THE EFFECTS OF MATERNAL DIETS, VARYING IN FAT CONTENT, ON PROXIMAL HEPATIC AND SKELETAL MUSCLE INSULIN SIGNALLING IN NEONATAL WISTAR RAT OFFSPRING

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KEYWORDS

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Free fatty acids

ABSTRACT

The incidence of type 2 diabetes (T2D) is persistently increasing globally. T2D is associated with pancreatic β cell dysfunction and insulin resistance in peripheral tissues such as the liver and skeletal muscle. Skeletal muscle is the major site for insulin stimulated glucose uptake. Maintenance on a gestational high fat diet may programme insulin resistance. Programming is induced by the exposure of organisms to either a stimulus or insult during foetal and/or early neonatal life and alters offspring physiology and metabolism. The aim of the present study was therefore to investigate the effects of maternal diets, varying in fat content, on neonatal hepatic and skeletal muscle gene (mRNA) and protein (immunoreactivity) expression of proximal insulin signalling factors: insulin receptor alpha (IRα), insulin receptor substrate 2 (IRS2) and phosphoinositide 3-kinase-p110 alpha (PI3K-p110α), and to assess the therapeutic potential of *Aspalathus linearis* extract after high fat programming.

Pregnant rats were randomised into groups maintained on diets with varying fat proportions: 10% (control), 20% (20F), 30% (30F) and 40% (40F) fat as energy throughout gestation. Neonatal liver and skeletal muscle were collected to determine the proximal insulin signalling expression profiles of the target factors: IRα, IRS2 and PI3K-p110α. Quantitative polymerase chain reaction (qPCR) was applied to determine mRNA expression of these target insulin signalling factors. Immunostaining of the target proteins in the liver and skeletal muscle was performed followed by relative quantification with image analysis software. Further, *Aspalathus linearis* (Al) extract was orally administered to mothers during gestation in the 10% (Control-Al) and 40% (HFD-Al) diets at a dose of 150 mg/kg. Body weight, food intake and blood glucose concentrations were monitored throughout gestation in mothers.

Maternal diets, varying in the percentage of fat content, showed no significant effect on neonatal hepatic IR and IRS2 mRNA expression. However, hepatic PI3K mRNA expression was elevated in 30F neonates compared to 20F neonates. Skeletal muscle IR and PI3K mRNA expression were reduced in the 30F and 40F neonates compared to 20F neonates. There was reduced hepatic IRα immunoreactivity in 40F neonates compared to control and 20F neonates. Further, skeletal muscle IRα immunoreactivity was significantly reduced in 30F and 40F neonates compared to control neonates. Therefore foetal high fat programming

reduced IRα in both the liver and skeletal muscle which may impair proximal insulin signalling in these glucose recipient organs. *Aspalathus linearis* had no effect on maternal serum insulin and glucagon concentrations. In addition, maternal caloric intake, body weight and organ weights (liver, brain and pancreas) were not altered amongst the groups. Further, HFD-Al neonates were heavier than control neonates. In conclusion, *Aspalathus linearis*, at a dose of 150 mg/kg, had neither harmful nor ameliorative effects in pregnant mothers fed high fat diet during gestation. In addition, *Aspalathus linearis* treatment had no ameliorative effects on neonates from mothers fed high fat diet throughout gestation.

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DEDICATION

This thesis is dedicated to my parents, Mr. Elliot D. Ndlovu and Mrs. Nokhaya Ndlovu, for their endless love, support and encouragement.

DECLARATION

I declare that the thesis The effect of maternal diets, varying in fat content, on proximal hepatic and skeletal muscle insulin signaling in neonatal Wistar rat offspring is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Signed: . . . . . May 2013

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APO B	Apo-lipoprotein B
ARC	Agricultural Research Council (South Africa)
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
CB1	Cannabinoid receptor type 1
CHOD-PAP	Cholesterol oxidase- phenol + aminophenazone method
CRK	Chicken tumour virus No.10 regulatory kinase
DAG	Diacylglycerol
EGSIR	European Group for the Study of Insulin Resistance
eIF2B	Eukaryotic initiation factors 2B
FFA	Free fatty acids
GABA	Gamma-aminobutyric acid
GCK	Glucokinase
GDM	Gestational diabetes
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GPO-PAP	Glycerol-3-phosphate oxidase- phenol + aminophenazone method
GRB2	Growth factor receptor-bound protein 2
GRK2	G-protein coupled receptor kinase 2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate

HDL	High-density lipoprotein
HFD	High fat diet
HOMA-IR	Homeostasis Model of Assessment - Insulin Resistance
HPLC	High-performance liquid chromatography
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-6	Interleukin 6
IR	Insulin receptor
IRR	Insulin related receptor
IRS	Insulin receptor substrate
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
IRα	Insulin receptor alpha
IRβ	Insulin receptor beta
LC-ACoA	Long-chain acyl-coenzyme A
LDL	Low-density lipoprotein
LGA	Large for gestational age
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MetS	Metabolic syndrome
Mtor	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acids
NGS	Normal goat serum
NHS	Normal horse serum
NPY	Neuropeptide Y

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pH	Protons of hydrogen
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
POMC	Pro-opiomelanocortin neurons
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5)-triphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase polymerase chain reaction
SEM	Standard error of the mean
SFA	Saturated fatty acids
SH2	Src homology 2
SOP	Standard operating procedure
SREBP	Sterol regulatory element-binding proteins
STZ	Streptozotocin
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TBP	TATA-binding protein
TBS	Tris buffered saline
TNF-α	Tumour necrosis factor alpha
US NIH	United States National Institute of Health
WHO	World Health Organization

CHAPTER 1

LITERATURE REVIEW

1.1 Diabetes

Diabetes is a chronic metabolic disorder characterised by an abnormal elevation in blood glucose concentrations (hyperglycaemia). Pancreatic β cells produce insulin, a hormone that stimulates glucose uptake from the blood stream into peripheral tissues. Diabetes may present due to β cell failure to synthesise and/or secrete insulin, or insulin resistance in peripheral (glucose recipient) tissues such as skeletal muscle, liver and adipose tissue (Lin and Sun, 2010). Type 1 (T1D), type 2 (T2D) and gestational diabetes mellitus (GDM) are the major forms of diabetes. T1D mostly occurs in children and adolescents and is an autoimmune disorder characterised by few or no pancreatic β cells that results in reduced insulin production (Ku *et al*., 2012). T2D is mainly due to β cell dysfunction and insulin resistance (Ku *et al*., 2012) although this association remains complex. GDM first occurs during pregnancy and may disappear upon delivery or progress to T2D. The symptoms of diabetes include thirst, excessive urination, unexplained weight loss, blue-red vision, lethargy and changes in energy production (Lin and Sun, 2010; Spellman, 2010).

1.1.2 Type 2 diabetes mellitus (T2D)

1.1.2.1 Overview on the prevalence of T2D

T2D has increased in all age groups globally. The number of people with diabetes was projected to increase from 171 million in 2000 to 366 million by 2030 (Wild *et al.,* 2004). This projection signals that the number of individuals with diabetes will be double by 2030. T2D is linked with an increase in obesity, one of the core factors in metabolic syndrome (MetS) and insulin resistance. An increase in T2D and obesity results due to nutritional transition to a westernised diet and sedentary lifestyle particularly in developing countries (Gupta *et al*., 2012). Nutritional transition can be driven by urbanisation, globalisation and social changes (Amuna and Zotor, 2008). People tend to change their traditional diets (plant based food) due to urbanisation to adopt more westernised diets. Traditional diets contain more fibre and low fat unlike westernised diets that contain low fibre, high fat and

carbohydrate content that may lead to obesity. Obesity is associated with development of T2D and cardiovascular diseases.

1.1.2.2 Overview of the pathophysiology of T2D

Physiologically, insulin is constantly synthesised by pancreatic β cells and stored in vacuoles regardless of circulating glucose concentrations (Lin and Sun, 2010; Spellman, 2010). Insulin release from the vacuoles is triggered by elevated circulating glucose concentrations. In addition to glucose uptake, insulin converts glucose to glycogen for internal storage in skeletal muscle and liver (Seino, 2012; Spellman, 2010). In response to hypoglycaemia, glucagon is released from the pancreatic α cells. On the other hand, the insulin is released from pancreatic β cells to maintain glucose homeostasis (Seino, 2012).

Glucose homeostasis is impaired in T2D. Apart from β cell failure, insulin resistance is the major pathophysiological event in T2D (Lin and Sun, 2010). Pancreatic β cell failure to synthesise and secrete insulin further exacerbates hyperglycaemia (Lin and Sun, 2010; Spellman, 2010). In addition, factors such glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum (ER) stress and adipokines can reduce pancreatic β cell mass and function (Weir and Bonner-Weir, 2013). These factors can directly or indirectly lead to pancreatic β cell failure and demise.

1.1.3 Gestational diabetes mellitus (GDM)

GDM is glucose intolerance first detected during pregnancy (Gobl *et al*., 2013). Pregnant women can be screened for clinical risk factors such as pre-diabetes, impaired glucose tolerance, maternity age (women >35 are more susceptible to GDM), overweight, obesity and family history of T2D (Gobl *et al*., 2013). GDM results from an inadequate supply of insulin to tissues for normal blood glucose regulation and it accounts for about 3-10% of pregnancies depending on the population (Buchanan and Xiang, 2005).

1.2 Metabolic Syndrome (MetS)

MetS is important for the identification and diagnosis of individuals at high risk for T2D and cardiovascular disease (Alberti *et al*., 2005). MetS refers to a cluster of metabolic risk factors, including central obesity, insulin resistance, dyslipidaemia, glucose intolerance and hypertension (Eckel *et al.*, 2010; Shen *et al*., 2003; Alberti *et al.*,2005) and is also known as syndrome X or the insulin resistance syndrome (Eckel *et al*. 2005).

