *et al*., 1994). Endotoxins activate these cells to release ROS mediators including H_2O_2, NO and superoxide anion, which are attributed to liver inflammation and tissue injury (de Luca and Olefsky, 2008; Landino *et al*., 1996). Nitric oxide is a small, lipophilic, multifunctional, diffusible and transcellular messenger implicated in numerous physiological and pathological conditions (Achike and Kwan, 2003; Choi *et al*., 2002). Its biological actions are mediated via signalling pathways such as activation of soluble guanylate cyclase (GC), interactions with superoxide anion and cysteine thiol-S-nitrosylation with a specific role of each of these signalling pathways influenced by a number of factors including concentrations of NO, cell type and surrounding redox state (Rockey and Shah, 2004). The NO dependent production by the iNOS plays a key role in inflammation induced by cytokines **(Figure 2.13)**, lipopolysaccharide (Chung *et al*., 2001; Taylor *et al*., 1998; Wang *et al*., 2000), bacterial products, viral proteins, lipid mediators and oxidants (Reviewed in Conner and Crisham, 1996). Moreover, herbal extracts including *Taraxacum afficinate* (Kim *et al*., 1999a), *Scutellaria baricalensis* (Kim *et al*., 1999b) and *Harungana madagascariensis* Lam. ex Poiret (Iwalewa *et al*., 2009) have also been reported to mediate iNOS induction.

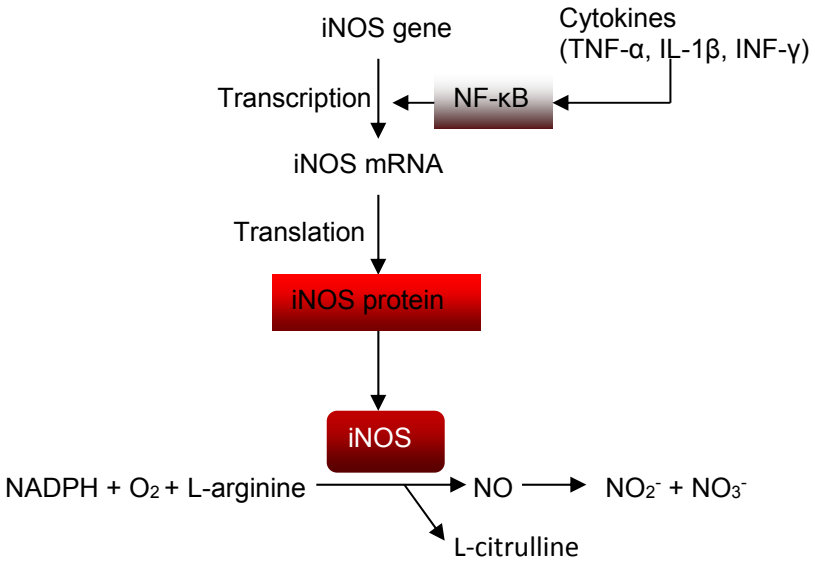


Figure 2.13 *Nitric oxide (NO) biosynthetic pathways redrawn from Taylor et al. (1998). The measured iNOS protein expression in the present study is highlighted in red. The iNOS enzyme catalysis oxidation-reduction*

reaction of L-arginine in the presence of molecular oxygen and NADPH to form citrulline and NO, the latter has a short half-life and is oxidized to the inactive end-products nitrite and nitrate (NO_2^- and NO_3^-). Induction of iNOS is upregulated by cytokines through the transcription factor NF-kB. In iNOS regulation, transcriptional and post-transcriptional mechanisms are important processes (Taylor et al., 1998).

2.6.1.3.2 Nitric oxide and liver anti-apoptosis

Possible pathways of apoptotic signal cascade (caspases) and NO-mediated anti-apoptosis are shown in **Figure 2.14**. Procaspase-3 (inactive form) is converted to caspase-3 (active form), in which leakage of pro-apoptotic proteins including cytochrome c from the damaged mitochondria is considered as a measure consequence of HFD induced NASH associated with increased apoptosis via increasing activated caspase-3 protein levels in the liver (Jiang *et al*., 2011). Activation of procaspase-3 is also associated with hyperglycaemia, GSH depletion and increased iNOS expression in the livers of HFD induced NASH rat models (Karmakar *et al*., 2011; Wang *et al*., 2008). Proapoptotic proteins from mitochondria that are involved in activation of procaspase-3 to caspase-3 are reviewed in Grattagliano *et al*. (2012). In addition, NO can induce pro-apoptosis in some cells, but inhibits apoptosis in the hepatocytes and endothelial cells. Its anti-apoptosis action in the hepatocytes and endothelial cells is via inhibition of caspase proteolytic activation and direct suppression of caspase activity. The physiological or low concentrations of NO can prevent the cells from apoptosis induced by trophic factor withdrawal, Fas, TNF-α, and lipopolysaccharide (**Figure 2.14**) (Choi *et al*., 2002; Chung *et al*., 2001).

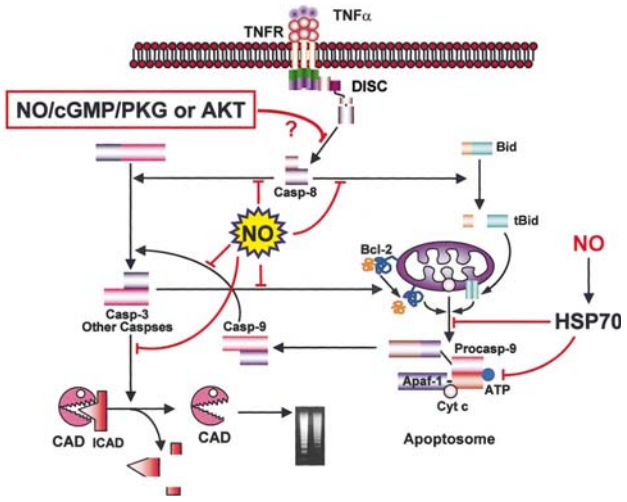


Figure 2.14 *Illustrative diagram of possible pathways of apoptotic signal cascade and NO-mediated anti-apoptosis (Taken from Chung et al, 2001). The main signalling components of apoptotic cell death as well as*

some possible antagonistic interventions by NO are depicted. In this way the arrows do not imply direct cause effect relations. CAD, caspase-activated DNase; DISC, death inducing signalling complex; ICAD, inhibitor of CAD; HSP70, heat shock protein 70; TNFR, tumour necrosis factor receptor (Chung et al., 2001).

2.6.1.3.3 iNOS produces NO in the interplay of insulin and inflammatory pathways

Increased liver oxidative stress and/or fatty liver pathway responses can result in activation of inflammatory pathways in both overnutrition and obesity conditions, which can lead to inflammation in the liver. Inflammation can also mediate the Kupffer cells (liver macrophages) activation, resulting in the release of local cytokines, which can further worsen liver insulin resistance (**Figure 2.15**) (de Luca and Olefsky, 2008).

Two distinct mechanisms by which NO directly or indirectly inhibits apoptosis in hepatocytes caspase-3 like activity have been reported including cGMP-dependent mechanism and by direct inhibition via interaction with protein *S*-nitrosylation (Kim *et al*., 1997). These anti-apoptotic mechanisms can also occur via expression of protective genes such as heat shock proteins, Bcl-2 as well as direct inhibition of the apoptotic caspase family proteases by S-nitrosylation of the cysteine thiol (Chung *et al*., 2001). Under the inflammatory conditions, hepatocyte survival is also dependent on NF-kB activation by resulting in the transcription of anti-apoptotic genes cIAP2, which inhibits both caspase-3 activity and apoptosis (Schoemaker *et al*., 2002). Altogether indicate that either NF-kB or iNOS activation can results in inhibition of liver caspase-3 activity and apoptosis. Alternatively, during nitrosative stress, high concentrations of NO can induce apoptosis via activation of the mitochondrial apoptotic pathways, including the release of cytochrome c to cytosol, an apoptosis-inducing factor, and endonuclease G as well as the suppression of NF-κB activity (Kim *et al*., 2002a). Cholesterol-rich diet enhances iNOS expression and HFD was reported to mediates an increase in plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDH-C) as well as NO and iNOS mRNA expression levels in the liver (Kim *et al*., 2002b), indicating that high calories may act as an iNOS activators. Therefore, in the present study, both iNOS and caspase-3 protein levels were analysed.

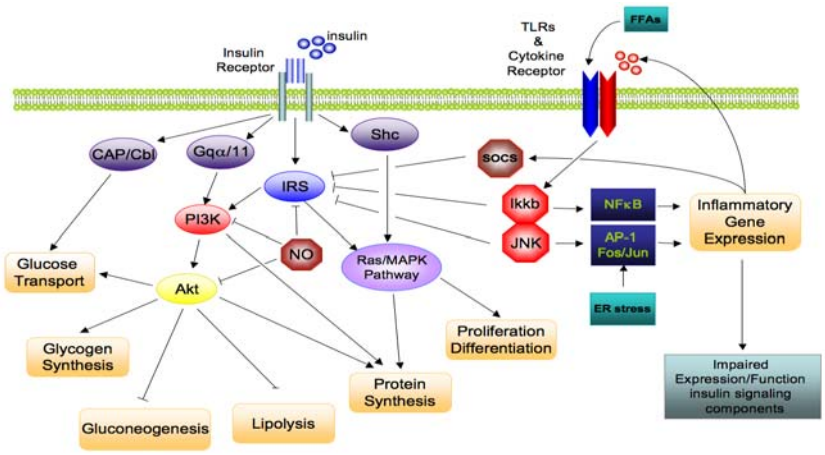


Figure 2.15 *Demonstrative diagram of a direct interplay of insulin signalling and inflammatory pathways (Taken from de Luca and Olefsky, 2008).*

Accumulation of lipids in the liver may lead to sub-acute hepatic inflammation via NF-κB activation and cytokine production including TNF-α, IL-6 and IL-1β, which cause both locally and systemically insulin resistance in the mice fed with HFD. In addition, the NF-κB activation has been attributed to a significant increase in the liver TG and serum FFAs induced by HFD (Cai *et al*., 2005). According to Sass *et al*. (2001), iNOS is a critical immune response that mediates inflammatory liver injury by produced NO that regulates proinflammatory genes in the concanavalin A (con A) mouse model. Furthermore, a study by Ma *et al*. (2008) demonstrated that the liver iNOS protein levels and NO overproduction are influenced by activation of NF-κB mediated by con A induced acute hepatitis in mice. Recently, iNOS has been recognized as a major factor in the regulation of liver glucose metabolism and insulin sensitivity by FFAs *in vivo* (Charbonneau and Marette, 2010). Insulin signalling can be impaired via interconnected action of three independent mechanisms designated by promoting inhibitory serine phosphorylation of IRS proteins, tyrosine nitration of IRβ and IRS-1/-2 and directly impairing Akt activity through its tyrosine nitration. Therefore, iNOS may be involved in mediation of liver insulin resistance (**Figure 2.15**).

Altogether, mechanisms second and third of the nitrosative modifications are most potentially triggered by iNOS-linked $ONOO^-$. Hence, limitation of $ONOO^-$ generation may be considered as a possible target for treating insulin resistance in obesity and metabolic conditions associated with excess lipid accumulation in the tissue (Charbonneau and Marette, 2010). In the present study, it remains vital to investigate iNOS protein levels in the

liver of HFD-induced fatty liver rat model. This is done to determine whether fatty liver can progress to NASH.

2.7 Pro-oxidants and oxidative stress

In the last two decades, free radicals in the form of ROS have been noted in the biological systems as the major players in the development of metabolic diseases including fatty liver (Aruoma, 1994; Kirkham and Rahman, 2006). ROS is a general term used to refer free radicals such as superoxide, hydroxyl ($^{\bullet}OH$), and peroxyl (ROO) and non-radicals including H_2O_2, hypochlorous acid (HOC), singlet oxygen and ozone (Aruoma, 1994). Superoxide and $^{\bullet}OH$ are unstable and most reactive radicals with unpaired electrons that are capable of initiating oxidation of proteins, lipids, and DNA. Oxidation of these molecules results in a direct tissue damage or induce a variety of cellular responses via production of secondary metabolic reactive species such as malondialdehydes (MDA) (Kirkham and Rahman, 2006).

Liver has a large surface area that exists in a rich oxygen environment and blood supply is susceptible to injury mediated by oxidative stress. Like other tissues, liver has several sources of ROS production including mitochondrial respiratory chain, NAD(P)H oxidase in the activated macrophages (Kupffer cells) and eosinophils, as well as hypoxanthine-xanthine oxidases (HXO and XO) system and FA oxidase. These oxidases generate ROS. Superoxide radical can react with NO molecule to form a highly reactive $ONOO^-$ or rapidly catalysed by SOD to form H_2O_2. This can lead to the non-enzymatic production of more damaging hydroxides radical from H_2O_2 in the present of metal cations such as Cu^{2+} or Fe^{2+} through the Fenton reaction (the iron salt-dependent decomposition of H_2O_2), that result in the oxidation of Fe^{2+} to Fe^{3+} (Aruoma, 1994; Kirkham and Rahman, 2006). Moreover, Fe^{3+} can directly generate $^{\bullet}OH$ from superoxide ($O_2^{\bullet-}$) and regenerate Fe^{2+} through the Haber-Weiss reaction (Halliwell and Gutteridge, 1990). The Haber-Weiss reaction of iron complex is accelerated by the involvement of $O_2^{\bullet-}$, ascobate and paraquat radical as depicted in **reactions 1a and b**, adapted from Aruoma (1994).

Fe^{3+}-chalate + (superoxide) $O_2^{\bullet-}$ $\longrightarrow$ Fe^{2+}-chalate + O_2 [**1a**]

Fe^{3+}-chalate + ascorbate $\longrightarrow$ Fe^{2+}-chalate +ascorbate radical [**1b**]

On the other hand, the redox cycling of Fe^{2+} to Fe^{3+} can rapidly lead to formation of more damaging $^{\bullet}OH$ from the initial supply of superoxide anion that may result in tissue damage. Other ROS producing enzymes including heme peroxidases, myeloperoxidase, or eosinophil peroxidase that catalyse the formation of hypochlorous and hypobromous acids from H_2O_2 in the presence of chlorine are located on the Kupffer cells of the liver. These ROS are potent and very damaging oxidants as they can interact with variety of molecules via electron donation in the biological systems that results in lipid peroxidation, enzyme inactivation and mediate proinflammatory cell signalling such cytokines production including, TNFα and NF-κB, and activator protein-1 (AP-1) pathways (Kirkham and Rahman, 2006).

2.7.1 PUFA depletion induced by oxidative stress during increased saturated FAs intake

Interrelationships between the levels of oxidative stress and insulin resistance, leading to fatty liver and its progression to NASH (steatohepatitis) are associated with overnutrition. In the liver, FAs are the major oxidative fuel under most circumstances (Videla, 2009; 2010). Alternatively, carbohydrate and lipid induce changes in liver intermediary metabolism. High glucose and insulin levels stimulate *de novo* FA synthesis from glucose and inhibit β-oxidation, redirecting FAs towards TG synthesis in the tissues. These conditions can also be influenced by chronic feeding on the high calorie diets such as HFD, resulting in the increased TG storage as lipid droplets within hepatocytes. Since non-adipose tissues have limited capacity for TG storage, excess lipids that accumulate under conditions of overnutrition determine high intracellular levels of SFA that cause cell dysfunction and/or cell death, a condition known as lipotoxicity. Higher rates of FA oxidation and ROS generation are achieved, which might explain an increase in oxidative stress related parameters and antioxidant depletion found in the liver of obese patients with fatty liver (Videla *et al*., 2004; 2009; Spadaro *et al*., 2008). Prolonged oxidative stress may favour one or both of the following two conditions (Spadaro *et al*., 2008; Videla *et al*., 2004):

i. Liver n-3 LCPUFA depletion that can be intensified by dietary imbalance and defective desaturation activity and
ii. Insulin resistance in association with the redox activation of multiple stress-sensitive serine/threonine kinases, which alters insulin signalling.

The latter condition is a membrane-mediated process that may be exposed by *n-3* LCPUFA depletion, due to loss of membrane polyunsaturation (Spadaro *et al*., 2008). Both insulin resistance and *n-3* LCPUFA depletion in the liver can determine fatty liver status by different mechanisms, including insulin resistant-dependent higher peripheral mobilization of FAs and glycerol to the liver, and *n-3* LCPUFA depletion induced changes in the DNA-binding activity of PPAR-α and SREBP-1c. These conditions determine the metabolic imbalance between FA oxidation and lipogenesis, favouring the lipogenesis pathways (Videla *et al*., 2004; 2009).

2.8 Antioxidants

2.8.1 Non-enzymatic antioxidant reduced glutathione

Pathways for reduced glutathione (GSH) synthesis, regeneration and utilization are depicted in **Figure 2.16**. The GSH is a non-protein thiol-containing tripeptide (L-gamma-glutamyl-L-cisteinglycine) widely distributed in animal tissues, plants and microorganisms (Perricone *et al*., 2009). It is the major antioxidant, redox and cell signalling regulator that guards cells against oxidative injury by scavenging ROS and nitrogen radicals such as NO and by reducing H_2O_2 (Ye *et al*., 2010; Yuan and Kaplowitz, 2009). Thus in many forms of liver diseases, GSH pool in the liver are compromised through impairment of synthesis, transport and/or over-consumption. Disruption of GSH homeostasis in the liver gives rise to ROS that oxidize lipids, DNA and proteins and alters multiple signalling pathways, which affect intermediary metabolism proliferation and survival (**Figure 2.16**) (Perricone *et al*., 2009). Superoxide is produced by the oxidases including XO system as mentioned above in **Section 2.6.1**. The produced superoxide reacts with GSH to cause an increase in oxygen consumption and GSSG formation, both of which are fully inhibited by SOD (Winterbourn and Metodiewa, 1994). Moreover, GSH is a potential antioxidant for the prevention of reperfusion injury after liver transplantation (Schauer *et al*., 2004). The liver total reduced thiol content was also measured in the present study to see if antioxidant capacity was affected by HFD.

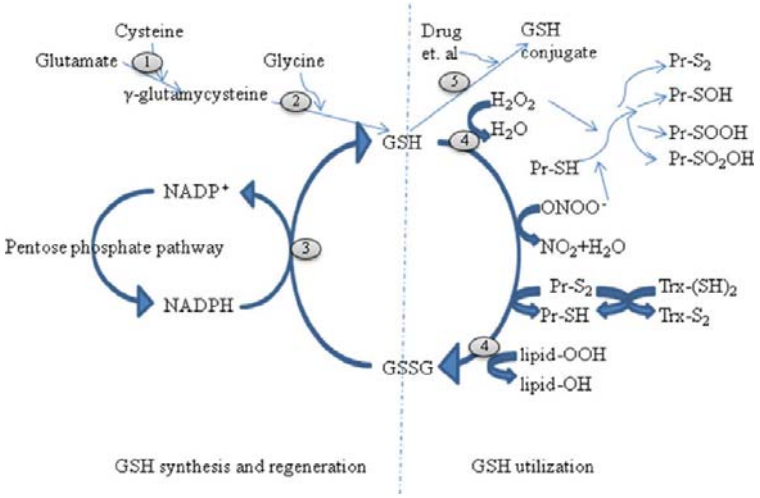


Figure 2.16 *Pathways for GSH synthesis, regeneration and utilization illustrative diagram taken from Yuan and Kaplowitz (2009)* ***(1)*** *C-glutamycysteine synthetase* ***(2)*** *GSH synthetase* ***(3)*** *Glutathione reductase* ***(4)*** *Glutathione peroxidase* ***(5)*** *Glutathione-S-transferase. Pr-SOH (sulfenic acid) reacts with GSH to form Pr-S-S-G (glutathionylation) is then specially reduced by glutaredoxin. Thus, glutathionylation prevents further oxidation of the sulfenic to sulfonic and sulfonic acid (Yuan and Kaplowitz, 2009).*

2.8.2 Enzymatic antioxidants

The enzymatic antioxidant enzymes including SOD, GP, GR, and CT are described under **Section 2.6** with an exception of glutathione-S-transferees (GST). The GST is a group of diametric enzymes that plays a critical role in the defence against oxidative stress products such as peroxides, electrophilic compounds and toxic metals via conjugation or reduction with GSH (Reszka and Wasowicz, 2001).

2.9 Factors responsible for NAFLD progression

Factors responsible for the progression of simple steatosis (fatty liver) to NASH are still not well understood. Two-hit have been hypothesized (**Chapter** 1, **Section 1.1.3** (Videla, 2009). As also depicted in **Figure 2.17**, 1st hit is excessive liver lipid primarily outstanding to insulin resistance, by increasing the efflux of FAs from the adipose tissue to the liver. Production of ROS, apoptosis dysregulation and imbalance between pro-inflammatory cytokines such as TNF-α, interleukin-6, leptin is common during insulin resistance condition as depicted in **Figure 2.17**. Insulin resistance also promotes hyperinsulinemia, which favours the accumulation of TG in the liver via decreasing its ability to re-esterify TG and allow them to leave the hepatocyte for storage in the adipose tissue. Thus, insulin resistance is a central

mechanism in the development of NAFLD (Mehta *et al*., 2002, Videla, 2009). Second (2nd) hit is oxidative stress owing to ROS, gut-derived bacterial endotoxin and the hormones from adipose tissue that are the anti-inflammatory response adipocytokines such as TNF-α, leptin and adiponectin that lead to hepatocellular inflammation, fibrosis and necroinflammatory change (Adams *et al*., 2005). However, insulin resistance may be directly implicated in liver fibrosis as a key complex interplay between the underlying mechanisms (Videla, 2009).

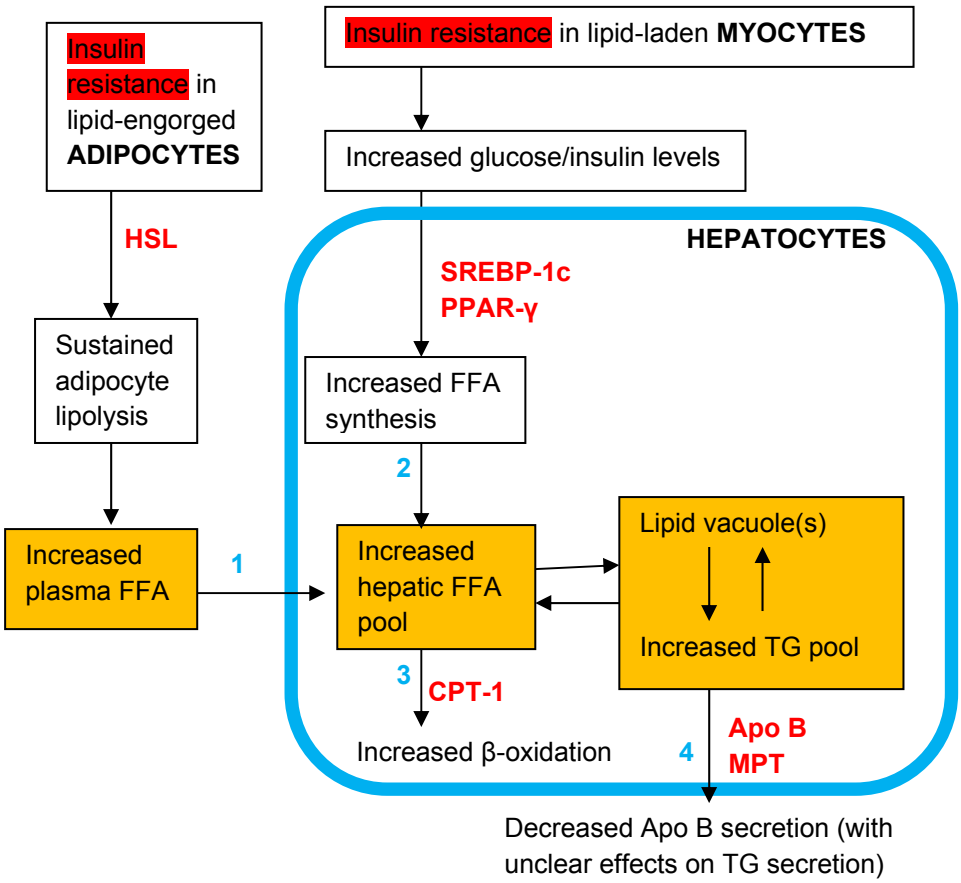


Figure 2.17 *Mechanisms of lipid secretion in the liver during insulin resistance-associated NASH redrawn from Fromenty et al. (2004). Insulin resistance in fat-engorged adipocytes hampers the inhibitory action of insulin on hormone-sensitive lipase (HSL), thus increasing the hydrolysis of triglycerides (TG) and the release of free fatty acids (FFA) from adipose tissue. Increased plasma FFA levels increase the hepatic uptake of FFA (**arrow 1**). Concomitantly, insulin resistance in fat-laden myocytes can increase glucose and/or insulin levels, which may increase hepatic fatty acid synthesis (**arrow 2**) through the up-regulation of sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor γ (PPARγ). Increased plasma FFA uptake and increased hepatic FFA synthesis cause the expansion of the FFA pool, which is in equilibrium with an expanded pool of triglycerides. Because fat cannot accrue indefinitely within hepatocytes, a new steady state is reached, whereby increased input pathways are finally compensated for by increased output pathways. A major compensatory pathway is the enhancement of mitochondrial FFA β-oxidation (**arrow 3**) due the increased resistance of carnitine palmitoyltransferase I (CPT-I) to the inhibitory effects of its endogenous inhibitor,*

*malonyl-CoA. VLDL secretion involves the intrahepatic lipidation of apolipoprotein B (apo B) by microsomal triglyceride transfer protein (MTP). Although apo B secretion seems to be reduced during insulin resistance, it is unclear yet whether this also affects hepatic TG secretion (**Arrow 4**) (Fromenty et al., 2004).*

2.9.1 Inflammatory and fibrotic mediators in fatty liver

Adipocytokines, FFAs, mitochondrial dysfunction, bacterial endotoxin and vascular disturbance are all attributed to the development of inflammation and fibrosis in patients with fatty liver (Reviewed in Adams *et al*., 2005; Calder, 2006). Amongst other adipocytokines, TNF-α promotes inflammation and insulin resistance in the liver (Adams *et al*., 2005). Recently, lipids content in the liver was reported not to be associated with plasma concentrations of retinol-binding protein 4, fetuin A, adiponectin, IL-6, and TNF-α in 9 subjects with fatty liver out of 25 in total. Moreover, univariate analysis revealed that liver lipid levels are significantly associated with BMI, visceral fat area, TG, VLDL-apoB-100 concentrations and secretion rate (Chan *et al*., 2010).

CHAPTER 3: INTRODUCTION TO THE PRESENT STUDY

Synopsis

In this Chapter, the present study is introduced including its brief background. Methodology used in this study is depicted in ***Chapter 4****.*

OUTLINE

3. Introduction to the present study
    3.1 Background to the study
        3.1.1 Animal maintenance and diets
        3.1.2 Gavage preparation and their dosages
            3.1.2.1 The *S. frutescens* leaves aqueous extract
            3.1.2.2 The *P. africana* bark aqueous extract
            3.1.2.3 Metformin
        3.1.3 Biological materials
        3.1.4 Previous laboratory findings obtained from the blood and growth curve of this HFD rat model

3. Introduction to the present study

The present study is about the elucidation of the lipid and glucose metabolic changes in fatty liver (NAFLD) induced by 16 weeks of chronic intake of 38.9% HFD enriched with SFA and MUFA mainly palmitic (40.9%) and oleic (29.8%) acids, respectively. This study also includes elucidation of efficacy of 4 weeks of gavaging the rats fed on HFD with either *S. frutescens* leaves (HF+Sf) or *P. africana* bark (HF+Pa) aqueous extracts or metformin (HF+Met) on the prevention of HFD-induced fatty liver, following feeding on same HFD for 12 weeks.

As previously mentioned in **Chapter 1**, fatty liver and its progression to NASH, cirrhosis, fibrosis, HCC and liver dysfunction are currently the major public health problems and have recently drawn the scientific attention worldwide (Wei *et al*., 2008). However, HFD-induced fatty liver is generally accepted, its induction and pathogenic mechanisms remain complex and elusive. This is due to differences in response of the tissues to the different types of FAs and their contents consumed at a given period.

To date there is no licensed drug for the treatment of fatty liver. Therefore, there is a need to study the selected lipid, glucose, pro-oxidant and antioxidant metabolic enzymes that play roles in fatty liver development by using the rats fed on HFD and/or on HFD plus either plant extract or metformin as the prevention strategies. This study is achieved with the aim to elucidate the efficacy of the selected plant extracts and metformin on the prevention of an HFD-induced fatty liver and to document the actual metabolic enzymes and their products, which are altered or influenced by a chronic intake of an HFD, as well as their improvement or worsened by gavaging the rats fed on an HFD with plant extract or metformin. The liver metabolic components at risk during energy overload and/or drug exposure are mainly enzymes, proteins, genes and lipid deposition and their secretion during the pathogenesis and prevention of fatty liver. Hence, the liver histology, enzyme activities and lipid profiles are presented in the present study.

The focus of this study is specifically on the liver with a view to measure the metabolic enzymes that play the vital roles in glucose, lipid and oxidants utilization and production mediated by an HFD. Thus, measuring the liver lipid profiles and microscopically observation of the liver histological structure among the rats fed on LFD, HFD or HFD plus either plant extract or metformin as a fatty liver prevention is the core of this study. Selected metabolic enzymes and lipids profile as well as the methodology used in the present study are described in **Chapter 4.**

3.1 Background to the present study

In this HFD rat model, the liver samples used in this project were available from two previous studies (Mackenzie, 2009; Karachi, 2009). In those two studies of rats fed on HFD for 16 weeks were gavaged for the last 4 weeks with either *S. frutescens* leaves aqueous extract or metformin (Mackenzie, 2009) or *P. africana* bark aqueous extract (Karachi, 2009). The rats on normal mice chow served as CL (after 12 weeks) or LF (after 16 weeks, lean) control group, whereas rats on HFD without gavage served as CH (after 12 weeks) or HF (after 16 weeks, fatty liver model) group.

3.1.1 Animal maintenance and diets

The animal protocol ethical clearance (A068BM-004) was approved and guided according to the Nelson Mandela Metropolitan University (NMMU) animal ethics committee for the animal care and use. The male Wistar rats, one week post weaning, were purchased from the Medical Research Council (MRC) animal unit (Cape Town, South Africa). The rats were housed under controlled temperature and lighting conditions (21-22˚C and 12 hours light and dark cycles). One week after transported to the NMMU's animal facility, the rats were divided into 5 groups of 6-7 per group. The CL and LF (lean) control groups were fed on low fat diet (LFD), and the CH and HF (fatty liver) groups were fed on an HFD (see **Table 3.1** for the dietary compositions) and the experimental groups were fed on HFD for 12 weeks (Karachi *et al*., 2009; Mackenzie *et al*., 2009). After 12 weeks, the experimental groups were fed an HFD and gavaged with *S. frutescens* leaves extract (HF+Sf) or *P. africana* bark extract (HF+Pa) or metformin (HF+Met) for another 4 weeks (see **sections 3.1.2.1**, **3.1.2.2** and **3.1.2.1** for the gavage dosage), while the control groups were kept on the constant diets.

The dietary nutritional contents and values are shown in **Table 3.1**. For the LFD, the percentage (%) of the mice pellets calories in protein, carbohydrate and fat were 21.3%, 72.4%, and 6.3% (expressed as % energy/100 g wet weight) (EPOL, South Africa). The HFD was laboratory prepared from 1 Kg grounded Mice pellets mixed with margarine (128 g), Holsum (190 g) and condensed milk (220 g), respectively. The percentage of an HFD calories in protein, carbohydrate and fat were 15.8%, 45.3% and 38.9% (expressed as % energy/100 g wet weight). The rats were allowed to have free access to food and water during the course of the study (Mackenzie, 2009).

The main increase in the lipid contents in the HFD was an increase from 13.5% to 40.9% for palmitic acid and from 24.1 to 29.8 for oleic acid as well as a decrease in PUFA content in an HFD from 55.2% to 14.7% as compared to that in the LFD (**Table 3.1**).

Table 3.1 *LFD and HFD nutritional contents and their values*, names and types*[$#]

*	*	LFD*	HFD*		
g/100 g wet weight	Ash	6.30	7.14		
	Protein	16.82	15.10		
	Carbohydrates	57.17	43.12		
	Fat	2.22	16.47		
	moisture	9.77	12.92		
KCal/100 g wet weight		**315.9**	**381.1**		
% of total energy	Carbohydrates	72.38	45.26		
	Protein	21.3	15.84		
	Fat	6.32	38.9		
	Numeric formula			Common name[#$]	Functions[#$¥δ]
% of total fatty acids	C10:0	-	0.6	Capric acid	SFA
	C12:0	-	5.1	Lauric acid	SFA
	C14:0	0.9	2.8	Myristic acid	SFA, linked to some proteins
	C15:0	0.3	0.1		SFA
	C16:0	13.5	40.9	Palmitic acid	SFA, Product of FAS
	C16:1 (n-7)	1.3	0.4	Palmitoleic acid	Non-essential MUFA
	C17:1	2.2	4.5	Cis-10-DHA	Non-essential MUFA
	C18:0	2.2	4.5	Stearic acid	SFA
	C18:1 (n-9 trans)	-	-	Elaidic acid	
	C18:1 (n.9 Cis)	24.1	29.8	Oleic acid	Non-essential MUFA
	C18:2 (n-6)	45.3	13.7	γ-Linoleic acid	Essential PUFA
	C18:3 (n-)3	2.1	0.6	α-Linolenic acid (omega 3)	Essential PUFA
	C20:0	0.3	0.3	Arachidic acid	SFA
	C20:1	1.0	0.1	Gondoleic acid	Non-essential MUFA
	C20:2	0.1	-	Cis-11,14 DA	
	C20:4 (n-6)	0.2	-	Arachidonic acid	Essential PUFA
	C20:5 (n-3)	2.5	0.2	Timnodonic acid	Essential PUFA
	C22:0	0.2	0.1	Behenic acid	SFA
	C22:1 (n-9)	0.4	-	Erucic acid	Non-essential MUFA
	C22:5 (n-3)	0.6	0.1	DPH (omega 3)	Essential PUFA

Table 3.1 Continued

% of total fatty acids	C22:6 (n-3)	4.5	0.3	DHA (omega 3)	Essential PUFA
	C24:0	0.3	0.1	Lignoceric acid	SFA
	C24:1	0.2	-	Nervonic acid	Non-essential MUFA
% of fat type	SFA	17.8	54.7		
	MUFA	27.0	30.3		
	PUFA	55.2	14.9		
	Trans FA	-	-		
	n3-FA	5.1	1.2		
	n6-FA	45.5	13.7		
	n6/n3 ratio	8.9	11.4		

**Mackenzie, 2009; [#]Zock et al, 1994; [$] Guillou et al, 2010; [¥]Grundy, 1994; [δ]Temme et al., 1996. Cbns, carbons; DHA, decoosahexaenoic acid (ervonic acid); DPA, decosapentaenoic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid, SFA, saturated fatty acid.*

3.1.2 Gavage preparation and their dosages

The plant extracts and metformin were prepared by Janine Mackenzie, and Jackie Karachi as described in the next three subsections.

3.1.2.1 *Sutherlandia frutescens* leaves aqueous extract

The dried commercially available *S. frutescens* leaves aqueous extract was a gift from the Value Added Life Health Products (South Africa, batch 671). The *S. frutescens* extract was dissolved in water at 25 mg/ml concentration. The rats were gavaged with 50 mg *S. frutescens* extract/kg bwt once per day for 4 weeks, while they were fed on an HFD (HF+Sf) (Mackenzie, 2009).

3.1.2.2 P*runus africana* bark aqueous extract

The dried *P. africana* bark aqueous extract (Tolo *et al*., 2007) was obtained from the Kenyan Medical Research Foundation (KEMRI) through Dr. Mathiu. The *P. africana* extract was

dissolved in water at 50 mg/ml. The rats were gavaged with 0.125 mg *P. africana* extract/kg body weight once per day for 4 weeks, while they were fed on an HFD (HF+Pa) (Karachi, 2009).

3.1.2.3 Metformin

The commercially available metformin tablets (Glucophage, Merck) were used. Metformin tablets were dissolved in water at 25 mg/mL concentration. The rats were gavaged with 16 mg metformin/kg bwt once per day for 4 weeks, while they were fed on an HFD (HF+Met) (Karachi, 2009; Mackenzie, 2009).

3.1.3 Biological materials

The rats were fasted overnight for 12 hrs and then anaesthetized by an intramuscular injection with 3 µL ketamine/g body weights (Centaur Labs, South Africa). The rats were sacrificed removing blood through heart puncture with a 22 gauge needle. The rats were dissected; the liver samples were immediately removed, snap frozen in liquid nitrogen (N_2) and kept at -80˚C freezer until used for this study (Karachi, 2009; Mackenzie, 2009).

3.1.4 Previous laboratory findings obtained from the blood and growth curve of our rat model

The previous findings obtained from the blood and growth curve of this HFD rat model as reported by Karachi (2009) and Mackenzie (2009) are shown in **Tables 3.2, 3.3** and **3.4**, and plotted in **Figure 3.1**, respectively. Other studied data are found on Karachi's dissertation (2009) and Mackenzie's thesis (2009).

Table 3.2 *Rat body weight, insulin sensitivity and blood parameters as reported by Mackenzie (2009).*

Diet	Body weight (g)	QUICKI	HOMA-IR	Glucose (mmol/L)	Insulin$^{\delta}$(ng/mL)	FFAs (µmol/L)	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL (mmol/L)
CL	503.8±13.9	0.389±0.001	5.8±0.2	6.60±0.40	0.70±0.19	ND	ND	ND	ND	ND
CH	511.4±14.7	0.270±0.07††	14.1±0.5†††	6.99±0.40	1.75±0.56†††	ND	ND	ND	ND	ND
LF	509.0±10.0	0.319±0.005	5.06±0.33	6.33±0.34	0.60±0.08	69.15±4.46	0.59±0.03	1.21±0.10	0.303±0.02	0.82±0.08
HF	591.8±20.9**	0.292±0.007*	10.12±2.10*	6.22±0.34	1.26±0.22*	85.27±4.40*	0.86±0.08*	1.50±0.06*	0.298±0.01	1.03±0.06
HF+Sf	547.3±8.1**$^{\#}$	0.304±0.003$^{\#}$	5.01±0.30$^{\#}$	6.53±0.2	0.70±0.05$^{\#}$	48.32±3.04**$^{\#\#\#}$	0.59±0.03$^{\#}$	1.31±0.05$^{\#}$	0.206±0.03**$^{\#}$	0.98±0.04
HF+Pa	-	-	-	-	-	-	-	-	-	-
HF+Met	Value not shown **	0.302±0.013	6.79±1.11	6.17±0.11	1.15±0.12*	51.12±6.51**$^{\#\#\#}$	0.73±0.13*	1.32±0.07$^{\#}$	0.408±0.04*$^{\#}$	0.85±0.007

ND*, not determined.* ***-****, Not studied,* ***CL****, low fat diet after 12 weeks;* ***CH****, high fat diet after 12 weeks;* ***LF****, low fat diet after 16 weeks;* ***HF****, high fat die after 16 weeks;* ***HF+Sf****, high fat diet plus 50 mg S. frutescens leaves extract/kg bwt per day;* ***HF+Pa****, high fat diet plus 0.125 mg P. africana bark extract/kg bwt/day;* ***HF+Met****, high fat diet plus 16 mg metformin/kg bwt/day; QUICKI, qualitative insulin sensitivity index; FFAs, free fatty acids; TG, triglycerides; TC, total cholesterol; total cholesterol,* ***LDL-C****, low density lipoprotein cholesterol; and* ***HDL-C****, high density lipoprotein cholesterol.* † *= significantly different from CL control,* $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$; * *= significantly different from LF control,* $*P < 0.05$, $***P < 0.01$, $***P < 0.001$ *and* $^{\#}$ *= significantly different from HF control,* $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. *LF: n=6; HF: n=7.*

Table 3.3 Rat body weight, insulin sensitivity and blood parameters as reported by Karachi (2009).

Diet	Body weight (g)	QUICKI	Glucose (mmol/L)	Insulin (ng/mL)	FFAs (µmol/L)	TG (mmol/L)	TC (mmol/L	HDL-C (mmol/L)
CL	504.6±25.679	0.307±0.015	3.96±0.472	0.622±0.133	70.0±20.0	0.662±0.175	1.271±0.131	0.223±0.032
CH	490.8±7.430	0.272±0.016††	5.36±0.05†	0.8±0.460†††	76.0±6.0	0.712±0.313	1.540±0.210	0.236±0.053
LF	511±23.927	0.306±0.024	5.58±0.476	0.575±0.217	69.0±11.0	0.585±0.073	1.212±0.248	0.303±0.05
HF	581±88.564**	0.283±0.024	5.76±0.643	1.593±0.857*	85.0±12.0*	0.860±0.196*	1.496±0.149*	0.298±0.032
HF+Sf	-	-	-	-	-	-	-	-
HF+Pa	523.2±24.631$^{\#}$	0.252±0.013*	5.36±0.619	4.363±1.286***$^{\#\#}$	75.0±9.0$^{\#}$	0.592±0.146$^{\#}$	1.175±0.074$^{\#\#}$	0.174±0.049*$^{\#}$
HF+Met	553.2±40.660	0.298±0.026	6.16±0.836	1.022±0.462$^{\#}$	51.0±16.0*$^{\#\#}$	0.733±0.267*	1.323±0.132$^{\#}$	0.408±0.092*$^{\#}$

***-**, Not studied, COL, low fat diet after 12 weeks; COH, high fat diet after 12 weeks;**LF**, low fat diet after 16 weeks; **HF**, high fat diet after 16 weeks; **HF+Sf**, high fat diet plus 50 mg S. frutescens leaves extract/kg bwt/day; **HF+Pa**, high fat diet plus 0.125 mg P. africana bark extract/kg bwt/day; **HF+Met**, high fat diet plus 3.3 mg metformin/kg bwt/day; QUICKI, qualitative insulin sensitivity index; FFAs, free fatty acids; TG, triglycerides; TC, total cholesterol; total cholesterol, **LDL-C**, low density lipoprotein cholesterol; and **HL-C**, high density lipoprotein cholesterol. † = significantly different from CL control, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$; * = significantly different from LF control, *P < 0.05, ***P < 0.01, ***P < 0.001 and $^{\#}$ = significantly different from HF control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. LF: n=6; HF: n=7.*

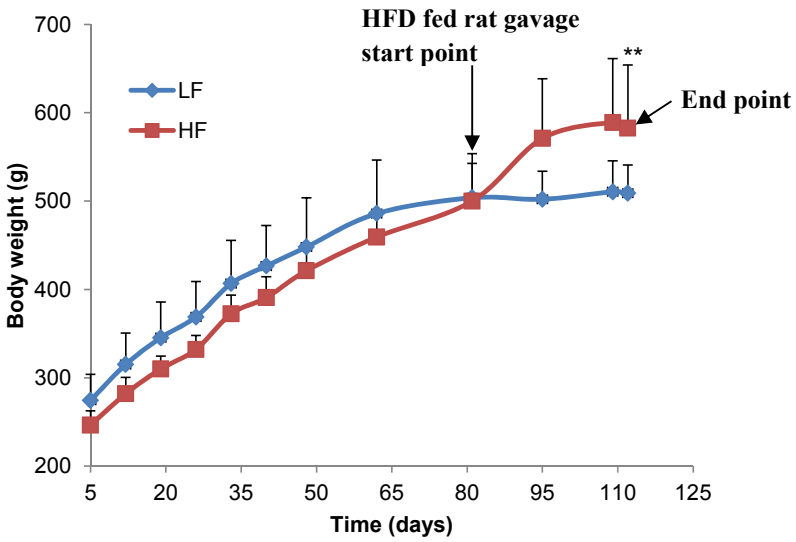


Figure 3.1 *Rats body weights (redrawn from Mackenzie, 2009). It should be noted that the prevention study was initiated at day 81 (12 weeks) up until 4 weeks on HF.* ***LF****, low fat diet;* ***HF****, high fat diet * = significantly different from LF control. ** P < 0.01. LF: n=6; HF: n=7 (Karachi, 2009; Mackenzie, 2009).*

Table 3.4 shows the rat's blood cytokines reported by Mackenzie (2009).

Diet	TNF-α (pg/mL)	MCP-1 (pg/mL)	IL-1α (pg/mL)	IL-4 (pg/mL)
CL	ND	ND	ND	ND
CH	ND	ND	ND	ND
LF	36.7±7.1	226.3±31.8	9.5±4.3	0.2±0.1
HF	18.3±5.8	213.2±15.6	30.8±17.1	2.1±1.0
HF+Sf	8.3±2.3**$^{\#\#\#}$	317.6±39.7	35.0±11.3	1.0±0.3
HF+Pa	-	-	-	-
HF+Met	62.5±13.7*$^{\#\#}$	350±36.6	44.5±16.6	1.3±0.4

ND, *not determined;* ***-****, Not studied.* ***CL****, low fat diet after 12 weeks;* ***CH****, high fat diet after 12 weeks;* ***LF****, low fat diet after 16 weeks;* ***HF****, high fat diet after 16 weeks;* ***HF+Sf****, high fat diet plus 50 mg S. frutescens leaves extract/kg bwt/day;* ***HF+Pa****, high fat diet plus 0.125 mg P. africana bark extract/kg bwt/day;* ***HF+Met****, high fat diet plus 16 mg metformin/kg bwt/day. † = significantly different from CL control, $^{\dagger}P < 0.05$;* = significantly different from LF control, * P < 0.05; ** P < 0.01 and $^{\#}$ = significantly different from HF control, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$. LF: n=6; HF: n=7.*

CHAPTER 4: METHODOLOGY

Synopsis

This Chapter describes all the methodologies that have been employed in the present study. The liver histology, lipid profiles, selected metabolic enzymes and their products were determined in all five rats groups (LF, HF, HF+Sf, HF+Pa and HF+Met). The obtained results were analysed statistically using the repeated measures one-way analysis of variance (ANOVA), followed by post-hoc analysis and Tukey test (STATISTICA version 9, 2010). P-values <0.05 were considered statistically significant. The results and discussion are depicted in ***Chapters 5****,* ***6****, 7 and* ***8****, separately.*

OUTLINE

4.14 Statistical analysis

4. Methodology

4.1 Chemicals

Amplex red, horseradish peroxidase (HRP), amplex red, reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GP), glutathione-S-Transferase (GSH), malondialdehyde (MDA), glucokinase (GK), glucose-6-phosphate dehydrogenase (G6PD), lactate dehydrogenase (LDH), ATP citrate lyase (ACL), glucose-6-phosphatase (G6Pase), xanthine oxidase (XO), hypoxanthine oxidase (HXO), iodonitrotetrazolium chloride (INT), ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), oxidised nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxidised nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), succinate dehydrogenase (SDH) and catalase (CT) were purchased from Sigma, β-actin, inducible nitric oxide synthase (iNOS) and caspase-3 antibodies were purchased from Sigma, Santa Cruz Biotechnology and Imgenex. All other chemicals and reagents were of the best analytical grade available.

4.2. Liver lipid profiles

Lipid accumulation in the liver is known to be induced by eating food with high fat calories. The liver total lipids (TL) were extracted to elucidate whether 16 weeks of feeding the rats on 38.9% HFD-induced fatty liver. The total lipids were extracted from the livers obtained from the LFD (CL) and HFD (CH) rats groups (after 12 weeks), and from the LFD (LF), HFD (HF), HF+Sf, HF+Pa and HF+Met rats groups (after 16 weeks).

4.2.1. Total lipid (TL) extraction

The liver total lipids (TL) were extracted according to the method described by Folch *et al*. (1957). Briefly, approximately 400 mg wet liver was homogenized in 3 mL chloroform: methanol (2:1) mixture (gas chromatography grade; SupraSolv, Merck, Germany) containing 0.01% (w/v) butylyted hydoxytuluene (BHT, lipid antioxidant) in a handheld ground glass

homogenizing tube. The homogenate was filtered through a glass fibre filter (GF/A, Whatman) to a glass vial and the filter was washed with 8 mL of extraction solvent mixture. Twenty-five (25) % (v/v, 3 mL) of 0.9% (w/v) NaCl containing 0.01% BHT saturated with chloroform/methanol mixture was added to 12 mL of filtrates and gently mixed to remove non-lipid components from solution. The mixture was allowed to stand for 3 min prior to centrifugation at 3000 rpm for 5 min to separate lipid (chloroform, bottom layer) from non-lipid (methanol:water, top layer) components. A top layer was discarded by gently suction with the use of a glass pasture pipette and the remaining bottom layer (chloroform) was filtered through approximately 3 mg anhydrous sodium sulphate (Na_2SO_4) packed on the Whitman filter paper to remove excess water. Lipids were collected in a new pre-weighed glass vials, dried under nitrogen gas (N_2) at 40 ˚C in a heating block until all solvents were evaporated and the vials were cooled to room temperature (RT) prior to weighing. Total lipid (TL) was calculated by subtracting the tube weight from that of tube plus lipids. The TL was dissolved in 1 mL of extraction solvent (chloroform: methanol; 2:1), blown under N_2 and stored at -20 ˚C.

4.3 Liver fractionation

To study the altered pathways that participate in the glucose and lipid metabolism of the liver, mitochondrial and cytosolic compartments were fractionated from the liver homogenate.

4.3.1 Liver homogenization and fractionation

All steps for the liver homogenization and fractionation were carried out at 4 ˚C, unless indicated otherwise. The homogenization buffer (50 mM Tris-HCl containing 250 mM sucrose, 2.5 mM HEPES and 5 mM EDTA, pH 7.5) was used to homogenize liver samples. Fresh commercially available protease inhibitors cocktail (500 µL) was added to 100 mL of buffer on the day of homogenization and fractionation, due to a low stability of the inhibitors at 4 ˚C in solvent other than 100% DMSO. Briefly, approximately 100 mg wet liver was homogenized in 1 mL of extraction buffer containing protease inhibitors cocktail, using a potter homogenizer with Telfon pestle at 400 rpm. The homogenate was used immediately for the enzymatic assay and 100 µL aliquots were taken and kept frozen at -80 ˚C freezer until used for other analysis.

4.3.2 Mitochondrial fractionation

Approximately 200 mg of wet liver was homogenized in 1.5 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose, 2.5 mM HEPES and 5 mM EDTA (extraction buffer). Hundred (100) µl aliquot was taken for the homogenate assays. The remaining homogenate was centrifuged at 1 000 xg for 10 min to remove the nuclear fraction. The supernatant was centrifuged at 8 000 xg for 20 min to collect the crude mitochondrial (pellets). Pellets were suspended in 1.5 mL of extraction buffer and centrifuged at 8 000 xg for 20 min (washing step). The washing step was sequentially repeated twice by suspension of the resultant pellets in the same volume of extraction buffer and centrifugation conditions. The washed pellets (mitochondrial-enriched fraction) were suspended in 500 µl of extraction buffer and used immediately for the enzymatic assays. Fifty (50) µL aliquots of the mitochondrial-enriched fraction were kept at -80 ˚C for other analysis. In this study, lower centrifugation speed was used to preserve the mitochondrial integrity, which is known to be interrupted by higher centrifugation speed, when using the frozen tissue (Navarro and Baveris, 2004).

4.3.3 Post-mitochondrial supernatant (PMS) fractionation

The nuclear and mitochondria compartments were removed as described in **Section 4.3.1**. The resultant supernatant from first step of mitochondrial sedimentation was collected and used to prepare the cytosolic fraction. Supernatant was centrifuged at 20 000 xg for 30 min to remove contaminating light mitochondria and peroxisomes. Pellets were discarded and the supernatant was collected as the post-mitochondrial supernatant (PMS) fraction. The PMS fraction was used immediately for the microsomal and cytosolic enzymatic assays. Fifty (50) µL aliquots from the PMS fraction were taken and kept frozen at -80 ˚C for other analysis.

4.3.3.1 Fraction enrichment and purity analysis

Succinate dehydrogenases (SDH, mitochondrial) and glucose-6-phosphatase (G6Pase, microsomal) specific activities relative to that of the homogenate were used to determine the mitochondrial fraction enrichment and contamination by the cytosolic fraction containing microsomes.

4.4 Lipids and protein peroxidation markers

Malonaldehyde (MDA), protein carbonyl and total hydroperoxides, which are the major indicators for oxidative stress were selected as the markers to determine both lipid and protein peroxidation, respectively.

4.4.1 Malonaldehyde

One of the end products for lipid oxidation is MDA, which is commonly used as a marker. The liver MDA levels were estimated to elucidate whether 16 weeks of feeding the rats on HFD-induced fatty liver is associated with oxidative stress.

4.4.1.1 Assay principle

The use of thiobarbarbituric acid (TBA) reagent is the method to assay lipid peroxidation in various animal tissue homogenates. Malonaldehyde is the end product of lipid breakdown caused by oxidative stress and it is determined by the TBA reactive substance method, which forms a fluorogenic 1:2 adduct (Qujeq *et al.*, 2005; Uchiyama and Mihara, 1978).

4.4.1.2 Assay procedure

Mitochondrial MDA was undetectable in all five groups; this may be due to little proteins or its availability at undetectable amount. Therefore, the homogenate MDA levels were determined according to the method described by Uchiyama and Mihara (1978) and Qujeq *et al.* (2005) with a minor modification. Briefly, 150 µL of sample was mixed with 300 µL 15% (w/v) of ice-cold TCA and allowed to stand on ice for 10 min to precipitate proteins. The precipitated proteins were removed by centrifugation at 3 000 xg for 15 min. The supernatant was treated with 400 µL 0.6% (w/v) TBA and placed in a boiling water bath for 15 min. After cooling on ice for 10 min, n-butanol (500 µL) was added, mixed to extract an MDA adduct and centrifuged at 3000 xg for 3 min. Aliquot (200 µL) was transferred to a black 96-well microtiter plate in duplicate and the fluorescence intensity of an MDA adduct was measured using the Fluoroskan Ascent FL microtiter plate fluorometer at excitation (Ex) and emission (Em) wavelength of 485 nm and 538 nm, respectively. The concentration of MDA was calculated by using a linear portion of the

MDA standard curve ranging from 0-180 μM (r^2 = 0.968). The levels of MDA are expressed as μM/mg protein.

4.4.2 Protein carbonyl levels

Oxidative stress can also mediate protein carbonylation resulting in protein damage. Therefore, the protein carbonyl groups were measured in the liver homogenate as a marker for oxidative stress.

4.4.2.1 Assay principle

Protein carbonyl groups are derivatized by 2,4-dinitrophenylhydrazine (DNPH) and react with guanidine hydrochloride to produce protein-hydrozone, which is detected by fluorescence (Talent *et al.*, 1998).

4.4.2.2 Assay procedure

Protein carbonyl levels of the liver homogenate were determined by the method described by Qujeq *et al*. (2005) with a minor modification. Briefly, 100 μL of homogenate was mixed with 300 μL of 8 mM DNPH dissolved in 3 M HCl. The tubes were incubated at 37 ˚C for 50 min in the dark and vortexed every 10 min. After incubation, 400 μL 25% (w/v) of ice cold trichloroacetic acid (TCA) was added and the mixture was allowed to stand on ice for 10 min to precipitate proteins. Solution was centrifuged at 3 000 xg for 3 min. The pellet was washed using 500 μL 15% (w/v) ice cold TCA using the above mentioned centrifugation conditions. Pellets were washed twice with 500 μL ethanol-ethyl acetate (1:1, v/v) by centrifugation at 3 000 xg for 3 min. The final pellets were air dried at RT for 10 min and dissolved in 450 μL of 5 M guanidine hydrochloride in 20 mM sodium phosphate buffer (pH 6.5). Insoluble materials were removed by centrifugation at the same conditions as described above. The supernatant was incubated for 15 min at 37 ˚C. Protein carbonyl levels were calculated from the fluorescence peak at Ex and Em of 355 and 390 nm, using a standard concentration 45.45 mmol/mL. The homogenate protein carbonyl levels results are expressed as mmol/mg protein.

4.4.3 Total hydroperoxides production

Under the oxidative stress conditions, the oxidase enzymes are activated because they are responsible for the ROS production in the body. Therefore, an H_2O_2 was used as a marker to confirm the presence of oxidative stress in the liver samples.

4.4.3.1 Assay principle

The ferric xylenol orange (Fox) method is based on the peroxide intermediate oxidation of Fe^{2+} to Fe^{3+} under acidic conditions, which then reacts with xylenol orange to form an Fe^{3+}-xylenol orange (blue-purple) complex detectable at an absorbance wavelength of 560 nm. Peroxides including H_2O_2, linoleic hydroperoxide, *t*-butyl hydroperoxide and cumene hydroperoxide have a mean extinction coefficient of 4.52 x 10^4 M^{-1} cm^{-1} and any of these peroxides can be used as a calibration standard (Jiang *et al*., 1991).

4.4.3.2 Assay procedure

The production of homogenate and mitochondrial total hydroperoxides was determined by the method described by Jiang *et al.* (1991) and Saravanan and Pari (2005). The Fox reagent was prepared by dissolving 88 mg butylated hydroxytoluene (BHT), 7.6 mg xylenol orange and 9.8 mg ammonium ferrous (iron) sulphate in 100 mL mixture of methanol (90%, v/v) plus 250 mM sulphuric acid (10%, v/v, H_2SO_4). The Fox reagent (200 µL) was added to 20 µL of diluted sample in duplicate with the use of the multichannel pipette, mixed and incubated at 37 ˚C for 30 min at which colour development is completed. The blue-purple colour was read at 560 nm. Concentrations of the total hydroperoxides were calculated from H_2O_2 standard curve ranging from 0-0.6 mM (r^2 = 0.994). The total hydroperoxides results are expressed as mM/mg protein.

4.4.4 The mitochondrial hydrogen peroxide production

The mitochondrial H_2O_2 production was assayed by the method described by Zhou *et al.* (1997) (**se Section 4.7.1**). The levels of H_2O_2 were calculated from standard curve ranges from 0-1 µM (r^2=0.996). The mitochondrial H_2O_2 results are expressed as µM/mg protein.

4.5 Total thiol group levels

4.5.1 Assay principle

A non-enzymatic antioxidant, namely, thiol groups such as GSH reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at RT to produce a yellow colour 5-thio-2-nitrobenzoic acid (TNB), which is detectable at an absorbance wavelength of 412 nm (Tietze, 1969; Hissin and Hilf, 1976).

4.5.2 Assay procedure

The liver homogenate total reduced thiol group content was determined as described by Ming *et al*. (2009). Briefly, thawed rat liver (100 mg) was homogenized in 1 mL of 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. The precipitate was removed by centrifugation at 10 000 xg for 15 min. After centrifugation, 120 µL 0.5 mM DTNB was added to 30 µL of supernatant and the total volume was made up to 200 µL by adding 50 µL of 100 mM sodium phosphate buffer (pH 7.0). The mixture was incubated at RT for 10 min and the developed colour (yellow) was read at 412 nm. The concentrations of reduced thiol groups were calculated by using a linear portion standard curve of GSH ranging from 0-1 mM (r^2 = 1.0). The homogenate thiol group results are expressed as mM GSH /mg protein.

4.6. Protein concentrations

Protein concentrations of the liver samples were determined according to the method described by Smith *et al*. (1985) and modified by Tshidino (2008). The bovine serum albumin (BSA) was used as a standard protein using the bicinchoninic acid (BCA) assay kit (Sigma). Protein concentrations were calculated from BSA standard curve ranging from 0-1 mg/mL (r^2=0.999).

4.7 Oxidant producing enzymes

Oxidase enzymes are the major producers of ROS as mentioned in **Section 2.7.3**. Hence, xanthine (XO), hypoxanthine (HXO) and NADH (NOX) oxidases were assayed in the liver homogenate and mitochondrial compartment.

4.7.1 Assay principle for the oxidases

Hydrogen peroxide (H_2O_2) is one of the oxidants produced by the oxidase enzymes. It is detectable by using the enzymatic coupled reaction method (Zhou *et al.* 1997), which is based on the oxidation of a most sensitive and stable fluorogenic probe, namely 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red also known as Ampliflue). Amplex res is a colourless and non-fluorescent derivative of dihydroresorufin, which is converted by horseradish peroxidase (HRP) enzyme to red fluorescent resorufin form that has Ex and Em wavelengths at 563 and 587 nm. Thus, the oxidase catalysed reaction with Amplex red results in an increase in the fluorescence (Zhou *et al.*, 1997).

4.7.1.1 Xanthine oxidase (EC 1.2.3.2) assay procedure

The reagents used for the assay reaction cocktail are shown in **Table 4.1**.

Table 4.1 Reagents used to prepare the reaction cocktail for the xanthine oxidase (XO) activity assay

Reagent	Volume (mL)	Final concentration
100 mM Tris-HCl buffer (pH 7.5)	4.88	100.0 mM
10 mM Amplex red in 100% Dimethyl sulfoxide (DMSO)	0.05	0.1 mM (1% DMSO)
100 U/mL Horseradish peroxidase (HRP)	0.02	0.4 U/mL
20 mM Xanthine or Hypoxanthine in 40 mM NaOH	0.05	0.2 mM

The activities of xanthine (XO) and hypoxanthine (HXO) oxidases were assayed as described by Zhou *et al.* (1997) with a minor modification. Briefly, 50 µL of reaction cocktail (100 mM Tris-HCl buffer pH 7.5 containing 0.2 mM xanthine or hypoxanthine, 0.1% Amplex red, 0.4 U/mL

and 1% DMSO) was added to 50 µL of XO standards, blank or diluted samples in black 96-well microtiter plate in duplicates, mixed and incubated at 37 ˚C for 30 min. An increase in fluorescence was measured kinetically at 544 and 590 nm in 5 min intervals. The activities of XO and HXO were calculated from a linear portion of the enzyme standard ranges from 0-0.4 U/mL using xanthine (XO, r^2 = 0.997) or hypoxanthine (HXO, r^2 = 1.0) as a substrate. The homogenate and mitochondrial XO and HXO results are expressed as U/mg protein. One unit (U) of XO activity is defined as the amount of enzyme that produced a change of one fluorescence unit per minute during oxidation of xanthine or hypoxanthine.

4.7.1.2 Mitochondrial NADH oxidase (EC 1.6.99.3)

4.7.1.2.1 Assay procedure

The reagents used for the NADH oxidase (NOX) assay reaction cocktail are shown in **Table 4.2**.

Table 4.2 Reagents used to prepare the reaction cocktail for the NADH oxidase (NOX) assay

Reagent	Volume (mL)	Final concentration (mM)
100 mM Tris-HCl (pH 7.5)	4.88	100.0 mM
10 mM Amplex red in 100% DMSO	0.05	0.1 mM in 1% DMSO
100 U/mL HRP in buffer (pH 7.5)	0.02	0.4 U/mL
10 mM NADH in cold 100 mM NaOH	0.05	0.1 mM

The activity of mitochondrial NOX was assayed as described by Zhou *et al.* (1997) with a minor modification. Briefly, 50 µL of reaction cocktail (100 mM Tris-HCl buffer pH 7.5 containing 0.1 mM Amplex red, 0.4 U/mL HRP, 0.1 mM NADH and 1% DMSO) was added in duplicate to 50 µL of diluted mitochondrial samples in black 96-well microtiter plate, mixed and incubated at 37 ˚C for 30 min. An increase in fluorescence was measured kinetically at Ex 544 and Em 590 nm for 30 min at 5 min intervals. The activity of NOX was calculated using 40 µM H_2O_2 as control. The NOX activity results are expressed as mU/mg protein. One unit (U) of NOX activity is defined as the amount of enzyme that produced a change of one fluorescence unit per minute during oxidation of NADH.

4.8 Antioxidant enzymes

The enzymatic antioxidants system is composed of superoxide dismutase (SOD), glutathione peroxide (GP), glutathione reductase (GR), catalase (CT) and glutathione-S-transferase (GST). These enzymatic antioxidants were measured in the liver homogenate and mitochondrial fractions to elucidate the effect of HFD on the activities.

4.8.1 Superoxide dismutase (EC 1.15.1.1)

4.8.1.1 Assay principle

Superoxide dismutase (SOD) is the enzyme that catalysis dismutation of superoxide anion to produce H_2O_2 and molecular oxygen. The activity of SOD was determined according to the SOD kit-WST (Fluka) protocol. The SOD activity assay uses the XO system that generates superoxide anions and water soluble tetrazolium salt to produce water soluble formazan dye upon reduction by superoxide anions. The SOD activity is determined by the colorimetric method as the percentage (%) inhibition of tetrazolium salt reduction using an equation provided on the kit manual (Fluka).

4.8.1.2 Assay procedure

The reagents used for preparation of the assay reaction mixture are shown in **Table 3.3**.

Table 4.3 Reaction mixture for SOD activity assay

Reagent	Sample volume (µL)	Blank 1 volume (µL)	Blank 2 volume (µL)	Blank 3 volume (µL)
Sample solution	10		10	
ddH_2O		10		10
WST working solution	100	100	100	100
Enzyme working solution	10	10		
Dilution buffer			10	10

The activity of SOD was assayed as described on the Fluka manual with minor modifications. The assay mixture was incubated at 37 ˚C for 20 min. The absorbance was read at 450 nm using microtiterplate reader.* The SOD activity was calculated using the following equation:

* SOD activity (% inhibition) = $\{[A_{blank1}-A_{blank2}] - A_{sample}-A_{blank2}\}/(A_{blank1}-A_{blank3})\}$ x100 (Fluka manual). One unit (U) of SOD activity is defined as the amount of enzyme that inhibited the tetrazolium salt reduction by XO taking to account the change in absorbance of both sample and blank solutions as shown in **Table 4.3**.

4.8.2 Glutathione peroxidase (EC 1.11.1.9)

4.8.2.1 Assay principle

Glutathione peroxidase (GP) is the enzyme that catalysis the reduction of FA hydroperoxides and H_2O_2 to produce water and oxidized glutathione (GSSG), the latter is coupled to an enzymatic reaction of glutathione reductase (GR), which is measured by a decrease in absorbance at 340 nm (Mates and Sanchez-Jimenez, 1999).

4.8.2.2 Assay procedure

The reagents used for the assay reaction cocktail are shown in **Table 4.4**.

Table 4.4 Reagents used to dissolve 4 mg NADPH to prepare reaction cocktail for the GP activity assay

Reagent	Volume (mL)	Final concentration
A. 50 mM potassium phosphate buffer with 1 mM DTT (pH 7.5)	0	50.0 mM
1 mM sodium azide solution in reagent A	38.6	1.0 mM
100 U/mL glutathione reductase (GR) in ddH_2O	0.4	1.0 U/mL
200 mM reduced glutathione (GSH) in ddH_2O	0.2	1.0 mM

The activity of GP was assayed as described by Mates and Sanchez-Jimenez (1999) with minor modifications. Briefly, 200 µL of reaction cocktail (50 mM potassium phosphate buffer with 1 mM EDTA pH 7.5 containing 1 mM sodium azide, 1 U/mL GR, 1 mM GSH and 0.1 mM

NADPH) was added in 5 µL of sample in duplicate, mixed and equilibrated to RT for 15 min in the microtiter palate. After equilibration, 5 µL of 50 mM H_2O_2 was added to each well and mixed. A decrease in absorbance was monitored at 340 nm for approximately 2 min. The activity of GP was calculated by using non-linear regression of the enzyme standard ranging from 0-4.8 U/mL ($r^2 = 0.971$). The GP results are expressed as U/mg protein. One unit (U) of GP activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during consumption of NADPH in the presence of GR as a coupling enzyme.

4.8.3 Glutathione reductase (EC 1.6.4.2)

4.8.3.1 Assay principle

Glutathione reductase (GR) is the enzyme that catalysis the reduction of oxidized glutathione (GSSH) in the presence of NADPH, which is oxidized to $NADP^+$ during the formation of reduced GSH (Mannervik and Carlberg, 1985).

4.8.3.2 Assay procedure

The assay reaction cocktail was prepared by dissolving 4 mg NADPH (reduced) in 37.2 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, 15% BSA, and 0.2 mL of 200 mM GSSG was added to the solution. The activity of GR was assayed according to the method described by Mannervik and Carlberg (1985). Briefly, 200 µL of reaction cocktail was added to 10 µL of diluted sample using the multichannel pipette and mixed. A decrease in absorbance was monitored at 340 nm for approximately 2 min. The activity of GR was calculated by using non-linear regression of enzyme standard ranging from 0-1 U/mL ($r^2 = 0.993$). The GR activity results are expressed as U/mg protein. One unit (U) of GR activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during consumption of NADPH.

4.8.4 Catalase (CT, EC 1.11.1.6)

4.8.4.1 Assay principle

Catalase (CT) is the enzyme that catalysis the breakdown of H_2O_2 (Mohanty *et al*., 1997) and its activity can be assayed using the Amplex red oxidation method described in **Section 4.10.1**, thereby measuring increase in the fluorescence following 30 min incubation of catalase and H_2O_2 reaction mixture at RT using a standard concentration of H_2O_2 as control.

4.8.4.2 Assay procedure

The reagents used for preparation of the CT assay reaction cocktail are shown in **Table 4.5**.

Table 4.5 Reagents used to prepare the reaction cocktail for catalase (CT) activity assay

Reagent	Volume (mL)	Final concentration
5 mM sodium phosphate buffer (pH 7.5)	2.465	5 mM
10 mM Amplex red in 100% DMSO	0.025	0.1 mM (1% DMSO)
100 U/mL HRP in buffer (pH 7.5)	0.01	0.4 U/mL

The activity of CT was assayed as described by Mohanty *et al*. (1997) and Zhou *et al.* (1997) with minor modifications. Briefly, 25 µL of 40 µM H_2O_2 (2.5 mL stock) was added to 25 µL of diluted sample in duplicate, mixed and incubated at RT for 30 min. After incubation, 50 µL of reaction cocktail (5 mM sodium phosphate buffer pH 7.5 containing 0.1 mM Amplex red, 0.4 U/mL HRP and 1% DMSO) was added and incubated at 37 ˚C for 30 min. An increase in fluorescence was measured at the Ex and Em wavelengths of 544 and 590 nm using 40 µM H_2O_2 as control. The activity of CT was calculated by using non-linear regression of the enzyme standard ranging from 0-0.4 U/mL (r^2 = 0.996). The CT results are expressed as U/mg protein. One unit (U) of CT activity is defined as the amount of enzyme that produced a change of one fluorescence unit per minute during reduction of H_2O_2 in the presence of HRP as a coupling enzyme.

4.8.5 Glutathione-S-transferase (EC 2.5.1.18)

4.8.5.1 Assay principle

Glutathione-S-transferase (GST) is the enzyme that catalysis the conjugation of GSH to 1-chloro-2,4dinitrobezene (CDNB) via the thiol group of GSH. An increase in absorbance is measured at 340 nm (Habig *et al*., 1974).

4.8.5.2 Assay procedure

The reagents used for preparation of the GST assay reaction cocktail are shown in **Table 4.6**.

Table 4.6 Reaction cocktail for GST activity assay

Reagent	Volume (mL)	Final concentration (mM)
(A) 100 mM potassium phosphate buffer with 1 mM EDTA (pH 6.5)	27	93.1
150 mM glutathione (GSH, reduced) in reagent A	1	5.0
30 mM CDNB in 95% ethanol (EtOH) [substrate]	1	1.0 (3.3% EtOH)

The activity of GST was assayed as described by Habig *et al*. (1974) with minor modifications. Briefly, 200 µL of reaction cocktail (100 mM potassium phosphate buffer pH 6.5 containing 1 mM EDTA, 5 mM GSH, 1 mM CDNB and 3.3% ethanol) was added to 10 µL of diluted sample and mixed. An increase in absorbance was monitored at 340 nm for approximately 2 min at RT. The activity of GST was calculated by using a linear portion of the enzyme standard ranging from 0-1 U/mL ($r^2 = 0.977$). The GST activity results are expressed as U/mg protein. One unit (U) of GST activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during conjugation of CDNB.

4.9 Cytosolic glucose metabolic enzymes

4.9.1 Glucokinase (GK, EC 2.7.1.2)

4.9.1.1 Assay principle

Glucokinase (GK) is the enzyme that catalysis D-glucose to G6P, which is detectable by the enzymatic coupling reaction of glucose-6-phosphate dehydrogenase (G6PD), by measuring an increase in absorbance at 340 nm due to the reduction of $NADP^+$ to NADPH (Goward *et al*., 1986).