Table 1: Metabolic syndrome as defined by the World Health Organization (WHO)

Core factors of MetS	Values
Diabetes or impaired fasting glycaemia, impaired glucose tolerance or insulin resistance	Hyperinsulinaemic, euglycaemic clamp-glucose uptake in lowest 25%
Obesity	BMI >30 or waist to hip ratio >0.9 (male) or >0.85 (females)
Dyslipidaemia	Triglycerides ≥1.7 mmol/L or HDL-cholesterol <0.9 (male) or 1.0 (female) mmol/L
Hypertension	Blood pressure ≥140/90 mmHg
Microalbuminuria	Albumin excretion >20µg/min
Fasting plasma glucose	Glucose concentration ≥6.1 mmol/L

Adapted from Eckel *et al* 2005

1.2.1 Factors contributing to MetS (Table 1)

1.2.1.1 Insulin resistance

The overabundance of circulating fatty acids is a major contributor to insulin resistance (Eckel *et al*., 2005). Plasma albumin-bound free fatty acids (FFA) are derived from adipose tissue triglyceride stores and released through the action of cyclic AMP-dependent enzyme hormone sensitive lipase (Eckel *et al*., 2005). Lipolysis of triglycerides in tissues by the action of lipoprotein lipase increases the concentrations of circulating of FFA (Wang and Eckel, 2009; Bruckert and Dejager, 1994). Insulin is important for both anti-lipolysis and the stimulation of lipoprotein. Plasma FFA can inhibit insulin stimulated glucose transport and phosphorylation in peripheral tissues and also inhibit glycogen synthase activity (Straczkowski and Kowalska, 2008). Elevated FFA inhibits insulin action on endogenous glucose production in the liver and enhances gluconeogenesis. The generation of excess acyl CoAs or acyl-CoA derivatives such as sphingolipid ceramide can reduce Akt1 activation

(Straczkowski and Kowalska, 2008; de la Monte *et al.*, 2010). Glycerolipids such as diacylglycerol (DAG) derived from long-chain acyl-coenzymes A (LC-ACoA) inhibit insulin receptor substrate 1 (IRS1) phosphorylation via activation of PKC-θ and PKC-ε, and phosphatidylinositol 3-kinase (PI3K) activity (Straczkowski and Kowalska, 2008; Erion and Shulman, 2010).

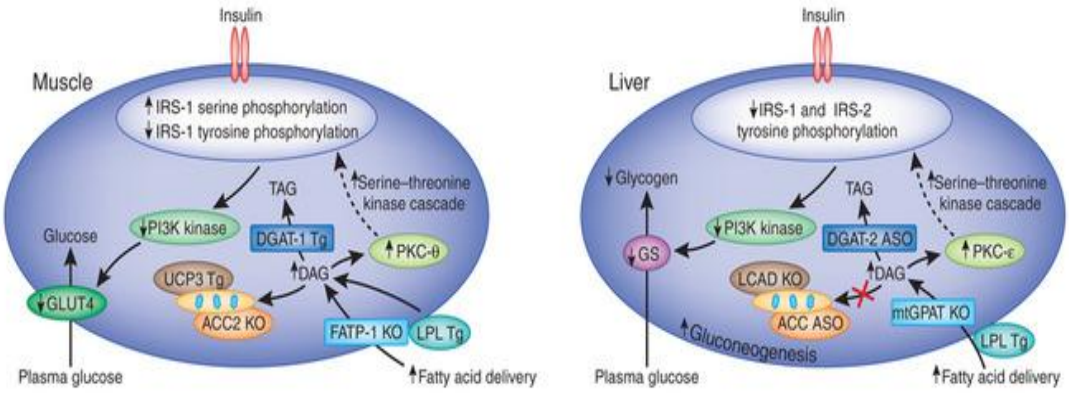


Figure 1.1: Molecular mechanism of insulin resistance in skeletal muscle and liver
Taken from Erion and Shulman, 2010

1.2.1.2 Central obesity

Central or abdominal obesity is defined by an increase in waist to thigh ratio, waist circumference and sagittal abdominal diameter (Iribarrene *et al.*, 2006). Obesity is linked to an increased risk of cardiovascular disease. Central obesity is associated with a higher risk of heart disease, hypertension, insulin resistance and T2D (Buettner *et al*., 2007).

Despite the importance of obesity in MetS, people of normal weight can also develop MetS (Ruderman *et al*., 1998; Hayes *et al*., 2010). Intra-abdominal adipose tissue-derived FFA can enter the liver through splanchnic circulation and have direct effects on hepatic metabolism (including glucose production and lipid synthesis) (Aubert *et al*., 2003).

1.2.1.3 Dyslipidaemia

Dyslipidaemia occurs with an increase in FFA flux to the liver and increased production of apo B-containing triglyceride-rich very low-density lipoproteins (Lewis *et al*., 1995; Darioli *et al*., 2002). Insulin resistance and increased flux of FFA to the liver increases hepatic triglyceride synthesis; however, under physiological conditions, insulin inhibits rather than increases the secretion of very low-density lipoproteins into the systemic circulation (Soska,

2003). This response is partially due to insulin's effect on the degradation of apo B (Eckel *at al*., 2005). MetS is also characterised by a reduction of high density lipoprotein (HDL) cholesterol. In the presence of hypertriglyceridaemia, a decrease in the cholesterol content of HDL results from decreases in the cholesteryl ester content of the lipoprotein core with variable increases in triglyceride rendering the particle small and dense (He *et al*., 2008). This change in lipoprotein composition also results in increased clearance of HDL from circulation (Tian *et al.*, 2010; He *et al.*, 2008).

1.2.1.4 Glucose intolerance

Glucose intolerance includes deficiencies in the ability of the hormone to suppress glucose production by the liver and kidney and to mediate glucose uptake and metabolism in insulin sensitive tissues such as the muscle, liver and adipose tissue (Eckel *et al*., 2005).

1.2.1.5 Hypertension

Insulin is a vasodilator when administered intravenously to people of normal weight (Eckel *et al*. 2005, Steinberg *et al*., 1994), with secondary effects on sodium reabsorption in the kidney (Eckel *et al*. 2005). In insulin resistant patients, the vasodilatory effect of insulin is usually lost (Tooke and Hannemann, 2000) whereas the renal effect on sodium reabsorption is preserved (Kuroda *et al.*, 1999). FFA can mediate relative vasoconstriction (Tripathy *et al.*, 2003). Insulin also increases the activity of the sympathetic nervous system, an effect that may be preserved in insulin resistance (Eckel *et al*., 2005).

1.3 Developmental programming

Growth and development *in utero* is a complex dynamic process requiring interacting components from the mother and foetus to sustain optimal foetal growth and survival throughout pregnancy (Warner and Ozanne, 2010). Programming refers to effects of an altered metabolic environment during a critical or sensitive period of development inducing immediate, transient or durable effects in offspring (O'Brien *et al*., 2008). The physiology and metabolism of the foetus and neonate can be altered by an insult or stimulus during critical developmental periods. Maternal nutrition plays an important role in the health of the offspring, with maternal high fat feeding inducing deleterious effects on pancreatic islet development and altering the expression, both at gene and protein level, of key genes involved in pancreatic β cell maintenance (Cerf *et al*., 2005; Cerf *et al*., 2006). An increase in

the prevalence of metabolic diseases in adult life is associated with an imbalance in energy intake and expenditure (Grant *et al*., 2011). Emerging evidence suggests that the response to metabolic challenges during postnatal life is modified by environment influences during foetal development (Grant *et al*., 2011). The exposure to an environmental insult at a critical stage has permanent effects on the structure of organs, tissue and systems in offspring (Grant *et al.*, 2011).

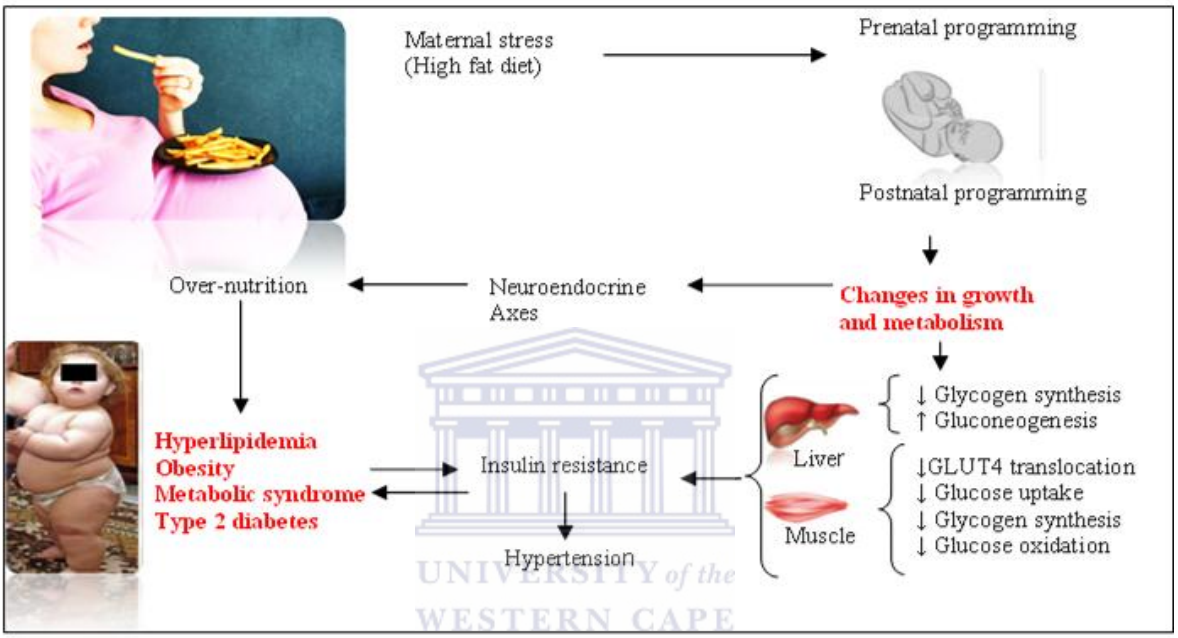


Figure 1.2: Developmental programming

Adapted from Alfaradhi and Ozanne, 2011

1.3.1 Maternal high fat diet

Strong evidence suggests that maternal nutrition during pregnancy can programme nutritional imbalances that may lead to obesity (Grant *et al.*, 2011). Among other factors, maternal obesity and GDM have been implicated in the development of metabolic disorders, impaired glucose tolerance and risk of developing obesity and diabetes in adulthood (Boney *et al*., 2005; Grant *et al*., 2011). An increase in the consumption of low nutrient dense foods (carbohydrates and saturated fat) was identified as major contributors of energy in a cohort of pregnant woman (Grant *et al*., 2011; Siega-Riz *et al*., 2002). The exposure to diets rich in animal fat during gestation and lactation may contribute to offspring obesity and the development of MetS (Ashino *et al*., 2011). Offspring from mothers fed a high fat diet

presented clinical features of MetS, lipid accumulation and activation of c-Jun N-terminus kinases consistent with the development of non-alcoholic fatty liver disease (Ashino *et al*., 2011). High fat diets directly insult β cells, which respond by increasing both replication and neogenesis resulting in increased β cell mass and hyperinsulinaemia (Gniuli *et al*., 2008). This dietary insult can be permanent on the β cell function of the foetus and also worsen in early neonatal life (Gniuli *et al*., 2008). Another study demonstrated that 12 week old offspring from mothers fed lard rich diets were insulin resistant compared to offspring from mothers fed polysaturated fat rich fish oil (Taylor *et al*., 2005).

1.3.2 Maternal obesity

Maternal high fat consumption often leads to maternal obesity. Maternal obesity has an independent effect on high fat feeding on programming adiposity, hyperphagia and insulin resistance (Alfaradhi and Ozone, 2011). Offspring exposed to maternal obesity during gestation and lactation while maintained on high fat diet displayed an increase in weight gain and fat mass (Shankar *et al.*, 2008). Maternal obese mothers displayed hyperleptinemia in both serum and milk during lactation compared to non-obese mothers (White *et al*., 2009). Offspring from maternal obese mothers were insulin insensitive compared to offspring from non-obese mothers suggesting that the exposure of neonates to maternal obesity induces an increase in body weight similar to the effect of offspring maintained on high fat diets in adult life (White *et al.*, 2009).

1.3.3 Impaired glucose tolerance and GDM

Obesity is a risk factor for the development of T2D and the development of glucose intolerance during gestation leading to GDM (Buchanan and Xiang, 2005). Reduced insulin sensitivity and inadequate insulin response are metabolic defects related to development of GDM (Buchanan and Xiang, 2005). Maternal hyperglycaemia leads to maternal insulin resistance that may lead to offspring obesity (Chandler-Laney and Bush 2011). Offspring from obese mothers with GDM developed intrahepatic fat which may lead to non-alcoholic fatty liver disease and insulin resistance (Brumbaugh *et al*., 2013). Insulin is known to induce adiposity; foetal hyperinsulinaemia is associated with adiposity in young and adult life (Chandler-Laney and Bush 2011). Therefore maternal glucose is hypothesised to increase foetal insulin concentrations that may alter foetal programming (Chandler-Laney and Bush 2011).

1.3.4 Hyperlipidaemia

Lipids also play a role in reducing insulin sensitivity as an increase in FFA concentrations impair the ability of insulin to suppress lipolysis in late gestation (Catalano, 2010). Increased FFA also provides energy in late gestation to meet maternal energy requirements. The increased FFA may also play a role in excessive foetal growth. Although foetal insulin production is stimulated by increased glucose production as observed in obese and gestational diabetic women, the nutrient substrates for foetal growth, particularly in adiposity, are less described (Catalano, 2010). Total cholesterol, HDL cholesterol and triglyceride concentrations increase in normal pregnancy compared to non-pregnant women (Chandler-Laney and Bush 2011). In comparison to lean pregnant women, obese pregnant women have higher triglyceride and lower HDL cholesterol concentrations (Chandler-Laney and Bush 2011). Elevated cholesterol and triglyceride concentrations either prior to or during pregnancy are associated with increased risk of complications during pregnancy such as preeclampsia, glucose intolerance and GDM (Chandler-Laney and Bush 2011). In a study in women with well controlled GDM, maternal FFA and triglyceride concentrations were positively associated with foetal abdominal circumference during the third trimester (Schaefer-Graf *et al.,* 2008). Those parameters measured close to delivery were positively associated with concentrations measured in cord blood, increased the risk of delivering large for gestational age (LGA) infants (Schaefer-Graf *et al.,* 2008). Elevated maternal triglyceride concentrations in these women were also associated with increased neonatal fat mass (Schaefer-Graf *et al.,* 2008).

1.3.6 Leptin

Maternal leptin is produced from adipose stores and the placenta (Henson and Castracane, 2006). During pregnancy and lactation, leptin modulates energy expenditure and metabolism (Stocker *et al*., 2005). During early life, both insulin and leptin function as trophic factors and have been shown to impact neuronal development during early life (Bouret and Simerly, 2004). The lack of leptin, as observed in *ob/ob* mice, has been shown to underlie the development of obesity in these mice (Bouret *et al*., 2004; Srinivasan *et al*., 2008). Abnormal insulin concentrations in the foetal or early postnatal periods induce alterations in the hypothalamic appetite regulating mechanisms resulting in adult onset obesity (Srinivasan *et al.,* 2008). The roles of leptin and insulin in regulating feeding behaviour in adults are well established. The functional leptin receptor, ObRb, is largely expressed in brain regions

known to control energy balance. Leptin administration in postnatal mice has been shown to act on neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons (Caron *et al*., 2010).

1.4 Insulin signalling

1.4.1 Insulin secretion

Circulating glucose is derived from three sources: (1) intestinal absorption during the fed state, (2) conversion of glycogen to glucose (glycogenolysis), and (3) generation of glucose (gluconeogenesis) from non-carbohydrate substrate such as lactate, glycerol and glucogenic amino acids in the liver (Aronoff *et al.,* 2004). Under physiological conditions, plasma glucose concentrations are determined by the rate at which glucose enters the circulation and balanced by the rate of glucose removal (glucose uptake) from blood (Aronoff *et al.,* 2004).

Pancreatic β cells are derived from neuroendocrine cells and equipped to sense nutrients, primarily glucose (Aronoff *et al.,* 2004). β cells function as glucose sensors with the important task of adjusting insulin release to control blood glucose concentrations (Maechler *et al*., 2005; Henquin, 2000). Insulin and glucagon are effective regulators of glucose metabolism (Aronoff *et al.*, 2004). The hormone, insulin, is synthesised and secreted by pancreatic β cells. Insulin is a small protein composed of two polypeptide chains containing 51 amino acids (Aronoff *et al*., 2004). The actions of insulin affect glucose metabolism, storage of ingested nutrients, glucose uptake by the cells and the use of glucose as the primary source of energy (Aronoff *et al.,* 2004). Insulin also promotes protein and fat synthesis (Aronoff *et al.,* 2004). Like other hormones, insulin's actions are mediated through binding to its receptors present on many cells of the body including myocytes, hepatocytes and adipocytes (Aronoff *et al*, 2004).

Insulin secretion occurs when glucose is metabolised in the β cell and the ATP/ADP ratio increases (Ashcroft *et al*., 1994; Winzell *et al*., 2006). This leads to closure of ATP-sensitive K^+-channels, depolarisation of the cell membrane and opening of voltage-gated Ca^{2+}-channels resulting in a rise in intracellular Ca^{2+} which, in turn, results in insulin excocytosis (Ashcroft *et al.,* 1994; Winzell *et al*., 2006). Insulin's primary action is to stimulate glucose clearance (Aronoff *et al*., 2004). The insulin signalling pathway is triggered when insulin stimulates insulin sensitive peripheral tissues such as the skeletal muscle and liver to increase

their glucose uptake (Aronoff *et al*., 2004). Insulin then acts on the liver to promote glycogenesis (Aronoff *et al*., 2004). Finally, insulin simultaneously inhibits glucagon secretion from pancreatic α cells and signals the liver to stop glycogenolysis and gluconeogenesis (Girard, 2006; Choukem and Gautier, 2008).

1.4.2 Overview of insulin signalling and insulin receptor (IR) phosphorylation

IR is a trans-membrane receptor (Ward and Lawrence, 2009; Patti and Kahn, 1998) that belongs to the sub-family of receptor tyrosine kinases that includes insulin like-growth factor (IGF) and insulin-related receptor (IRR) (Patti and Kahn, 1998; Saltiel and Kahn, 2001). IR is composed of two extracellular α subunits and two trans-membrane β subunits linked by disulphide bonds (Ward and Lawrence, 2009). Binding of insulin to the α-subunit causes a conformational change resulting in autophosphorylation of tyrosine residues present on β subunits (Van *et al.,* 2001). Adaptor proteins such as members of the insulin receptor substrate (IRS) family have phosphotyrosine binding domains that recognise the tyrosine residues present in the β subunit (Saltiel and Kahn, 2001; Lizcano and Alessi, 2002). IR activation leads to phosphorylation of the key tyrosine residues on IRS proteins, some of which are recognised by the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K; a lipid kinase) (Saltiel and Kahn, 2001; Ande and Mishra, 2009). The catalytic subunit of PI3K, p110, then phosphorylates PtdIns(4,5)P_2 on the plasma membranes of cells to generate the second messenger PtdIns(3,4,5)P_3 (Ande and Mishra, 2009). The key downstream effector of PtdIns(3,4,5)P_3 in insulin signalling is Akt or protein kinase B (PKB) (Lizcano *et al*., 2002). Akt binds to PtdIns(3,4,5)P_3 via a pleckstrin homology domain located at its amino terminus (Ande and Mishra, 2009; Scheid and Woodgett, 2003). This results in the recruitment of Akt from the cytosol to the plasma membrane where PtdIns(3,4,5)P_3 is located. The interaction between Akt and PtdIns(3,4,5)P_3 does not directly activate Akt, instead it recruits the other protein kinases that directly phosporylate Akt at Thr308 and Ser473 (Scheid and Woodgett, 2003). When Akt is activated, it dissociates from the plasma membrane and phosphorylates numerous substrates in the cytoplasm and nucleus, which play important roles in regulating insulin dependent processes that include glucose uptake, protein synthesis and the regulation of lipid synthesis (Lizcano *et al* 2002).

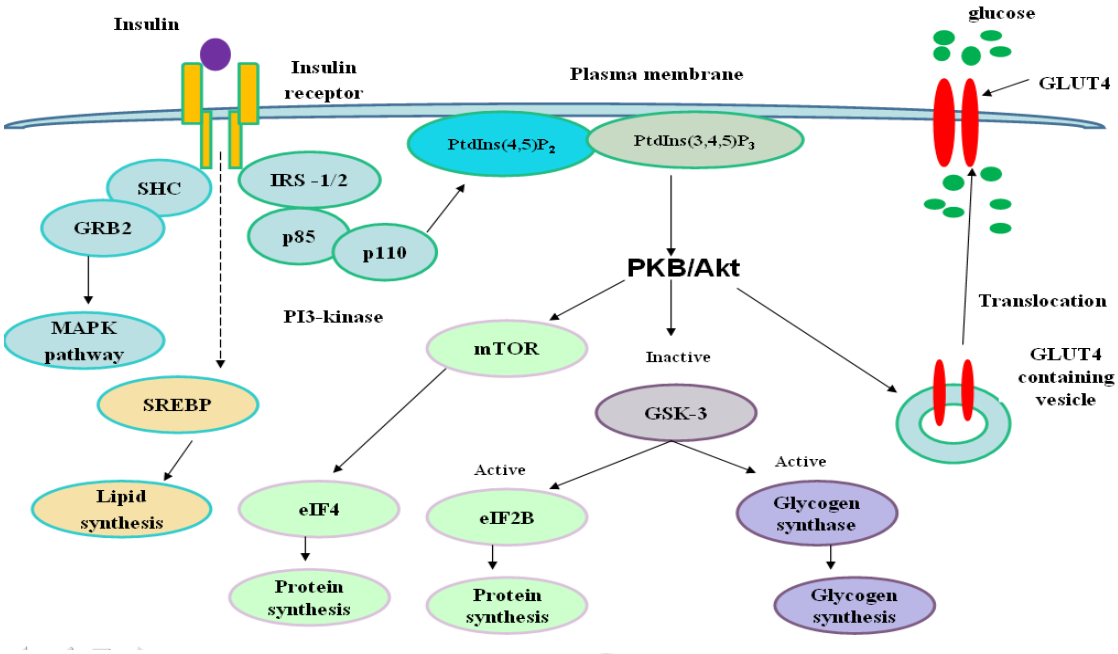


Figure 1.3: Insulin signalling pathway

Adapted from http://www.cellsignalling.com

Insulin is the primary hormone that controls both glucose and lipid metabolism. Insulin activates insulin receptor (IR) a tyrosine kinase that phosphorylates adaptor proteins that include the IRS family proteins. Phosphorylated IRS displays binding sites for PI3K (a lipid kinase). PI3K phosphorylates PtdIns(4,5)P_2 at the plasma membrane to generate a second messenger PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 indirectly activates Akt. Akt induces glycogen synthesis by inhibiting GSK-3, protein synthesis via mTOR and GLUT4 translocation to the plasma membrane.