4.9.1.2 Assay procedure

The reagents used for the GK assay reaction cocktail are shown in **Table 4.7**.

Table 4.7 Reagents used to prepare reaction cocktail for GK activity assay

Reagent	Volume (mL)	Final concentration (mM)
75 mM Tris-HCl (pH 9)	12.0	64.3
600 mM $MgCl_2$ in cold ddH_2O	0.5	21.4
120 mM ATP in cold ddH_2O	0.5	4.3
360 mM D-glucose in cold ddH_2O	0.5	12.9
27 mM NADP (oxidized) in cold ddH_2O	0.5	1.0

The activity of GK was assayed as described by Goward *et al.* (1986) using a G6PD as coupling enzyme. Briefly, 5 µL of 100 U/mL G6PD (from 0.5 mL stock) solution was added to 140 µL of reaction cocktail using the multichannel pipette and incubated at 30 ˚C for 15 min monitoring the absorbance at 340 nm until a stable baseline was reached. The final concentration of G6PD in the mixture was 3.6 U/mL. To the incubated solution (145 µL), 5 µL of blank or diluted sample was added, mixed and immediately read at 340 nm for 5 min at 1 min interval. Hexokinase was used as enzyme standard. The activity of GK was calculated using a linear potion of hexokinase ranges from 0-1 U/mL. The GK activity results are expressed as U/mg protein. One unit (U) of GK activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during formation of NADPH in the presence of G6PD as a coupling enzyme.

4.9.2 Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

4.9.2.1 Assay principle

Glucose-6-phosphate dehydrogenase (G6PD) is the enzyme that catalysis G6P and oxidized $NADP^+$ to produce NADPH (Broad and Shepherd, 1970). The activity of G6PD is determined by measured by an increase in absorbance at 340 nm due to reduction of $NADP^+$ to NADPH.

4.9.2.2 Assay procedure

The activity of G6PD was assayed as described by Broad and Shepherd (1970). Briefly, thawed rat liver (100 mg) was homogenized in 1 mL of 20 mM Tris-HCl buffer (pH 8.0) containing 250 mM sucrose and 1 mM EDTA and centrifuged at 1 000 xg for 15 minutes at 4 ˚C. Diluted supernatant (10 µL) was added to 190 µL of 60 mM Tris-HCl, 20 mM $MgCl_2$ buffer (pH 8.5) containing 0.25 mM G6P and 0.25 mM $NADP^+$. An increase in absorbance was read at 340 nm at RT for 5 min at 1 min interval. The G6PD activity results are expressed as U/mg protein. One unit (U) of G6PD activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during reduction of $NADP^+$.

4.9.3 Glucose-6-phosphatase (EC 3.1.3.9)

4.9.3.1 Assay principle

Glucose-6-phosphatase (G6Pase) is the enzyme that hydrolysis G6P to release glucose and Pi (Van Schiftingen and Gerin, 2002), the latter reacts with molybdate and ascorbate (vitamin C) to form phospho-molybdate complex, which is detected at absorbance of 815 nm (Gawronski and Benson, 2004).

4.9.3.2 Assay procedure

The reagents used for the G6Pase assay reaction cocktail are shown in **Table 4.8**.

Table 4.8 Shows reagents used for the G6Pase activity assay

Reagent	Volume (mL)
A. 12% ascorbic acid in (w/v)1 N HCl	100
B. 2% ammonium molybdate(w/v) in ddH_2O	100
C. 2 parts of A with 1 part of B (2A+1B, v+v)	30
D. 2% Na citrate (w/v), 2% acetic acid (v/v) and 2% SDS (w/v)	100
0.5 mM G6P in 20 mM HEPES buffer (pH 6.8)	1
0.5 mM β-glycerolphosphate (βGP) in 20 mM HEPES buffer (pH 6.8)	1

The activity of G6Pase was assayed as described by Van Schiftingen and Gerin (2002) and Gawronski and Benson (2004), with a minor modification. The microsomes in the cytosolic fraction were disrupted by the freeze-thaw method at -20 ˚C and 4 ˚C, thereby repeating the steps 3 times. Either glucose-6-phosphate (G6P) or β-glycerolphosphate (βGP) substrate (45 µL) was added separately to 5 µL of blank or dilute samples in duplicate using the multichannel pipette. A blank or each phosphate standard (50 µL) was also added to wells in duplicate. Both standard and sample mixtures were incubated at 37 ˚C for 20 min. The reaction was terminated by adding 150 µL of reagent C to each well and the mixture was incubated at RT for 5 min. Reagent D (150 µL) was added to each sample and incubated at 37 ˚C for 15 min to develop a blue-green colour. The developed phospho-molybdate complex was read at 815 nm. The activity of G6Pase was calculated from a linear portion of Pi standard ranging from 0-50 nM (r^2 = 1.0). The G6Pase activity results are expressed as µmol Pi/min/mg protein = U/mg protein.

4.9.4 Lactate dehydrogenase (EC 1.1.1.27) activity

4.9.4.1 Principle

Lactate dehydrogenase (LDH) is the enzyme that catalysis the inter-conversion of lactate and pyruvate with an NADH as a coenzyme (Tsujibo *et al*., 1985). This LDH assay is based on the pyruvate-to-lactate reaction that yields a greater change in absorbance per unit time, which

allows more accurate spectrophotometric readout at lower reactant concentrations (Howell *et al*., 1979).

4.9.4.2 Assay procedure

The reagents used for preparation of the LDH assay reaction cocktail are shown in **Table 4.9**.

Table 4.9 shows the reaction cocktail for the LDH activity assay

Reagent	Volume (mL)	Final concentration (mM)
50 mM Tris-HCl (pH 7.6)	12.0	46.3
7.3 mM NADH in cold ddH_2O	0.5	0.3
62.4 mM sodium pyruvate in cold ddH_2O	0.5	2.4

The activity of LDH was determined as described by Howell *et al*. (1979) with minor modifications. Briefly, 140 µL of reaction cocktail (46.3 mM Tris-HCl buffer pH 7.6 containing 0.3 mM NADH and 2.4 mM sodium pyruvate) was added to 5 µL blank or diluted sample and mixed. A decrease in absorbance was monitored at 340 nm for 1 min. The LDH activity results are expressed as U/mg protein. One unit (U) of LDH activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during oxidation of NADH to NAD^+.

4.10 Cytosolic enzyme for the endogenous lipids synthesis

4.10.1 ATP citrate lyase (EC 4.1.3.8)

Adenosine triphosphate citrate lyase (ATP citrate lyase, ACL) is one of the cytosolic enzymes that play important roles in both the *de novo* FA and cholesterol synthesis pathways (Wells, 1991; Wang, 2009a). The ATP citrate lyase is the enzyme that catalysis the cleavage of the cytosolic citrate with hydrolysis of ATP to form oxaloacetate, acetyl-CoA, ADP and Pi, a reaction that links glucose and lipid metabolism.

4.10.1.1 Assay principle

The activity of ACL is determined by the enzymatic coupling method, the oxaloacetate formed from the cleavage of citrate is measured by its reduction with NADH in the presence of a coupling enzyme malate dehydrogenase (MDH) (Takeda *et al*. 1969).

4.10.1.2 Assay procedure

The reagents used for preparation of the ACL assay reaction cocktail are shown in **Table 4.10**.

Table 4.10 Shows the reagents used to prepare reaction cocktail for ACL activity assay

Reagent	Volume (mL)	Final concentration (mM)
50 mM Tris-HCl (pH 8.4)	10.0	27.4
200 mM $MgCl_2$	1.25	13.7
200 mM β-mercaptoethanol	1.25	13.7
200 mM potassium citrate	2.5	27.4
100 mM ATP	2.5	13.7
10 mM NADH	0.5	0.3
20 U/mL malate dehydrogenase	0.25	0.3

The activity of ACL was assayed as described by Takeda *et al*. (1969) with minor modifications. Briefly, 175 µL of reaction cocktail (27.4 mM Tris-HCl buffer pH 8.4 containing 13.7 mM $MgCl_2$, 13.7 mM β-mercaptoethanol, 27.4 mM potassium citrate, 13.7 mM ATP, 0.3 mM NADH and 0.3 U/mL MDH) was added to 10 µL of diluted sample using the multichannel pipette. Acetyl-CoA and ATP were not added to the blanks. The reaction was started by addition of 25 µL of 2 mM acetyl-CA to the sample mixture and 25 µL of ddH_2O to the blank, mixed and incubated at 37 ˚C for 10 min. A decrease in absorbance was measured at 340 nm as a result of NADH oxidation to NAD^+. The ACL activity was calculated by using a linear portion of the enzyme standard curve ranges from 0-0.3 U/mL (r^2 = 0.997). The ACL activity results are expressed as U/mg protein. One unit (U) of the ACL activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during oxidation of NADH in the presence of MDH as a coupling enzyme.

4.11 Mitochondrial TCA cycle and respiratory chain enzymatic assays

The citrate synthase (CS, EC 4.1.3.7) is the first enzyme in the TCA cycle that was assayed to determine the rate of citrate synthesis in the mitochondria. The mitochondrial electron chain enzymes including complexes I, II and III were measured to investigate ROS production from complexes I and III.

4.11.1 Citrate synthase activity assay

4.11.1.1 Mitochondrial disruption

The liver mitochondrial disruption was done by freeze-thaw method at -20 ˚C and 4, respectively. The method was repeated 3 times to disrupt mitochondria for the citrate synthase (CS), NADH dehydrogenase and NADH oxidase activities (**Section 4.10.1.2**).

4.11.1.2 Assay principle

The activity of CS is determined by the chemical coupling of Coenzyme-ASH liberated from acetyl-CoA during the synthesis of citrate to Ellman's reagent to form a mercaptide ion, which is released and detected at an absorbance of 412 nm (Parvin, 1969; Sere, 1969).

4.11.1.3 Assay procedure

The reagents used for preparation of the CS assay reaction cocktail are shown in **Table 4.11**.

Table 4.11 Shows the reagents used for preparation of the reaction cocktail

Reagent	Volume (mL)	Final concentration (mM)
100 mM Tris-HCl (pH 8.0)	12.0	85.8
2.8 mM DTNB	0.5	0.1
8.4 mM Acetyl Coenzyme A	0.5	0.3
93.2 mM k_2HPO_4	0.5	3.3
14 mM oxaloacetate	0.5	0.5

The activity of CS assay was determined as described by Shepherd and Garland (1969) with minor modifications. Briefly, 150 µL of reaction cocktail (85.8 mM Tris-HCl buffer pH 8.0 containing 0.1 mM DTNB, 0.3 mM Acetyl CoA, 3.3 mM K_2HPO_4, 0.5 mM oxaloacetate) was added to 10 µL of blank or diluted samples and mixed. An increase in absorbance was monitored at 412 nm for 5 min at RT. One unit (U) of the CS activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during formation of citrate in the presence of DTNB as a coupling chemical.

4.11.2 NADH dehydrogenase (EC 1.6.5.3)

The mitochondrial NADH dehydrogenase (complex I, EC 1.6.5.3) and succinate dehydrogenate (SDH, complex II) receive electrons from the oxidation of both NADH and succinate, and pass them to a lipid electron carrier, which is coenzyme Q (Labbea *et al*., 2008). The mitochondrial SDH integrates the TCA cycle and electrons transport chain (Rustin *et al*., 2002).

4.11.2.1 Assay procedure

The activity of complex I was determined as described by Audi *et al*. (2008). Briefly, 180 µL of 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM KCl, 1 mM EDTA, 1 mM sodium azide (NaN_3), 10 µM antimycin-A in the presence or absence of 20 µM rotenone was added to 10 µL of samples. Reaction was initiated by addition of 10 µL of 2 mM NADH and 10 µL of 2 mM Coenzyme Q1. A decrease in absorbance at 340 nm was read for 3 min as a rate of NADH oxidation. The activity of complex I was calculated by subtracting the NADH oxidation in the absence of inhibitor from that in the presence of inhibitor. One unit (U) of complex I activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during oxidation of NADH.

4.11.3 Succinate dehydrogenase (EC 1.3.5.1)

The intactness of mitochondrial and its functional were measured by succinate dehydrogenase (SDH, complex II, EC 1.3.5.1) activity. Mitochondrial complex II is the enzyme that catalysis

oxidation of succinate to fumarate in the TCA cycle, and flows electrons to the respiratory chain ubiquinone in the form of $FADH_2$ (Rustin *et al*., 2002).

4.11.3.1 Assay principle

The activity of complex II (SDH) is measured by the reduction of iodonitrotetrazolium chloride (INT) to formazan, which is stabilized by Cremophor EL and read at an absorbance of 500 nm. This assay also gives an opportunity to study the relative time by using the kinetic measurement (Munujos *et al*., 1993). In the present study, Cremophor EL was replaced by 1% DMSO.

4.11.3.2 Assay procedure

A reaction cocktail composed of 100 mM Tris-HCl (pH 8.3) containing 0.5 mM EDTA, 2 mM KCN, 2 mM INT, 1% DMSO and 20 mM succinate was used to assay the activity of complex II. The activity of complex II was determined as described by Munujos *et al*., (1993) with a minor modification. Briefly, 245 µl of reaction cocktail (100 mM Tris-HCl buffer pH 8.3 containing 0.5 mM EDTA, 2 mM KCN, 2 mM INT, 1% DMSO and 20 mM succinate) was added to 5 µl of diluted samples in the clear 96-microtiter plate using the multichannel pipette. Blanks were prepared in the absence of substrate (succinate). The developing colour at 30 ˚C was measured kinetically at 500 nm at 1 min interval for 10 min, in which the reaction maintains its linearity in the presence of DMSO. The activity of complex II was calculated by subtracting the activity without the substrate from that with the substrate. The complex II activity results are expressed as U/mg protein. DMSO stabilizes the formed formazan in this study, suggesting that it is a good solvent to be used at 1% concentration without affecting the enzyme activity. One unit (U) of SDH activity is defined as the amount of enzyme that produced a change of one absorbance unit per minute during formation of INT-formazan.

4.11.4 Cytochrome c reductase (EC 1.10.2.2) activity

Cytochrome c reductase (complex III, EC 1.10.2.2) is the enzyme that oxidizes the reduced form of cytochrome Q and in turn cytochrome c, which is a protein electron carrier that is also mobile in the inner mitochondrial membrane (Labbea *et al*., 2008).

4.11.4.1 Assay procedure

The activity of complex III was determined as described by Zhou *et al.* (1997) using the HRP as a coupling enzyme. Reaction cocktail composed of 100 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM Amplex red, 0.4 U/mL HRP, 0.1 mM cytochrome c and 1% DMSO (50 µL) was added to 50 µL of sample. An increase in the fluorescence was measured kinetically at Ex and Em of 544 and 590 nm for 30 min at 5 min intervals at 37˚C. The activity of complex III was calculated using 40 µM H_2O_2 as control. The complex III activity results are expressed as U/mg protein. One unit (U) of complex III activity is defined as the amount of enzyme that produced a change of one fluorescence unit per minute during catalysis of cytochrome c in the presence of HRP as a coupling enzyme.

4.12 Histological study

The liver histological analysis was done to determine the structural changes and the lipid droplets by using the routing stain method, namely, Haematoxylin and eosin stain (H&E).

4.12.1 Haematoxylin and eosin staining

The frozen liver samples were embedded in the OCT medium. The liver samples were sectioned at 8 µm thick and placed on the slides. The sections were dried at RT for 30 min, fixed in ice cold 95% ethanol for 30 sec and air dried. The slides were transferred to 70% ethanol for 3 min and to 40% for another 3 min. The slides were then transferred to dH_2O for 3 min and air dried to check if the OCT is completely removed. The sections were placed in haematoxylin for 5 min. The sections were washed in dH_2O and blued in tap H_2O for 3 min. The washed sections were placed in 1% acid alcohol solution (75% ethanol containing 1% HCl) for 30 sec and washed in tap H_2O for 1 min. The slides were dipped 10 times in 95% ethanol and then placed in eosin for 5 min. The slides were washed in tap H_2O and dehydrated by dipped 10 times in 95% ethanol and placed in 100% ethanol for 10 min. The sections were cleared in 100% xylene for 10 min, drained and mounted using the Vector mounting medium (H-5000). The liver histology was visualized under the light microscope.

4.13 Western blotting

The procaspase-3 and iNOS protein levels were determined in the liver cytosolic fractions to elucidate whether 16 weeks of feeding the rats on HFD induced apoptotic and inflammatory responses in the liver. Buffers used for the western blotting are shown in **Table 4.12**.

Table 4.12 Western blot reagents

0.2% Ponceau S	10 X Tris-HCl buffer (pH 8.0, TBS stock)	1XTBS	1XTBS added 0.1% Tween-20 (TBS-T)	Blocking buffer (10% milk in 1% TBS-T)
0.2 g Ponceau S	250 mM Tris-HCl	25 mM Tris-HCl (pH 8.0)	1 mL Tween-20	10 g non-fat dry milk
100 mL 3% TCA	1250 mM NaCl	125 mM NaCl	999 mL 1X TBS	100 mL TBS-T

***TBS**, Tris-buffer saline; **NaCl**, Sodium chloride, **TCA**, Trichloroacetic acid, **T**, Tween*

4.13.1 Procedure

The liver procaspase-3 and iNOS protein levels were determined by the Western blot method described by Towbin *et al*. (1979). Briefly, 5 µL of the pre-stained protein markers (Promega) and the rat liver cytosolic proteins 30 µg/ lane (30 µg proteins/10 µL) in the Laemmli sample buffer (Laemmli, 1970) were loaded to 12.5% (procaspase-3) and 7.5 % (iNOS) SDS-PAGE under the reducing conditions. The electrophoresis was carried out at 100 V and 50 mA for 60 min at RT. The membranes, gels and blotting papers were equilibrated in ice-cold protein blotting buffer for 30 min. Proteins were transferred to the immobilon-P transfer membrane (0.45µm, Millipore) using the semi-dry blot system (Bio-Rad) at 25 V and 2 mA for 2 hrs. The transferred proteins were visualized on the membranes by 2% poceau S stain (Sigma) in 3% TCA and destained by 1x TBS containing 0.1% tween-20 for 20 min. The membranes were blocked in 50 mL of 10% non-fat milk (SPAR instant fat free milk powder, South Africa) in 1x TBS containing 0.1% Tween-20 for 2 hrs at RT with gentle shaking on the Labnet rocker 25 at 20 rpm. The blocked membranes were washed 3 times in 25 mL 1x TBS containing 0.1% Tween-20 at 10 min interval. The washed membranes were incubated with the primary antibodies for β-actin 1:5000 (anti-actin, Sigma) and caspase-3 1:2500 (Anti-caspase-3 active/cleaved isotype rabbit IgG, IMGENEX) or iNOS 1:2000 (iNOS rabbit polyclonal IgG,

Santa Cruz Biotechnology) in 5% non-fat milk TBS containing 0.1% Tween-20 for 2 hrs at RT. The membranes were washed 3 times for 10 min in 25 mL of 1x TBS containing 0.1% Tween-20. The washed membranes were incubated with the secondary antibody, anti-rabbit (whole molecule) alkaline phosphatase conjugated (Sigma) 1:30 000 in 10 mL 10% non-fat milk TBS containing 0.1% Tween-20 for 2 hrs at RT. The membranes were washed 3 times in 25 mL 1x TBS containing 0.1% Tween-20 at 10 min interval. The washed membranes were incubated in 5 mL of 5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium (BCIP/NTB) colour developer (alkaline phosphatase substrate, Promega) for 1 hr at RT in the dark. The developed membranes were washed with dH_2O for 1 min and dried on between the filter membranes in the dark. The visualized bands were photographed using the Gel Doc (AlphaImager 3400, Alpha innotech). The bands were quantified and normalized by β-actin protein using the Gel Doc.

4.14 Statistical analysis

Results are presented as mean ± standard deviation (SD). The comparisons between groups were made using the repeated measures one-way analysis of variance (ANOVA), followed by post-hoc analysis and Tukey test (STATISTICA version 9, 2010). P-values <0.05 were considered statistically significant.

CHAPTER 5: Effect of HFD on the development of fatty liver

Synopsis

This study was undertaken to characterize the biochemical changes in lipid and glucose metabolism, as well as oxidative stress, which occur during the development of high fat diet (HFD) induced fatty liver. The findings discussed in this Chapter serve to confirm the development of fatty liver and define the biochemical alterations that occur in the current HFD model.

Fourteen male Wistar rats were fed a high fat diet (CH group) or normal rat chow (CL group) for 12 weeks. Feeding the rats on a HFD for 12 weeks (CH group), increased lipid accumulation in the liver, but with a total lipid (TL) content of <5% per liver weight. However, feeding the rats for an additional 4 weeks (HF group) increased the lipid content to >5% per liver weight, signifying the induction of fatty liver. Liver histology suggests the presence of microsteatosis and macrosteatosis after 16 weeks. This was associated with increased ($P<0.05$) malonaldehyde (MDA) levels and activities of glucose-6-phosphate dehydrogenase (cG6PD), xanthine oxidase (XO), and mitochondrial NADH oxidase (mNOX), and decreased ($P<0.05$) activities of homogenate superoxide dismutase (hSOD) and mitochondrial complex II, when compared to the LF group. Since cACL activity was unchanged in the HF group, this may imply no change in acetyl-CoA influx to the TCA cycle and to the de novo synthesis of fatty acids. Increased cG6PD activity in the HF group suggests that there is an increased NADPH demand, which may possibly include cholesterol and TG synthesis from NEFAs taken up by the liver from circulation and for maintenance of the NADPH-dependent antioxidants and pro-oxidants in the liver, however this was not confirmed. Increased mitochondrial FA oxidation in the HF group was shown by significant increased mNOX and decreased complex II (succinate dehydrogenase, SDH) activities, without altering activities of complexes I (NADH dehydrogenase) and III (cytochrome c reductase) when compared to that in the LF group. In addition, the homogenate reduced thiol groups, total hydroperoxides, glutathione peroxidase (GP), glutathione reductase (GR), and glutathione-S-transferase in the HF group was similar to that in the LF group, suggesting that HFD-induced fatty liver is not associated with oxidative stress nor does there appear to be any association with an inflammatory or apoptotic response.

In conclusion, biochemical changes mediated by HFD-induced fatty liver appear to have partitioned cytosolic G6P towards NADPH production from PPP accompanied by a decrease in mitochondrial complex II activity. Together these alterations may contribute to the development of macrovesicular and microvesicular steatosis. These findings support that biochemical changes in the liver are useful in the characterization of fatty liver induced by the present HFD.

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5.9 Proposed mechanism(s) of action by which a HFD-induced fatty liver in Male Wistar rats.

5. Introduction

This chapter reports the effect of HFD on rat liver glucose, lipid, pro-oxidant and antioxidant enzymes. Together these play a major role in the induction of fatty liver and consequent progression to via mitochondrial oxidative stress, respiratory chain dysfunction and decreased antioxidant capacity, all characteristic features in the development of simple fatty liver. The liver is uniquely poised in lipid metabolism as it can synthesize substances like FAs, TG and cholesterol, and also removing elevated TG, glycerol and FFAs from the circulation, especially when adipose tissue becomes dysfunctional (Iozzo *et al*., 2004). In the liver, FAs can be completely oxidized in the mitochondria to produce energy or used to synthesize glucose, lipids, ketone bodies and in the process produce oxidative stress mediators (Bradburg, 2006). An imbalance in glucose and lipid metabolism mediated by chronic consumption of a HFD may lead to pathological conditions such as fatty liver and metabolic syndrome (Gauthier *et al*., 2003). Fatty liver is characterized by an increase in lipid accumulation (>5% per liver weight) in the hepatocytes (Yu *et al*., 2009). Accumulated lipid in the liver can cause damage, which may give rise to an inflammatory response, oxidative stress and abnormal cellular signalling depending on the types of FAs consumed as well as the amount and time of dietary intake (Mensink and Katan, 1990; Tessari *et* a*l*., 2009; Timmers *et al*., 2011).

5.1 Evaluation of fatty liver

5.1.1 Liver histology

To determine the status of the rat liver after 16 weeks on a HFD, lipid accumulation was investigated histologically. Representative histology images of the liver are shown in **Figure 5.1**.

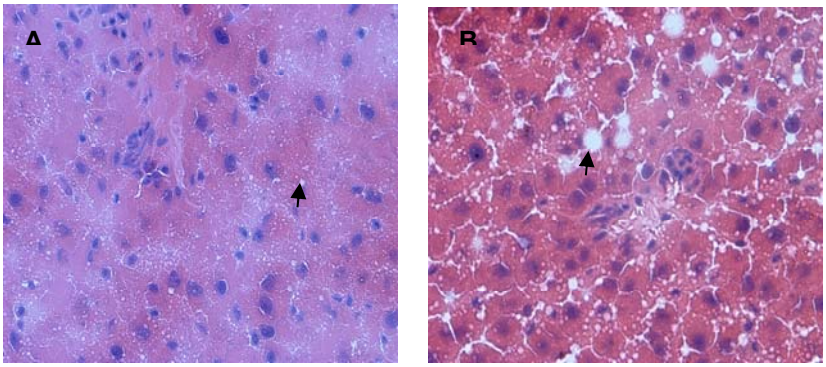

Figure 5.1 *Rat liver histology (**A**) LF (rats fed on a low fat diet, lean) (**B**) HF (rats fed on a high fat diet, fatty liver) (Original magnification X40). Arrow indicates a lipid droplet.*

Haematoxylin and eosin (H&E) stained sections showed an increase in lipid droplet content in liver of the HF group when compared to the LF group (**Figure 5.1**). In the present study, a HFD induced both microvesicular and macrovesicular fatty liver forms, whilst LFD showed limited microvesicular without macrovesicular droplets. These results clearly indicate that the HFD induced increased lipid accumulation in the liver. Lipid accumulation in the liver was also confirmed by the Nile red stain (data not shown).

5.1.2 Results

To confirm the development of fatty liver in response to a HFD relative to a LFD, the total lipid (TL) content at weeks 12 (coded CH and CL, respectively) and 16 (coded HF and LF, respectively) were extracted and quantified (**Table 5.1**).

Liver TL content was significantly increased in the CH group when compared to the CL group ($P < 0.05$), indicating that feeding the rats a HFD mediated a modest, but significant accumulation of lipid in the liver after 12 weeks. However, the TL content from the CH group was 27.6 mg/g wet liver weight, which is equivalent to 2.76% of lipids by liver weight (**Table 5.1**), implying that fatty liver had not yet developed at week 12, since the benchmark for fatty liver is >5% of lipids by liver weight.

After 16 weeks, the liver TL content was significantly increased in the HF group when compared to both CH ($P < 0.001$) and LF ($P < 0.01$) groups, respectively. Thus at week 16, both the HF and LF groups had accumulated lipids approximately 2-fold of that in the CH and CL groups (**Table 5.1**). The TL content in the HF group was 67.3 mg/g liver weight,

which is equivalent to 6.73% of lipids by liver weight; therefore this group can be classified as "fatty liver" (**Table 5.1**) as also suggested from the liver histology (**Figure 5.1B**) and thus confirms the data obtained by Mackenzie (2009).

Table 5.1 Liver total lipids

	CL (lean)	CH (fatty liver)	LF (lean)	HF (fatty liver)
TL (mg/g liver wet weight)	22.6±3.5	27.6±2.9$^{\$}$	48.0±1.1$^{\$\$\$}$	67.34±6.0***$^{\#\#}$
Percentage lipid	2.2	2.76	4.8	6.73
Fold difference (CL vs CH and LF vs HF)		1.22		1.40
Ratio 16 week/12 week			2.1	2.4
Percentage increase HF vs LF		22.1		40.3
Percentage increase 16 weeks vs 12 weeks			112	144

***CL**, low fat diet fed rats after 12 weeks; **CH**, high fat diet fed rats after 12 week; **LF**, low fat diet fed rats after 16 weeks; **HF**, high fat diet fed rats after 16 weeks; **TL,** total lipid. $^{\$}$ = significantly different from **CL**, $^{\$}P < 0.05$; $^{\$\$\$}P < 0.001$ * = significantly different from the LF control, *** $P < 0.001$; and $^{\#}$ = significantly different from the CH, $^{\#\#\#}P < 0.001$. **ND**, not determined; LF: n=6; HF: n=7.*

5.1.3 Discussion

According to Buettner *et al*. (2007) there is much controversy about the metabolic disorders of rats fed HFD. A possible explanation for this wide variety of results in the rats fed HFD could be due to a lack of standardization of the HFD used in these various studies. As reviewed by Buettner *et al*. (2007), many studies do not describe the exact quantity of fats in the HFD, its energy density (isocaloric or hypercaloric), the time of exposure to the diet, or the types of fat used (whether a vegetable or an animal fat). Many studies have employed well-defined, semi-purified HFDs in which the fat component replaces carbohydrate and/or protein. However, other studies have added fat to a standard rodent chow that results in an unbalanced diet composition with respect to all macro- and micronutrients (Reviewed in Buettner *et al*., 2007; Gajda *et al*., 2007; Picchi *et al*., 2011). The HFD used in the present study falls under those that added fat to a standard rodent chow (Xu *et al*., 2010), but is well characterised as described in **Chapter 3** (**Table 3.1**). In this study, the HFD enriched with palmitic acid (40.9%) in the presence of oleic acid (29%) and relatively low in carbohydrates,

induced simple fatty liver, which manifested as a mix of microvesicular and macrovesicular steatosis (**Figure 5.1)**.

Since, this HFD was originally designed to induce insulin resistance and investigated as such (Mackenzie 2009); it was deemed of interest to further investigate biochemical changes that accompany the development of fatty liver in this rat model.

Thus, it remains important to define the present HFD's metabolic effects in the context of fatty liver development and its suitability as a model to investigate potential treatments for this disease. At week 12, no significant change in plasma lipid parameters were observed, whereas after 16 weeks, significant increased plasma TG, TC and FFAs levels are reported in these same rats fed a HFD compared to those fed a LFD ($P < 0.05$) (Karachi, 2009; Mackenzie, 2009; **Tables 3.1** and **3.2**). The present study confirmed that the liver TL content was also significantly elevated in the HF group when compared to the LF group ($P < 0.01$) (**Table 5.1**). The study by Mackenzie, (2009) showed that these rats were insulin resistant (IR) at 12 weeks. The present study has confirmed that these HFD fed rats also develop fatty liver after 16 weeks, but not at week 12 (**Figure 5.1** and **Table 5.1**), suggesting that the onset of insulin resistance occurs prior to and/or is independent of the development of fatty liver. It should however be emphasised that cut off values used to define these two pathological conditions are arbitrary and subsequently. Thus, it may also be expected that the actual biochemical events leading to the development of fatty liver may indeed overlap significantly despite the absence of any clear and defined pathological characteristics to indicate such. Furthermore, there is uncertainty as to whether such changes in blood lipid parameters are a cause or simply a consequence of metabolic alterations in the liver due to a HFD. It is also of interest to note that in the LF group, lipid values closely approach the cut-off limit for fatty liver (**Table 5.1**), further indicating that these cut off values lack absolute sensitivity as a defining feature for fatty liver in response to HFD feeding.

5.2 Markers of the inflammatory and apoptotic response

5.2.1 Results

Considering that inflammation is associated with the progression of fatty liver, it was deemed of interest to determine whether 16 weeks of feeding the rats a HFD can mediate

inflammatory and concomitant apoptotic responses. Liver iNOS and procaspase-3 protein levels were measured as markers of inflammation and apoptosis, respectively (**Figure 5**).

Unfortunately the levels of active caspase-3 protein was below the detection limit and thus not detectable on the Western blot (**Appendix A**). However, procaspase-3 protein levels were similar in both the HF and LF groups, suggesting the absence of apoptosis (**Figures 5.2**). Similarly iNOS levels remained unchanged (**Appendix B)**; indicating that in the present experimental HFD system, fatty liver developed independent of inflammation via the iNOS pathway and concomitant apoptotic cell death. Such a finding is perhaps not surprising considering the relatively modest level of fatty liver in the HF group as defined by a total lipid content just exceeding the cut-off value of 5% (**Table 5.1**). Inflammation and apoptotic cell death are considered progressive features in the pathogenesis of fatty liver (Tolman and Dalpiaz, 2007).

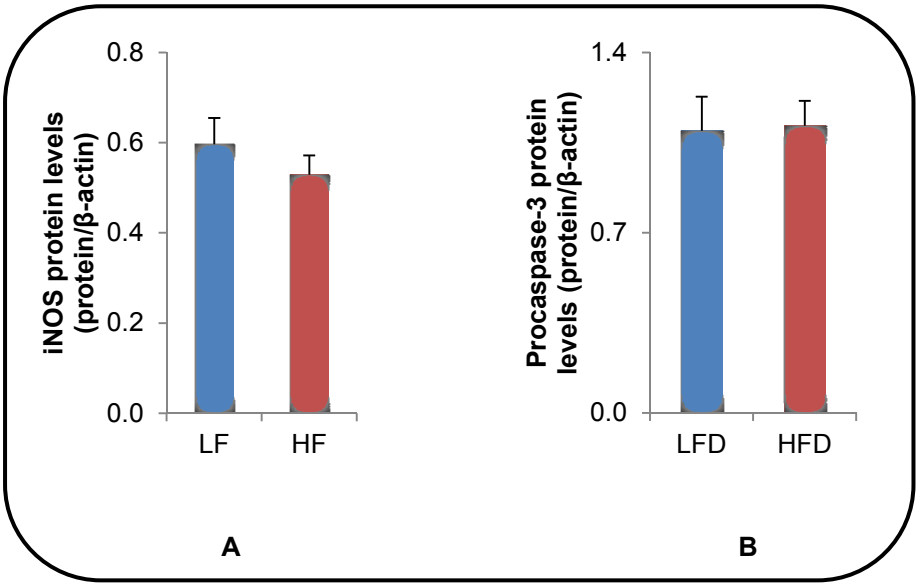

Figure 5.2 *Rat liver inducible nitric oxide (iNOS) and procaspase-3 protein expression levels (A) iNOS protein levels (B) Procaspase-3 protein levels.* ***LF****, low fat diet fed rats,* ***HF****, high fat diet fed rats. LF: n=6; HF: n=7. Arrows indicate the protein bands.*

5.2.2 Discussion

Inflammation and apoptosis play an important role in the progression of simple fatty liver to NASH (Koteish and Diehl, 2002). The HFD effects on the liver in the present study is similar to that mediated by a choline deficient (CD) diet that had induced fatty liver without

inflammation, despite that CD diet with undefined % fat (Grattagliano *et al*., 2000) induced fatty liver in 30 days (Petrosillo *et al*., 2007).

5.3 Properties of isolated liver mitochondria and PMS fraction

The liver homogenate was fractionated to provide mitochondrial and cytosolic fractions. Mitochondria were isolated to investigate whether oxidative stress, if present, differs between the mitochondria and the cytosol. These fractions were first analysed for mitochondrial- and microsomal [cytosolic, post-mitochondrial supernatant (PMS)]-enrichment, recovery and contamination by using enzymatic markers that are known to be associated with these organelles. Succinate dehydrogenase (SDH) is integrally located in the inner mitochondrial membrane; hence SDH activity was used for the analysis of the mitochondrial-enrichment fraction as well as the cytosol contamination by the mitochondria. Glucose-6-phosphatase (G6Pase) is integrally located in the microsomes (Bandsma *et al*., 2001). Therefore, G6Pase was used to analyse the PMS fraction as well as the mitochondria contamination with microsomes. The homogenate as well as the mitochondria and microsome recoveries and enrichments data are summarized in **Table 5.2**.

Table 5.2 Distribution of marker enzymes in various sub-cellular fractions obtained by differential centrifugation of liver tissue derived from the HF group. Seven liver samples of the HF group were used to determine the organelle enrichment and purity. It is assumed that diet and treatment would not significantly alter enrichment or purity of the isolated fractions, however this was not tested.