1.4.3 Insulin receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) phosphorylation

1.4.3.1 IRS1

IRS1 is one of the key members of IRS family proteins (Gual *et al.*, 2005). IRS1 is tyrosine phosphorylated in response to insulin, cytokines and IGF-1 (Patti and Kahn, 1998). In response to insulin, IRS1 becomes tyrosine phosphorylated and recruits a number of SH2 containing signal transducers including PI3K (Gual *et al.*, 2005). Tyrosine phosphorylation of

IRS1 initiates insulin signalling. Reductions in tyrosine phosphorylation of IRS1 have been reported in 30% of subjects at high risk of obesity and type 2 diabetes (Gual *et al.,* 2005). Reduced IRS1 tyrosine phosphorylation and PI3K activation severely impairs insulin signalling and glucose transporter 4 (GLUT4) translocation in skeletal myocytes and adipocytes (Rains and Jain, 2011). The activation of serine/threonine kinases impairs IRS1 downstream signalling to PI3K and Akt which reduces GLUT4 expression and translocation to the plasma membrane (Rains and Jain, 2011). This subsequently reduces glucose uptake and metabolism leading to insulin resistance and eventually T2D (Rains and Jain, 2011).

Regarding insulin resistance, several studies have suggested that agents that induce insulin resistance such as pro-inflammatory cytokines (Kanety *et al*., 1995), FFA, cellular stresses and hyperinsulinaemia can also activate serine/threonine kinases that inhibit phosphorylation of IRS1 and inhibit its function (Gual *et al.,* 2005). Pro-inflammatory cytokine gene expression of IL6, TNF-α and MCP1 may also lead to insulin resistance and T2D (Rains and Jain, 2011). Several studies have reported that TNF-α inhibits tyrosine phosphorylation by promoting serine phosphorylation that changes conformation (Rains and Jain, 2011). Conformation changes reduce recruitment of PI3K and also stimulate the IRS1 degradation pathway (Rains and Jain, 2011).

1.4.3.2 IRS2

IRS2 is a cytoplasmic signal molecule that mediates the effects of insulin, IGF-1 and other cytokines by acting as a molecular adaptor between receptor tyrosine kinase and downstream effectors (Jiang *et al*., 2000; Valverde *et al*., 1998). IRS2 was initially identified as an alternative substrate in animals with targeted disruption of IRS1 (Sesti *et al.,* 2001). IRS2 contains 22 potential tyrosine phosphorylation sites with only 13 conserved in IRS1 (Sesti *et al.,* 2001). Both IRS1 and IRS2 may regulate unique signalling pathways in different tissues, subcellular locations, kinetics activation/deactivation or specific interactions with downstream effectors (Sesti *et al.,* 2001). In addition, both IRS1 and IRS2 mediate metabolic pathways. Hepatic IRS1 functions immediately after feeding and IRS2 functions mainly during fasting (Kubota *et al.,* 2008). IRS2 does not fully compensate for reduced levels of IRS1 and only partially compensates for improving insulin action on IRS2 (Hennige *et al.,* 2003). In mice, systemic failure of IRS2 causes peripheral insulin resistance followed by β cell failure and diabetes (Hennige *et al* 2003). IRS2 protects β cells from destruction by

streptozotocin and improves function in isolated β cells used in transplantation (Hennige *et al* 2003). Upregulation of IRS2 in β cells may help in treatment of diabetes (Hennige *et al* 2003).

1.4.4 Phosphatidylinositol 3-kinase (PI3K) signalling

PI3K is a heterodimetric lipid kinase with important functions in metabolic, cell growth, proliferation, differentiation and mitogenic actions of insulin (Foukas and Shepherd, 2004). PI3K comprises a heterodimer between the p110 catalytic subunit and p85 regulatory subunit with two SH2 domains that bind to phosphotyrosine motifs on the tyrosine kinase receptor or substrate (Foukas and Shepherd, 2004). The family is divided into three classes (I-III) (Foukas and Shepherd, 2004). The insulin sensitive tissues that include skeletal muscle, liver and adipose tissue express variations of regulatory subunits such as p50α and p55α (Chen *et al.*, 2004). IRS1 and IRS2 dock with p85 regulatory subunits that activate the p110α catalytic subunits (Chen *et al*., 2004). PtdIns(4,5)P_2 is phosphorylated on the 3-OH position of the inositol ring to produce PtdIns(3,4,5)P_3 and formation of this lipid recruits Akt and protein kinase C (PKC) (Chen *et al.*, 2004). The insulin action of PI3K is impaired in skeletal muscle from obese insulin resistant and T2D patients (Krook *et al.*, 2000; Cusi *et al.*, 2000).

PI3K is involved in insulin stimulated glucose uptake, basal glucose transporter recycling and phosphorylation of other intracellular second messengers (O'Brien *et al*., 2008). The association of p85 with IRS1 is an indirect marker of insulin stimulated PI3K activation and therefore insulin sensitivity (LeRoith *et al*., 1995; Rordorf-Nikolic *et al*., 1995; White, 1997).The catalytic p110α subunit plays a critical role in hepatic insulin/PI3K signalling and is required for normal glucose and lipid homeostasis (Sopasakis *et al*., 2010). A lack of p110α results in impaired insulin signalling with a decrease in activation of downstream molecules including Akt (Sopasakis *et al*., 2010).

1.4.5 Biological actions of insulin in metabolism

1.4.5.1 Glucose uptake

IR activation leads to the phosphorylation of cannabinoide receptor type 1 (Cbl), which is associated with the adaptor protein, catabolite activator protein (CAP) following phosphorylation of the Cbl-CAP complex and translocation to lipid rafts in the plasma membrane (Saltiel and Kahn 2001; Gupte and Mora 2006). Cbl then interacts with the

adaptor protein, chicken tumor virus no. 10 (CT10) regulator of kinase (Crk) that is constitutively associated with the Rho-family guanine nucleotide exchange factor, C3G (Gupte and Mora 2006). C3G in turn activates members of the guanosine triphosphate (GTP)-binding protein family, TC10, which promotes GLUT4 translocation to the plasma membrane (Saltiel and Kahn 2001, Lizcano and Alessi 2002). Glucose is then taken up into the insulin sensitive tissues for metabolism and/or storage.

1.4.5.2 Regulation of lipid synthesis

Insulin promotes the uptake of FFA and the synthesis of lipids whilst inhibiting lipolysis. Lipid synthesis requires an increase in the transcription factor, steroid regulatory element binding protein (SREBP) (Shimomura *et al*., 1999). Insulin inhibits lipid metabolism by decreasing the cellular concentrations of cAMP by activating a cAMP specific phosphodiesterase in adipocytes (Kitamura *et al.,* 1999).

1.4.5.3 Protein synthesis

Insulin stimulates amino acid uptake into cells, inhibits protein degradation and promotes protein synthesis (Saltiel and Kahn 2001). Under basal conditions the constitutive activity of glycogen synthase kinase (GSK3) leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor, eIF2B, which regulates the initiation of protein translation (Asnaghi *et al*., 2004). Therefore, upon receiving an insulin signal, inactivation of glycogen synthase kinase 3 GSK3 by Akt leads to the dephosphorylation of eIF2B thereby promoting protein synthesis and the storage of amino acids (Lizcano and Alessi, 2002). Akt also activates mammalian target of rapamycin (mTOR) that promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) (Asnaghi *et al.,* 2004).

1.4.6 Insulin resistance

1.4.6.1 Skeletal muscle insulin signalling and resistance

Skeletal muscle is the major site for insulin stimulated glucose uptake (Lin and Sun, 2010; Abdul-Ghani and DeFronzo, 2010). Approximately 75% of insulin stimulated glucose uptake occurs in the skeletal muscle (Lin and Sun, 2010). Skeletal muscle utilises both glucose and FFA as energy sources (Abdul-Ghani and DeFronzo, 2010). Plasma insulin concentration is the co-factor for glucose uptake in skeletal muscle and suppresses lipolysis (Abdul-Ghani and

DeFronzo, 2010). During fasting, skeletal muscle glucose uptake is low and plasma FFA concentrations are increased; FFA therefore serve as the main energy source during fasting (Abdul-Ghani and DeFronzo, 2010). T2D patients may have impaired insulin stimulated tyrosine phosphorylation of IRS1 in skeletal myocytes that may not be related to a decrease in protein expression of IRS1 (Lin and Sun 2010). Similar observations were evident at the level of PI3K in fatty obese rats (Asano *et al*., 2007). IR and IR protein family dysregulation is the common feature of insulin resistance in skeletal muscle. This dysregulation may include TNFα mediated and kinase mediated serine/threonine phosphorylation, proteasome mediated degradation and phosphatase mediated phosphorylation, all resulting in impaired glucose transport in skeletal myocytes. Excessive intake of a palatable cafeteria (but low fat) diet induces obesity and insulin resistance both at whole body and skeletal muscle levels (Brandt, De, Richter, and Hespel, 2010). G-protein coupled receptor kinase 2 (GRK2) is a key modulator of insulin sensitivity in vivo (Garcia-Guerra *et al.,* 2010). In cultured myoblasts and adipocytes, increased GRK2 levels inhibited insulin stimulated glucose uptake and signalling in a kinase activity independent manner by mechanisms involving the formation of dynamic GRK2/IRS1 complexes (Garcia-Guerra *et al* 2010).

1.4.6.2 Hepatic insulin signalling and resistance

In the liver, insulin regulates fasting glucose concentrations by inhibiting hepatic glucose production and stimulating glycogen synthesis. Glycogenolysis and gluconeogenesis are two different mechanisms involved in hepatic glucose production (Weickert and Pfeiffer, 2006). Glycogenolysis produces glucose in a relative short period within several hours of fasting (Weickert and Pfeiffer, 2006). Glycogenolysis is then suppressed by insulin after 1-2 hours after food intake (Weickert and Pfeiffer, 2006). During longer periods of fasting, the liver glycogen stores become depleted and gluconeogenesis kicks in by producing glucose from precursors such as pyruvate, lactate, glycerol and glucogenic amino acids (Weickert and Pfeiffer, 2006).

At the molecular level, the IRS2/PI3K pathway is the major signal transduction pathway in the liver; abnormal IRS2 signalling is closely associated with hepatic insulin resistance (Valverde *et al.,* 2003). In the liver, insulin also stimulates glycolysis, glycogen synthesis and the synthesis of long chain fatty acids while suppressing lipolysis (Rebrin *et al*., 1996). When the amount of p85α or p85β was increased in the liver of obese and diabetic *ob/ob* mice,

glucose tolerance was substantially increased and blood glucose concentrations reduced to normoglycaemic levels (Park *et al*., 2010). Tyrosine phosphorylation of IRβ, IRS1, IRS2 and the interactions between IRS1 or IRS2 and PI3K declined significantly in hepatic ischemia/reperfused rats suggesting that insulin signalling was impaired after hepatic ischemia/reperfusion (Liu *et al.,* 2008). Hepatic ischemia/reperfusion inhibited insulin secretion and induced insulin resistance via reduced tyrosine phosphorylation of IRβ, IRS1, IRS2 and the interactions between IRS and PI3K in rats (Liu *et al*., 2008).

1.5. *Aspalathus linearis* (rooibos)

1.5.1 Taxonomy

Rooibos tea is a popular South African herbal tea and is derived from Afrikaans translated as red bush. Rooibos tea is produced from the leaves of the indigenous South African Cape fynbos plant called *Aspalathus linearis* (Figure 1.4) (Beltran-Debon *et al.,* 2011). *Aspalathus linearis* is a member of the family *Fabaceae* under the tribe *Crotalaria* (Joubert *et al*., 2008). Rooibos grows naturally in certain areas in the Western Cape Province of South Africa (Beltran-Debon *et al.,* 2011).

Figure 1.4: *Aspalathus linearis* (rooibos)

1.5.2 History and the discovery of rooibos

In 1771, the Swedish botanist Carl Thunberg reported that the South African Khoisan tribe drank tea from a "good plant" (rooibos) for health purposes (Figure 1.4) (Joubert *et al.*, 2008). Rooibos was also later discovered by Dutch settlers as a cheap alternative to black tea imported from China and India. In the 1900s, the Russian settler and tea tradesman Benjamin Ginsberg developed an interest in rooibos brewing (Joubert *et al*., 2008) and applied the method of traditional Chinese fermenting of Keemun tea.

In recent years, green tea and herbal teas have gained popularity due to their health promoting, particularly antioxidant, properties. Currently rooibos tea consumption has gradually increased since its introduction to the domestic market in 1904 by Ginsberg (Joubert *et al*., 2008). In 2010, rooibos tea secured 23% of the South African tea market exceeding 500 tons (Joubert and de Beer, 2012). Rooibos tea is estimated to be consumed in 10.9 million households (Joubert *et al*., 2008)

1.5.3 Major chemical constituents of rooibos

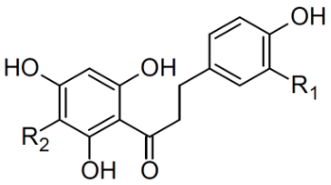
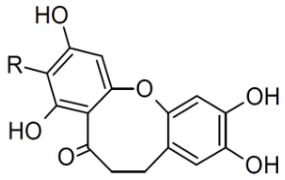
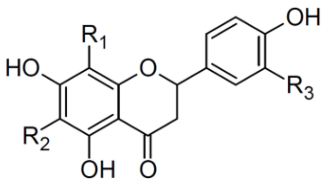
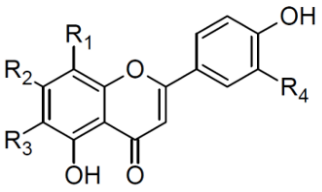
Flavonoids hold a remarkable range of biochemical and pharmaceutical properties that include anti-inflammatory, anti-oxidant, anti-carcinogenic and anti-thrombotic properties (Snijman *et al*., 2007). Rooibos is the only known natural source of a C-C linked dihydrochalcone glucoside, aspalathin and a cyclic dihydrochalcone, aspalinin (Joubert *et al.*, 2008). These rare compounds are unique to rooibos. Nothofagin, a 3-dehydroxy dihydrocholcone glucoside, has previously been shown to be present in *Nothofagus fusca*

(heartwood) and is the second most abundant flavonoid in rooibos after aspalathin (Joubert *et al.,* 2008). Rooibos also contains several C-C linked β-D-glucopyranosides, namely, the flavones orientin, iso-orientin, vitexin and iso-vitrxin; and the flavanones dihydro-orientin, dihydro-iso-orientin and hemiplorin (Joubert *et al.*, 2008).

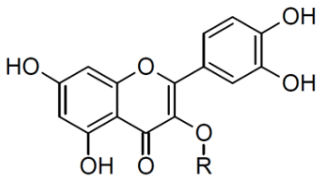
1.5.4 Health benefits of rooibos

Oxidative stress is caused by an imbalance in the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Bansal and Bilaspuri, 2010). The elevated production of ROS has been suggested to play a role development of insulin resistance (Kawano *et al*., 2009). In a physiological state, ROS is removed by internal enzymatic and non-enzymatic processes and the failure of these two mechanisms may lead to an elevation of ROS (Ulicna *et al*., 2006), thus leading to pathological complications such as insulin resistance (Ulicna *et al*., 2006). Compounds that possess anti-oxidant properties can prevent insulin resistance and T2D by lowering oxidative stress and hence maintain glucose homeostasis (Kawano *et al*., 2009). Numerous studies have been conducted on the anti-oxidant, anti-cancer and anti-diabetic properties of rooibos tea. Rooibos tea, particularly non-fermented (green rooibos), contains large amounts of flavonoids and other anti-oxidants with potent scavenging for ROS (Bramati *et al*., 2003; Joubert *et al*., 2010). Studies have reported on the anti-oxidant activity of rooibos tea using different extracts and assays. Aspalathin, a major flavonoid of rooibos tea, improved glucose tolerance in type 2 diabetic model *db/db* mice (Kawano *et al*., 2009). In humans, a recent study provided clinical evidence that the chronic consumption of rooibos tea significantly improved several biomarkers of blood lipid status (Marnewick *et al*., 2011). Rooibos tea reduced oxidative stress by lowering lipid peroxidation and improved redox status in adults at risk for cardiovascular diseases (Marnewick *et al*., 2011).

Table 1.2: Major chemical constituents of rooibos (Joubert *et al.*, 2008; Joubert *et al.*, 2012)

Compound type, names and substituents	General structure
Dihydrochalcones Aspalathin: R1 = OH, R2 = C-β-D-glucosyl Nothofagin: R1 = H, R2 = C-β-D –glucosyl	
Cyclic dihydrochalcone Aspalalinin: R = C-β-D-glucosyl	
Flavanones Hemiphlorin: R1 = C-β-D-glucosyl, R2 = R3 = H *(R)/(S)*-eriodictyol-8-glucoside : R1 = C-β-D-glucosyl, R2 = H, R3 = OH *(R)/(S)*-eriodictyol-6-glucoside: R1 = H, R2 = C-β-D-glucosyl, R3 = OH	
Flavones Orientin: R1 = C-β-D-glucosyl, R2 = R4 =OH, R3 = H Isoorientin: R1 = H, R2 = R4 =OH, R3 = C-β-D-glucosyl Vitexin: R1 = C-β-D-glucosyl, R2 = OH, R3 = R4 = H Isovitexin: R1 = R4 = H, R2 = OH, R3 = C-β-D-glucosyl Luteolin: R1 = R3 = H, R2 = R4 = OH Luteolin-7-O-glucosided: R1 = R3 = H, R2 = O-β-D-glucosyl, R4 = OH Chrysoeriol: R1 = R3 = H, R2 = OH, R4 = OCH3	

Flavonols



Quercetin: R = H

Isoquercitrin: R = O-β-D-glucosyl

Hyperoside: R = O-β-D-galactosyl

Rutin: R = O-β-D-rutinosyl

Quercetin-3-O-β-D-robinosideg: R = O-robinosyl

CHAPTER 2

STUDY AIMS

2.1 Study aim

To investigate the effects of maternal diets varying in fat content on neonatal gene and protein expression profiles of proximal insulin signalling factors (IRα, IRS2 and PI3K-p110α).

2.2 Specific objectives

- To determine the maternal circulating total cholesterol, triglyceride and free fatty acid concentrations.
- To determine the relative gene and protein expression profiles of IRα, IRS2 and the p110α subunit of PI3K in neonatal skeletal muscle and liver.
- To assess the therapeutic potential of *Aspalathus linearis* (green rooibos) on the programming effects of a high fat diet.

2.3 Expected outcomes

- Exposure to varying dietary fat content, *in utero*, may have differential effects on the expression profiles of insulin signalling factors in neonatal skeletal muscle and liver.
- A high fat diet may programme neonatal insulin resistance characterised by reduced expression of proximal insulin signalling factors.
- *Aspalathus linearis* supplementation may ameliorate the adverse programming effects of a maternal high fat diet in neonatal offspring.

CHAPTER 3

MATERIALS AND METHODS

3.1 Main study

3.1.1 Experimental design

The animal experiments were carried out according to the protocol approved by the Medical Research Council ethics committee (Ref. 07/11). Virgin Wistar rats (3 months old), weighing 220-275 g, were individually housed and mated overnight with pregnancy confirmed by presence of vaginal plug(s). The pregnant rats were then randomly assigned to groups. The groups (n = 4 rats per group) were 10% fat (as energy) diet (control), 20% fat (as energy) diet (20F), 30% fat (as energy) diet (30F) and 40% fat (as energy) (40F or high fat diet) (Table 3.1). Therefore the groups were control (10% fat diet), 20F, 30F and 40F mothers and their one-day-old neonatal offspring (Table 3.1). The pregnant rats had free access to food and water and were housed at a temperature of 22-25ºC, humidity of 45-55% and a 12 hour light/dark (light daily from 06h00-18h00). The rats were treated in accordance with the United States National Institutes of Health (NIH) guidelines for animal care and usage.