	Homogenate	**Mitochondria**	**PMS**
Protein (mg/g liver)	205.172±2.221	17.642±0.829	111.287±2.291
SDH activity (ΔA/min/g liver)	1.565±0.178	14.169±2.028	0.017±0.002
Mitochondrial-enrichment (relative SDH activity) (fold)	1	9.011	0.011
G6Pase activity (mM Pi/min/g liver)	230.045±20.962	32.694±5.056	304.849±78.085
Microsomal-enrichment (relative G6Pase activity) (fold)	1	0.142	1.325

The homogenate showed presence of each of the assayed enzymes, which was determined in the selected liver compartments namely, mitochondria and cytosol/microsomes (**Table 5.2**). The mitochondria enrichment and recovery after centrifugation steps were evaluated by measuring the SDH specific activity in the liver homogenate and in the final mitochondrial

fraction. The mitochondrial recovery was 68.9% relative to that in the homogenate, which is assumed to be 100% (**Table 5.2**). The mitochondria contamination with microsomes was also assessed by measuring the G6Pase specific activity in the PMS relative to that in the crude homogenate. The mitochondrial fraction showed contaminated by 0.12% microsomes. The microsomes recovery in the PMS fraction was 72.8% relative to that in the homogenate (100%). The PMS fraction was contaminated by 11% mitochondria. These results revealed that 31.1% mitochondria were lost during the fraction preparations (**Table 5.2**).

5.4 Lipogenic enzymes activities

5.4.1 Results

To further investigate the altered lipid metabolic pathways mediated by HFD-induced fatty liver as shown in **Sections 5.1.1** and **5.1.2**, the lipogenic enzyme activities were measured in the mitochondrial and PMS fractions. The assayed lipogenic enzymes are cytosolic glucose-6-phosphate dehydrogenase (cG6PD), mitochondrial citrate synthase (mCS) and cytosolic ATP citrate lyase (cACL) as shown in **Table 5.3**.

Table 5.3 Lipogenic enzyme activities

	LF (lean)	HF (fatty liver)	Fold difference
cG6PD (ΔA/min/mg protein)	3.959±1.025	6.679±0.893*	1.7
cACL (ΔA/min/mg protein)	32.011±4.905	29.252±3.897	1.1
mCS (ΔA/min/mg protein)	18.991±1.723	17.693±1.712	1.1

LF, *low fat diet fed rats;* ***HF****, high fat diet fed rats;* ***cG6PD****, glucose-6-phosphate dehydrogenase*; ***cACL****, ATP citrate lyase; and* ***mCS,*** *citrate synthase. * = significantly different from the LF control, * P < 0.05. LF: n=6; HF: n=7.*

The activity of cG6PD was significantly increased 1.7-fold in the HF group as compared to the LF group ($P < 0.05$), while the activities of cACL and mCS remained unchanged (**Table 5.3**). These data indicate that despite a significant increase in activity of cG6PD in the HF group; the concomitant enzymatic capacity required for *de novo* lipogenesis remains unchanged, suggesting that the expected elevation in NADPH synthesis may not relate to the increased lipid accumulation in the liver (**Table 5.1**). Since NADPH is a universal cofactor

for a host of biochemical processes, it may be assumed that the elevated G6PD activity observed in response to HFD is linked to a pathway other than de novo lipogenesis. Like *de novo* fatty acid synthesis, elongation of dietary fatty acids will also require cACL and mCS for malonyl-CoA synthesis, the essential component required for elongation, and too is unlikely to contribute to lipogenesis. In contrast however, esterification of readily available NEFAs taken up from the blood circulation by the liver does not depend on the activation of cACL and mCS enzymes for lipid accumulation, thus fatty liver development can occur independent of de novo lipogenesis and consequently the elevated G6PDH activity most likely indicates the involvement of some other biochemical process such as resistance to oxidative stress.

5.4.2 Discussion

Current literature suggests that fatty acids in the diet and feeding period may determine the metabolic pathway followed in the liver. It has been reported that 16 weeks of feeding mice on either 45% HFD or 60% HFD induce hyperleptinemia and hyperinsulinemia, leading to suppression of the *de novo* palmitic acid synthesis in fatty liver (Jiang *et al*., 2009). A cafeteria HFD (13.6%) mediated *de novo* palmitic acid synthesis in the female Wistar rats, which developed fatty liver (Baiges *et al*., 2010). According to Li *et al*. (2009b), saturated fatty acids (SFAs) desaturation and elongation processes play a key role in the partitioning of excess SFA and enables adequate lipid storage under fatty liver conditions. Thus re-esterification of circulating SFAs represents a major pathway contributing to TG disposal to the liver (Oosterveer *et al*. 2009a). Therefore, dietary FAs type, content and duration of their intake are suggested to be the contributing factors of the reported contradicting data in the HFDs induced fatty liver in animal studies, however no formal studies have been reported, which define the precise role of each component of the diet in the metabolic adaptation occurring during the development of fatty liver. In the present study, HFD differs from LFD mainly by an increase in palmitic acid and oleic acid content as well as a decrease in the carbohydrate content. The contribution of each component of the diet towards the development of fatty liver was not the aim of the study and therefore, this study just reports the effect of a HFD on the development of fatty liver.

5.5 Glucose metabolism

Since *de novo* lipogenesis is also dependent on glucose metabolism for both the production of reducing equivalents and to provide the carbon source for lipid synthesis, it was deemed of interest to assess the effects of HFD on hepatic glucose metabolism.

5.5.1 Results

To investigate the potential involvement of glucose metabolism in this HFD-induced fatty liver model, the liver LDH, GK and G6Pase activities were assayed and the data shown in **Table 5.4**. The activity of cLDH (LDHA) was not significantly changed in the HF group, suggesting that pyruvate formation from lactate was unaltered by feeding the rats a HFD (**Table 5.4**). In addition, the activities of hGK, hG6Pase, and microsomal G6Pase were not affected as shown by the insignificant differences between the HF and LF groups (**Table 5.4**).

Table 5.4 Glucose metabolic enzyme activities

	LF (lean)	**HF (fatty liver)**	**Fold difference**
cLDH (ΔA/min/mg protein)	42.735±3.779	49.140±3.983	1.2
hGK (ΔA/min/mg protein)	1.070±0.175	1.262±0.331	1.2
hG6Pase (mM Pi/min/mg protein)	1.190±0.180	1.027±0.123	1.2
miG6Pase (mM Pi/min/mg protein)	2.593±0.367	2.433±0.250367	1.1

LF, *low fat diet fed rats;* ***HF,*** *high fat diet fed rats;* ***LDH****; lactate dehydrogenase;* ***hGK,*** *homogenate glucokinase;* ***cGK,*** *cytosolic glucokinase;* ***hG6Pase,*** *homogenate glucose-6-phosphatase;* ***miG6Pase,*** *microsomal glucose-6-phosphatase. LF: n=6; HF: n=7.*

5.5.2 Discussion

As previously reported by Karachi (2009) and Mackenzie (2009), the HFD used in the present study mediated insulin resistance with compensatory hyperinsulinemia and normoglycemia. In addition, the present study confirms the findings of Mackenzie (2009) that this HFD-induced fatty liver after 16 weeks, albeit rather modest. The data presented in **Table 5.4** indicate that despite the hyperinsulinemia and increased liver lipid content, the key enzymes involved in glucose metabolism remain unchanged. This finding is somewhat unexpected considering that elevated levels of circulating insulin, which accompany the

insulin resistant state, would be expected to lead to a stronger repression of the expression of gluconeogenic enzymes such as G6Pase. However, it should be noted that the dephosphorylation of G6P, as measured in the present study, is not the rate limiting component of the G6Pase complex. Together these results indicate that in the present model, glucose metabolism is not significantly altered in response to HFD, adding further evidence that lipid accumulation does not rely heavily on *de novo* lipogenensis and supports the data reflecting a similar trend in lipogenic enzymes.

Liver LDH is an enzyme that catalysis the conversion of pyruvate to lactate and vice versa. The liver LDHA isoform converts pyruvate to lactate, while the LDHB isoform converts lactate to pyruvate, which may increase pyruvate formation from lactate. In the present study, LDHA isoform was measured in the liver. An increase in the LDHA isoform (cLDH) activity may partition pyruvate away from the TCA cycle, resulting in a decrease in pyruvate entering mitochondria for oxaloacetate and acetyl-CoA generation via the pyruvate carboxylase and pyruvate dehydrogenase enzymes. Generated pyruvate from lactate, glycerol, glutamate and/or alanine also serves as an intermediate of the endogenous G6P synthesis, which is a substrate for glucose synthesis (Devlin, 2006; Postic *et al*., 2004). Therefore, in the present study, it was of interest to measure the cLDH activity to elucidate whether the glycolytic pathway and oxaloacetate recycling were affected by HFD intake. A decrease in the cLDH activity can also increase pyruvate entry to the TCA cycle for acetyl-CoA synthesis in the mitochondria as illustrated in **Chapter 2**, **Figure 2.1**.

Based on the absence of any significant differences in the enzymes relating to glucose metabolism, it appears that the impact of this HFD-induced fatty liver does not severely alter the manner in which glucose is handled. Furthermore, absence of any significant enhancement of the capacity to increase glucose uptake in response to HFD implies that glucose is an unlikely carbon source for the increased lipid accumulation in the HF group and provides further evidence that lipid accumulation in response to this HFD does not involve *de novo* lipogenesis. Marchesini *et al*. (2001) also observed that glucose production from the liver is lowered in the fatty liver patients compared to the T2DM patients. Recently, the liver GK mRNA expression has been reported to be strongly associated with lipid accumulation in the human liver (Peter *et al*., 2011), however the present study does not support this finding as the GK activity remained unchanged.

5.6 Oxidative stress and antioxidants

5.6.1 Results

5.6.1.1 The liver homogenate

The liver homogenate MDA, protein carbonyl, total hydroperoxide levels, XO activity and antioxidants data are summarized in **Table 5.5**.

Table 5.5 Homogenate oxidative stress markers, pro-oxidants and antioxidants

	LF (lean)	HF (fatty liver)	Fold difference
Oxidative stress markers			
hMDA (μM/mg protein)	189.648±47.879	423.205±177.365*	2.2
Protein carbonyl levels (mM/mg protein)	0.382±0.042	0.378±0.049	1.0
hTotal hydroperoxides level (mM/mg protein)	6.570±1.712	6.041±1.519	1.1
Pro-oxidant enzyme			
hXO (ΔA/min/mg protein)	122.125±16.769	160.761±22.290*	1.3
Enzymatic antioxidants			
hSOD (sod1 plus sod2) (ΔA/min/mg protein)	17.607±8.778	2.872±0.041*	6.0
hGP (ΔA/min/mg protein)	21.988±2.347	21.736±1.500	1.0
hCT (ΔA/min/mg protein)	0.755±0.070	0.787±0.055	1.0
hGR (ΔA/min/mg protein)	13.459±1.249	14.056±0.984	1.0
hGST (ΔA/min/mg protein)	85.279±10.348	86.351±5.722	1.0
Non-enzymatic antioxidant			
Total reduced thiol (mM/mg protein)	0.988±0.067	0.987±0.077	1.0

LF, *low fat diet fed rats;* ***HF,*** *high fat diet fed rats;* ***hMDA****, homogenate malondialdehyde,* ***hXO****; homogenate xanthine-xanthine oxidase;* ***hSOD****; homogenate superoxide dismutase;* ***hGP****, homogenate glutathione peroxidase;* ***hCT****, homogenate catalase;* ***hGR****, homogenate glutathione reductase;* ***hGST****, homogenate glutathione-S-transferase;* ***GSH****, reduced glutathione was used as standard to measure thiol groups. * = significantly different from the LF group, * P < 0.05.* ***U*** *= ΔA/minute unless otherwise indicated in* ***Chapter 4****. LF: n=6; HF: n=7.*

The liver homogenate showed significant increase in the hXO activity (1.3-fold) and hMDA levels (2.2-fold) in the HF group as compared to the LF group (P < 0.05) (**Table 5.5**),

suggesting the possibility that the present HFD mediated lipid peroxidation, a marker for oxidative stress, which may involve hXO, leading to a higher level of hMDA. In contrast, other markers for oxidative stress namely; total hydroperoxides including lipid hydroperoxides (1.1-fold) and protein carbonyl (1.0-fold) levels were unaltered in the HF group as compared to the LF group (**Table 5.5**).

Considering that the levels of hMDA, hydroperoxides and protein carbonylation are well known and interrelated markers for oxidative stress, it is somewhat surprising that only the MDA levels reveal a significant increase. However, sensitivity and specificity of different assay methods may have contributed to this observation. Monitoring oxidative stress often involves measurement of lipid hydroperoxides and related oxidative secondary products like MDA and protein carbonylation. The MDA-TBA assay is one of the most popular assays for studies related to lipid peroxidation, and it is still currently used widely to evaluate oxidative stress despite clear warnings of its accuracy. TBA reacts with many different carbonyl compounds and their resultant adducts absorb at the same UV wavelength absorbed by the MDA-TBA adduct. Therefore, the simple spectrophotometric TBA assay is not specific to MDA, and results in an overestimation of the MDA concentration. To overcome this drawback, modern approaches include the use of HPLC and GC to accurately evaluate MDA (Palmieri and Sblendorio, 2007). Taken this into consideration, and the absence of any significant change in the other two markers for oxidative stress (hydroperoxides and protein carbonylation); there appears no clear indication for severe oxidative stress in this HFD rat model, despite the known correlation between fatty liver and lipid peroxidation. One possible explanation for this observation may relate to the relatively modest difference in lipid content between the LF and HF diet groups and subsequently oxidative stress is still well maintained by the antioxidant system of the liver.

Looking at the liver homogenate defence system, the hSOD (total SOD) activity was significantly decreased 6-fold in the HF group compared to the LF group ($P < 0.05$) (**Table 5.5**). While superoxide is not a strong oxidant, its reaction with divalent metal ions or nitric oxide may lead to the formation of the potent oxidants, the hydroxyl radical and peroxynitrate. Therefore, a 6-fold decrease in SOD activity would predispose the tissue to increased lipid peroxidation due to increase superoxide anion. Furthermore, such a decrease in the hSOD activity may lead to a decrease in production of H_2O_2 from superoxide anion. However, hXO also produces both H_2O_2 and superoxide anion, implying the need to

neutralize superoxide anion in the HF group. In addition, the total hydroperoxides were not changed; hence the antioxidant defence system responsible for this would remain unchanged as was indeed found to be in that total reduced thiol content and GSH dependent enzymes, namely hGP, hGR, and hGST activities as well as hCT remained unaltered. Furthermore, the presence of increased lipid peroxidation marker, hMDA and the absence of any other significant markers to signify server oxidative stress as well as the lack of apoptosis and inflammation induction, characteristic feature of sever oxidative stress, suggest that under the current experimental conditions, this HFD model involves modest oxidative stress and that some changes in the production of ROS are efficiently counteracted by the antioxidant network of the liver.

5.6.1.2 The liver mitochondria

The liver mitochondrial total hydroperoxides, oxidases and antioxidants data are summarized in **Tables 5.6**. The activities of mSOD, mGP, mGR, mCT and mGST were similar in both HF and LF groups (**Table 5.6**). The mitochondrial total reduced thiol content was not determined. The inability to detect mMDA in the mitochondrial preparation is most likely due to the relatively high protein to lipid ratio of mitochondrial membranes, and consequently lipid peroxides and mMDA levels will be radically less when analysed on a protein basis in both lean and fatty liver samples, as in the present study. Indeed, it is important to emphasise that undetectable does not imply either absence nor unchanged, at this stage one can make no definitive conclusion regarding this feature, however the absence of any changes in the hydroperoxide levels suggest the absence of oxidative stress in the mitochondria of the liver.

In the present study, the liver mNOX and mXO activities were significantly increased 2.3- and 1.6-fold in the HF group as compared to the LF group ($P < 0.05$) (**Table 5.6**), both enzymes have a potential to increase the mitochondrial superoxides, subsequent mH_2O_2 levels as also shown by a significant increase of 4.1-fold (mH_2O_2) in the HF group compared to the LF group ($P < 0.01$). However, the total hydroperoxides, which include H_2O_2, were not significantly increase as shown by 1.3-fold difference in the HF group compared to the LF group. Altogether, these limitations in the data make it difficult to draw any meaningful conclusion regarding mitochondrial oxidative stress as measured in this liver compartment. These data suggest that, despite a decrease in the antioxidant defence (hSOD), unchanged mSOD and an increase in prooxidant-producing enzymes mNOX and mXO, which may

contribute to cellular oxidative stress, the antioxidant defence system was sufficient to eliminate mitochondrial oxidants (**Table 5.6**). Perhaps it is the relatively modest degree of lipid peroxidation (2.2-fold) and fatty liver (6.73% of lipids by liver weight) induced by the current HFD, which allows the fatty liver system to remain resistant to oxidative stress and differs from other studies that link fatty liver to sever oxidative stress.

Table 5.6 Mitochondrial total hydroperoxides, oxidases and antioxidants

	LF (lean)	HF (fatty liver)	Fold difference
Oxidative stress markers			
mMDA (μM/mg protein)	Undetectable	Undetectable	-
mTotal hydroperoxides level (mM/mg protein)	1.693± 0.164	2.159±0.204	1.3
mH_2O_2 levels (μM/mg protein)	0.183± 0.09	0.747±0.08**	4.1
Pro-oxidant enzymes			
mNOX (ΔA/min/mg protein)	0.212±0.069	0.497±0.104*	2.3
mXO (ΔA/min/mg protein)	1.670±0.246	2.642±0.383*	1.6
Antioxidant enzymes			
mSOD (ΔA/min/mg protein)	16.241±1.181	17.457±2.303	1.1
mGP (ΔA/min/mg protein)	17.985±1.368	18.805±0.650	1.1
mCT (U/mg protein)	0.101±0.009	0.105±0.003	1.0
mGR (ΔA/min/mg protein)	1.369±0.117	1.433±0.156	1.1
mGST (ΔA/min/mg protein)	7.659±0.727	7.392±0.965	1.0

LF, *low fat diet fed rats;* ***HF,*** *high fat diet fed rats;* **mH_2O_2**, mitochondrial hydrogen peroxides; ***mNOX****, mitochondrial reduced nicotinamide adenine dinucleotide oxidase;* ***mXO****; mitochondrial xanthine oxidase;* ***mSOD****; mitochondrial superoxide dismutase;* ***mGP****, mitochondrial glutathione peroxidase;* ***mCT****, mitochondrial catalas;* ***mGR****, mitochondrial glutathione reductase;* ***mGST****, mitochondrial glutathione-S-transferase. * = significantly different from the LF control, * P < 0.05, ** P < 0.01. LF: n=6; HF: n=7.*

5.6.2 Discussion

Oxidative stress and mitochondrial dysfunction play a critical role in the pathogenesis of fatty liver and its progression (Oliveira *et al*., 2006). In addition, fatty liver and its progression are associated with mitochondrial oxidative changes (Reviewed in Esposti *et al*., 2012). To determine whether HFD-induced fatty liver is associated with oxidative stress in the present

rat model, the oxidative stress markers, namely protein carbonylation, MDA and total hydroperoxides levels as well as the enzymatic and non-enzymatic antioxidants were measured in the homogenate and mitochondrial fraction. Cellular proteins and lipid membranes are considered to be the main target of ROS-induced oxidative stress (Noeman *et al*., 2011). The MDA is a product generated from oxidative stress due to increase lipid oxidation mediated by ROS during a process termed lipid peroxidation (Liu *et al*., 2003).

To reduce oxidative stress, cells may implement a number of effective mechanisms to prevent ROS induced tissue damage. Such antioxidant mechanisms include enzymes such as GP, GR, CT, GST and two forms of SOD (a cytosolic Cu/Zn-SOD and a mitochondrial Mn-SOD, mSOD), and non-enzymatic antioxidants such as GSH. Maintenance of reduced thiol group such as GSH and GST GP and GR also depends on NADPH produced from the PPP (Spolarics, 1998; Noeman *et al*., 2011). Oxidative metabolism in mitochondria represents the main source of energy for the cells. Changes of specific mitochondrial functions may result in an impaired generation of ATP and a decreased uptake of substrates for mitochondrial metabolism (Vendemiale *et al*., 2001). According to Rus *et al*., (2007) mitochondrial XO activity plays an important role in several physiological and pathological conditions via its production of an electron carrier, NADH and an oxidants, superoxide and H_2O_2, respectively.

In the present study, even though the activity of hSOD was significantly reduced in the HF group, changes in the activities of mSOD, hGP, mGP and cG6PD are suggested to have prevented chain oxidation of GSH. Although mMDA were not detected, this most likely represents a limitation in the assay sensitivity and not an indication of the absence of MDA in this fraction. It can also be suggested that mitochondrial superoxides and H_2O_2 were readily detoxified by mitochondrial antioxidants to prevent lipid peroxidation in this liver compartment. In the HF group, a significant increase in the homogenate and mitochondrial XO activities may reveal that purine synthesis and degradation pathways were affected by HFD-induced fatty liver, however there is to date no conclusive evidence for a gene product encoding a mitochondrial specific form of xanthine oxidase nor that the consequent putative mitochondrial purine metabolism is independently regulated.

In the present study, the assayed enzymes are the major antioxidants that play a direct critical role in neutralization of ROS such as superoxide, H_2O_2 and hydroperoxides. Basically, SOD catalysis the dismutation of superoxide to H_2O_2 by reduction, which is a first line of defence

against ROS. The formed H_2O_2 is converted into water and oxygen by GP or CT enzymes. The enzyme GP detoxifies H_2O_2 by using it to oxidize GSH into oxidized glutathione (GSSG). The enzyme GR regenerates GSH from GSSG, with NADPH as a source of reducing power. The enzyme GP also reduces non-lipid and lipid hydroperoxides, while oxidizing glutathione (GSH) (Review in Pham-Huy *et al*., 2008). GSH comprises another defence against free radicals in the liver and is also responsible for the maintenance of protein thiols, while acting as a substrate for both GP and GST enzymes (Hsu and Yen, 2007).

It is documented that superoxide radicals can react with NO to form a highly reactive peroxynitrite molecule ($ONOO^-$), only if they are not rapidly catalysed by SOD to form H_2O_2 (Droge, 2002; Kirkham and Rahman, 2006). Alternatively, an increase in superoxide conversion to H_2O_2 can also lead to the non-enzymatic production of more damaging hydroxyl radical in the present of metal cations such as Cu^{2+} or Fe^{2+}, through the Fenton reaction (the iron salt-dependent decomposition of H_2O_2) that lead to the oxidation of Fe^{2+} to Fe^{3+} as previously depicted in **reactions 1a** and **b** (**see Chapter 2**) and decreased H_2O_2 catalysing antioxidant capacity (Aruoma, 1994; Kirkham and Rahman, 2006). In the present study, there was no change in iNOS protein levels as shown in **Section 5.1.3**, while the activity of hSOD was significantly decreased in the HF group, suggesting that the production of $ONOO^-$ from interaction of NO and superoxide may not be of major importance in the present rat model, further supporting the lack of any significant changes in markers of oxidative stress

Interestingly, in the present study, only hSOD activity was significantly decreased in the HF group as mention above, whilst the activities of other measured homogenate antioxidant enzymes were not affected. A decrease in the hSOD activity may result in an increased level of superoxides, as generated by increased XO activity and other oxidases such as NOX and NADPH oxidase and superoxide anions may initiate lipid peroxidation through Fenton and Haber-Weiss reactions as mentioned above. Even though XO is one of the ROS producing enzymes in fatty liver (Videla *et al*., 2009), its increased activity may only mediate lipid peroxidation via production of superoxides, but not via H_2O_2, since hGP and hCT activities were unaltered in the present HFD rat model. This condition is confirmed by no significant change in the homogenate total hydroperoxide content between the HF and LF groups. Importantly, the measured homogenate total hydroperoxides levels represents their production from all superoxide and H_2O_2 producing enzymes in the liver and also include that

produced by the microsomal CYP2E1 (Mantena *et al*, 2009) and phagocyte cells NADPH oxidase.

Normal total reduced thiol content (measured using GSH as a standard) in the HF group is consistence with the function of its metabolism by GP, GR and GST enzymes in both homogenate and mitochondria. Overexpression of G6PD and an increase in its activity have been attributed to maintain the GSH levels to protect sulfhydryl groups and cellular integrity from rising oxidative radicals (Salvemini *et al*., 1999; Puskas *et al*., 2000). This mechanism is similar to the findings from the present study, where an increase in G6PD activity also maintains the total reduced thiol content in the HFD-induced fatty liver rat model. Furthermore, G6PD is one of the 10 protective response enzymes against superoxide anions that are regulated by Sox under oxidative stress; other enzymes include mSOD and NADPH: ferredoxin oxidoreductase (reviewed in Droge, 2002).

Recently, 46% HFD enriched with 25.5% corn oil and 20.5% beef tallow has been reported to significantly induce liver oxidative stress after 16 weeks in rats. In their study, the liver homogenate antioxidant enzymes, including hGP and hGST activities and GSH content were significantly decreased, while the protein carbonyl and MDA levels were significantly increased in the HF group compared to control group. The elevated protein carbonyl was found to be significantly correlated to a decrease in the liver GSH levels and hGP and hGST activities, but the MDA was not significantly correlated to the decreased GSH levels (Noeman *et al*., 2011).

In the present study, the liver total reduced thiol content and hGP, hGR, hGST activities and protein carbonyl levels were similar in the HF and LF groups. Therefore, the present study agrees with Noeman *et al*. (2011), who observed a correlation showing that protein damage is influenced by a decrease in the levels of liver GSH and its dependent antioxidant enzymes, whereas lipid peroxidation is influence by a decrease in SOD activity as in the present HF group, wherein only the activity of hSOD was significantly decreased in the HF group. In addition, 10 weeks of feeding HFD enriched with beef tallow (400 g/kg dietary weight) has been reported to induce macrovesicular fatty liver associated with a significant increase in the liver TG and TC and a significant decrease in the homogenate GSH levels and its dependent enzyme activities include GP, GR and GST in the male Wistar rats (Hsu and Yen, 2007), conditions which differ from that of the present HFD rat model. Taken together, these results

suggest that lipid peroxidation may be an early maker of modest oxidative stress, while sever oxidative stress may be a relatively late marker in the development of fatty liver in the present HFD rat model.

5.7 Mitochondrial respiratory chain enzyme activities

5.7.1 Results

Mitochondrial dysfunction is believed to play a pivotal role in the development of NASH from simple fatty liver (NALFD). To determine whether a HFD can mediate mitochondrial respiratory chain dysfunction, mitochondrial complexes I, II and III activities were assayed and the data summarized in **Table 5.7.** The activities of mitochondrial complexes I and III were similar in both HF and LF groups (**Table 5.7**). Surprisingly, the activity of mitochondrial complex II was significantly reduced 1.4-fold in the HF group as compared to the LF group ($P < 0.05$) (**Table 5.7**), suggesting that conversion of succinate to fumarate in the mitochondria was impaired by a HFD intake. At this stage, it is difficult to conclude to what extent this change represents mitochondrial dysfunction. Taking into consideration that mitochondrial dysfunction is a primary driving force in the development of NAFLD, the absence of inflammation and apoptosis suggests that the decreased SDH activity and modest oxidative stress in this rat model may not represent mitochondrial dysfunction *per se*, but may simply be the metabolic reprogramming due the altered diet.

Table 5.7 Mitochondrial respiratory chain complexes

	LF (lean)	HF (fatty liver)	Fold difference
Complex I (ΔA/min/mg protein)	16.332±3.428	18.522±3.262	1.1
Complex II (ΔA/min/mg protein)	12.124±1.768	8.794±0.870*	1.4
Complex III (ΔA/min/mg protein)	1.519±0.188	1.629±0.492	1.1

***LF**, low fat diet fed rats; **HF**, high fat diet fed rats; **Complex I**, NADPH dehydrogenase; **Complex II**, succinate dehydrogenase**, Complex III,** cytochrome c reductase. * = significantly different from the LF control, * $P < 0.05$. LF: n=6; HF: n=7.*

5.7.2 Discussion

Lipid peroxidation and other ROS products can impair mitochondrial respiratory chain, either indirectly or directly through oxidative damage to the mitochondrial genome. These characteristics can lead to increase production of ROS and a vicious cycle results, ultimately ending in cell death due to necrosis or apoptosis (Noeman *et al*., 2011). High mitochondrial β-oxidation rates can assist to metabolize surplus FFAs in the liver, even though large amounts of electrons entering the respiratory chain may cause abnormal reduction of oxygen. An influx of electrons can then lead to increased mitochondrial ROS production. This phenomenon may indicate that the impaired respiratory chain function causes an increase in mitochondrial ROS production in addition to other ROS producing compartments (Matsuzawa-Nagata *et al*., 2008).

Complex I passes electrons to complex III, which also receives electrons from complex II and $FADH_2$ (Castellani *et al*., 2010). Thus in the present study, despite a significant reduced complex II activity, complex III activity was not changed in the HF group. This situation may reflect that the functional complex I activity and increased FA oxidation can maintain activity of complex III to compensate for an impaired activity of complex II. Recently, complex I activity has been reported to be significantly reduced with no change in complex II activity, whereas activity of complex III was significantly increased in the Male heterozygous transgenic n-3 enriched fat-1 mice, which is the animal model that converts n-6 to n-3 fatty acids. In addition, a decrease in complex I activity was found to lead to a decrease in H_2O_2 production from mitochondria (Hagopian *et al*., 2010).

5.8 Conclusion

The aim of this study was to investigate the effect of HFD-induced fatty liver on the metabolic regulation of liver glucose, lipid, oxidants and antioxidant capacity. The use of enzyme activity measurements to denote changes in metabolism is well accepted; however it is paramount that the data be interpreted within the context of the experimental design and assay limitations. The main objective of this study was to investigate the effects of this HFD on the development of fatty liver. However, the HFD rat model used in this study does not represent fatty liver in isolation, but rather a combination of various metabolic deviations including insulin resistance. Thus, it is critical to interpret the data in a context that represents

NAFLD and other metabolic changes such as insulin resistance, since other factors may equally be responsible for the observed differences. It is with this in mind that the discussions of the present study are merely intended to highlight potential changes in the metabolic state of fatty liver mediated by HFD and as a result, further studies are recommended to confirm the interpreted findings.

The liver TL content (2.76% by liver weight) was within the normal physiological range at week 12, whereas at week 16, lipid accumulation had increased to 6.73% lipids by liver weight, consistent with the characteristics of fatty liver. This relatively modest increase in the lipid content, and the absence of inflammatory or apoptotic response (**Figure 5.1A**, **Table 5.1** and **Figures 5.2A** and **B**), suggests that the current model represents an early stage development of the disease. Furthermore, the induction of fatty liver at week 16 does not appear to be associated with notable oxidative stress as judged by the markers for oxidative stress and enzymes involved in the antioxidant defence system (**Tables 5.5** and **5.6**).

Lipid accumulation in the liver is a dynamic process regulated through a number of mechanisms including an increase in the *de novo* lipogenesis, increased NEFA uptake, decreased β-oxidation and increased esterification of fatty acids. At first glance the increased G6PD activity may suggest that the *de novo* synthesis of fatty acids contributes to the observed lipid accumulation, however the activities of mCS and cACL, which are key enzymes that contribute carbon in the form of acetyl-CoA for the *de novo* synthesis of fatty acids, were unchanged. Therefore the observed results of unchanged mCS and cACL activities in both HF and LF groups (**Table 5.3**) suggest that the increased levels of total lipid (TL) in the liver were not mediated by HFD induced *de novo* FAs synthesis. Since *de novo* fatty acids synthesis was similar in both HF and LF groups, another mechanism such as increased esterification of the readily available NEFAs in the liver may be involved in the increased lipid accumulation in the HF group. These results are in accord with the mainstream hypothesis that lipid accumulation is primarily the result of elevated circulating FFA levels, which were also shown to significantly increase in this HFD model (Mackenzie 2009). However, despite a significant increase the FFA levels at week 12, lipid accumulation was considerably less than that at 16 weeks. Therefore there appears no clear relationship between the development of fatty liver and other factors (insulin resistance, hyperinsulinemia, and oxidative stress) suspected to contribute to this pathology. The observed decreased hSOD activity, maintenance of the liver redox state and iNOS protein

levels and absence of caspase-3 activation levels, protein oxidation and total hydroperoxides levels in the HF group may support the modest effect of the present HFD on the liver glucose and lipid metabolism as well as oxidative stress.

Taken together, these findings suggest that with respect to fatty liver development, the male Wistar rats were able to adapt to this HFD until week 12, since induced peripheral insulin resistance was associated with normal blood glucose and plasma lipid parameters (Karachi, 2009; Mackenzie, 2009) and less than 5% lipid/ liver weight as observed in the present study. However, this HFD may not be tolerated by rats after week 16 of its intake, since the induced fatty liver was associated with elevated plasma lipid parameters with no change in HDL-C levels. Hence, the rats receiving HFD also maintained normal plasma glucose and elevated insulin levels at week 16 (Karachi, 2009; Mackenzie, 2009). It is documented that inability to adapt to HFD by decreasing *de novo* FA synthesis, results in increase fasting plasma TG and lipid accumulation in the liver (Bassilian *et al*., 2001). The present HFD may not induce fatty liver via altering *de novo* FA synthesis in the liver in the fasting state, as shown by the measured glucose and lipid metabolic enzyme activities (**Tables 5.3** and **5.4**).

Therefore, this study recommends that the present HFD-induced fatty liver rat model at week 16 be employed in the future studies to investigate fatty liver prevention and treatment remedies. This is supported by observation that HFD induce fatty liver with modest oxidative stress and with an increase in activities of the G6PD and XO enzymes, and a decrease in the mitochondrial complex II activity without *de novo* FA synthesis from glucose.