Table: 3.1 Experimental diets

Macronutrient	Control	20F	30F	40F
Fat (%)	10.69	20.68	31.00	40.17
Protein (%)	15.13	15.09	15.77	15.09
Carbohydrate (%)	74.16	64.22	53.23	44.73
Total kcal/100g	453.37	525.51	554.08	600.81

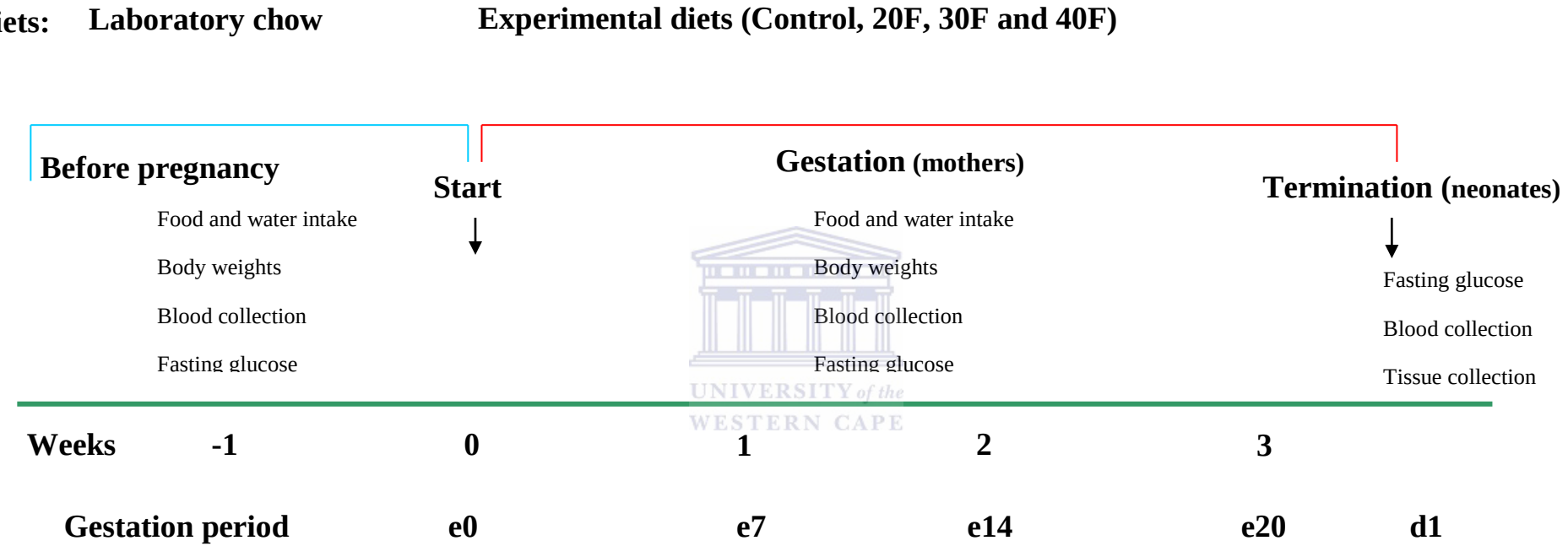


Figure 3.1 Experimental timeline

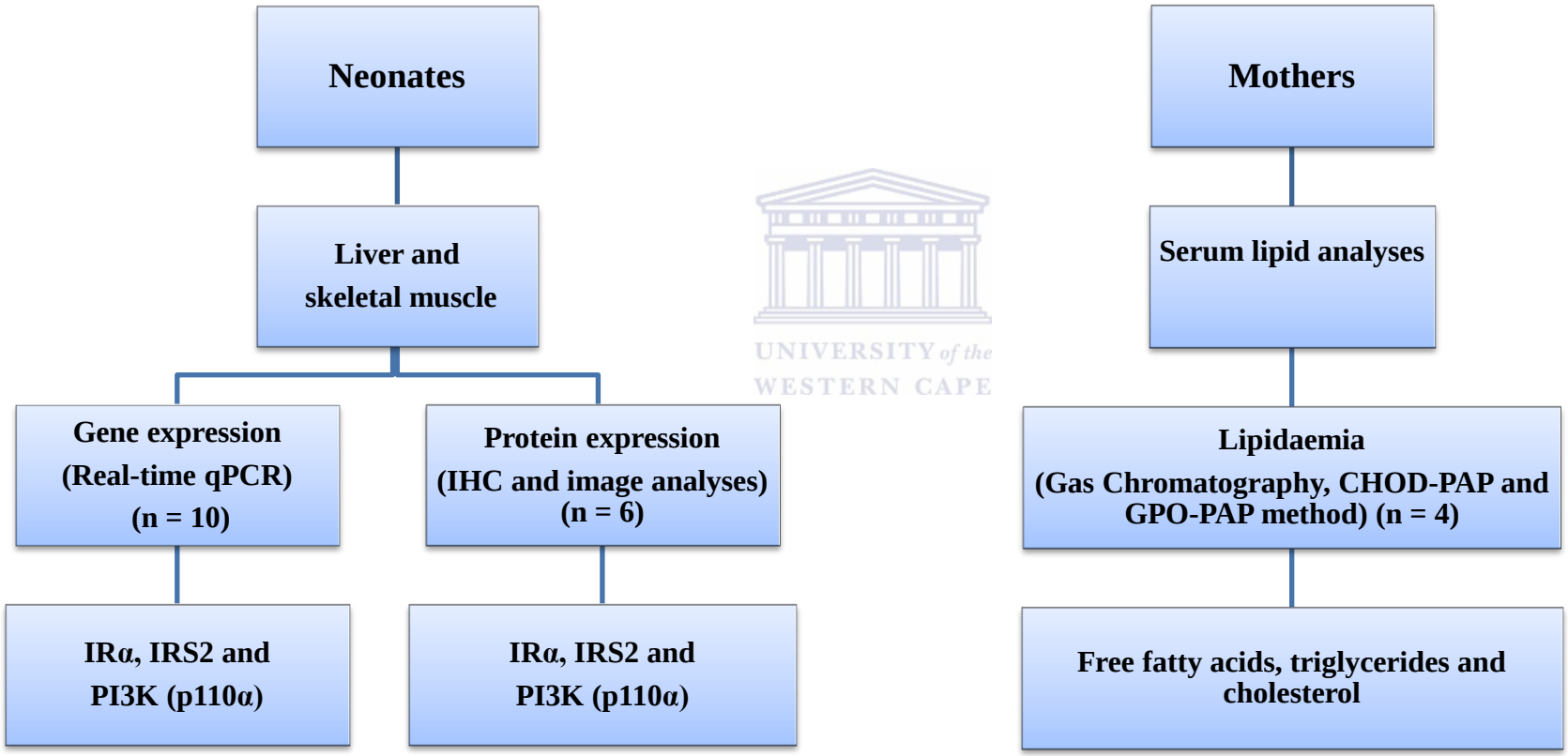


Figure 3.2 Experimental design (main study)

3.1.2 Maternal and neonatal blood collection

Blood analyses were performed to determine the circulating lipid profiles in mothers and neonates. Pregnant rats were fasted for four hours; a volume of 0.5 ml blood was collected weekly from the tail vein. Blood was collected before mating, at days 7, 14 and 20 of gestation (e7, e14 and e20 respectively) and on the day of delivery (d1). On d1, the mothers were euthanized and blood was immediately collected from the aorta with 6 ml syringes and transferred to tubes (BD Vacutainer blood collection tubes, BD, New Jersey, USA) for the determination of serum insulin, glucagon, free fatty acid (FFA; total and individual), total triglyceride and total cholesterol concentrations. Neonatal offspring were euthanized on postnatal day one (d1). Trunk blood from the litter of neonates per dam was pooled for sampling (a minimum of 500 µl per sample) for each experimental group.

3.1.3 Determination of serum cholesterol and total triglyceride concentrations

In mothers, serum triglyceride concentrations were determined by the GPO-PAP method with the enzymatic colorimetric test for triglycerides with clearing factor (LCF) and measured in an autohumalyzer A5 (Human Biochemical and Diagnostics, Wiesbaden, Germany). Serum total cholesterol concentrations were determined by the CHOD-PAP method with the enzymatic colorimetric test for cholesterol with LCF and measured with an autohumalyzer A5 (Human Biochemical and Diagnostics).

3.1.4 Determination of total free fatty acids (FFA) concentrations

FFA were extracted from maternal serum samples. A volume of 200 µl of serum was added to 3 ml methanol + butylated hydroxytoluene and 50 µl of internal standard for quantification of FFA then vortex mixed for 15 seconds. A volume of 6 ml of chloroform was added and the mixture was centrifuged at 2500 rpm for 10 min. The lower chloroform layer was removed and re-suspended into a clean test tube. The chloroform layer was then evaporated completely in a water bath at 37°C for 15 min. When dried completely, 2 ml of transmethylating reagent was added. Hexane (HPLC grade) was used for FFA extraction. The hexane phase was re-suspended into a clean test tube and evaporated completely in a water bath at 37°C. Carbon disulphide was added to re-dissolve lipids and injected in gas chromatography.

3.1.5 Relative PCR quantification

3.1.5.1 Housekeeping gene selection

In skeletal muscle, β-actin and TBP displayed the most stable expression in rat gastrocnemius and soleus muscles (Yuzbasioglu *et al*., 2010). In the liver, β-actin and TATA-binding protein (TBP) were the most stable housekeeping genes in the regenerating mouse model (Tatsumi *et al*., 2008). Therefore β-actin and TBP were selected as housekeeping genes for qRT-PCR analyses.

3.1.5.2 RNA preparation and quantitative RT-PCR

Skeletal muscle and liver were harvested; snap frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from 100 mg of skeletal muscle and liver using QIAzol lysis reagent (Qiagen, Hilden, Germany). RNA was purified using RNeasy mini kits according to the manufacturer's instructions (Qiagen, Hilden, Germany). The RNA samples were then treated with TURBO DNA-free kits (Ambion, Applied Biosystems, Foster City, California, USA) to remove any contamination with genomic DNA. The RNA yield and quality were assessed using a nanodrop spectrophotometer and RNA integrity was assessed using an Agilent Bioanalyser and Agilent RNA 600 nano kit (Agilent technologies, Santa Clara, CA, USA). A total of 1 µg total RNA per sample constituted to 10 µl with sterile water was reverse transcribed into first strand complementary DNA (cDNA) using a high capacity cDNA kit (Applied Biosystems). A positive control was prepared by constituting 1 µg of Amb rat liver RNA control template to 10 µl with sterile water and the negative control by adding 10 µl of sterile water to a PCR tube. The PCR tubes were prepared in duplicate and placed in a thermal cycler (Applied Biosystems 2720). Taqman probes for insulin receptor, insulin substrate 2 and PI3K were used along with the housekeeping genes β-actin mRNA and TBP mRNA. Quantitative PCR was performed using the applied Biosystems 7500 RT-PCR system and the Power SYBER Green PCR kit (Applied Biosystems) using a cycler programme consisting of an activation step of 10 min at 95°C, 40 cycles with a 15 second denaturing step at 95ºC and 1 min at 60ºC for annealing and extension. Data analysis was performed with 7500 SDS software to run a relative quantification plate and to analyse the RT-PCR results. The relative mRNA expression was expressed as the quantity mean of IR, IRS2 and PI3K divided by an average quantity mean of β-actin and TBP.

3.1.6 Tissue collection and processing for immunohistochemistry (IHC)

Skeletal muscle and liver were collected in 10% buffered formalin and processed in paraffin wax for IHC followed by image analysis.

3.1.7 IHC

Neonatal skeletal muscle and liver were fixed in 10% buffered formalin (pH 7.4) for 12 hours, processed using Leica TP11020 Automatic Tissue Processor (Leica, Wetzlar, Germany) and embedded in paraffin wax. Sections of 4 µm thick were cut using a Leica RM2125 RT rotary microtome (Leica) and mounted onto APES-coated microscope glass slides. Serial wax sections of skeletal muscle and liver were rehydrated through xylene, alcohol and distilled water. The sections were then incubated in 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature, placed in heat resistant staining jars filled with 1.01 M citrate buffer (pH 6.0) and cooked in a Dako Pascal pressure chamber (Dako cytomation, DK-2600, Glostrup, Denmark) (125°C for 3 minutes; 90°C for 30 seconds). All sections were then jet washed with 50mM Tris buffer (pH 7.2) for 5 minutes at room temperature.

Sections immunostained for IRα (1:500; Abcam, Cambridge, UK) were blocked with normal goat serum for 20 minutes, incubated with IRα overnight at 4°C then incubated in 1:200 biotinylated anti-rabbit IgG (Vector, Laboratories, Burlingame, CA, USA) for 30 minutes. Sections immunostained for IRS2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were blocked with normal goat serum for 20 minutes, incubated with insulin receptor substrate 2 overnight at 4°C then incubated in 1:200 biotinylated anti-rabbit IgG (Vector, Laboratories) for 30 minutes. Sections immunostained for PI3K-p110α antibody (1:10; Cell Signalling Technology, Danvers, MA, USA) were blocked with normal horse serum for 20 minutes, labelled with PI3K-p110α overnight at 4°C, then incubated in 1:200 biotinylated anti-rabbit IgG (Vector Laboratories) for 30 minutes. All sections were then washed with 50 mM Tris buffer (pH 7.2) for 5 minutes and incubated with ABC complex (Vector Laboratories) for 60 minutes at room temperature, washed in 50 mM tris buffer (pH 7.2) for 5 minutes and stained with 0.05% diaminobenzadine containing 0.01% H_2O_2 for 5-10 minutes at room temperature. All sections were counterstained with haematoxylin for 2 minutes, left to dry and mounted with entellan.

3.1.8 Image analysis

Images were captured with a Nikon-DS Fi1 digital camera mounted onto an Olympus BX50 light microscope (Olympus, Hamburg, Germany). All images were captured at X20 and stored in tiff format at a final resolution of 1024 X 796. Stored images were analysed with Leica QwinPro V3.0 image analysis software (Leica Microsystems GmbH, Wetzlar, Germany) to differentiate between immunohistochemical positive and negative areas using RGB colour thresholding. Immunoreactivity was expressed as the area of immunostained target protein, namely, IRα, IRS2 and PI3K-p110α, in the skeletal muscle and liver per total tissue area. This generated a relative quantification as a percentage.

3.2 Pilot study

3.2.1 Mothers

3.2.1.1 Experimental design

The pilot study was separately approved by the Medical Research Council ethics committee (Ref. 07/11). Twenty virgin female Wistar rats (3-month-old) with an average weight of 234.75 g (weight range of 205 - 252 g) were individually housed and mated overnight with pregnancy confirmed by presence of vaginal plug(s). The pregnant rats were then randomly assigned to groups (n = 5 rats per group), namely: 10% fat (as energy) diet (control), 10% fat (as energy) diet supplemented with *Aspalathus linearis* enriched aqueous extract (control + Al), 40% fat (as energy) diet (40F; high fat diet) and a 40% fat (as energy) diet supplemented with *Aspalathus linearis* enriched aqueous extract (40F + Al) (Table 3.2). Therefore the groups were control (10% fat diet), control + Al, 40F (40% fat diet) and 40F + Al mothers and their neonatal offspring (Table 3.2). The mothers had free access to food and water and were housed at a temperature of 22-25ºC, humidity of 45-55% and a 12 hour light/dark (light daily from 06h00-18h00). The rats were treated in accordance with the United States NIH guidelines for animal care and usage.

Table 3.2: Experimental groups

Group	Diet	Nomenclature
1	10% fat	Control
2	10% fat + *Aspalathus linearis*	Control + Al
3	40% fat	HFD
4	40% fat + *Aspalathus linearis*	HFD + Al

3.2.1.2 *Aspalathus linearis* extraction and formulation

Powdered green rooibos extract was obtained from the Raps Foundation (Freising-Weihenstephan, Germany). The extract is an aspalithin-enriched solvent-based extract prepared according to a patented process (Grüner-Richter *et al.*, 2008). The basic preparation entailed extraction of the plant material with an 80% ethanol-water mixture at room temperature, filtration and vacuum-drying. The powder was then extracted with ethyl acetate to reduce the chlorophyll content, filtered and vacuum-dried.

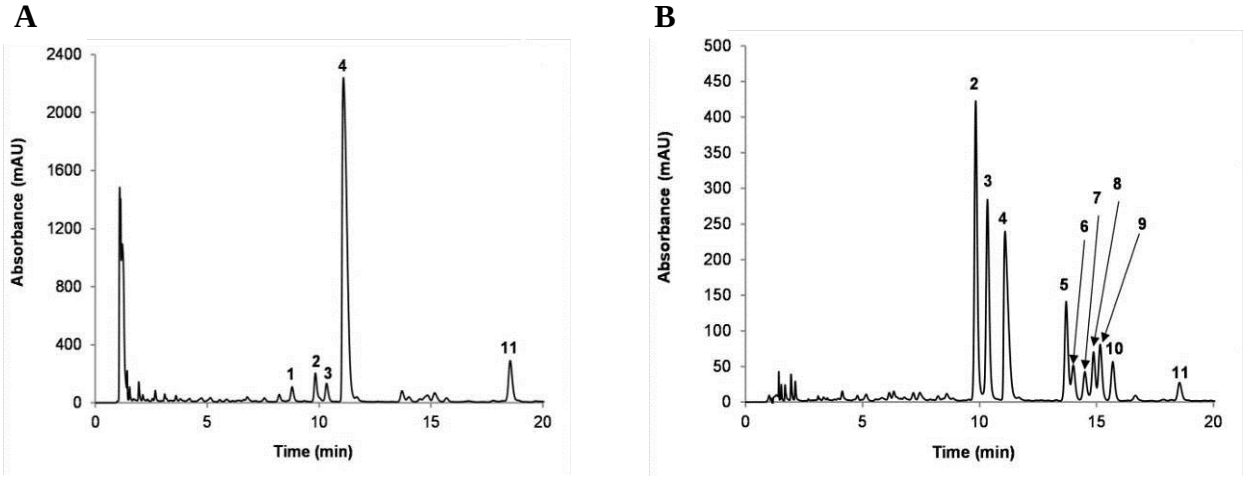


Figure 3.3: HPLC chromatogram of an unfermented green rooibos 80% ethanol-water extract at 288 nm (A) and at 350 nm (B) [1, enolic phenylpyruvic acid-2-O-glucoside; 2, iso-orientin; 3, orientin; 4, aspalathin; 5, quercetin-3-O-robinobioside; 6, vitexin; 7, hyperoside; 8, rutin; 9, isovitexin; 10, isoquercitrin; 11, nothofagin] (Muller *et al.*, 2012).

Table.3.3 Chemical characterization of *Aspalathus linearis* (Muller *et al*., 2012)

Compound	Concentration in g/100 g *Aspalathus linearis* extract
PPAG	0.491
Aspalathin	18.440
Nothofagin	1.292
Iso-orientin	2.054
Quercetin-3-O-robinobioside	1.053
Vitexin	0.270
Hyperoside	0.266
Rutin	0.536
IsoVitexin	0.389
Isoquercitrin	0.377

3.2.1.3 HPLC chromatography and phenolic acid content of a *Aspalathus linearis* extract

The unfermented *Aspalathus linearis* water extract revealed that aspalathin (peak 4) had the highest peak at 280 nm (Figure 3.3A) and at 350 nm iso-orientin (peak 2), orientin (peak 3) and aspalathin (peak 4) had the highest peaks, in decreasing order (Figure 3.3B). Unfermented rooibos also had the highest concentration of aspalathin followed by iso-orientin in g/100g of green rooibos extract (Table 3.3) (Muller *et al*., 2012).

3.2.1.4. Extract preparation and administration

The extract was orally administered to the 10% (control) and 40% (40F or HFD) diet groups via jelly cubes according to an established Diabetes Discovery Platform SOP for administering treatment to rats. *Aspalathus linearis* enriched aqueous extract was obtained from the Agricultural Research Council (ARC). The extract was added and dissolved in jelly. Jelly stock comprised 80 g jelly, 7 g gelatin (Pick and Pay, Rosmead Avenue, Kenilworth, South Africa)

and 300 ml of boiled distilled water. The *Aspalathus linearis* dose of 150 mg/kg per day was transferred into ice cube trays corresponding to the experimental rat numbers. Quality control procedures were to maintain a clean working environment, servicing pipettes and calibrating scales. The *Aspalathus linearis* extract was freshly prepared daily to prevent the degradation of unstable compounds, bacterial and fungal contamination.

Table 3.4: Experimental outline

Time	Phase	Diet
1 week prior to mating (e-7 to e-1)	Habituation: jelly habituation	Control
Weeks 1-3 gestation period (e0-e21)	Experiments: • Pregnancy confirmed • Mothers assigned to diets • Maternal food intake • Blood collection	Control and high fat diets
End of week 3 (delivery; d1)	Terminations: • Neonatal blood and tissue collection • Maternal blood and tissue collection	Control and high fat groups

3.2.1.3 Maternal body weight and food intake

Maternal body weights were recorded prior to mating (e0), on days 7, 14 and 20 of gestation (e7, e14 and e20) and on the day of delivery (d1). Food consumption was recorded daily.

3.2.1.4 Maternal blood collection

Pregnant rats were fasted for four hours and 0.5 ml blood was collected a week prior to mating (e-7), e0, e7, e14, e20 and d1. Further, on d1 the rats were terminated and maternal blood was immediately collected from the aorta using 6 ml syringe and transferred to BD Vacutainer blood collection tubes (BD, Franklin Lakes, NJ, USA) for the determination of serum insulin, glucagon, FFA, total triglyceride and total cholesterol concentrations.

3.2.1.5 Maternal blood glucose concentrations

Four hour fasted blood glucose concentrations were measured weekly (e0, e7, e14, e20 and d1). A drop of blood was used to determine maternal blood glucose concentrations using a One Touch Ultra glucometer (Life Scan, Milpitas, CA, USA).

3.2.1.6 Serum insulin and glucagon concentrations

Radioimmunoassays were applied to determine the serum insulin (^{125}I-labelled rat anti-insulin; Linco, St. Charles, MO, USA) and glucagon (^{125}I-labelled rat anti-glucagon; Linco) concentrations. I-labelled samples were counted in a Perkin Elmar 1470 automatic gamma counter (Perkin Elmar, Turku, Finland).