5.9 Proposed mechanism(s) of action by which HFD-induced fatty liver in Male Wistar rats (see next page).

Diagram 5.1: Proposed model of mechanism for HFD-induced fatty liver in rats

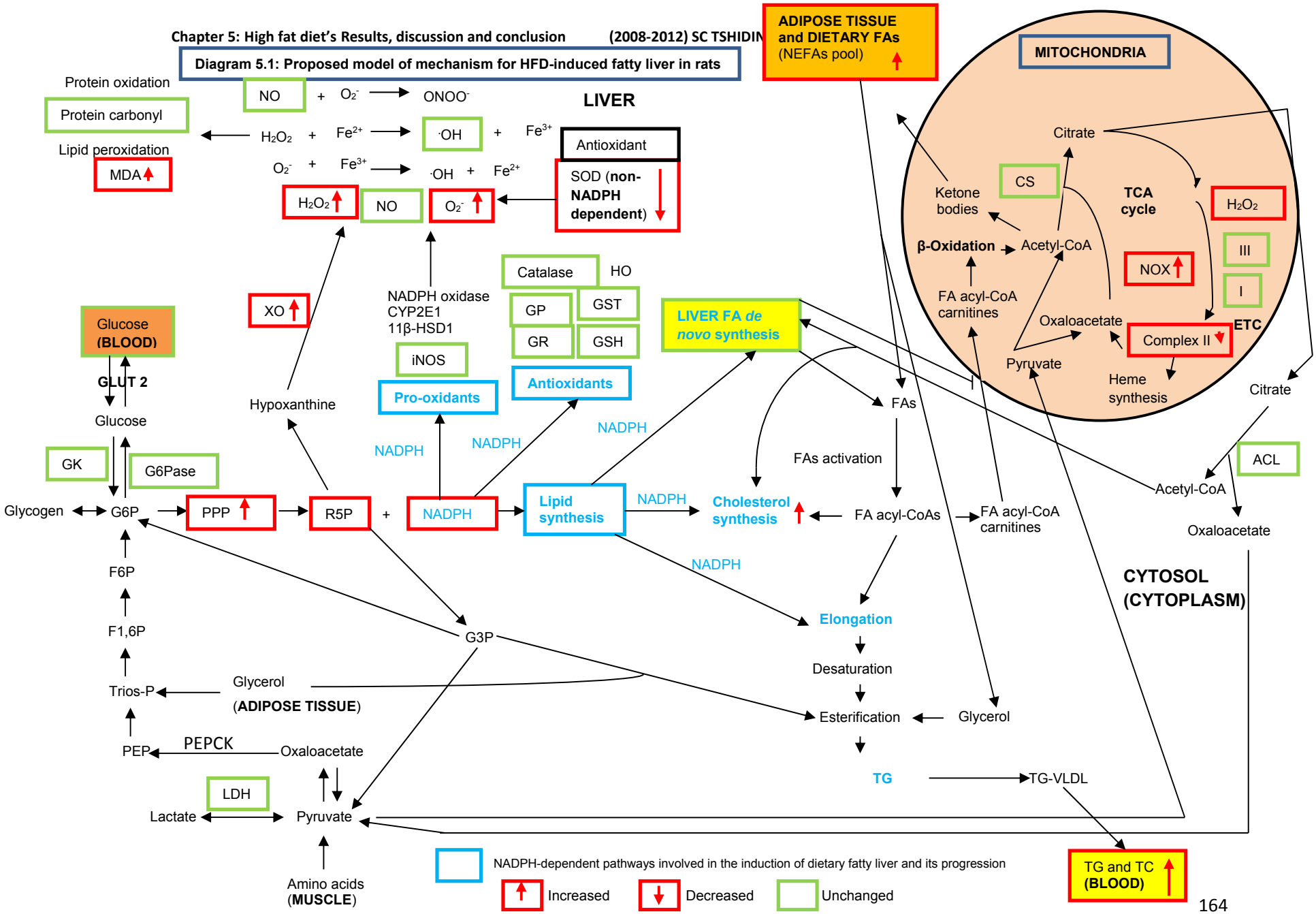

Diagram 5.1 *depicts the proposed mechanism of action by which 16 weeks of chronic feeding on a 38.9% HFD enriched with palmitic acid (40%) in the presence of oleic acid (29%) induced fatty liver associated with modest oxidative stress in the male Wistar rat in comparison to the used LFD. The metabolic changes induced by HFD are shown in Red square, those unaffected by HFD are shown in Green Square and the NADPH-dependent pathways are shown in blue colour. Abbreviations:* ***ACL****, ATP citrate lyase;* ***cICD****, cytosolic isocitrate dehydrogenase;* ***CoA****, Coenzyme A;* **CYP2E1**, cytochrome P450 2E1; ***CS****, citrate synthase;* ***ETC****, electron transport chain;* ***FAs****, fatty acids;* ***FFAs****, free fatty acids;* ***Fe****, iron,* ***GSH****, reduced glutathione;* ***GLUT2****, glucose transporter 2; G3P, glyceraldehyde-3-phosphate,* ***G6P****, glucose-6-phosphate; G6Pase; glucose-6-phosphostase;* ***cG6PD****, glucose-6-phosphate dehydrogenase;* ***GP****, glutathione peroxidase;* ***GR****, glutathione reductase;* ***GST****, glutathione-S-transferase;* ***11βHSD1****, 11β-hydrosteroid dehydrogenase-1;* ***H_2O_2****, hydrogen peroxide;* ***HFD****, high fat diet;* ***I****, complex I;* ***III****, complex III;* ***iNOS****, inducible nitric oxide synthase;* ***LDH****, lactate dehydrogenase;* ***MDA****, malondialdehydes*; ***NADPH****, reduced nicotinamide adenine dinucleotide phosphate;* ***NO****, nitric oxide;* ***NOX****, reduced nicotinamide adenine dinucleotide oxidase,* ***O_2^-****, superoxide*; ***$^{\cdot}OH$****, hydroxyl;* ***$ONOO^-$****, peroxynitrite molecule****;* ***PPP****, pentose phosphate pathway;* ***R5P****, ribose-5-phosphate;* ***SOD****, superoxide dismutase;* ***TC****, total cholesterol,* ***TG****, triglycerides;* ***TCA****, tricarboxylic acid.*

CHAPTER 6: Efficacy of 4 weeks of *Sutherlandia frutescens* aqueous extract in the prevention of a HFD-induced fatty liver.

Synopsis

The present study was undertaken to investigate the effects of Sutherlandia frutescens aqueous extract on high fat diet (HFD)-induced fatty liver. Male Wistar rats were fed a high fat diet (HF group) or normal rat chow (LF group) for 12 weeks. Although hepatic lipid accumulation had not progressed to the limits defining fatty liver, 7 rats of the HF group were treated with 50 mg S. frutescens extract/kg bwt/day (HF+Sf group) for an additional 4 weeks on the same HFD. At this stage, untreated HFD-fed animals had progressed to represent fatty liver.

The S. frutescens treatment decreased mitochondrial complex II (succinate dehydrogenase, SDH), mitochondrial glutathione-S-transferase (mGST) and homogenate superoxide dismutase (hSOD) activities and activated mitochondrial citrate synthase (mCS) and cytosolic glucose-6-phosphate dehydrogenase (cG6PD) activity. Efficacy of 4 weeks of S. frutescens extract in the prevention of HFD-induced fatty liver demonstrates that this plant possesses an antioxidant activity via increasing the liver total reduced thiol content. Moreover, S. frutescens extract may not prevent this HFD-induced fatty liver, suggesting that this plant leaves crude aqueous extract does not limit HFD to increase lipid accumulation in the liver, when used without changing the dietary habits in this rat model.

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6.8 Proposed mechanism of actions by which *S. frutescens* leaves aqueous extract could improve antioxidant capacity without preventing HFD-induced fatty liver (see next page).

6. Introduction

This chapter focuses on the effect of crude aqueous extract of *S. frutescens* leaves on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity in a HFD-induced fatty liver. Chadwick *et al*. (2007) and Mackenzie (2009) showed that insulin resistance can be attenuated by treatment with an aqueous extract of *S. frutescens*. However, the effects on the hepatic metabolic changes remain to be fully investigated. Therefore, this study investigated the effect of 4 weeks treatment of HFD-fed rats with 50 mg *S. frutescens* extract/kg bwt/day (HF+Sf) on the induction of fatty liver.

6.1 Evaluation of *S. frutescens* to prevent HFD-induced fatty liver

6.1.1 Liver histology

To determine the effect of *S. frutescens* extract on hepatic lipid accumulation, lipid accumulation and histological changes were investigated microscopically. Representative histology images of the liver are shown in **Figure 6.1**.

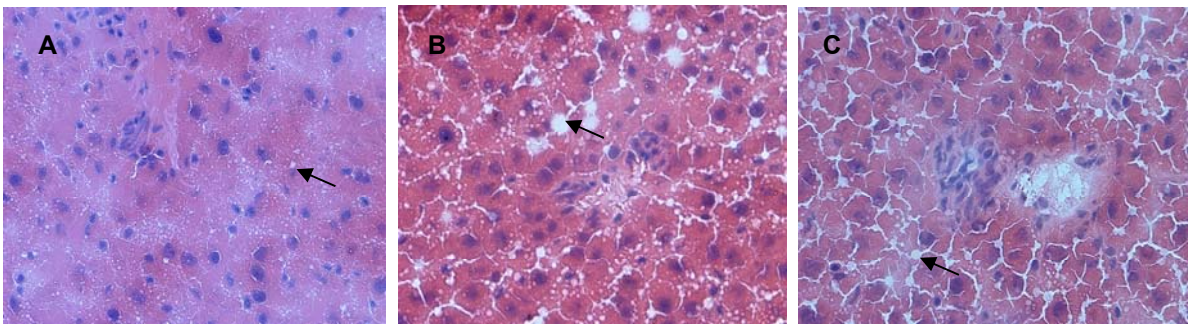

Figure 6.1 *Rat liver histology (**A**) LF (lean) (**B**) HF (fatty liver) (**C**) HF+Sf (gavaged), (Original magnification X40). Arrow indicates a lipid droplet.* ***LF****, low fat diet-fed rats;* ***HF****, high fat diet-fed rats;* ***HF+Sf****, high fat diet-fed rats gavaged with S. frutescens extract.*

The H&E staining showed a decreased macrovesicular steatosis in the HF+Sf group compared to the HF group, as observed under the light microscope (**Figures 6.1B** and **C**).

6.1.2 Liver lipid content

The lipid content results of the liver are summarized in **Table 6.1**.

Table 6.1 Effect of *S. frutescens* extract on liver total lipids

	CF	CH	LF	HF	HF+Sf
TL (mg/g liver wet weight)	22.6±3.5	27.6±2.9	48.0±1.1	67.34±6.0***	61.8±3.9**
Percentage lipid	2.2	2.76	4.8	6.73	6.18
Fold difference (HF vs HF+Sf)					-1.1
Percentage decrease (HF vs HF+Sf)					8.9
Percentage increase (16 weeks vs 12 weeks)			112	144	124

***CL**, low fat diet fed rats after 12 weeks; **CH**, high fat diet fed rats after 12 weeks; **LF**, low fat diet-fed rat after 16 weeks; **HF,** high fat diet-fed rats after 16 weeks (**see Chapter 5**, **Table 5.1** for their statistical changes); **HF+Sf,** high fat diet-fed rats for 16 weeks while gavaging with S. frutescens extract last four weeks; **TL,** total lipid, **vs**, versus. * = significantly different from the LF group, ** P < 0.01; *** P < 0.001. LF: n=6; HF: n=7; HF+Sf: n=7).*

The liver TL content in the HF+Sf group was not significantly different from that found in the HF group, but significantly elevated when compared to the LF group ($P < 0.01$) (**Table 6.1**). These findings are in agreement with that obtained by Mackenzie (2009).

6.1.3 Discussion

Microscopic examination indicates that hepatic lipid droplets were accumulated mostly on the portal areas in the HF+Sf group as compared to the HF group, which has both microvesicular and macrovesicular steatosis. From the extracted total lipids, there was 8.9% less accumulation in the HF+Sf group relative to the HF group, suggesting that this plant extract attenuates hepatic lipid accumulation, but could not prevent fatty liver development as per the standard definition.

In the HF+Sf group, significant lowered fasting plasma insulin, FFAs, TG, TC and HDL-C levels with no change in the blood glucose levels when compared to the HF group have been reported by Mackenzie, (2009). Furthermore, circulating HDL-C in the HF+Sf group has also

been reported to be significantly decreased when compared to both HF and LF groups (Mackenzie, 2009).

6.2 Effect of *S. frutescens* extract on liver markers of inflammatory and apoptotic responses

6.2.1 Results

To determine whether gavaging the HFD-fed rats with *S. frutescens* extract has any effects on iNOS (an inflammatory marker) or procaspase-3 (an apoptosis marker), their proteins levels were analysed by the Western blotting and the results are shown in **Figure 6.2**. Surprisingly, iNOS protein levels were significantly increased in the HF+Sf group when compared to both LF and HF groups ($P < 0.001$) (**Figure 6.2A**, **Appendix A**), but no change in the procaspase-3 protein in all groups (**Figure 6.2B**, **Appendix B**). These data suggest that *S. frutescens* extract may either indirectly or directly mediate an inflammatory response.

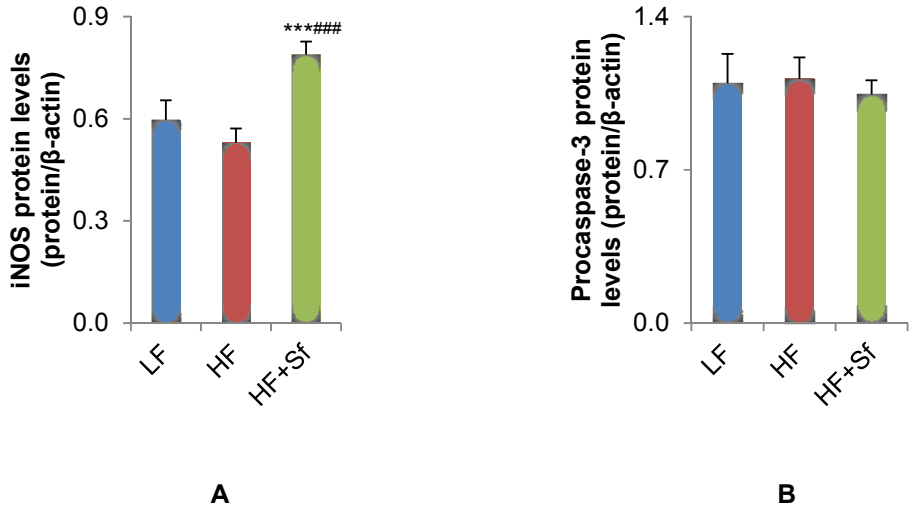

***Figure* 6.2** *Rat liver inducible nitric oxide (iNOS) and procaspase-3 protein levels. (**A**) The iNOS protein levels (**B**) Procaspase-3 protein levels. **LF**, low fat diet-fed rats, **HF**, high fat diet-fed rats; **HF+Sf**, high fat fed rats gavaged with S. frutescens extract * = significantly different from LF, *** $P < 0.001$ and # = significantly different from HF, ### $P < 0.001$. LF: n=6; HF: n=7; HF+Sf: n=7.*

6.2.2 Discussion

The present study is first to demonstrate an effect of *S. frutescens* leaves aqueous extract on the liver iNOS protein levels, which may influence NO production in this tissue of the HFD rat model.

6.3 Effect of *S. frutescens* leaves extract on the liver lipogenic enzymes

6.3.1 Results

The activities of liver cG6PD, mCS and cACL were measured and data summarized in **Table 6.2**. Cytosolic G6PD activity was significantly increased in the HF+Sf (1.9-fold, $P < 0.01$) and HF groups (1.7-fold, $P < 0.05$) when compared to that in the LF group (**Table 6.2**), however, no significant difference between the HF+Sf and HF groups (1.1-fold). The cACL activity was similar in all three groups. In addition, the mCS activity was significantly increased in the HF+Sf group compared to the HF group (1.2-fold, $P < 0.05$), but not to the LF group (**Table 6.2**).

Table 6.2 Effect of *S. frutescens* extract on the lipogenic enzyme activities

	LF (lean)	HF (fatty liver)	HF+Sf	Fold difference (HF vs HF+Sf)
cG6PD (ΔA/min/mg protein)	3.959±1.025	6.679±0.893*	7.451±0.893**	1.1
cACL (ΔA/min/mg protein)	32.011±4.905	29.252±3.897	32.939±3.159	1.1
mCS (ΔA/min/mg protein)	18.991±1.723	17.693±1.712	21.275±0.664$^{\#}$	1.2

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Sf**, high fat fed rats gavaged with S. frutescens leaves extract; **cG6PD**, cytosolic glucose-6-phosphate dehydrogenase; **cACL**, cytosolic ATP citrate lyase; and **mCS**, mitochondrial citrate synthase; **vs**, versus. * = significantly different from the LF control, * $P < 0.05$; ** $P < 0.01$ and $^{\#}$ = significantly different from HF control, $^{\#}P < 0.05$. LF: n=6; HF: n=7; HF+Sf: n=7.*

Although statistically significant, these differences in the enzyme activity are considered too small to represent any meaningful metabolic impact. In the present study, enzyme activities were determined according to well established methods, which have been optimised to assess the maximum rate of the individual enzyme catalysed reactions. Thus activities are determined under conditions of extreme substrate excess and optimal pH, which may not always be physiological, and in the absence of factors that remove the product formed. It is clear that such

conditions are far removed for the *in vivo* steady state and consequently these measurements cannot be expected to directly reflect *in vivo* changes of the metabolic flux. The fundamental principles of enzymology dictate that enzyme reactions are not linear but follow a Michaelis-Menton or sigmoidal kinetic pattern, which therefore implies that in the presence of excess substrate the reaction rate will be directly correlated with the quantity of enzyme present and not the in vivo metabolic rate. It thus follows that the enzymatic rates measured in the present study reflect changes in the relative amount of the specified enzyme. Although changes in the quantity of enzyme can alter the metabolic flux, other parameters such as substrate availability, allosteric regulation, covalent enzyme modifications and many other factors also contribute to the regulation of metabolic flux.

It is therefore, reasonable to expect that subtle changes in the quantity of an enzyme can easily be overshadowed by this myriad of factors, which collectively regulate metabolic rates. Hence, only when the differences in enzyme quantity reach substantial levels, can it be reasoned that there exist meaningful alterations in the metabolic capacity. Considering that allosteric regulation alone can induce a several fold change in the enzymatic rate, therefore a 1.1 and 1.2 fold differences in the enzyme quantity shown for mCS and cACL respectively, are too small to make definite conclusions on the physiological effects, despite that these measurements are statistically significant.

6.3.2 Discussion

The absence of any meaningful changes in the quantities of lipogenic enzymes upon *S. frutescens* treatment indicates that the attenuation of lipid accumulation is independent of *de novo* lipogenesis. The significant changes in blood lipid profile reported by Mackenzie (2009), suggest that these parameters may contribute to the lower hepatic lipid accumulation, although lipid accumulation was not statistically significant. It is well documented that a decrease in circulating FFAs by increasing their incorporation to form TG in the liver protects tissues from FFA-induced lipotoxicity such as oxidative stress and progressive liver damage (Reviewed in Choi and Diehl, 2008; Lam *et al*., 2003; Yamaguchi *et al*., 2007).

6.4 Effect of *S. frutescens* leaves extract on the liver glucose metabolic enzymes

6.4.1 Results

Glucose metabolic enzymes, LDH, GK and G6Pase activities were measured in the liver homogenate and PMS fraction and their results are summarized in **Table 6.3**. The liver cLDH activity was not significantly changed in the HF+Sf group compared to the HF group. The activities of hGK, hG6Pase and miG6Pase were similar in all three groups (1.1 fold) (**Table 6.3**).

Table 6.3 Effect of *S. frutescens* extract on the glucose metabolic enzyme activities

	LF (lean)	HF (fatty liver)	HF+Sf	Fold difference (HF vs HF+Sf)
LDH (ΔA/min/mg protein)	42.735±3.779	49.140±3.983	45.893±3.565	1.1
hGK (ΔA/min/mg protein)	1.070±0.175	1.262±0.331	0.964±0.253	1.3
hG6Pase (mM Pi/min/mg protein)	1.190±0.180	1.027±0.123	1.130±0.614	1.1
miG6Pase (mM Pi/min/mg protein)	2.593±0.367	2.433±0.250	2.750±0.614	1.1

LF, *low fat diet-fed rats;* ***HF,*** *high fat diet-fed rats;* ***HF+Sf,*** *high fat diet-fed rats gavaged with S. frutescens leaves extract group;* ***cLDH****, lactate dehydrogenase;* ***hGK,*** *homogenate glucokinase;* ***hG6Pase,*** *homogenate glucose-6-phosphatase;* ***miG6Pase,*** *microsomal glucose-6-phosphatase;* ***vs****, versus. LF: n=6; HF: n=7; HF+Sf: n=7.*

6.4.2 Discussion

Since the HF+Sf group has been reported to normalise blood insulin levels (Mackenzie, 2009), the present study reveals that fatty liver may be resulted from a chronic intake of HFD, but not due to increase in the blood insulin levels of the HF group or vice versa. Interestingly, the cG6PD activity was significantly increased in both HF+Sf ($P < 0.01$) and HF ($P < 0.05$) groups as previously shown in **Table 6.2**, however this does not imply that there was increased G6P flux towards PPP for the NADPH formation.

6.5 Effect of *S. frutescens* leaves extract on the liver oxidative stress and antioxidants status

Oxidative stress is defined as an imbalance between the factors that produce free radicals and those that protect from free radical damage. Unfortunately, there is no single marker that can

unequivocally confirm oxidative stress. Consequently, it is standard practice to measure multiple markers to reliably demonstrate oxidative stress.

6.5.1 Results

In the present study, various free radical metabolites (hydroperoxides, MDA, protein carbonyl modification and total reduced thiols) as well as pro- and anti-oxidant enzymes were measured to quantify oxidative stress. However, not all known enzymes involved in oxidative stress were measured.

6.5.1.1 The liver homogenate

The liver homogenate MDA, protein carbonyl, total hydroperoxides, XO activity and antioxidants data are summarized in **Tables 6.4**. No significant different in the hMDA levels (1.1-fold) and hXO activity between the HF+Sf and HF groups (**Table 6.4**). The liver protein carbonyl and total hydroperoxides levels (1.2-fold) were similar in all three groups (**Table 6.4**). The hSOD activity was significantly decreased 10.4-fold in the HF+Sf group as compared to the HF group ($P < 0.05$) and 63.6-fold as compared to the LF group ($P < 0.01$). However, this enzyme activity was also decreased 6.1-fold in the HF group when compared to the LF group ($P < 0.05$). No change in the activities of other measured antioxidant enzymes, namely, hGP, hGR, hCT and hGST in all three groups (1.0 fold), while the total reduced thiol content increased 1.2-fold in the HF+Sf group as compared to both HF and LF groups ($P < 0.01$). The latter effect shows that antioxidant defence against ROS may be increased in the HF+Sf group (**Table 6.4**).

Table 6.4 Effect of *S. frutescens* extract on the homogenate oxidative stress markers, pro-oxidants and antioxidants

	LF (Lean)	HF (Fatty liver)	HF+Sf (gavaged)	Fold difference (HF vs HF+Sf)
Oxidative stress markers				
hMDA (μM/mg protein)	189.648±47.879	423.205±177.365*	384.252±112.653	1.1
Protein carbonyl levels (mM/mg protein)	0.382±0.042	0.378±0.049	0.312±0.017	-1.2
Total hydroperoxides levels (mM/mg protein)	6.570±1.712	6.041±1.519	7.316±1.080	1.2
Pro-oxidant enzymes				
hXO (ΔA/min/mg protein)	122.125±16.769	160.761±22.290*	114.380±21.544$^{\#}$	-1.4
Enzymatic antioxidants				
hSOD (sod1 plus sod2) (ΔA/min/mg protein)	17.607±8.778	2.872±0.041*	0.277±0.055**$^{\#}$	-10.4
hGP (ΔA/min/mg protein)	21.988±2.347	21.736±1.500	22.109±1.391	1.0
hCT (ΔA/min/mg protein)	0.755±0.070	0.787±0.055	0.804±0.070	1.0
hGR (ΔA/min/mg protein)	13.459±1.249	14.056±0.984	14.351±1.240	1.0
hGST (ΔA/min/mg protein)	85.279±10.348	86.351±5.722	84.582±10.481	1.0
Non-enzymatic antioxidant				
Total reduced thiol (mM/mg protein)	0.988±0.067	0.987±0.077	1.134±0.027**$^{\#\#}$	1.2

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Sf**, high fat diet-fed rats gavaged with S. frutescens leaves extract; **hMDA**, homogenate malondialdehyde, **hXO**; homogenate xanthine-xanthine oxidase; **hSOD**; homogenate superoxide dismutase; **hGP**, homogenate glutathione peroxidase; **hCT**, homogenate catalase; **hGR**, homogenate glutathione reductase; **hGST**, homogenate glutathione-S-transferase; **vs**, versus. * $P < 0.05$, ** $P < 0.01$ and $^{\#}$ = significantly different from the HF control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. LF: n=6; HF: n=7; HF+Pa: n=6-7.*

6.5.1.2 The liver mitochondria

The liver mitochondrial total hydroperoxides, oxidases and antioxidants data are shown in **Tables 6.5**

Table 6.5 Effect of *S. frutescens* on the mitochondrial oxidative stress markers, pro-oxidants and antioxidants

	LF (Lean)	HF (Fatty liver)	HF+Sf (gavaged)	Fold difference (HF vs HF+Sf)
Oxidative stress markers				
mMDA (μM/mg protein)	Undetectable	Undetectable	Undetectable	-
mTotal hydroperoxides level (mM/mg protein)	1.693± 0.164	2.159±0.204	2.178±0.130	1.0
mH_2O_2 levels (μM/mg protein)	0.183± 0.09	0.747±0.08*	1.958±0.371**#	2.6
Pro-oxidant enzymes				
mNOX (ΔA/min/mg protein)	0.212±0.069	0.497±0.104*	0.559±0.143**	1.1
mXO (ΔA/min/mg protein)	1.670±0.246	2.642±0.383*	2.647±0.638*	1.0
Antioxidant enzymes				
mSOD (ΔA/min/mg protein)	16.241±1.181	17.457±2.303	16.508±1.657	1.1
mGP (ΔA/min/mg protein)	17.985±1.368	18.805±0.650	17.557±1.554	1.1
mCT (ΔA/min/mg protein)	0.101±0.009	0.105±0.003	0.096±0.009	1.1
mGR (ΔA/min/mg protein)	1.369±0.117	1.433±0.156	1.324±0.121	1.1
mGST (ΔA/min/mg protein)	7.659±0.727	7.392±o.965	5.427±0.853*#	-1.4

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Sf**, high fat fed rats gavaged with S. frutescens leaves extract;* **mH_2O_2**, mitochondrial hydrogen peroxides; ***mCT**, mitochondrial catalase; **mNOX**, mitochondrial reduced nicotinamide adenine dinucleotide oxidase; **mXO**; mitochondrial xanthine oxidase; **mSOD**; mitochondrial superoxide dismutase; **mGP**, mitochondrial glutathione peroxidase; **mGR**, mitochondrial glutathione reductase; **mGST**, mitochondrial glutathione-S-transferase; **vs**, versus. * $P < 0.05$), ** $P < 0.01$ and # = significantly different from the HF control, #$P < 0.05$. LF: n=6; HF: n=7; HF+Sf: n=6.*

The mMDA level was undetectable in all three groups as also explained in **Chapter 5**. In addition, the mitochondrial total hydroperoxide levels were similar 1.0-fold in both HF+Sf and HF groups (**Table 6.5**). The mH_2O_2 levels in the HF+Sf group were significantly increased compared to the HF (2.6-fold; $P < 0.05$) and LF (10.7-fold; $P < 0.01$) groups, respectively. The mNOX activity was significantly increased in both HF+Sf (2.6-fold, $P < 0.01$) and HF (2.3-fold, $P < 0.05$) groups (**Table 6.5**) compared to that in the LF group. The mXO activity was similarly

significantly increased in both HF+Sf and HF groups compared to the LF group ($P < 0.05$). Looking at the liver mitochondrial antioxidant defence systems, the mGP, mGR, mCT and mSOD activities were similar in all three groups (1.1 fold). In addition, the mGST activity was significantly decreased 1.4-fold in the HF+Sf group as compared to both HF and LF groups ($P < 0.05$). Altogether, these data showed that *S. frutescens* extract has antioxidant potential in this HFD rat model.

6.5.1.3 Discussion

Very little is known about the antioxidant potential of *S. frutescens* leaves aqueous extract *in vivo*. Therefore, the present study reports for the first time, an increase in the liver total reduced thiol content. On the other hand, the *S. frutescens* extract antioxidant activity has been reported in the cell free systems and to stimulate neutrophil (Fernandes *et al*., 2004), but the types of antioxidants involved in the superoxide and H_2O_2 elimination were not elucidated in their study. The present study demonstrates some pro-oxidant and antioxidant capacities of *S. frutescens* extract in the liver of this HFD rat model.

Unchanged protein carbonyl content in the HF+Sf group may be due to no change in the cellular protein damage by produced ROS, as this plant extract can increase the total reduced thiol content, revealing its antioxidant potential in this HFD model (**Table 6.4**). Even though the measured ROS producing enzyme activities (mXO and mNOX) were increased in the HF+Sf group, GSH can neutralize the effect that may be caused by ROS. In addition, an increase in mNOX activity may be as a result of increased both FA oxidation and TCA cycle, producing more NADH and $FADH_2$ electron carriers. Increased FA oxidation mediated by this plant may also be supported by elevated mH_2O_2 concentrations in the HF+Sf group compared to that in both HF and LF groups, despite that mH_2O_2 was also significantly increased in the HF group. Interestingly, in HF+Sf and HF there were no changes in the activities of enzymes that catalyse H_2O_2 to H_2O. However, a significant decrease in hSOD and mGST activities in the HF+Sf may indicate that superoxide produced from the hXO could not be sufficiently catalysed to H_2O_2 in both groups. Therefore, other measured liver homogenate and mitochondrial antioxidant

enzymes that were not affected in the HF+Sf group are confirmed by no change in the homogenate total hydroperoxides content.

Based on these observations, it can be suggested that accumulated superoxides were involved in the induction of modest lipid peroxidation, while an H_2O_2 was neutralised to H_2O by GP and CT in both HF+Sf and HF groups. Furthermore, significant increased total reduced thiol content in the HF+Sf group is consistent with normal functions of GSH dependent enzymes, namely hGP, hGR, hGST and mGP with an exception of mGST, which its activity was reduced. Notably, the cG6PD activity in the HF+Sf was higher than in the HF group, which may increase the GSH content, a component of total reduced thiols (**Tables 6.2** and **6.4**).

6.6 Effect of *S. frutescens* leaves extract on the liver mitochondrial respiratory chain enzymes

6.6.1 Results

The mitochondrial respiratory chain enzymes, complexes I, II and III activities were assayed and data summarized in **Table 6.6**.

Table 6.6 Effect of *S. frutescens* extract on the mitochondrial respiratory chain complexes

	LF (lean)	HF (fatty liver)	HF+Sf	Fold difference (HF vs HF+Sf)
Complex I (ΔA/min/mg protein)	16.332±3.428	18.522±3.262	20.539±2.508	1.1
Complex II (ΔA/min/mg protein)	12.124±1.768	8.794±0.870*	6.682±1.648***	-1.3
Complex III (ΔA/min/mg protein)	1.519±0.188	1.629±0.492	2.056±0.282	1.3

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats, **HF+Sf**, high fat diet-fed rats gavaged with S. frutescens extract; **Complex I**, NADH dehydrogenase; **Complex II**, succinate dehydrogenase (SDH), **Complex III,** cytochrome c reductase; **vs**, versus. * = significantly different from LF control, *P < 0.05 and ***P < 0.001. LF: n=6; HF: n=7; HF+Sf: n=7.*

The liver mitochondrial complexes I and III activities were not significantly affected in all groups (1.1 fold) (**Table 6.6**). However, the mitochondrial complex II activity was significantly decreased in both HF+Sf (1.8-fold, $P < 0.001$) and HF (1.4-fold, $P < 0.05$) groups when compare

to the LF group, while decreased 1.3 in the HF+Sf group relative to the HF group; revealing that *S. frutescens* extract may have an inhibitory effect on the activity of complex II.

6.6.2 Discussion

Despite the statistically significant differences in the enzymes involved in the mitochondrial TCA cycle and respiratory chain in the *S. frutescens* treated rats, the relatively small change (fold less than 2) do not suggest that such differences will have significant metabolic impact. An increase in FA oxidation and TCA cycle result in an increase formation of both NADH and $FADH_2$, which are the electron carriers for the mitochondrial respiratory chain to produce energy in the form of ATP (Cole *et al*., 2011). In the present study, *S. frutescens* extract showed to influence an increase in the mNOX activity and mH_2O_2 production.

6.7 Conclusion

The aim of this study was to investigate the effects of 4 weeks of gavaging the HFD-fed rats with 50 mg of *S. frutescens* leaves aqueous extract/kg bwt/day on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity during prevention of a HFD-induced fatty liver in week 16. The *S. frutescens* extract mechanism of actions on the liver metabolic changes during prevention of a HFD-induced fatty liver in the male Wistar rats remain to be investigated. The present study elucidated that lipid accumulation in the liver may not be prevented by treating the HFD-fed rats with *S. frutescens* extract. This was shown by the liver TL content in the HF+Sf group (6.2% lipids by liver weight) compared to the HF group (6.7% lipids by liver weight) (**Table 6.1**) that was at the fatty liver range i.e. > 5% lipids by liver weight. In the HF+Sf group, the developed fatty liver was associated with a significant increase in iNOS protein and total reduced thiol levels. Considering that oxidative stress is a contributing factor to the induction of apoptosis, the absence of any significant indication of oxidative stress (**Tables 6.4** and **6.5**) is in line with the absence of apoptotic marker, caspase-3 (**Figure 6.2**). The liver TL in the HF+Sf group was less by 8.9% compared to that in the HF group, indicating that *S. frutescens* extract may not significantly prevent lipid accumulation in the liver (**Table 6.1**) to an extent of limiting a HFD-induce fatty liver in the present rat model. From the established results in this study, it can be concluded that *S. frutescens* extract may not successfully prevent HFD-induced fatty

liver. However, this plant extract possesses antioxidant potential through increasing the liver total reduced thiol content.

The *S. frutescens* leaves aqueous extract also contains 35% polysaccharides (Van Wyk and Albrecht, 2008), which may play a role on glucose and lipid metabolism in the liver. Some compounds that are contained in this plant leaves extract were isolated using organic solvents (Fu *et al.,* 2010b; Avula *et al*., 2010) and their contents in aqueous extract has been reported by Muller *et al*. (2012), who demonstrated that both aqueous and methanol extracts contain the flavonol glycosides and triterpenoids together with the sutherlandiosides and sutherlandins, respectively. Therefore, altogether their effects as well as that of other known compounds in the *S. frutescens* leaves aqueous extract may not be ruled out from the observed data in the HF+Sf group responses to this plant extract.

In addition, the elucidated effects of *S. frutescens* extract include increasing the iNOS protein levels (which may also be beneficial depending on the status of the liver as an increased NO plays a role on wound healing) and inhibition of the hSOD, mGST and complex II activities, increased total reduced thiol content in the liver. Therefore, the present study demonstrates that *S. frutescens* extract may not prevent HFD to induce fatty liver without changing the dietary habits during treatment, but can enhance antioxidant capacity in the liver. The use of this plant extract in the prevention of HFD-induced insulin resistant and fatty liver with change in the dietary habits is herein proposed to be conducted in the future studies, to validate its harmful and/or beneficial effects.

6.8 Proposed mechanism of actions by which *S. frutescens* leaves aqueous extract could improve antioxidant capacity without preventing HFD-induced fatty liver (see next page)

Diagram 1: Proposed model of mechanism for *S. frutescens* on the prevention of HFD-induced fatty liver in rats
Chapter 6: *Sutherlandia frutescens's leaves aqueous extracts*'s results, discussion and conclusion (2008-2012) SC TSHIDINO NMMU

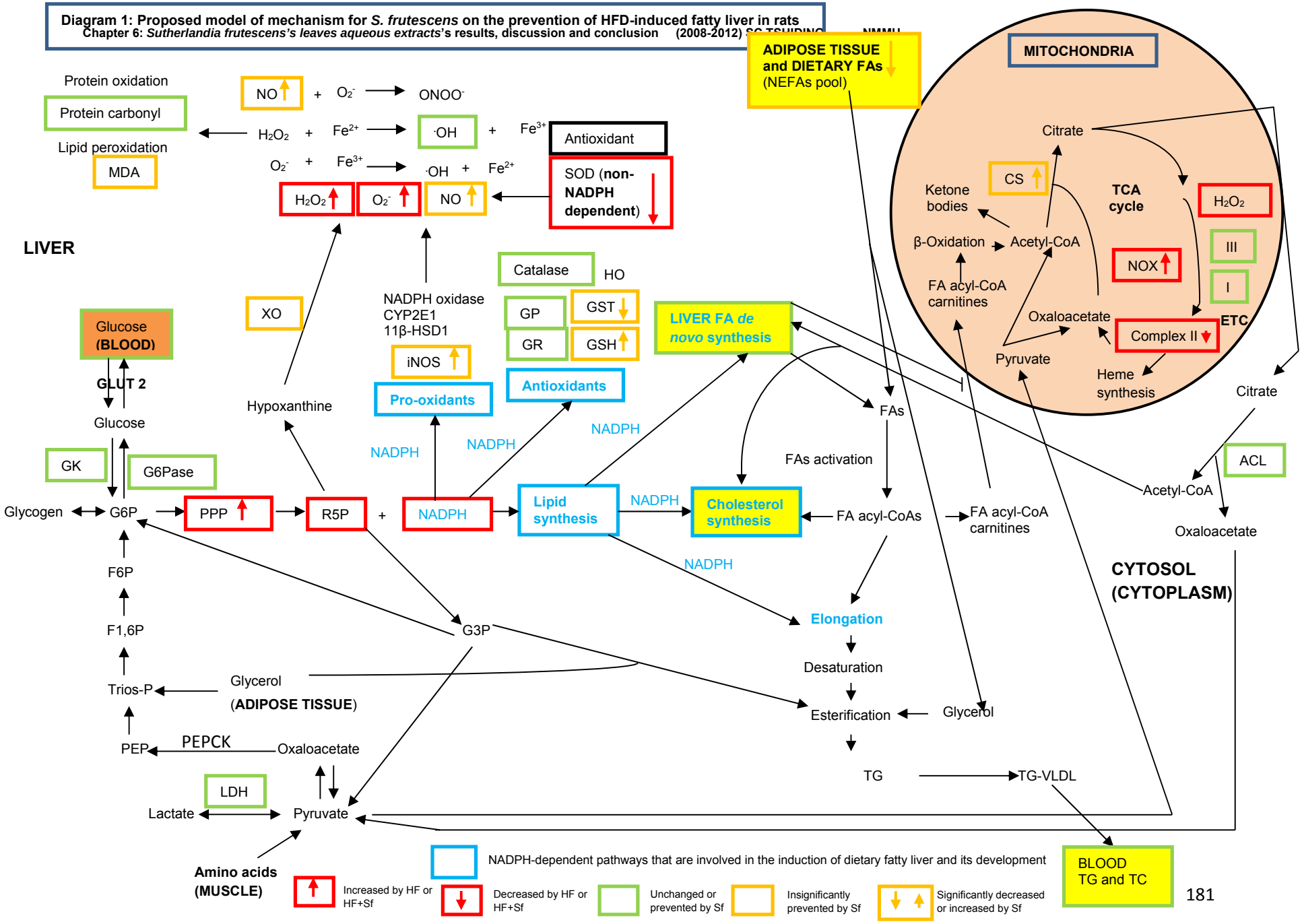

Diagram 6.1 *Depicts the proposed mechanism of action by which a 4 week of gavanging the HFD-fed rats with S. frutescens extract affected fatty liver development in rats Orange square. Abbreviations:* ***ACL****, ATP citrate lyase;* ***cICD****, cytosolic isocitrate dehydrogenase;* ***CoA****, Coenzyme A;* ***CYP2E1****, cytochrome P450 2E1;* ***CS****, citrate synthase;* ***FAs****, fatty acids;* ***FFAs****, free fatty acids;* ***Fe****, iron,* ***GSH****, reduced glutathione;* ***GLUT2****, glucose transporter 2; G3P, glyceraldehyde-3-phosphate,* ***G6P****, glucose-6-phosphate; G6Pase; glucose-6-phosphostase;* ***cG6PD****, glucose-6-phosphate dehydrogenase;* ***GP****, glutathione peroxidase;* ***GR****, glutathione reductase;* ***GST****, glutathione-S-transferase;* ***11βHSD1****, 11β-hydrosteroid dehydrogenase-1;* ***H_2O_2****, hydrogen peroxide;* ***HFD****, high fat diet;* ***I****, complex I;* ***III****, complex III;* ***iNOS****, inducible nitric oxide synthase;* ***LDH****, lactate dehydrogenase;* ***MDA****, malondialdehydes;* ***NADPH****, reduced nicotinamide adenine dinucleotide phosphate;* ***NO****, nitric oxide;* ***NOX****, reduced nicotinamide adenine dinucleotide oxidase,* ***O_2^-****, superoxide;* ***$^{\cdot}OH$****, hydroxyl (hydroperoxydes);* ***$ONOO^-$****, peroxynitrite molecule****;* ***PPP****, pentose phosphate pathway;* ***R5P****, ribose-5-phosphate;* ***SOD****, superoxide dismutase;* ***TC****, total cholesterol,* ***TG****, triglyceride;* ***TCA****, tricarboxylic acid.*

CHAPTER 7: Efficacy of 4 weeks of *Prunus africana* bark crude aqueous extract in the prevention of a HFD-induced fatty liver

Synopsis

This study was undertaken to investigate the role of Prunus africana bark extract on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity in the prevention of high fat diet (HFD) induced fatty liver. Male Wistar rats were fed a high fat diet (HF group) or normal rat chow (LF group) for 12 weeks. Although hepatic lipid accumulation had not progressed to the limits defining fatty liver, 6 rats of the HF group were gavaged with 0.125 mg P. africana extract/kg bwt/day (HF+Pa group) for an additional 4 weeks on the same HFD. At this stage, untreated HFD-fed animals had progressed to represent fatty liver.

A significant reduction ($P<0.05$) in the liver TL content and hGK activity and an increase ($P<0.01$) in the total reduced thiol content and mH_2O_2 level, and activities of cACL and complex III were observed in the HF+Pa group, when compared to the HF group. Thus, P. africana extract possesses hepatic antioxidant potential that may attenuate HFD-induced fatty liver through increasing FA oxidation; however β-oxidation was not investigated. Together these effects of plant extract may enhance hepatic antioxidant capacity to compensate for the unconfirmed increase in mitochondrial FA oxidation and ROS production, due to increased energy availability in the form of FFA. Therefore, P. africana bark aqueous extract may have therapeutic potential in the treatment of NAFLD, but further investigations are needed to fully elucidate its efficacy and safety.

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7.8 Proposed mechanism of actions by which *P. africana* bark aqueous extract prevented HFD-induced lipid accumulation in the liver of the male Wistar rats.

7. Introduction

This chapter focuses on the effect of aqueous extract of *P. africana* bark on the metabolic regulation of liver glucose, lipid, pro-oxidant and antioxidant capacity in the prevention of HFD-induced fatty liver. Karachi (2009) reported that aqueous extract of *P. africana* bark may possess insulin secregogue effects or mediate a decrease in insulin degradation; thereby increasing the fasting plasma insulin levels in this HFD-induced fatty liver and insulin resistance model. However, insulin degradation in this HFD rat model has been ruled out in a study reported by Suleiman (2009). Hence, whether aqueous extract of *P. africana* bark can prevent HFD-induced fatty liver remains unknown.

7.1 Evaluation of *P. africana* to prevent HFD-induced fatty liver

7.1.1 Liver histology

To determine the effect of *P. africana* extract on hepatic lipid accumulation, lipid accumulation and histological changes were investigated microscopically. Representative histology images of the liver are shown in **Figure 7.1**.

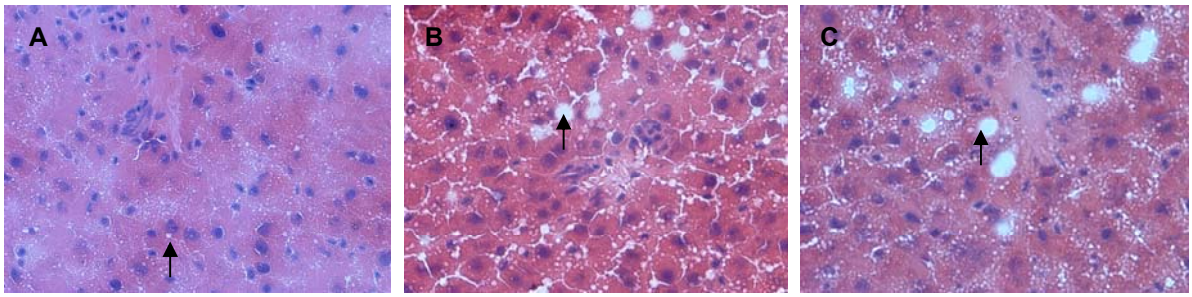


Figure 7.1 *Rat liver histology (**A**) **LF** (lean liver) (**B**) **HF** (fatty liver) (**C**) **HF+Pa** (gavaged), (Original magnification X40). Arrow indicates a lipid droplet. **LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Pa**, high fat diet-fed rats gavaged with P. africana extract.*

The H&E staining showed an increase in lipid droplet content with no liver lesions in the HF+Pa group when compared to the HF group (**Figures 7.1B** and **C**).

7.1.2 Liver lipid content

To determine whether gavaging the HFD-fed rats with *P. africana* extract can prevent HFD-induced fatty liver, the TL content was extracted and quantified (**Table 7.1**).

Table 7.1 Effect of *P. africana* bark extract on the liver total lipids

	CF	CH	LF	HF	HF+Pa
TL (mg/g liver wet weight)	22.6±3.5	27.6±2.9	48.0±1.1	67.34±6.0***	59.0±6.0**#
Percentage lipid	2.2	2.76	4.80	6.73	5.90
Fold difference (HF vs HF+Pa)					-1.1
Percentage decrease (HF vs HF+Pa)					12.3
Percentage increase (16 weeks vs 12 weeks)			112	144	114

***CL**, low fat diet fed rats after 12 weeks; **CH**, high fat diet fed rats after 12 weeks; **LF**, low fat diet-fed rat after 16 weeks; **HF,** high fat diet-fed rats after 16 weeks (**see Chapter 5**, **Table 5.1** for their statistical changes); **HF+Pa,** high fat diet-fed rats for 16 weeks while gavaging with P. africana extract last four weeks; **TL,** total lipid, **vs**, versus. * = significantly different from the LF control, ** $P < 0.01$; *** $P < 0.001$ and # = significantly different from the HF control, #$P < 0.05$. LF: n=6; HF: n=7; HF+Pa: n=6).*

The liver TL content was significantly lowered by 12.3% in the HF+Pa group when compared to the HF group ($P < 0.05$), but remained significantly higher than that in the LF group ($P < 0.01$) (**Table 7.1**). These data suggest that *P. africana* bark aqueous extract may slow down the development of fatty liver.

7.1.3 Discussion

The liver histology images clearly revealed that *P. africana* extract may increase the macrovesicular steatosis, but not microvesicular steatosis (**Figures 7.1**C). However, macrovesicular steatosis by itself in the absence of other liver lesions is a relatively benign condition, although only in the short term (Labbea *et al*., 2008). The present study suggests that this plant extract may prevent HFD to mediate fatty liver. Together these data indicate that *P. africana* extract significantly lowered TL accumulation in the liver ($P < 0.05$), i.e. 5.9% of lipids

per liver wet weight in the HF+Pa group relatively to 6.7% of the HF group, thus yet not to a level of limiting HFD to induce fatty liver, since the TL content per liver weight remained > 5%. Hence, *P. africana* extract may attenuate HFD-induced fatty liver.

In this HFD rat model, Karachi (2009) reported that the fasting plasma FFA, TG and TC levels are significantly lowered in the HF+Pa group compared to the HF group. In addition, fasting plasma HDL-C levels in the HF+Pa group have been found to be significantly lowered than that in both HF and LF groups, with no change in the LDL-C levels (Karachi, 2009; **Table 3.3**). It has been reported by other authors that chloroform extract of *P. africana* bark (Tadenan) contains the ferulic acid esters, which have potent anti-hypercholesterolemia effects in the prostate as a potent treatment of BPH (Cristoni *et al*., 2000; Stewart, 2003b), however the limited solubility of ferulic acid esters in water makes it an unlikely component in the aqueous extract prepared for this study.

7.2 Effect of *P. africana* bark extract on the markers of inflammatory and apoptotic responses

7.2.1 Results

The iNOS and procaspase-3 protein levels were analysed and shown in **Figure 7.2**. The iNOS and procaspase-3 protein levels were similar in all groups (**Appendixes A** and **B**), suggesting that aqueous extract of *P. africana* may not promote neither apoptotic nor inflammatory marker in this HFD rat model.

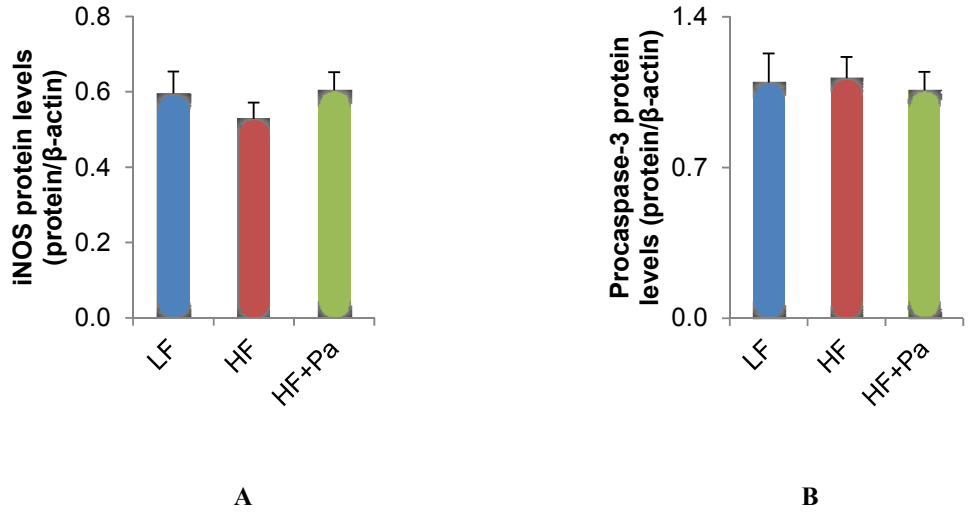


Figure 7.2 *Effect of P. africana extract on rat liver inflammatory and apoptotic response protein levels. (**A**) iNOS protein levels (**B**) Procaspase-3 protein levels.* ***LF****, low fat diet-fed rats,* ***HF****, high fat diet-fed rats;* ***HF+Pa****, high fat fed rats gavaged with P. africana extract, LF: n=6; HF: n=7; HF+Pa: n=6.*

7.2.2 Discussion

Chloroform extract of *P. africana* bark (Tadenan) has been reported to decrease the number of iNOS positive cells in the diabetic bladder (Wang *et al*., 2009b). It is noteworthy that these rats were diabetic, while the rats in this study were only insulin resistant and fatty liver. Furthermore, the constituents of a chloroform extract are expected to differ significantly from that used in the present study. Importantly, liver is a central organ that plays a role in lipid metabolism, importing serum FFAs and synthesizing, storing and exporting lipids and lipoproteins (Adams *et al*., 2005). Therefore, it can be proposed that the rate at which cholesterol is secreted from the liver in the HF+Pa group needs to be investigated to elucidate its effects on HDL-C synthesis. In the liver, accumulation of cholesterol rather than TG may play a vital role in the progression from simple fatty liver to NASH (Enjoji *et al*., 2012). In addition, inhibition of TG synthesis can ameliorate fatty liver, but has been shown to aggravate liver damage and fibrosis in obese mice with NASH (Yamaguchi *et al*., 2007). Therefore, both cholesterol and TG may play the major role in the progression of fatty liver. Since the fasting plasma insulin levels

in the HF+Pa group were found to be significantly higher than that in the HF group (Karachi, 2009), it should be emphasized that although insulin may modulate molecules and pathways that restore insulin sensitivity, chronic elevated blood insulin levels can enhance insulin resistance and/or tissue inflammation (Iida *et al*., 2001). However in the study by Karachi (2009) it is evident that despite the increased fasting insulin levels, there was no indication of such an adverse effect since prominent counter regulatory mechanisms such as hypoglycaemia, insulin resistance and body weight increase did not occur, suggesting that adequate insulin sensitivity is somehow maintained. Nonetheless, prolonged treatment with *P africana* may eventually lead to adverse effects including the demise of the pancreatic beta-cell as observed in diabetes, and consequently the chronic use should be approached with vigilance. Therefore, a long term study on the effect of *P. africana* extract on the prevention of HFD-induce fatty liver is needed, to further elucidate its safety and efficacy.

7.3 Effect of *P. africana* extract on the lipogenic enzymes

7.3.1 Results

The hepatic lipogenic enzymes, cG6PD, mCS and cACL activities were measured and the results presented in **Table 7.2**

Table 7.2 Effect of *P. africana* extract on the lipogenic enzyme activities

	LF (lean)	**HF (fatty liver)**	**HF+Pa**	**Fold difference (HF vs HF+Pa)**
cG6PD (ΔA/min/mg protein)	3.958±1.025	6.679±1.357*	4.521±1.000$^{\#}$	-1.5
cACL (ΔA/min/mg protein)	32.011±4.905	29.252±3.897	41.636±5.946*$^{\#\#}$	1.4
mCS (ΔA/min/mg protein)	18.991±1.723	17.693±1.712	18.078±1.918	1.0

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Pa**, high fat diet-fed rats gavaged with P. africana extract; **cG6PD**, cytosolic glucose-6-phosphate dehydrogenase; **cACL**, cytosolic ATP citrate lyase; and **mCS**, mitochondrial citrate synthase; **vs**, versus. * = significantly different from the LF group, * $P < 0.05$ and $^{\#}$ = significantly different from the HF group, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$. LF: n=6; HF: n=7; HF+Pa: n=6.*

The cG6PD activity was significantly reduced 1.5-fold in the HF+Pa group relatively to the HF group ($P < 0.05$), but not significantly different when compared to the LF group (**Table 7.2**). The mCS activity was similar in all groups, indicating no change in entrance of acetyl-CoA into the TCA cycle (**Table 7.2**). However, activity of cACL was significantly increased in the HF+Pa group compared to both HF (1.4-fold, $P < 0.01$) and LF (1.3-fold, $P < 0.05$) groups (**Table 7.2**).

7.3.2 Discussion

It is generally accepted that G6PD is unique in the group of lipogenic enzymes in that it participates in multiple metabolic pathways. In addition to lipogenesis, G6PD also contributes to cell growth, protection against oxidative stress and the production of ROS (Salati and Amir-Ahmady, 2001). Therefore, changes in the activity of this enzyme are difficult to directly interpret; unless the full domain of its involvement has been investigated. ACL plays a significant role in catalysis of the cytosolic citrate to release acetyl-CoA and oxaloacetate, the former is used for the *de novo* FA and cholesterol synthesis; and the latter used for the gluconeogenesis (Leonhardt and Langhans, 2002).

7.4 Effects of *P. africana* extract on the liver glucose metabolic enzymes

7.4.1 Results

The glucose metabolic enzymes, LDH, GK and G6Pase were measured and the results shown in **Table 7.3**. LDH activity was similar in the HF+Pa and HF groups (**Table 7.3**). Considering that increased LDH activity is usually associated with an increased glycolytic rate and/or mitochondrial dysfunction, constant LDH activity suggests that *P. africana* treatment does not exceed the normal capacity to metabolise glucose. This is further supported by a significant decrease in hGK activity, a prominent pacemaker of glycolysis, which was decreased 1.6-fold in the HF+Pa group relatively to the HF group ($P < 0.05$). No change in the homogenate and microsomal G6Pase activities in HF+Pa and HF groups was evident (**Table 7.3**).

Table 7.3 Effect of *P. africana* bark extract on the liver glucose metabolic enzymes

	LF (lean)	HF (fatty liver)	HF+Pa	Fold difference (HF vs HF+Pa)
cLDH (ΔA/min/mg protein)	42.735±3.779	49.140±3.983	49.923±4.664	1.0
hGK (ΔA/min/mg protein)	1.070±0.175	1.262±0.331	0.784±0.108$^{\#}$	1.6
hG6Pase (mM Pi/min/mg protein)	1.190±0.180	1.027±0.183	0.985±0.111	1.0
miG6Pase (mM Pi/min/mg protein)	2.593±0.367	2.433±0.250	2.112±0.651	1.2

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Pa**, high fat diet-fed rats gavaged with P. africana extract**; cLDH**, lactate dehydrogenase; hGK, homogenate glucokinase; **hG6Pase**, homogenate glucose-6-phosphatase; **miG6Pase**, microsomal glucose-6-phosphatase; **vs**, versus. $^{\#}$ = significantly different from the HF control, $^{\#}P < 0.05$. LF: n=6; HF: n=7; HF+Pa: n=6.*

7.4.2 Discussion

Taken together, the results indicate a decrease in the metabolism of glucose in *P africana* treated animals. Although these findings appear to contradict the effects on glucose metabolism expected for the dramatic increase in serum insulin levels, the relatively small changes in enzyme quantity may not reliably reflect the *in vivo* situation. Further, the absence of a robust elevation in glucose disposal suggests that tissues other than the liver may be more involved in maintaining normal blood glucose levels. However, further confirmation to support these observations remains essential in the future studies.

7.5 Effect of *P. africana* extract on the liver oxidative stress and antioxidant status

The oxidative stress markers, protein carbonyl content and MDA levels were determined. In addition, the enzymatic and non-enzymatic pro-oxidants and antioxidants were also measured in the liver homogenate and mitochondria; and the results presented in **Tables 7.4** and **7.5**.

7.5.1 Results

7.5.1.1 The liver homogenate

The liver homogenate MDA, protein carbonyl, total hydroperoxides, XO activity and antioxidants data are summarized in **Table 7.4**.

Table 7.4 Effect of *P. africana* extract on the homogenate pro-oxidants and antioxidants

	LF (lean)	**HF (fatty liver)**	**HF+Pa (gavaged)**	**Fold difference (HF vs HF+Pa)**
Oxidative stress markers				
hMDA (µM/mg protein)	189.648±47.879	423.205±177.365*	351.344±81.202	-1.2
hProtein carbonyl level (mM/mg protein)	0.382±0.042	0.378±0.049	0.339±0.043	-1.1
hTotal hydroperoxides level (mM/mg protein)	6.570±1.712	6.041±1.519	6.683±1.157	1.1
Pro-oxidant enzyme				
hXO (ΔA/min/mg protein)	122.125±16.769	160.761±22.290*	148.208±11.660	-1.1
Enzymatic antioxidants				
hSOD (sod1 plus sod2) (ΔA/min/mg protein)	17.607±8.778	2.872±0.041*	17.877±7.131$^{\#}$	6
hGP (ΔA/min/mg protein)	21.988±2.347	21.736±1.500	20.839±1.293	-1.1
hCT (ΔA/min/mg protein)	0.755±0.070	0.787±0.055	0.794±0.092	1.0
hGR (ΔA/min/mg protein)	13.459±1.249	14.056±0.984	14.171±1.633	1.0
hGST (ΔA/min/mg protein)	85.279±10.348	86.351±5.722	85.003±8.714	-1.0
Non-enzymatic antioxidant				
Total reduced thiol group (mM/mg protein)	0.988±0.067	0.987±0.077	1.232±0.090**$^{\#\#}$	1.3

LF, *low fat diet group;* ***HF,*** *high fat diet group;* ***HF+Pa,*** *high fat diet plus P. africana bark extract;* ***hMDA****, homogenate malondialdehyde,* ***hXO****; homogenate xanthine-xanthine oxidase;* ***hSOD****; homogenate superoxide dismutase;* ***hGP****, homogenate glutathione peroxidase;* ***hCT****, homogenate catalase;* ***hGR****, homogenate glutathione reductase;* ***hGST****, homogenate glutathione-S-transferase;* ***GSH****, reduced glutathione was used as standard to measure thiol groups;* ***vs****, versus. * = significantly different from the LF group, * $P < 0.05$), ** $P < 0.01$ and # = significantly different from the HF group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. LF: n=6; HF: n=7; HF+Pa: n=6.*

Although the HFD-induced a significant increase in hMDA, no significant changes in the other oxidative stress markers (protein peroxidation as measured by protein carbonyl and total hydroperoxide levels (**Table 7.4**) were evident. It is therefore concluded that neither the HFD nor the treatment with *P africana* leads to significant oxidative stress. Considering that both protein carbonylation and total hydroperoxides remain unchanged, no conclusive interpretations should be made on the increase in hMDA. Looking at the antioxidant enzymes, the hSOD activity was significantly decreased by the HFD (6-fold; $P < 0.05$), but was preserved/restored in the HF+Pa group (**Table 7.4**), suggesting that *P. africana* extract may counteract the effect of HFD on hSOD activity. The effect of HFD on SOD activity is somewhat unique as the hGP, hGR, hCT and hGST activities were similar in all groups (**Table 7.4**), indicating that enzymes responsible for the homogenate superoxides and H_2O_2 neutralization were not affected (**Table 7.4**).

Surprisingly, the liver total reduced thiols were not significantly altered despite the dramatic decrease in SOD activity, suggesting that its overall involvement in the induction of oxidative stress is relatively flexible. This is further supported by the increase in total reduced thiol content of *P. africana* treated rats when compared to the untreated LFD control. Hence, this plant extract demonstrates an antioxidant effect in the liver, via increasing the reduced thiol content, an effect which may relate to an increased availability of reducing equivalents in the form of NADPH, which is predominantly generated by cG6PD. However, as described in **Section 7.4.1**, glucose metabolism appears to be unchanged, and perhaps even decreased due to a reduction in GK activity, indicating that the pentose phosphate pathway is an unlikely contributing factor in the improved hepatic redox state of *P africana* treated animals.

7.5.1.2 The liver mitochondria

The liver mitochondrial total hydroperoxides, oxidases and antioxidants data are summarized in **Tables 7.5**. The mXO and mNOX activities were not significantly increased in the HF+Pa (**Table 7.5**) as compared to the LF group. Therefore, *P. africana* extract may not prevent HFD to mediate activation of these two enzymes. In addition, the mH_2O_2 levels were significantly increased in the HF+Pa group, when compared to both HF (6.7-fold) and LF (27.4-fold) ($P < 0.001$) groups (**Table 7.5**). An increase in the production of ROS in the mitochondrial fraction is

uprising, as mitochondria and in particular the electron transport chain constitutes the major source of ROS production within the cell. Furthermore, all the measured mitochondrial antioxidants include mSOD, mGP, mGR and mGST activities were similar in all three groups (**Table 7.5**).

Table 7.5 Effect of *P. africana* on the mitochondrial pro-oxidants and antioxidants

	LF (lean)	**HF (fatty liver)**	**HF+Pa**	**Fold difference (HF vs HF+Pa)**
Oxidative stress markers				
mMDA (μM/mg protein)	Undetectable	Undetectable	Undetectable	-
mTotal hydroperoxides levels (mM/mg protein)	1.693± 0.164	2.159±0.204	2.240±0.143	1.0
mH_2O_2 levels (μM/mg protein)	0.183± 0.09	0.747±0.08*	5.007±0.708***$^{\#\#\#}$	6.7
Pro-oxidant enzymes				
mNOX (ΔA/min/mg protein)	0.212±0.069	0.497±0.104*	0.515±0.157*	1.0
mXO (ΔA/min/mg protein)	1.670±0.246	2.642±0.383*	2.606±0.492*	1.0
Antioxidant enzymes				
mSOD (ΔA/min/mg protein)	16.241±1.181	17.457±2.303	17.231±2.030	1.0
mGP (ΔA/min/mg protein)	17.985±1.368	18.805±0.650	17.593±1.254	1.1
mCT (U/mg protein)	0.101±0.009	0.105±0.003	0.097±0.007	1.1
mGR (ΔA/min/mg protein)	1.369±0.117	1.433±0.156	1.334±0.088	1.1
mGST (ΔA/min/mg protein)	7.659±0.727	7.392±o.965	8.232±1.431	1.1

LF, *low fat diet-fed rats;* ***HF,*** *high fat diet-fed rats;* ***HF+Pa,*** *high fat diet-fed rats gavaged with P. africana bark extract;* **mH_2O_2**, mitochondrial hydrogen peroxides; ***mNOX****, mitochondrial reduced nicotinamide adenine dinucleotide oxidase;* ***mXO****; mitochondrial xanthine oxidase;* ***mSOD****; mitochondrial superoxide dismutase;* ***mGP****, mitochondrial glutathione peroxidase;* ***mGR****, mitochondrial glutathione reductase;* ***mGST****, mitochondrial glutathione-S-transferase,* ***vs****, versus. * = significantly different from the LF group, * P < 0.05; ** P < 0.01; *** P < 0.001 and # = significantly different from the HF group, ###P < 0.001. LF: n=6; HF: n=7.*

Considering the absence of any significant indication of cellular oxidative stress (**Table 7.4**) and that the mMDA levels were undetectable (presumably unchanged) in all three groups, it is

perplexing that a 6.7 fold elevation in mitochondrial H_2O_2, a prooxidant known to induce oxidative stress, is without effect on established markers of oxidative stress. Furthermore, the total hydroperoxide level remains unchanged despite the fact that H_2O_2 is in itself a hydroperoxide and was subsequently used as a positive control in the FOX assay to quantify hydroperoxides (**Chapter 4**). These discrepancies raise concern as to the accuracy of the mH_2O_2 determination and possibly indicate and artefact, while the data suggests that *P. africana* extract may promote ROS production from the mitochondria, further studies are required to fully understand the precise relevance of these findings.

7.5.2 Discussion

The significant increase in total reduced thiol content in the HF+Pa group is consistence with the normal function of GSH dependent enzymes including GP, GR and GST, indication that the redox status of the hepatocytes was not only maintained; but improved by *P africana* treatment. However, despite this clear absence in any significant oxidative stress, a dramatic elevation in the level of the superoxide producing enzyme SOD is apparent. This raises a fundamental discrepancy in that excessive mH_2O_2 will accumulate, since the enzymes responsible for neutralising H_2O_2 remain unchanged and levels of reduced thiols are in fact increased. Although this may suggest that an alternative pathway may be operational, there is no known metabolic alternative identified to date. A further discrepancy is apparent with regards to the mH_2O_2 levels, which were increased 6.7-fold when assayed using the Amplex red assay, but remained unchanged when measured using the FOX assay for total hydroperoxides. Considering these unexplainable discrepancies and the host of other unmeasured enzyme activities, which may also contribute to oxidative stress, it is difficult to make any meaningful conclusion without further studies.

7.6 Effect of *P. africana* bark extract on the mitochondrial respiratory chain enzyme complexes

7.6.1 Results

The mitochondrial respiratory chain enzymes, complexes I, II and III activities were measured and are presented in **Table 7.6**

Table 7.6 Effect of *P. africana* bark extract on the mitochondrial respiratory chain complexes

	LF (lean)	HF (fatty liver)	HF+Pa	Fold difference (HF vs HF+Pa)
Complex I (ΔA/min/mg protein)	16.332±3.428	18.522±3.262	18.560±4.078	1.0
Complex II (ΔA/min/mg protein)	12.124±1.768	8.794±0.870*	7.708±1.156**	-1.1
Complex III (ΔA/min/mg protein)	1.519±0.188	1.629±0.492	2.803±0.379***$^{\#\#\#}$	1.7

***LF**, low fat diet-fed diet; **HF**, high fat diet-fed rats, **HF+Pa**; high fat diet-fed rats gavaged with P. africana extract; **Complex I**, NADPH dehydrogenase; **Complex II**, succinate dehydrogenase (SDH), **Complex III**, cytochrome c reductase; **vs**, versus. * = significantly different from the LF control, * P < 0.05, ** P < 0.01, *** P < 0.001. # = significantly different from the HF control, ###P < 0.001. LF: n=6; HF: n=7; HF+Pa: n=6.*

Complex I activity was similar 1.0-fold in all three groups (**Table 7.6).** However, complex II activity was significantly decreased in the HF+Pa (2.2-fold, P < 0.01) and HF (1.4-fold, P < 0.05) groups, when compared to the LF group (**Table 7.6**), suggesting that *P. africana* extract exacerbates the HFD-induced decrease in complex II activity. In addition, complex III activity was significantly increased 1.8- and 1.7-fold in the HF+Pa group, when compared to the HF and LF (P < 0.001) groups, respectively (**Table 7.6**).

7.6.2 Discussion

An increase in complex III activity may result in an elevated mitochondrial superoxide production in the HF+Pa group. However oxidative stress markers indicate that the overall burden of ROS is well maintained and dose not lead to tissue damage. Thus, *P. africana* extract may improve fatty liver without promoting liver damage as observed by no change in iNOS and procaspase-3 levels, both are inducible proteins.

7.7 Conclusion

The aim of this study was to investigate the effect of 4 weeks treatment of HFD-fed rats with 0.125 mg *P. africana* bark aqueous extract/kg bwt/day on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity. Hence, *P. africana* bark aqueous extract mechanism of actions on the liver metabolic function in the prevention of HFD-induced fatty liver in the male Wistar rat remains to be investigated. The present study is first to demonstrate the effect of aqueous extract of *P. africana* on some of the liver glucose and lipid metabolic enzymes and other parameters including oxidants and antioxidants.

The present study indicates that *P. africana* extract may attenuate HFD-induced fatty liver. Liver TL accumulation in the HF+Pa group was significantly decreased by 12%, but it was still at a range of fatty liver i.e. 5.9% lipids by liver weight when compared to that in the HFD ($P< 0.05$; 6.7% lipids by liver weight) group (**Table 7.1**). Therefore, this plant extract revealed to improve rather than to completely prevent HFD-induced fatty liver. The alleviated fatty liver by this plant extract was independent of iNOS (inflammatory marker) and procaspase-3 (apoptosis marker) protein levels (**Figure 7.2**). In addition, there was a low level of lipid peroxidation, a marker of oxidative stress in the HF+Pa group, revealing that *P. africana* may enhance antioxidant capacity. *P. africana* extract has been demonstrated to prevent an increase in the fasting blood FFAs, TG and TC levels, while HDL-C levels were severely decreased, with no change in the LDL-C levels (Karachi, 2009, **Table 3.3**). Altogether, these data may raise concerns in the effect of *P. africana* extract on the cholesterol synthesis and its elimination from the liver. Thus, cholesterol metabolism including its deposition to extrahepatic tissues and its convection to bile acid and steroids for elimination from blood via faeces may have been enhanced by this plant extract. However, studies are needed to investigate the effect of this plant extract on the elimination of cholesterol metabolites on the faeces.

On the other hand, dramatically elevated plasma fasting insulin levels may be expected to lead to hypoglycaemia, due to insulin induced suppression of glucose output from the liver and its increase intake by other tissues. However, rats treated with *P. africana* extract demonstrate normal blood glucose levels as reported by Karachi (2009). The significant elevated liver

reduced thiol levels with no change in iNOS protein levels in the HF+Pa group may indicate that more NADPH was involved in the increase biosynthesis of GSH to enhance antioxidant capacity of this plant extract.

Significant increased TL content in the HF+Pa and HF groups when compared to the LF group may reveal an involvement of the FFAs taken up from the circulation by the liver in this HFD-induced fatty liver rat model. In addition, an increase in cACL activity observed in the HF+Pa group may show that the transcriptional regulation of SREBP-1c, which is upregulated by insulin, was altered in this rat group. Hence it can be suggested that in the present study, the cACL activity was transcriptionally regulated, a condition supported by Karachi (2009) who reported that in the HF+Pa group, the blood insulin levels were increased by 2-fold higher than in the HF group. SREBP-1c is likely to be activated by hyperinsulinaemia (Paschos and Paletas, 2009), indicating that this plant extract may influence sufficient hyperinsulinemia to upregulate the cACL activity via SREBP-1c activation, while HFD alone may not achieve this effect. Karachi (2009) reported a decrease in the blood HDL-C levels in the HF+Pa compared to that in both control groups. The effect of this plant extract on increasing the blood insulin levels renders further investigations on both insulin sensitivity and insulin resistance pathways in the future studies. In the present study, the mMDA was undetectable and no change in antioxidant enzyme activities in the HF+Pa group, even though the measured ROS producing enzyme activities were elevated in the mitochondria (**Table 7.5**). However, no mitochondrial damage is expected since complex II activity was significantly reduced with no change in that of complex I in both HF+Pa and HF groups, when compared to LF group (**Table 7.6**).

From the established results in this study, it can be concluded that *P. africana* extract may attenuate HFD induce fatty liver; as illustrated by the 12% less accumulated TL in the liver. However, this plant extract possesses antioxidant potential via increasing the liver total reduced thiol content. Therefore, the present study reports that *P. africana* extract may improve HFD-induced fatty liver without changing the dietary habits during treatment. Thus, the use of this plant extract on the prevention of HFD-induced fatty liver with changing the dietary habits is also proposed to be conducted in the future studies to validate its harmful and/or beneficial effects on fatty liver. Moreover, a long term study on the use of this plant extract in the

prevention of present HFD induce fatty liver rat model needs to be investigated in the future, to determine whether this plant extract may promote fatty liver progression to NASH as it has shown to reduce TL accumulation.

7.8 Proposed mechanism of actions by which *P. africana* bark aqueous extract prevented HFD-induced lipid accumulation in the liver of the male Wistar rats (see next page)

Diagram 1: Diagram 1: Proposed model of mechanism for *P. africana* on the prevention of HFD-induced fatty liver in rats

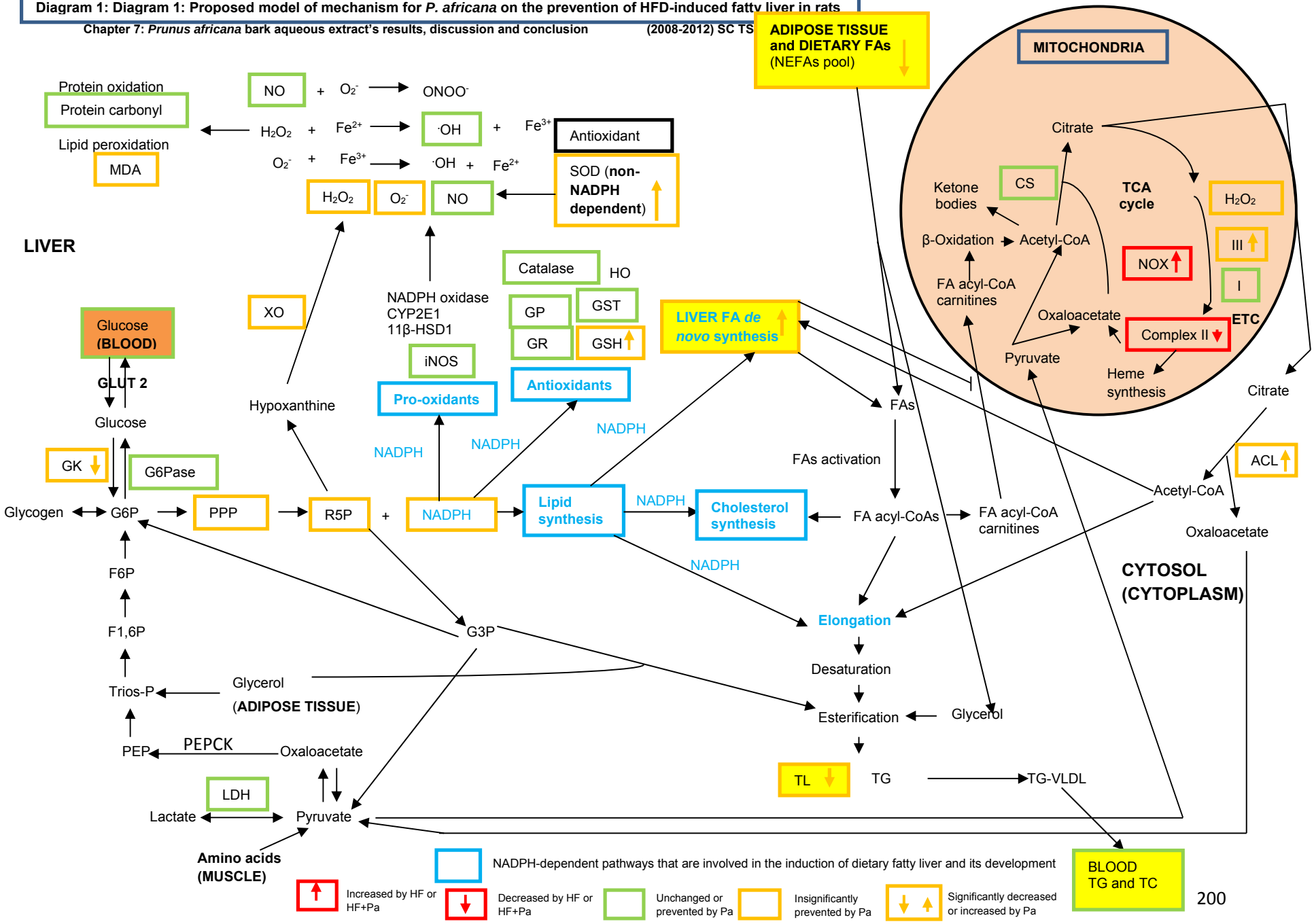


Diagram 7.1 *Depicts the proposed mechanism of action by which a 4 week of gavaging the HFD-fed rats with P. africana extract affected fatty liver development in rats through various metabolic processes. The liver metabolic changes induced by HFD or HFD plus P. africana extract are marked in Red square, those unaffected by HFD or improved by HFD plus P. africana extract are marked in Green square and those prevented or lowered or increased by gavage in P. africana extract are marked with Orange square. Abbreviations:* ***ACL****, ATP citrate lyase;* ***cICD****, cytosolic isocitrate dehydrogenase;* ***CoA****, Coenzyme A;* **CYP2E1**, cytochrome P450 2E1; ***CS****, citrate synthase;* ***FAs****, fatty acids;* ***FFAs****, free fatty acids;* ***Fe****, iron, thiol groups, reduced glutathione;* ***GLUT2****, glucose transporter 2; G3P, glyceraldehyde-3-phosphate,* ***G6P****, glucose-6-phosphate; G6Pase; glucose-6-phosphostase;* ***cG6PD****, glucose-6-phosphate dehydrogenase;* ***GP****, glutathione peroxidase;* ***GR****, glutathione reductase;* ***GST****, glutathione-S-transferase;* ***11βHSD1****, 11β-hydrosteroid dehydrogenase-1;* ***H_2O_2****, hydrogen peroxide;* ***HFD****, high fat diet;* ***I****, complex I;* ***III****, complex III;* ***iNOS****, inducible nitric oxide synthase;* ***LDH****, lactate dehydrogenase;* ***MDA****, malondialdehydes*; ***NADPH****, reduced nicotinamide adenine dinucleotide phosphate;* ***NO****, nitric oxide;* ***NOX****, reduced nicotinamide adenine dinucleotide oxidase,* ***O_2^-****, superoxide*; ***·OH****, hydroxyl (hydroperoxydes);* ***$ONOO^-$****, peroxynitrite molecule****;* ***PPP****, pentose phosphate pathway;* ***R5P****, ribose-5-phosphate;* ***SOD****, superoxide dismutase;* ***TC****, total cholesterol,* ***TG****, triglycerides;* ***TCA****, tricarboxylic acid.*

CHAPTER 8: Efficacy of 4 weeks of metformin on the prevention of a HFD-induced fatty liver

Synopsis

This study was undertaken to investigate the effect of metformin on liver glucose and lipid metabolism, as well as the antioxidant capacity during the high fat diet (HFD)-induced fatty liver. Male Wistar rats were fed a high fat diet (HF group) or normal rat chow (LF group) for 12 weeks. Although rats had not developed fatty liver yet, 6 rats of the HF group were gavaged with 16 mg metformin/kg bwt/day (HF+Met group) for an additional 4 weeks and maintained on the same HFD.

Treatment with metformin showed a significant decrease the liver lipid accumulation by 16% when compared to the HF ($P<0.05$). However, liver TL content in the HF+Met group remained within fatty liver range i.e. >5% lipids by liver wet weight. This may be due to the activation of cACL and cG6PD as was accompanied with an increase in iNOS protein levels. Mitochondrial complex II activity was further decreased, while that of complex III was significantly increased in the HF+Met group when compared to the HF group ($P<0.01$). Increased iNOS protein levels mediated by metformin may suggest a cause of an inflammatory response in the liver, which can be attributed to increase in the production of NADPH from the PPP and ME, as supported by a significant increase in the activities of both cG6PD and cACL enzymes in the HF+Met group. Thus, metformin may enhance antioxidant capacity to compensate for increased mitochondrial FA oxidation as observed by increased total reduced thiol contents, mH_2O_2 and complex III activity and decreased complex II activity. Altogether, metformin may attenuate HFD-induced fatty liver in the male Wistar rat model.

OUTLINE

8. Introduction

This chapter focuses on the effect of metformin on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity during the progression to fatty liver. The mechanism of action of metformin in the treatment of T2DM is well documented (Gad *et al*., 2010; Viollet *et al*., 2012; Yki-Jarvinen and Westerbacka, 2005). Similarly the role of metformin on the management of fatty liver have been reviewed, but the precise conclusion on its treatment guideline has not been reached, due to variation in findings from several reported studies (Mezza *et al*., 2012). Although rare, hepatotoxicity cases have been reported during metformin treatment (Aksay *et al.,* 2007; Kutoh, 2005). However, whether metformin can prevent the present HFD to induce fatty liver remains complex and not fully investigated. In this chapter, the efficacy of metformin treatment was investigated by treating rats after 12 weeks on HFD (**see Chapter 5**) with 16 mg metformin/kg bwt/day for an additional 4 weeks, while maintaining the HFD.

8.1 Evaluation of efficacy of metformin on HFD-induced fatty liver

8.1.1 Liver histology

To determine whether metformin treatment can prevent lipid accumulation in the liver of the HFD fed rats, lipid accumulation was investigated microscopically. Representative histology images of the liver are shown in **Figure 8.1**. The H&E staining showed a decrease in the liver lipid droplet content in the HF+Met group when compared to the HF group (**Figures B** and **C**), but higher than in the LF group (**Figure 8.1A**). The liver images demonstrate that lipids were accumulated mostly in the form of macrovesicular steatosis in the HF+Met group, when compared to the HF group (**Figures 8.1**).

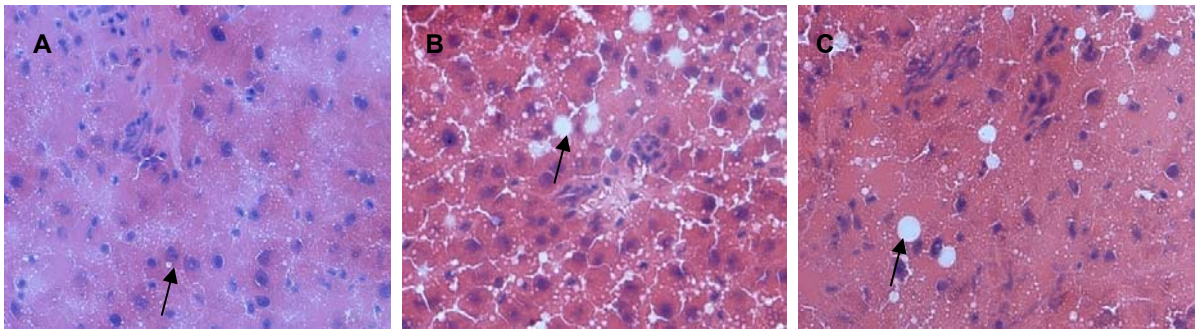


Figure 8.1 *Rat liver histology (**A**) LF (lean) group (**B**) HF (fatty liver) group (**C**) HF+Met (gavaged), (Original magnification X40). Arrow indicates a lipid droplet. **LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Met**, high fat diet-fed rats gavaged with* metformin

8.1.2 Liver lipid content

The total lipid content of the liver is summarized in **Table 8.1**.

Table 8.1 Effect of metformin on the liver lipid profiles

	CF	CH	LF (lean)	HF (fatty liver)	HF+Met (gavaged)
TLs (mg/g liver wet weight)	22.6±3.5	27.6±2.9	48.0±1.1	67.34±6.0***	56.45±1.60*#
Percentage TL	2.2	2.76	4.8	6.73	5.65
Ratio HF+Met/HF			-	-	0.84
Percentage decrease HF vs HF+Met			-	-	16.2
Percentage change in TL 16 weeks vs 12 weeks			112	144	105

***CL**, low fat diet fed rats after 12 weeks; **CH**, high fat diet fed rats after 12 weeks; **LF**, low fat die-fed rats after 16 weeks; **HF**, high fat diet-fed rats after 16 weeks (**see Chapter 5**, **Table 5.1** for their statistical changes); **HF+Met**, high fat diet-fed rats after 16 weeks after treating with metformin for the last 4 weeks; **TLs**, total lipids. * = significantly different from the LF group, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; # significantly different from the HF group, #$P < 0.05$. LF: n=6; HF: n=7; HF+Met: n=6.*

Lipid accumulation in the liver was significantly less in the HF+Met group, when compared to the HF group, but remained significantly higher than in the LF group ($P < 0.05$) (**Table 8.1**). Thus metformin contributed to a 16% reduction of TL content, when compared to the HFD (**Table 8.1**).

8.1.3 Discussion

The data obtained in this study may reveal that metformin can attenuate lipid accumulation in the liver, resulted. The liver is the primary organ for cholesterol elimination from the body through synthesis of bile acid from oxidised cholesterol, but this process is complex and not fully understood (Smith *et al*., 2009). Although the HFD did not decrease the blood HLD-C levels in the present HFD rat model, metformin mediated a significant increase in the blood HLD-C levels. This renders further investigation in the future studies in order to elucidate metformin actions on increasing fasting plasma HDL-C, thereby looking at the cellular bile acid metabolism. This mechanism is important because the plasma HDL-C plays a significant role in ferrying cholesterol to the liver, resulting in reduced risk of atherosclerosis. Thus HDL-C helps to keep the blood vessels dilated (widened), while it can also reduce the blood vessel injury via its anti-inflammatory and antioxidant effects, respectively (Tosh, 2005).

Metformin has been described as a safe drug when precisely used in properly selected patients to avoid its severe side effects (Bernal-Lopez *et al*., 2010; Vecchio and Protti, 2011). In the last decade, metformin has been reported to reverse fatty liver in leptin-deficient obese mice (Lin *et al*., 2000). Mice fed on HFD-enriched with lard fat containing 2-3 g/kg metformin for 8 weeks showed to have normal levels of fasting plasma TG, LDL-C, TC, but TG and TC in the liver were significantly decreased than in the HF group, while still significantly higher than in the LF control. In addition, metformin has also been demonstrated to prevent body weight gain induced by HFD alone in their mice model, and its metabolic action was suggested to be through activation of AMPK and suppression of FAS and SREBP-1c expression, respectively (Kim *et al*., 2010). Furthermore, AMPK is known to increase FAs oxidation and to prevent TG synthesis, thereby leading to a decrease in lipogenesis and glucose production in the liver. However, the AMPK function in the liver is also enhanced by the adipose-derived adiponectin (Reviewed in Gruzman *et al*., 2009).

In comparison to their study, metformin reduced lipids in the livers of the T2DM patients (Gupta *et al*., 2009; Reviewed in Mazza *et al*., 2012). Hence, metformin is suggested to have significant hypolipidemic effects in the liver of this HFD rat model, but not to a level of completely

preventing HFD to mediate lipid accumulation in the liver. Metformin has also been reported not to reduce accumulation of lipid in the liver, but increased the liver insulin sensitivity, without increasing peripheral glucose uptake in the T2DM patients (Tiikkainen *et al*., 2004). Several studies indicated that metformin may be of benefit in the treatment of NAFLD in both non-diabetic and T2DM patients, when consociated to weight control and hypocaloric diet (Reviewed in Mazza *et al*., 2012). Although the present study could not show that metformin can prevent the development of fatty liver after insulin resistance has developed and the diet does not change it showed that metformin could sifnificantly lower the hepatic TL. These ties in with the results of Karachi (2009) and Mackenzie (2009) who found a significant decrease in blood FFAs and TC as well as the significant increase in blood HDL-C. Thus, metformin may attenuate HFD-induced fatty liver in this rat model.

8.2 Effect of metformin on the markers of inflammatory and apoptotic responses

8.2.1 Results

To determine the effect of treating the HFD-fed rats with metformin on the inflammatory and apoptotic markers in the liver, iNOS protein levels and activation of procaspase-3 were analysed by Western blotting. The results are shown in **Figure 8.2**. The iNOS protein levels were significantly increased in the HF+Met group, when compared to both HF and LF groups ($P < 0.01$) (**Appendix A**). Procaspase-3 protein levels were similar in all three groups (**Appendix B**). A significant increase in the iNOS protein levels of the HF+Met group was similar to that observed in the HF+Sf group (**see Section 6.1.3**), when compared to both control groups ($P < 0.01$).

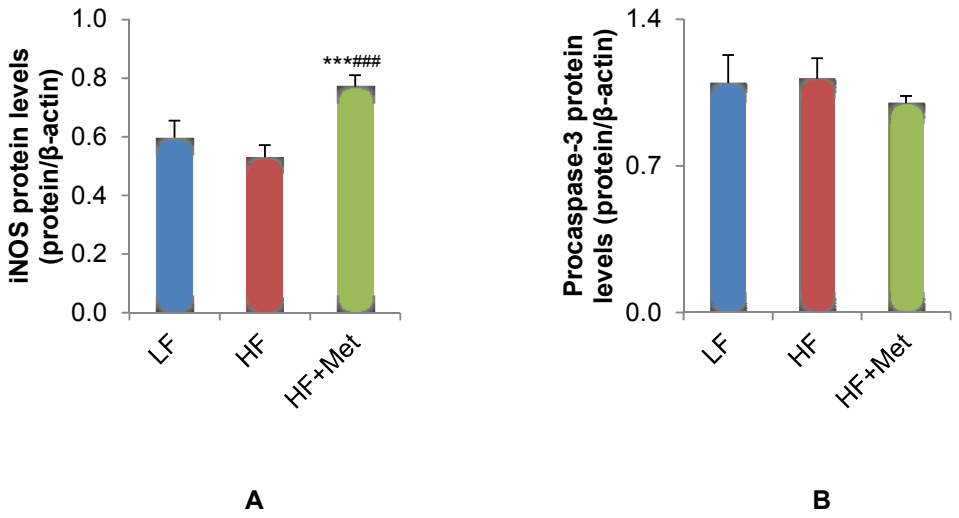


Figure 8.2 *Effect of metformin on the rat liver iNOS and procaspase-3 protein levels (**A**) iNOS protein levels (**C**) Procaspase-3 protein levels. * = significantly different from LF group, *** P < 0.001 and # = significantly different from HF group, ###P < 0.00. LF: n=6; HF: n=7; HF+Met: n=6.*

8.2.2 Discussion

Metformin significantly reduced the TL content of the liver in this rat model, which means that this significant increase in iNOS is may not be due to lipid accumulation. Metformin's antidiabetic effects has been reported to accompany an increased insulin sensitivity and normoglycaemia, but Tang *et al.* (2009b) found metformin to increase the activity of eNOS in the diabetic rats, resulted in significantly increase in serum NO levels.

8.3 Effect of metformin on the lipogenic enzyme activities

8.3.1 Results

To determine the effect of treating the HFD-fed rats with metformin on the lipogenic enzymes, the liver cG6PD, MSc and cACL activities were measured as shown in **Table 8.2**.

Table 8.2 Effect of metformin on the liver lipogenic enzyme activities

	LF (lean)	HF (fatty liver)	HF+Met	Fold difference (HF vs HF+Met)
cG6PD (ΔA/min/mg protein)	3.959±1.025	6.679±1.357*	7.305±2.230**	1.1
cACL (ΔA/min/mg protein)	32.011±4.905	29.252±3.897	41.127±2.271*$^{\#\#}$	1.4
mCS (ΔA/min/mg protein)	18.991±1.723	17.693±1.712	19.664±1.890	1.1

LF, *low fat diet-fed rats;* ***HF,*** *high fat diet-fed rats;* ***HF+Met,*** *high fat diet-fed rats gavaged with metformin;* ***cG6PD****, cytosolic glucose-6-phosphate dehydrogenase; c****ACL,*** *cytosolic ATP citrate lease; and* ***mCS,*** *mitochondrial citrate synthase;* ***vs****, versus. * = significantly different from the LF group, * P < 0.05; ** P < 0.01 and # = significantly different from the HF group, ##P < 0.01. LF: n=6; HF: n=7; HF+Met: n=7.*

The cG6PD activity was significantly increased in the HF+Met group (1.8-fold, P< 0.01) similarly to that in the HF group (1.7-fold, P < 0.05) (**Table 8.2**), when compared to the LF group. This change of the cG6PD activity in the HF+Met is similar to that observed in the HF+Sf group (**see Section 6.2**), when compared to the LF group (P < 0.01). No difference (1.1-fold) in the cG6PD activity between the HF+Met and HF groups. The cACL activity was significantly increased in the HF+Met group, when compared to both HF (1.4-fold, P < 0.01) and LF (1.3-fold, P < 0.05) groups (**Table 8.2**). This change in the cACL activity of the HF+Met is similar to that observed in the HF+Pa group (**see Section 7.3**), when compared to both HF (P < 0.01) and LF (P < 0.05) groups. The mCS activity was not changed (1.1-fold) in all groups (**Table 8.2**), indicating that acetyl-CoA entrance into the TCA cycle may not be altered.

8.3.2 Discussion

Amongst the three lipogenic enzyme activities measured in this study, the data clearly show that metformin increased the cACL activity in this HFD rat model. The cG6PD activity was also increased significantly compared to LF group, but it is clear that this must be an effect of the HFD and not of metformin because the cG6PD was also significantly increased in the HF group. Metformin could however not attenuate the effect of the HFD on this enzyme activity. Both cG6PD and cACL and their dependent-pathways are contributors for lipid synthesis, FAs elongation and desaturation through supply of NADPH and acetyl-CoA (Ntambi and Kim, 2001;

Horton, 2002; Liu *et al*., 2010, 2011). In the HF+Met group the hepatic TL content decreased significantly as compared to the HF group, indicating that the fold increase did not reach a level sufficiently robust to contribute to lipogenesis. It has to be noted that metformin prevented HFD to increase the plasma FFAs, thereby increasing FFAs uptake by the liver and their oxidation in mitochondria and other tissues. Increased mitochondrial FA oxidation potential may relate to the increased complex III activity in the HF+Met group as compared to both HF and LF groups.

8.4 Effect of metformin on the liver glucose metabolic enzymes

8.4.1 Results

To determine the effect of treating the HFD-fed rats with metformin on the glucose metabolic enzymes, LDH, GK and G6Pase activities were measured as shown in **Table 8.3**. The cLDH and hGK activities were similar 1.0-fold in all groups (**Table 8.3**). There was no significant difference (1.2-fold) in the activity of hGK in all groups. However, the miG6Pase catalytic subunit activity was significantly lowered in the HF+Met group compared to both HF (2-fold, $P < 0.05$) and LF (2.2-fold, $P< 0.01$) groups (**Table 8.3**).

Table 8.3 Effect of metformin on the liver glucose metabolic enzyme activities

	LF (lean)	HF (fatty liver)	HF+Met (gavaged)	Fold difference (HF vs HF+Met)
cLDH (ΔA/min/mg protein)	42.735±3.779	49.140±3.983	50.221±5.829	1.0
hGK (ΔA/min/mg protein)	1.070±0.175	1.262±0.331	1.047±0.142	-1.2
hG6Pase (mM Pi/min/mg protein)	1.190±0.180	1.027±0.123	1.037±0.105	1.0
miG6Pase (mM Pi/min/mg protein)	2.593±0.367	2.433±0.250	1.192±0.265**$^{\#}$	-2

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Met**, high fat diet-fed rats gavaged with metformin; **cLDH**, lactate dehydrogenase; **hGK**, homogenate glucokinase; **hG6Pase**, homogenate glucose-6-phosphatase; **miG6Pase**, microsomal glucose-6-phosphatase; **vs**, versus; **-**, decrease. * = significantly different from the LF group, ** $P < 0.01$ and $^{\#}$ = significantly different from the HF group, $^{\#}P < 0.05$. LF: n=6; HF: n=7; HF+Met: n=6.*

8.4.2 Discussion

These data show that metformin decreased the phosphatase activity of miG6Pase activity; however this is not the rate limiting step in the conversion of glucose-6-phosphate to glucose. This metformin effect agrees with the study that reported a significant decrease in the hG6Pase activity in the HFD-fed rat model of insulin resistance, where the increased liver hG6Pase activity was reduced by metformin treatment, resulting in normal blood glucose levels from hyperglycaemic conditions (Mithieux *et al*., 2002; Levere *et al*., 2003). Since hG6Pase activity was not decreased (1.0-fold), while that of miG6Pase was decreased (2-fold) in the present study, it remains difficult to rule out the effect of isolation procedure, because the assay used corrected the non-G6Pase activity as described in **Chapter 4**. However, unchanged hG6Pase activity in the present study may be attributed to the low dosage (i.e. 16 mg metformin/kg bwt/day) used; whereas other study used about 3 times higher dosage (i.e. 50 mg metformin/kg bwt/day). It has been concluded that metformin decreases glucose output from liver in the insulin resistant HFD-fed rats mainly through inhibition of the G6Pase activity, promoting glycogen sparing, and that additional mechanisms may involve the diversion of G6P into the PPP and an inhibition of lactate uptake by the liver (Mithieux *et al*., 2002). Metformin decreases the liver glucose output in the T2DM patients without causing hypoglycaemia (Kirpichnikov *et al*., 2002; Reviewed in Viollet *et al*., 2012). Indeed, metformin does not decrease the blood glucose levels in the non-diabetic subjects (Klip and Leiter, 1990), an effect that was also noted by Mackenzie 2009, on the same animals used in this study.

Metformin may reduce glucose output from the liver via a long-term, which is genetic and a short-term, which is metabolic effects (Fulgencio *et al*., 2001). This drug also increases insulin sensitivity, but is only effective in the presence of insulin (Patel, 2003). The present HFD rat model was hyperinsulinemia; therefore metformin may have enhanced insulin sensitivity of the liver as observed by its increasing the cACL activity (**Table 8.2**), while decreasing the miG6Pase activity without affecting that of the hG6Pase (**Table 8.3**). A decrease in the miG6Pase activity could enhance an influx of G6P to PPP for the production of NADPH. Metformin has been reported to increase the liver GK and cG6PD activities, which were significantly decreased in the neonatal streptozotocin (nSTZ) non-insulin dependent diabetic (Chakrabarti *et al*., 2003; Natarajan and Pari, 2005) and in the HFD-induced glucose intolerance rat models (Gad *et al*., 2010). Metformin inhibits gluconeogenesis independently of AMPK and LKB1 actions, but via

decreasing expression of the gene encoding the catalytic subunit of G6Pase, without affecting the cytosolic PEPCK gene expressions in the wild-type, AMPK-deficient, and LKB1-deficient hepatocytes (Foretz *et al.*, 2010). It needs to be noted that Mithieus *et al*. (2002) did not observe a change in the G6Pase protein levels of the insulin resistant HFD-fed rats. This mechanism needs more research in the future.

8.5 Effect of metformin on the liver oxidative stress and antioxidants

To determine the effect of treating the HFD-fed rats with metformin on the oxidative stress, the markers protein carbonyl and MDA levels and the enzymatic and non-enzymatic antioxidants were measured in the liver homogenate and mitochondria as shown in **Tables 8.4** and **8.5**.

8.5.1 Results

8.5.1.1 The liver homogenate

The liver homogenate MDA, protein carbonyl, total hydroperoxides, XO activity and antioxidants data are summarized in **Table 8.4**. The liver hXO activity was not significantly different between the HF+Met and HF groups (1.0-fold) (**Table 8.4**), but increased in both groups as compared to the LF group ($P < 0.05$). The homogenate total hydroperoxide levels were similar in the HF+Met and HF groups. Protein carbonyl levels were significantly increased in the HF+Met group, when compared to both HF (1.3-fold) and LF groups (1.6-fold, $P < 0.05$ (**Table 8.4**), respectively. Hence, metformin demonstrated to influence oxidative stress, thereby mediating protein peroxidation but not lipid peroxidation, which was not altered by HFD alone (**Table 8.4**). Interestingly, the hSOD activity was restored in the HF+Met, when compared to the HF group. Thus hSOD activity was significantly decreased 6-fold in the HF group, when compared to the LF group ($P < 0.05$). The hGP, hCT, hGR and hGST activities were similar 1.0-fold in all three groups. In contrast, the total reduced thiol levels were significantly increased 1.2-fold in the HF+Met group, when compared to both control groups ($P < 0.01$) (**Table 8.4**). A significant increase in the total reduced thiol content observed in the HF+Met was similar to that in both HF+Sf and HF+Pa groups (see **Sections 6.4** and **7.4**), when compared to both HF and LF

groups ($P < 0.01$), revealing metformin and *S. frutescens* leaves and *P. africana* bark extracts antioxidant effects in the livers of this HFD rat model.

Table 8.4 Effect of metformin on the homogenate pro-oxidants and antioxidants

	LF (lean)	**HF (fatty liver)**	**HF+Met (gavaged)**	**Fold difference (HF vs HF+Met)**
Oxidative stress markers				
hMDA (µM/mg protein)	189.648±47.879	423.205±177.365*	324.964±90.540	-1.3
Protein carbonyl level (mM/mg protein)	0.382±0.042	0.378±0.049	0.488±0.078*#	1.3
hTotal hydroperoxides level (mM/mg protein)	6.570±1.712	6.041±1.519	5.925±0.778	-1.0
Pro-oxidant enzymes				
hXO (ΔA/min/mg protein)	122.125±16.769	160.761±22.290*	149.425±10.627	1.1
Enzymatic antioxidants				
hSOD (sod1 plus sod2) (ΔA/min/mg protein)	17.607±8.778	2.872±0.041*	17.464±3.099#	6
hGP (ΔA/min/mg protein)	21.988±2.347	21.736±1.500	23.065±2.096	1.1
hCT (ΔA/min/mg protein)	0.755±0.070	0.787±0.055	0.840±0.077	1.1
hGR (ΔA/min/mg protein)	13.459±1.249	14.056±0.984	14.987±1.375	1.1
hGST (U/mg protein)	85.279±10.348	86.351±5.722	85.965±15.110	-1.0
Non-enzymatic antioxidant				
Total thiol group (mM/mg protein)	0.988±0.067	0.987±0.077	1.158±0.063**##	1.2

LF, *low fat diet-fed rats;* ***HF,*** *high fat diet-fed rats;* ***HF+Met,*** *high fat diet-fed rats gavaged with metformin;* ***hMDA****, homogenate malondialdehyde,* ***hXO****; homogenate xanthine-xanthine oxidase;* ***hSOD****; homogenate superoxide dismutase;* ***hGP****, homogenate glutathione peroxidase;* ***hCT****, homogenate catalase;* ***hGR****, homogenate glutathione reductase;* ***hGST****, homogenate glutathione-S-transferase;* ***GSH****, reduced glutathione was used as standard to measure thiol groups;* ***vs****, versus;* ***-****, decrease . * = significantly different from the LF group;* ***vs****, versus, * P < 0.05; ** P < 0.01 and # = significantly different from the HF group, ##P < 0.01. LF: n=5-6; HF: n=6-7; HF+Met: n=6-7.*

8.5.1.2 The liver mitochondria

The liver mitochondrial total hydroperoxides, oxidases and antioxidants data are summarized in **Tables 8.5**.

Table 8.5 Effect of metformin on the mitochondrial pro-oxidants and antioxidants

	LF (lean)	HF (fatty liver)	HF+Met (gavaged)	Fold difference (HF vs HF+Met)
Oxidative stress markers				
mMDA (μM/mg protein)	Undetectable	Undetectable	Undetectable	-
mTotal hydroperoxides level (mM/mg protein)	1.693± 0.164	2.159±0.204	2.591±0.602**	1.2
mH_2O_2 levels (μM/mg protein)	0.183± 0.09	0.747±0.08*	3.856±0.789***$^{\#\#\#}$	5.2
Pro-oxidant enzymes				
mNOX (ΔA/min/mg protein)	0.212±0.069	0.497±0.104*	0.496±0.150*	1.0
mXO (ΔA/min/mg protein)	1.670±0.246	2.642±0.383*	2.606±0.492*	1.1
Antioxidant enzymes				
mSOD (ΔA/min/mg protein)	16.241±1.181	17.457±2.303	19.467±2.198	1.1
mGP (ΔA/min/mg protein)	17.985±1.368	18.805±0.650	19.844±1.739	1.1
mCT (ΔA/min/mg protein)	0.101±0.009	0.105±0.003	0.106±0.009	1.0
mGR (ΔA/min/mg protein)	1.369±0.117	1.433±0.156	1.460±0.144	1.0
mGST (ΔA/min/mg protein)	7.659±0.727	7.392±o.965	6.472±0.530	1.1

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats; **HF+Met**, high fat diet-fed rats gavaged with metformin; **mH_2O_2**, mitochondrial hydrogen peroxides; **mNOX**, mitochondrial reduced nicotinamide adenine dinucleotide oxidase; **mXO**; mitochondrial xanthine oxidase; **mSOD**; mitochondrial superoxide dismutase; **mGP**, mitochondrial glutathione peroxidase; **mCT**, mitochondrial catalase; **mGR**, mitochondrial glutathione reductase; **mGST**, mitochondrial glutathione-S-transferase; **vs**, versus; **-**, decrease. * = significantly different from the LF group, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and $^{\#}$ = significantly different from the HF group, $^{\#\#\#}P < 0.001$. LF: n=6; HF: n=7; HF+Met: n=6.*

The mNOX was similarly significant increase 2.3-fold in the HF+Met and HF groups, when compared to the LF group ($P < 0.05$) (**Table 8.5**). The mH_2O_2 levels were significantly elevated 5.2 in the HF+Met group, when compared to the HF group ($P < 0.001$) (**Table 8.5**). It is assumed that the increase in mH_2O_2 production may be mostly driven from mitochondrial complex III as shown in **Table 8.6**. This is based on the discrepancy between the considerable elevation in hSOD activity (6 fold), and the contrasting increase in the mH_2O_2 levels together with unchanged activities of other H_2O_2 neutralising enzymes. However, mitochondrial total hydroperoxide levels were not significantly increased in the HF+Met group, when compared to the HF group (1.2-fold), but significantly increased, when compared to the LF group (1.5-fold, $P< 0.01$). These data revealed that metformin may increase ROS production in the mitochondria; however the levels are not sufficient to induce oxidative stress and can be counteracted by both cytosolic and mitochondrial antioxidant enzymes.

8.5.1.3 Discussion

Together the results of both the parameters for liver oxidative stress and antioxidants in the homogenate and the mitochondria it is noteworthy that the H_2O_2 levels in the mitochondria increased 5.2 fold in the HF+Met group versus the HF group, while mSOD did not change significantly. On the other hand in the homogenate the hSOD increased 6-fold and the htotal hydroperoxides and hMDA did not increase in the HF+Met group versus HF. The total reduced thiol groups including GSH may be responsible for the superoxides scavenging in the tissues (Droge, 2002). These discrepancies highlight the importance to exercise caution when extrapolating changes in the enzyme quantity to imply direct alterations in the metabolic flux. Despite that the changes in SOD activity which increased 6-fold, the product of this reaction, H_2O_2, reflects an opposite change 5.2 fold decrease. Furthermore, ROS production may be expected to decrease the reduced thiol content as H_2O_2 directly targets the oxidation of SH-groups, yet the results indicate that the level of total reduced thiols is increased. Taken together these data suggest that metformin has antioxidant potential on fatty liver.

This study reports for the first time, an increase in the liver total reduced thiol group contents by metformin in a HFD-induced insulin resistance and fatty liver model. An increase in the cG6PD

activity is well known to maintain the GSH levels among other reduced thiol groups, whereas its inhibition results in the GSH depletion (Jain *et al*., 2003). Therefore, in the present study, metformin actions on the activity of cG6PD may show both its antioxidant and inflammatory response potentials in the liver of HFD-induced fatty liver. Furthermore, this provides an explanation as to the elevated G6PDH activity in the absence of lipogenesis. The data in the present study agrees with the observation reported by Carvalho *et al*. (2008) that metformin may not prevent H_2O_2 production from the rat liver mitochondria, a condition that can influence mitochondrial impairment, if this drug is used in the presence of severely decreased mitochondrial antioxidant enzymes. It is also well documented that Metformin is an inhibitor of mitochondrial complex-1, which may contribute to the elevation in mH_2O_2 levels. However, all measured enzymatic antioxidants were not altered and they may protect mitochondria from ROS damage in the present HFD rat model.

8.6 Effect of metformin on the mitochondrial respiratory chain enzyme complexes

8.6.1 Results

To determine the effect of treating the HFD-fed rats with metformin on the liver mitochondrial function, complexes I, II and III activities were measured as shown in **Table 8.6**.

Table 8.6 Effect of metformin on the liver mitochondrial respiratory chain complexes

	LF (lean)	HF (fatty liver)	HF+Met (gavaged)	Fold difference (HF vs HF+Met)
Complex I (ΔA/min/mg protein)	16.332±3.428	18.522±3.262	21.969±2.363	1.2
Complex II (ΔA/min/mg protein)	12.124±1.768	8.794±0.870*	6.500±1.830***$^{\#}$	-1.4
Complex III (ΔA/min/mg protein)	1.519±0.188	1.629±0.492	2.456±0.414**$^{\#}$	1.5

***LF**, low fat diet-fed rats; **HF**, high fat diet-fed rats, **HF+Met**, high fat diet-fed rats gavaged with metformin; **Complex I**, NADH dehydrogenase; **Complex II**, succinate dehydrogenase (SDH), **Complex III**, cytochrome c reductase; **vs**, versus. * = significantly different from the LF group, * $P < 0.05$ and ** $P < 0.01$ and $^{\#}$ = significantly different from the HF group, $^{\#}P < 0.05$. LF: n=6; HF: n=7; HF+Met: n=6.*

Complex I activity was not significantly affected in all groups. However, complex II activity was significantly decreased 1.4-fold in the HF+Met group ($P < 0.001$) more than in the HF group ($P<0.05$) and 1.9-fold when compared to the LF group ($P < 0.001$). Complex III activity was significantly increased in the HF+Met group, when compared to both HF (P 1.5-fold, < 0.05) and LF (1.6-fold, $P < 0.01$) groups (**Table 8.6**). A significant increase in complex III activity observed in the HF+Met was similar to that in the HF+Pa group (see **Section 7.5**), when compared to the LF groups ($P < 0.01$).

8.6.2 Discussion

In this study, the miG6Pase activity was decreased while complex I activity was not altered in the HF+Met. On the other hand, *in vitro* study also demonstrated that a decrease in mitochondrial complex I activity in the presence of a decrease in the G6Pase expression mediated by metformin treatment, was independent from AMPK activation in the rat H4IIE cells (Ota *et al*., 2009). It may be speculated that since metformin is a direct inhibitor of complex-I activity, homogenization and dilution into the assay buffer could have negated its inhibitory effect when investigated *ex-vivo*. In the present study, metformin mechanism of actions on the increase of mitochondrial complex III activity may be attributed to its ability to enhance β-oxidation, which leads to a decrease in the activity of complex II and an increase in the activity of mNOX and mH_2O_2 production, while maintaining complex I activity (**Tables 8.5** and **8.6**). However considering the known inhibitory effect of metformin on complex I activity, such hypothesis appears unlikely. In the T2DM patients, inhibition of complex I has been attributed to lead to a decrease in the blood glucose levels (Reviewed in Miller and Bimbaum, 2010), but the rats in this study were not diabetic but only insulin resistance and therefore did not have elevated blood glucose levels that need to be corrected. The exact metformin mechanism to prevent HFD-induced insulin resistance and to slow down the development of fatty liver remains unclear as not more studies have been done on the selected metabolic changes measured in this study. Hence the present study presented some of the metabolic changes which are improved or influenced by metformin in this HFD rat model.

8.7 Conclusion

The aim of this study was to investigate the effect of 4 weeks of treating HFD-fed insulin resistant rats with 16 mg metformin/kg bwt/day on the metabolic regulation of liver glucose, lipids, oxidants and antioxidant capacity in the treatment of insulin resistance and the prevention of fatty liver. The present study is the first to demonstrate the effect of metformin on some of the liver metabolic glucose and lipogenic enzymes and other parameters including oxidants, which play roles in the dietary induced fatty liver and its progression, and antioxidants, which play roles in the prevention of liver damage from oxidants.

The present study elucidated that lipid accumulation in the liver of the HF (6.7% lipids by liver weight) and HF+Met (5.6% lipids by liver weight) groups were within a range considered as fatty liver, when compared to the LF group (4.8% lipids by liver weight). Hence, the liver TL content in the HF+Met group was 16% lower, when compared to that in the HF group ($P < 0.05$), indicating that metformin may slow down accumulation of lipid in the liver (**Table 8.1**), but not to an extent of preventing HFD to induce fatty liver. In the HF+Met group, the developed fatty liver was accompanied by a significant increase in the iNOS, but not procaspase-3 protein levels (**Figure 8.2**). Although the significant increase in protein peroxidation appears to suggest an increase in oxidative stress, all other markers indicate the ROS production is well contained and is in agreement with no apoptosis in the liver of the HF+Met group, when compared to both controls.

Even though the ROS producing enzymes, namely, mXO and mNOX activities were significantly elevated in the mitochondria, the antioxidant enzymes were unchanged in the HF+Met group (**Table 8.5**). Therefore, mitochondria may not be severely damaged in both HF+Met and HF groups as caspase-3 protein was not detectable in both groups. Complex II activity was significantly reduced, whilst that of complex III was increased in the HF+Met group (**Table 8.6**) than that in the LF group, indicating that metformin may influence mitochondrial oxidation, including fatty acids. A decrease in complex II activity may also promote heme synthesis that begins in the mitochondrial matrix with the decarboxylative condensation of glycine with succinyl-CoA to form 5-aminolevulinic acid (ALA) (Fleming, 2011).

In the liver, PGC-1α is strongly induced in fasting state and turns on the gene programs of fatty acid oxidation, heme synthesis, ketogenesis, oxidative phosphorylation and gluconeogenesis in response to glucagon and different nutrient signals, respectively (Lustig *et al*., 2011; Schmidt and Mandrup, 2011). There is increasing evidence that heme metabolites have cytoprotective roles including anti-apoptosis, anti-proliferation and anti-inflammation, and that both bilirubin and biliverdin have anti-inflammatory and anti-oxidative properties (Kinobe *et al*., 2004; Sass *et al*., 2012). However, chronic induction of heme oxygenase-1 (inducible form) protein may have both beneficial and damaging effects (Reviewed in Abraham and Kappas, 2008).

From the established results in this study, it can be concluded that metformin may attenuate HFD induce fatty liver, resulting in significantly decreased TL content by 16% ($P < 0.05$). However, metformin demonstrated to influence HFD to mediate protein damage, but not lipid oxidation. Thus metformin possess antioxidant potential via increasing the liver thiol group content and by preserving the hSOD and cG6PD activities. The observed significant increased cACL activity in the HF+Met group is suggested to be the effects of enhanced liver insulin sensitivity in this rats group, as metformin increases insulin sensitivity is revealed by decreased miG6Pase and increased G6PD activities. The effects of metformin on the prevention of elevated blood insulin on the HFD-induced fatty liver remains contradictory in the present rat model as mentioned above.

In the present study, the elucidated metformin effects include increased cACL, cG6PD and complex III activities, protein peroxidation, iNOS protein, mitochondrial total hydroperoxides and mH_2O_2 and thiol group levels and preserving hSOD activity in the liver of this HFD rat model. Metformin also demonstrated to counterbalance some of its harmful effects, thereby enhancing the antioxidant defence, despite its increasing protein carbonyl levels in the HF+Met group. Therefore, the present study demonstrates that metformin may attenuate HFD-induced fatty liver, without changing the dietary habits. However, usage of metformin in the prevention of HFD-induced fatty liver with changing the dietary habits is proposed to be conducted in the future studies, to validate its harmful and/or beneficial effects observed in the present study. Moreover, a long term study of the usage of metformin on the prevention of present HFD induce fatty liver still needs to be investigated in future, to determine whether it may promote fatty liver

progression to NASH as it has shown to stimulate an increase in the iNOS protein and protein carbonyl levels among other measures parameters in this study.

8.8 Proposed mechanism of actions by which metformin prevented HFD-induced lipid accumulation in the liver of the male Wistar rat (see next page)

Diagram 1: Proposed model of mechanism for metformin on the prevention of HFD-induced fatty liver

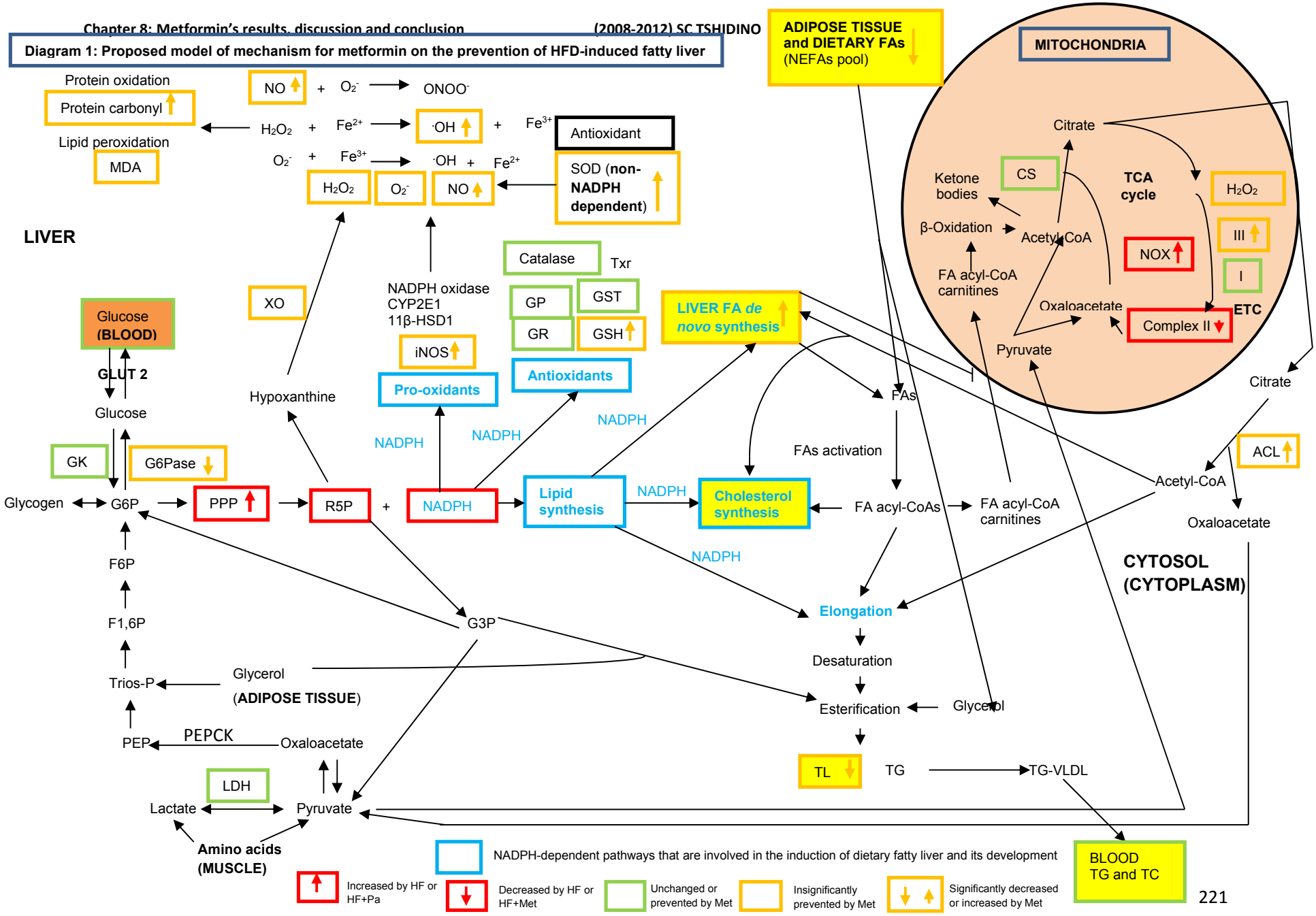


Diagram 8.1 *depicts the proposed mechanism of actions by which a 4 week of treating the HFD-fed rats with metformin affected fatty liver development in rats through various metabolic processes. Abbreviations:* ***ACL****, ATP citrate lyase;* ***cICD****, cytosolic isocitrate dehydrogenase;* ***CoA****, Coenzyme A;* **CYP2E1**, cytochrome P450 2E1; ***CS****, citrate synthase;* ***FAs****, fatty acids;* ***FFAs****, free fatty acids;* ***Fe****, iron,* ***GSH****, reduced glutathione;* ***GLUT2****, glucose transporter 2; G3P, glyceraldehyde-3-phosphate,* ***G6P****, glucose-6-phosphate; G6Pase; glucose-6-phosphostase;* ***cG6PD****, glucose-6-phosphate dehydrogenase;* ***GP****, glutathione peroxidase;* ***GR****, glutathione reductase;* ***GST****, glutathione-S-transferase;* ***11βHSD1****, 11β-hydrosteroid dehydrogenase-1;* ***H_2O_2****, hydrogen peroxide;* ***HFD****, high fat diet;* ***I****, complex I;* ***III****, complex III;* ***iNOS****, inducible nitric oxide synthase;* ***LDH****, lactate dehydrogenase;* ***Met****, metformin,* ***MDA,*** *malondialdehydes*; ***NADPH****, reduced nicotinamide adenine dinucleotide phosphate;* ***NO****, nitric oxide;* ***NOX****, reduced nicotinamide adenine dinucleotide oxidase,* ***O_2^-****, superoxide*; ***$^{\cdot}OH$****, hydroxyl;* ***$ONOO^-$****, peroxynitrite molecule****;* ***PPP****, pentose phosphate pathway;* ***R5P****, ribose-5-phosphate;* ***SOD****, superoxide dismutase;* ***TC****, total cholesterol,* ***TG****, triglycerides;* ***TCA****, tricarboxylic acid.*

CHAPTER 9: FINAL CONCLUSION AND RECOMMENDATIONS

This Chapter summarises the main outcomes of the research described in the thesis as well as recommendations for the future studies.

OUTLINE

9. Introduction

    9.1 Implications of the study

        9.1.1 Final conclusions

    9.2 Recommendations

9. Introduction

Fatty liver (NAFLD) is increasing at an alarming rate worldwide. Lipid accumulation in the liver is associated with the development of T2DM, where impaired insulin sensitivity results in increased fasting blood glucose levels. This study was undertaken to investigate the metabolic changes occurring during fatty liver development as well as the therapeutic potential of metformin and aqueous extracts of *S. frutescens* leaves and *P. africana* bark. Fatty liver was induced in rats by a HFD where 39% of the energy value was derived from fat. The fat content in the HFD contained mainly palmitic acid (C16:0; 41%), oleic acid (C18:1; 29%) and a relative low amount of PUFAs (14.9%). Fatty liver was evaluated histologically and by determining total lipids. Selected hepatic enzyme activities in the glucogenic, lipogenic, pro-oxidant and anti-oxidant pathways in the mitochondrial fraction and tissue homogenate were determined.

This is the first study to describe the effect of aqueous extract *S. frutescens* leaves and *P. africana* bark and metformin to prevent fatty liver development after insulin resistance has been induced. *In vivo* metabolic studies are very complicated because it is difficult to determine what could be the primary change due to the HFD and which metabolic changes are compensatory mechanisms. The results of a study, consisting of a lean rat model, a fatty liver rat model and the fatty liver groups treated with three different treatments, give another dimension to the analysis of the development of fatty liver and its possible prevention and/or treatment.

9.1 Implications of the study

The rate limiting enzymes of lipogenesis (cACL), mitochondrial TCA cycle (mCS) and respiratory chain (complexes I, II and III), PPP (cG6PD), gluconeogesis and glycogenolysis (LDH and G6Pase) and glycolysis (GK) pathways were investigated in the present study, to identify changes in metabolic activity in the liver in response to HFD or treatment with metformin and two plant extracts. **Table 9.1** gives a summary of all the statistically significant changes of the liver parameters that are documented in this study. The values for the body weight blood parameters, liver FFA and TC are also presented in the table for comparison, although they were determined and documented by co-researchers (Karachi, 2009; Mackenzie, 2009). The strength of this study is the comparison that could be drawn

between LFD; untreated HFD induced fatty liver and the effect of two plant extracts or metformin in the prevention of fatty liver development. A further strength was that the HFD model was insulin resistance, but did not have fatty liver when treatment started. It is observed that these two plant extracts and metformin differ in their capacity to treat the development of HFD induced fatty liver.

In order to gain a proper perspective on the data presented in **Table 9.1** it is imperative to consider the exact meaning of the values indicated and how differences can be expected to impact on metabolism. As previously stated, the enzyme activities reported in this study represent a measure of the quantity of enzyme and do not reflect the *in vivo* metabolic activity per se. Enzyme activity assays were conducted under conditions of substrate saturation, which are far removed from the actual metabolite level in steady state. However, no method can reflect the complete physiological process, since metabolic processes are depending on substrates, cofactors, allosteric regulators, etc and active enzyme resulting in the liver, an important metabolic organ of the body, the combination of products is extremely complex due to the number of divergent metabolic processes. As with any technology, there are limitations which need to be taken into consideration, even proteomics cannot give a complete answer since it indicates the change in proteins and does not indicate the sequence of events leading to such change. Since this study only evaluated lipid content enzyme quantity using ex-vivo activity measurements as a surrogate, it is considered an explorative study to probe for potential metabolic alterations during the development of fatty liver or its treatment. It is however understandable that such a simplified approach does not provide sufficiently robust information to allow definitive conclusions regarding complex metabolic implications. It covers a period after the development of insulin resistance, four weeks at the beginning of the development of fatty liver. Therefore, the strength of this study is the fact that more than one treatment was used and different factors or changes could be evaluated against the development of HFD induced fatty liver. Altogether, the number of rats per studied groups also provided evidence of the metabolic enzyme changes and other measured parameters mediated by HFD and/or treatments in the liver of this model.

The aim of the study was to investigate whether metformin and aqueous extracts from *S. frutescens* leaves and *P. africana* bark can prevent fatty liver development after insulin resistance has been induced and to determine the contribution of the best known lipogenic, glycogenic and oxidative stress parameters that are affected by the specific HFD or treatment.

Table 9.1 Summary of parameters where one or more groups showed a significant change

	LF	HF	HF+Sf	HF+Pa	HF+Met
Body weight†	511±24.0	581±88.7**	547.3±8.1**#	523.2±24.6#	553.2±40.7
Blood parameters†					
Insulin (ng/mL)	0.6±0.2	1.6±0.9*	0.7±0.1#	4.4±1.3***##	1.0±0.5#
TG (mmol/L)	0.6±0.1	0.9±0.2*	0.6±0.0#	0.6±0.2#	0.7±0.3*
TC (mmol/L)	1.2±0.2	1.5±0.2*	1.3±0.1#	1.2±0.1##	1.3±0.1#
HDL-C (mmol/L)	0.3±0.1	0.3±0.0	0.2±0.0**#	0.2±0.1*#	0.4±0.1*#
FFA (µmol/L)	69.0±11.0	85.0±12.0*	48.3±3.0**###	75.0±9.0#	51.0±16.0*##
Liver parameters¥†					
TL (mg/g liver wet weight)¥	48.0±1.1	67.3±6.0***	61.8±3.9**	59.0±6.0**#	56.5±1.6*#
TG (mmol/g liver)†	2892 ± 142	4767.9 ± 369***	3443.4 ± 182.*#	ND	3004 ± 231.7#
FFA (mmol/g liver)†	45.8 ± 2.1	110.3 ± 11.5***	55.5 ± 3.9###	ND	70.4 ± 29.4##
Lipogenic enzymes¥					
cG6PD (ΔA/min/mg protein)	4.0±1.0	6.7±1.4*	7.5±0.9**	4.5±1.0#	7.3±2.2**
cACL (ΔA/min/mg protein)	32.0±4.9	29.3±3.9	32.9±3.1	41.6±6.0*##	41.1±2.3*##
mCS (ΔA/min/mg protein)	19.0±1.7	17.7±1.7	21.3±0.7#	18.0±1.9	20.0±1.9
Glucogenic enzymes¥					
hGK (ΔA/min/mg protein)	1.1±0.2	1.2±0.3	1.0±0.3	0.8±0.1#	1.1±0.1
miG6Pase (mM Pi//min/mg protein)	2.6±0.4	2.4±0.3	2.7±0.6	2.1±0.7	1.2±0.3**#
Oxidative stress markers¥					
hMDA (µM/mg protein)	190.7±47.9	423.2±177.4*	384.3±112.7	351.3±81.2	325.0±90.5
hProtein carbonyl level (mM/mg protein)	0.4±0.0	0.4±0.1	0.3±0	0.3±0.0	0.5±0.1*#
mTotal hydroperoxides level (mM/mg protein)	1.7± 0.2	2.2±0.2	2.1±0.1	2.2±0.1	2.6±0.6**
mH_2O_2 levels (µM/mg protein)	0.2± 0.1	0.8±0.1*	2.0±0.4**#	5.0±0.7***###	3.9±0.8***###
Pro-oxidant enzymes¥					
hXO (ΔA/min/mg protein)	122.1±16.8	160.8±22.3*			
mNOX (ΔA/min/mg protein)	0.2±0.1	0.5±0.1*	0.6±0.1**	0.5±0.2*	0.5±0.2*
mXO (ΔA/min/mg protein)	1.7±0.2	2.6±0.4*	2.7±0.6*	2.6±0.5*	2.6±0.5*
Enzymatic antioxidants¥					
hSOD (ΔA/min/mg protein)	17.6±8.8	2.9±0.0*	0.3±0.1**#	17.9±7.1#	17.5±3.1#
mGST (ΔA/min/mg protein)	7.7±0.7	7.4±1.0	5.4±0.9*#	8.2±1.4	6.5±0.5
Non-enzymatic antioxidant¥					
Total reduced thiol (mM/mg protein)	1.0±0.1	1.0±0.1	1.1±0.0**##	1.2±0.1**##	1.2±0.1**##
Respiration chain enzymes¥					
Complex II (ΔA/min/mg protein)	12.1±1.8	8.8±0.9*	6.7±1.7***	7.8±1.2**	6.5±1.8***#
Complex III (ΔA/min/mg protein)	1.5±0.2	1.6±0.5	2.1±0.3	2.8±0.3***###	2.5±0.4**#

*†, Indicates that body weight and blood parameters were reported by Karachi (2009) and Mackenzie (2009), respectively (**See Chapter 3 for the full data**). ¥, indicates that the liver parameters are reported in the present study (**See Chapters 5, 6, 7 and 8 for the full data**). Numbers in this table are rounded to one decimal place. **ND**, not*

*determined; **h**, homogenate; and **m**, mitochondria. * = significantly different from the LF control, ** P < 0.05, ** P < 0.01, *** P < 0.001; and # = significantly different from the HF, #P < 0.05, ##P < 0.01, ###P < 0.001.*

Table 9.2 Summary of liver parameters that showed no significant changes either with HFD or treatment when compared to LFD

	LF	HF	HF+Sf	HF+Pa	HF+Met
Glucogenic enzymes¥					
cLDH (ΔA/min/mg protein)	42.7±3.8	49.1±4.0	45.3±3.6	49.9±4.7	50.2±5.9
hG6Pase (mM Pi/min/mg protein)	1.2±0.2	1.0±0.1	1.1±0.6	1.0±0.1	1.0±0.1
Oxidative stress markers¥					
hTotal hydroperoxides level (mM/mg protein)	6.6±1.7	6.0±1.5	7.3±1.1	6.7±1.2	5.9±0.8
mMDA	-	-	-	-	-
Enzymatic antioxidants¥					
hGP (ΔA/min/mg protein)	22.0±2.4	21.7±1.5	22.1±1.4	20.8±1.3	23.1±2.1
hCT (ΔA/min/mg protein)	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1
hGR (ΔA/min/mg protein)	13.5±1.3	14.1±1.0	14.4±1.2	14.2±1.6	15.0±1.4
hGST (ΔA/min/mg protein)	85.3±10.3	86.4±5.7	84.6±10.5	85.0±8.7	86.0±15.1
mSOD (ΔA/min/mg protein)	16.2±1.2	17.5±2.3	16.5±1.7	17.2±2.0	19.5±2.2
mGP (ΔA/min/mg protein)	18.0±1.4	18.8±0.7	17.6±1.6	17.6±1.3	19.8±1.7
mCT (U/mg protein)	0.1±0.0	0.1±0.4	0.1±0.01	0.1±0.01	0.1±0.01
mGR (ΔA/min/mg protein)	1.3±0.1	1.43±0.2	1.3±0.1	1.3±0.1	1.5±0.1

*¥, Indicates that the liver parameters are reported in the present study (**See Chapters 5, 6, 7 and 8 for the full data**). Numbers in this table are rounded to one decimal place. -, undetectable; **h**, homogenate, and **m**, mitochondria.*

According to **Table 9.1** none of the treatments could successfully prevent the development of fatty liver, but all three treatments slowed down the development. Although there is no significant difference between the different treatments, one could suggest that efficacy of the treatments would be metformin>*P. africana* bark extract>*S. frutescens* leaves extract if the significant differences from LF and HF is taken into account. Hence, this outcome needs to be taken into account in the interpretation of all the results reflected in **Table 9.1**. Mackenzie (2009) measured the total hepatic TG and FFA on the same rats, except for the rats treated with *P. africana* bark extract and the same pattern as TL was reflected. TG reflects the same pattern, but

not FFA. This confirms the expectation that the lipids stored in the liver, contributing to fatty liver, is mainly TG.

At 12 weeks of feeding the rats on a HFD were insulin resistant, but did not have fatty liver. At what point they developed insulin resistance is not yet elucidated. At 16 weeks, the HFD rats had developed significantly fatty liver ($p<0.001$) comparing to LF, however looking at the three lipogenic enzymes measured in this study only cG6PD changed significantly in HF rats comparing to LF rats, but reflecting only a significance of $p<0.05$. It needs to be noted that the *S. frutescens* and metformin treated rats showed even a more significant increase in activity of cG6PD ($p<0.01$), but a lower lipid accumulation, and the *S. frutescens* treated rats had a further increase in activity of mCS ($p<0.05$) though less lipid accumulation than HF group. Activity of cACL was also significantly increased ($p<0.01$) in rats treated with *P africana* and metformin, but not in HF group, although the HF rats developed the most severely fatty liver. Suggesting that both metformin and *S. frutescens* activation of cG6PD may play a role in the clearance of FFA and glycerol imposed by excess intake of HFD, since glycerol serves as a substrate for gluconeogenesis that can contribute glucose towards the PPP, while FFA oxidation can support this process by providing ATP molecules leading to lower TG accumulation in the liver. These mechanisms indicate that fatty liver is clearly influenced by other factors than only these lipogenic enzymes. The extracted TL from the liver of the HFD-fed rats and LFD-fed rats validated that although HFD induced significant insulin resistance by week 12, but developed fatty liver only after insulin resistance by week 16. This may indicate that fatty liver development is a consequence of HFD induced insulin resistance, while its treatment with metformin or two plants' extracts improved insulin sensitivity, which led to lower fatty liver development in the presence of chronic HFD intake.

Findings presented in **Chapter 5** also agree with that reported by Ciapaite *et al*. (2011), that a long term of feeding on HFD leads to an incorporation of FFAs into lipids in the liver of the male Wistar rats. Sunny *et al.* (2011) established that a HFD induced insulin resistance is not accompanied initially by impaired mitochondrial β-oxidation. However, the data presented in **Table 9.1**, provides no information on the rate limiting step (CPT-1) of β-oxidation per se. Even if the changes in the mitochondrial electron transport chain were sufficiently substantial to

produce a restriction in the β-oxidation flux, such changes would impact on other oxidation processes as well, including glucose and amino acid metabolism as they also share the same metabolic endpoint. Furthermore, as discussed above, the magnitude of these alterations require further studies to establish the true meaning of such subtle changes. In this study no indication of NASH or fibrosis was observed, supporting the absence of significant oxidative stress. However, a long term study (e.g. feeding on HFD for 32 weeks) is needed to see wherther the present HFD can induce fatty liver progression to NASH and concomitant oxidative stress.

It is also clear that the different treatments used in this study work with different mechanisms, however the study showed that aqueous extracts of S. *frutescens* leaves and *P. africana* bark, as well as metformin prevented with different levels of efficiency, the development of fatty liver mediated by HFD. The one aspect that was unexpectedly noticed was that total reduced thiol content correlated inversely with the hepatic lipid accumulation with three treatments. However, HFD could not affect the total reduced thiol, suggesting that this diet may not induce hepatic oxidative stress, nor could the metformin and respective plant extract treatments. There rather appears to be an enhancement in the antioxidant capacity and/or redox status of the cell. In this regard, it is important to also take into account the common parameters which could not change as a result of treatment (**Tables 9.1**). In the present study considerable effort was invested to characterise the involvement of oxidative stress in both the development of fatty liver as well as the potential treatment with different agents. A number of oxidative stress markers, pro-oxidant enzymes and anti-oxidant enzymes were measured. Given that ROS can in addition to DNA, also damage proteins and lipids, various endpoint markers were evaluated. For lipid peroxidation, both hydroperoxides and MDA levels were investigated; while protein damage was measured as protein carbonylation. In addition, the levels of total reduced thiols, a reflection of the redox capacity and thus a marker of the overall reducing or antioxidant capacity of the cellular environment were also determined. With the exception of MDA levels, none of these oxidative stress markers were elevated in response to HFD and neither did the redox capacity decline (**Table 9.1**).

Considering that ROS has the propensity to interact with various biological molecules, one may expect that if the levels of ROS were sufficiently elevated to produce MDA (the end product of

lipid peroxidation), then it would also be sufficiently high to elevate lipid hydroperoxides and protein carbonylation. Excessive ROS would also deplete GSH with a concomitant decrease in the reduced thiol content and cG6PD activity. This is however clearly not the case as is evident from the stable lipid hydroperoxide and protein carbonylation as well as a total reduced thiol content. The assay used to quantify MDA is based on the reaction between MDA and thiobarbituric acid, a reaction that lacks absolute specificity towards MDA and can react with a number of other aldehyde groups making the assay susceptible to false positives. The fact that the elevation in MDA is not observed in the respective treatments may indicate that whatever substance is responsible for this effect is normalised upon treatment. The absence of significant oxidative stress is further evident by the lack of either a significant increase or decrease in the antioxidant enzymes catalase and glutathione peroxidase, both enzymes which respond to changes in the redox status (**Table 9.2**). Taken together these findings indicate that despite the induction of insulin resistance and fatty liver, the antioxidant capacity is maintained and oxidative stress avoided.

At first glance this may seem contradictory to the well published notion that oxidative stress is associated with the development of both insulin resistance and the progression of fatty liver (**See Chapter 2**). However, an alternative hypothesis which has been proposed is that in conditions of insulin resistance the increased lipolysis in fat storage tissues generates an overload of FFA and glycerol to the liver that are "cleared" via increased TG synthesis and gluconeogenesis. Since the oxidation of glucose may inevitably lead to the production of ROS via mitochondrial oxidation, the major cellular source of ROS, limiting glucose uptake and consequent oxidation may prevent the associated production in ROS. Similarly, excess FFA may be diverted from oxidation and concomitant ROS production by esterification and storage as TG, giving rise to fatty liver (Choi and Diehl, 2008; Gaggini *et al* 2013; Yamaguchi *et al*., 2007), indicating that in the chronic intake of HFD, fatty liver is a consequence of insulin resistance. The findings of the present study support such a hypothesis. Thus, HFD induced insulin resistance and fatty liver with normoglycemia precedes induction of hepatic oxidative stress (**Tables 9.1** and **9.2**). The absence of oxidative stress also appears to contradict the substantial increase in the mH_2O_2 levels. Although the relative increase in this oxidant is considerable, metformin treatment has a 5-fold higher level than the HFD-fed rats, the absolute levels remain in the micro-molar range, which

may be too small to have an impact on oxidative stress. This may be relevant in the sense that low ROS levels such as H_2O_2 play an important role in cell signalling pathways and it is only when the levels become excessive, which can represent a challenge to the antioxidant capacity of the cell (Kirkham and Rahman, 2006).

9.1.1 Final conclusions

More studies on the evaluation of the efficiencies of exact dosages that can be used in the prevention or in the treatment of insulin resistance and fatty liver are still needed. The present study is first of its kind to report that *S. frutescens* and *P. africana* extracts and metformin possess antioxidant capacity via increasing the reduced thiol levels in the liver of the present HFD rat model. Hence, *P. africana* bark aqueous extract is herein reported for the first time that it may attenuate HFD induced fatty liver, through a significant reduction of the TL accumulation. It can also be mentioned that both plant extracts and metformin may be used to enhance antioxidant capacity as shown in this HFD rat model. Therefore, the present study serves as the baseline in this HFD induced fatty liver and the promising effects of *P. africana* bark extract in the prevention of its induced fatty liver without changing the dietary habits.

There are number of parameters that are the same between *P. africana* extract and metformin and seem as if both are strong antioxidants. Both *P. africana* extract and metformin increased mitochondrial complex III and mH_2O_2 the most. Thus *P. africana* extract and metformin significantly lowered lipid accumulation in the liver by 12% and 16%, respectively. Elucidation of their most effective dosages remains vital in the future studies. *S. frutescens* delayed development of fatty liver by 8.9% reduction of lipid accumulating in the liver, but to a lesser efficacy relative to metformin and *P. africana* extract.

In the present study HFD induced fatty liver after insulin resistance has developed, which revealed that NAFLD may become a great threat to public health in South Africa as also demonstrated by Kruger *et al*. (2010) in the Western Cape Province. Educational campaigns about lifestyle related diseases is necessary and may help to decrease the risk of NAFLD and its associated liver complications in South Africa, as this liver disease is emerging in both non-obese and obese T2DM patients as well as in the asymptomatic patients.

9.2 Recommendations

The current explorative study highlighted the possible pathways that may be affected by a HFD to develop fatty liver and the possibilities of *S. frutescens* and *P. africana* extracts and metformin in preventing the development of fatty liver. The results in this study give some more clarity and possibilities, but the following questions arose and are recommended to be addressed in the future studies.

a) This study indicated that the lipid accumulation in the liver of HFD-fed rats is not the results of oxidative stress and therefore the question how the genes regulating lipid and glucose homeostasis should be further investigated in rats on the same diet. The specific genes that should be further investigated are: SREBP-1a and 1c, SREBP-2, ChREBP, LXR, PPAR-α, PPAR-γ, PGC-1α, PGC-1β, PPAR-δ
b) Do *S. frutescens* leaves and *P. africana* bark aqueous extracts reduced cholesterol secretion or decreased its absorption or elimination as bile acid in the liver or increased its use for biosynthesis of other tissue-specific steroid hormones when gavage the HFD-fed rats with insulin resistance?
c) Does metformin increased collagen synthesis in the liver as it has demonstrated to increased protein peroxidation?
d) Which are the *P. africana* extract's compound(s) involved in controlling the accumulation of lipid in the liver of HFD induced insulin resistance and fatty liver?

Elucidation of SREBP-1a, 1c, and 2 as well as ChREBP, LXR, PPAR-α, PPAR-γ1 and 2, PGC-1α, PGC-1β and PPAR-δ genes may provide novel knowledge on which of these genes are involved in the onset of this HFD induced fatty liver after week 12. A standard reference with known hepatoprotective effects, namely silymarin (active flavonoid complex from milk thistle) (Lin *et al*., 2009) is recommended to be used as positive control during the prevention of HFD induced fatty liver in the future studies.

Studying bile acid and steroids metabolism may be helpful to understand cholesterol metabolism of this HFD rat model. Moreover, FFAs, TG and glyceride-3-phosphate and FA oxidation need to be investigated in the liver of the HF+Pa group to validate *P. africana* extract's effects on TL

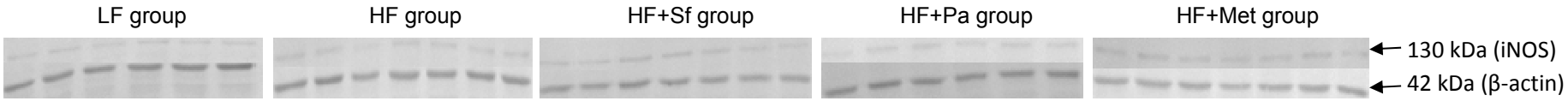
accumulation and FA oxidation in this tissue. Elongases (Elovls) 1 and 6 need to be elucidated at week 16 in order to study their expression during fatty liver development. Elovl 6 is known to be declined at birth and induced at weaning, while its expression during early postnatal parallels SREBP-1 nuclear is abundance (Wang *et al*., 2005). Both Elov-6 and SCD-1 are induced in response to excess SFA such as palmitic acid or carbohydrate as outline in **Chapter 2**, **Figure 2.5** (Ntambi and Kim, 2001). Hence CPT-1, Elovl-6 and SCD-1 are also recommended to be studied in the liver of the present rat model in the future studies. These may be achieved by measuring the Elovls-1, and -6, CPT-1 and SCD-1 mRNA expression with the use of the quantitative polymerase chain reaction (qPCR) techniques and enzymatic assays to confirm their activations.

The effects of *S. frutescens* and *P. africana* extracts on the VLDL, ApoB and MTP synthesis in the liver also need to be studied, to elucidate their mechanisms of action on how these plant extracts severely decreased the blood HDL-C levels of this rat model (Karachi, 2009; Mackenzie, 2009). The iNOS (inflammatory marker) and activated procaspase-3 (caspase-3, apoptotic marker) activities need to be assayed in the future studies to confirm their elucidated protein levels in the present study. Circulating glycerol, leptin, glucagon, adiponectine, lactate and ketone bodies levels remain elusive, and render to be measured in the present HFD rat model. These metabolites are also known to partake in the induction and/or attenuation of dietary induced insulin resistance and fatty liver. Insulin secretion and its degradation remain to be elucidated in both fed and fasted conditions. Furthermore, collagen stain can also be done on the liver sections to quantify fibrosis levels, especially the HFD-fed rats treated with metformin that showed increased in protein carboxylation. Aqueous extract of *P. africana* demonstrated to play a role in the regulation of lipid and glucose metabolic enzymes cACL and GK among other measured parameters, resulting in the improvement of fatty liver in this HFD rat model. Hence analysis of compounds of this plant extract that inhibit lipid accumulation in the liver and insulin resistance in the presence of HFD intake remains to be investigated in the future studies. These can be achieved by fingerprinting *P. africana*'s aqueous extract.

CHAPTER 10: List of appendixes

10. Appendixes

Appendix A: Western blots of iNOS



Appendix B. Western blots of procaspase-3

CHAPTER 11: List of references

10. References

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