3.2.2 Neonates

3.2.2.1 Anthropometric measurements

At birth, all neonates were removed from the mothers and separated according to gender. The anthropometric measurements were conducted within 24 hours of birth (postnatal day 1). Neonatal body weights were measured on a calibrated scale. Neonatal body dimensions were measured with a measuring tape. The head length (from the tip of the snout to the base of the skull), head width (distance from ear to ear), head circumference, crown-rump (distance from the top of the head to the rump) and crown-tail (distance from the top of the head to the tip of the tail) were recorded.

3.2.2.2 Neonatal blood collection

One-day-old neonates were removed from mothers and fasted for four hours. For neonatal blood collection, neonates were decapitated and trunk blood was collected from each neonatal group. Trunk blood from each litter per mother was pooled for sampling (a minimum of 500 µl per sample) for each experimental group. The neonatal blood samples were separated according to gender.

3.2.2.3 Neonatal blood glucose concentrations

Neonatal glucose concentrations were measured on the day of birth. A drop of blood (trunk blood) was used to determine blood glucose concentrations with a glucometer (Life Scan).

3.2.2.3 Neonatal serum insulin concentrations

All neonatal blood samples were collected and centrifuged at 4000 rpm for 15 minutes and stored at -20°C for analysis. Radioimmunoassays were applied to determine the serum insulin concentrations. I-labelled samples were counted in a Perkin Elmar 1470 automatic gamma counter (Perkin Elmar, Turku, Finland).

3.2.2.4 Homeostasis model assessment for insulin resistance (HOMA-IR)

HOMA-IR was calculated as HOMA-IR = [Fasting glucose (mmol/L)/Fasting insulin (mU/L)]/22.5 (Lisa *et al*., 2009).

3.3 Statistical analysis

All data are presented as means ± SEM. Comparisons of groups were performed by One-way ANOVA followed by Bonnferroni's post-test using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Significance was established at $p < 0.05$.

CHAPTER 4

RESULTS

4.1 MAIN STUDY

4.1.1 Mothers

4.1.1.1 Maternal total triglyceride, cholesterol and fatty acid concentrations

Serum total triglyceride, cholesterol and fatty acid concentrations were not significantly different before gestation, during gestation or on the day of delivery amongst the groups (Table 4.1).

Table 4.1 Maternal total serum triglyceride, cholesterol and fatty acid concentrations

	Control	20F	30F	40F
Triglycerides (mmol/l)				
Before pregnancy	1.49 ± 0.28	1.16 ± 0.16	1.27 ± 0.20	1.13 ± 0.14
Day 7 of gestation	1.20 ± 0.12	0.95 ± 0.17	1.24 ± 0.29	1.15 ± 0.16
Day 14 of gestation	1.57 ± 0.20	1.31 ± 0.50	1.64 ± 0.20	1.11 ± 0.04
Day 20 of gestation	1.93 ± 0.26	1.36 ± 0.41	3.38 ± 0.68	4.32 ± 1.06
Day of delivery	0.82 ± 0.17	1.22 ± 0.42	0.83 ± 0.31	0.81 ± 0.10
Cholesterol (mmol/l)				
Before pregnancy	1.11 ± 0.11	1.17 ± 0.09	1.38 ± 0.08	1.32 ± 0.12
Day 7 of gestation	1.49 ± 0.16	1.16 ± 0.08	1.53 ± 0.07	1.36 ± 0.10
Day 14 of gestation	0.98 ± 0.14	0.92 ± 0.08	1.22 ± 0.17	0.82 ± 0.08
Day 20 of gestation	1.59 ± 0.12	1.24 ± 0.11	1.82 ± 0.16	1.50 ± 0.32
Day of delivery	2.36 ± 0.49	1.58 ± 0.07	1.74 ± 0.08	2.03 ± 0.27

Table 4.1 continued on page 35

	Control	20F	30F	40F
Fatty acid (µg/ml)				
Before pregnancy	1035 ± 209	8734 ± 149	881 ± 80	1530 ± 540
Day 7 of gestation	1465 ± 66	1695 ± 350	1291 ± 92	1247 ± 92
Day 14 of gestation	1071 ± 145	1537 ± 95	1565 ± 365	1690 ± 181
Day 20 of gestation	2208 ± 162	1887 ± 247	1543 ± 494	2627 ± 196
Day of delivery	1340 ± 79	1255 ± 92.	1424 ± 132	1467 ± 141

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. All groups were compared with One-way ANOVA.

4.1.1.2 Maternal total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), omega 3 polyunsaturated fatty acids (n-3 PUFA) and omega 6 polyunsaturated fatty acids (n-6 PUFA)

On the day of delivery, both 30F and 40F mothers had reduced total SFA compared to control mothers (Table 4.2). On the day of delivery, 40F mothers had increased total MUFA compared to 20F and 30F mothers (Table 4.2). At day 7 of gestation, total n-3 PUFA were reduced in 30F and 40F mothers compared to control mothers; further, the 30F mothers had reduced total n-3 PUFA compared to 20F mothers (Table 4.2). At day 7 of gestation, there was an increase in total n-6 PUFA in 30F mothers compared to control mothers (Table 4.2).

Table 4.2 Maternal total SFA, MUFA, n-3 PUFA and n-6 PUFA compositions (%)

	Control	20F	30F	40F
SFA (%)				
Before pregnancy	49.49 ± 2.74	46.43 ± 1.40	58.82 ± 4.334	61.67 ± 3.53
Day 7 of gestation	39.62 ± 0.70	40.78 ± 1.46	42.68 ± 0.92	42.89 ± 1.53
Day 14 of gestation	41.95 ± 1.50	38.76 ± 1.41	53.31 ± 7.32	43.45 ± 4.14
Day 20 of gestation	36.43 ± 1.28	38.50 ± 2.15	43.54 ± 2.44	36.85 ± 2.33
Day of delivery	43.17 ± 0.84	40.44 ± 0.16	39.20 ± 0.30*	38.37 ± 1.22*
MUFA (%)				
Before pregnancy	20.41 ± 1.90	16.73 ± 1.32	12.08 ± 1.65	16.42 ± 5.80
Day 7 of gestation	26.77 ± 1.21	22.96 ± 2.76	18.54 ± 0.81	19.99 ± 1.11
Day 14 of gestation	26.99 ± 1.58	25.40 ± 1.86	20.81 ± 2.46	25.01 ± 1.05
Day 20 of gestation	29.95 ± 1.70	27.75 ± 0.69	24.67 ± 3.68	26.93 ± 1.81
Day of delivery	11.96 ± 0.60	10.39 ± 0.42	10.69 ± 0.99	14.78 ± 0.96†‡
n- 3 PUFA (%)				
Before pregnancy	2.33 ± 0.40	3.40 ± 0.50	2.93 ± 0.17	3.33 ± 0.63
Day 7 of gestation	4.52 ± 0.21	4.16 ± 0.11	2.76 ± 0.15*†	3.46 ± 0.19*
Day 14 of gestation	5.51 ± 0.59	4.62 ± 0.29	2.42 ± 0.58	3.83 ± 0.79
Day 20 of gestation	5.61 ± 0.66	5.45 ± 0.97	3.18 ± 0.67	5.45 ± 0.28
Day of delivery	6.23 ± 0.42	7.08 ± 0.28	5.74 ± 0.44	5.87 ± 0.62
n- 6 PUFA (%)				
Before pregnancy	27.76 ± 4.19	33.44 ± 2.51	26.17 ± 3.46	26.73 ± 2.33
Day 7 of gestation	29.09 ± 0.67	32.10 ± 1.32	36.02 ± 0.96*	33.67 ± 2.06
Day 14 of gestation	25.55 ± 0.79	31.22 ± 1.93	23.46 ± 4.57	27.73 ± 3.06
Day 20 of gestation	28.02 ± 2.32	28.31 ± 1.86	28.61 ± 3.49	30.78 ± 0.25
Day of delivery	28.02 ± 2.32	28.31 ± 1.86	28.61 ± 3.49	30.78 ±0.25

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. 20F, $^{\ddagger}p < 0.05$ vs. 30F. All groups were compared with One-way ANOVA.

4.1.1.3 Individual SFA and MUFA percentages

SFA

On day 7 of gestation, 30F mothers had increased behenic acid (22:0) compared to 20F mothers (Table 4.3). On day 14 of gestation and on the day of delivery, 40F mothers had reduced behenic acid (22:0) compared to 30F mothers.

Table 4.3 Maternal serum saturated fatty acids compositions (%)

	Control	20F	30F	40F
Myristic acid (14:0)				
Before pregnancy	4.01 ± 2.10	2.21 ± 1.79	6.75 ± 3.19	9.07 ± 2.62
Day 7 of gestation	0.83 ± 0.10	1.08 ± 0.22	1.09 ± 0.20	0.89 ± 0.04
Day 14 of gestation	0.76 ± 0.12	0.86 ± 0.10	7.41 ± 2.97	3.10 ± 1.80
Day 20 of gestation	0.75 ± 0.12	1.17 ± 0.16	1.45 ± 0.61	1.88 ± 0.45
Day of delivery	0.37 ± 0.10	0.24 ± 0.07	0.49 ± 0.11	0.51 ± 0.05
Palmitic acid (16:0)				
Before pregnancy	24.75 ± 0.96	23.38 ± 1.29	24.46 ± 2.90	23.52 ± 1.97
Day 7 of gestation	18.82 ± 0.79	18.85 ± 1.08	18.24 ± 0.30	18.16 ± 0.88
Day 14 of gestation	21.43 ± 0.77	19.03 ± 1.41	24.20 ± 2.73	20.25 ± 1.57
Day 20 of gestation	19.01 ± 0.01	19.27 ± 1.52	22.13 ± 1.79	18.02 ± 1.04
Day of delivery	21.72 ± 0.31	19.10 ± 0.56	19.14 ± 1.21	19.26 ± 0.61
Stearic acid (18:0)				
Before pregnancy	19.56 ± 0.96	20.71 ± 1.15	25.69 ± 0.92	21.16 ± 2.13
Day 7 of gestation	18.65 ± 0.48	19.72 ± 1.77	21.67 ± 1.04	22.40 ± 22.40
Day 14 of gestation	18.67 ± 1.38	18.04 ± 0.25	20.53 ± 1.68	18.99 ± 0.99
Day 20 of gestation	15.70 ± 1.11	17.07 ± 0.71	18.59 ± 0.48	16.22 ± 1.74
Day of delivery	19.80 ± 0.90	19.92 ± 0.49	18.33 ± 1.10	17.68 ± 0.62

Table 4.3 continued on page 38

	Control	20F	30F	40F
Arachidic acid (20:0)				
Before pregnancy	0.33 ± 0.03	0.30 ± 0.05	0.31 ± 0.06	0.40 ± 0.17
Day 7 of gestation	0.32 ± 0.03	0.33 ± 0.10	0.34 ± 0.04	0.32 ± 0.08
Day 14 of gestation	0.42 ± 0.11	0.21 ± 0.06	0.42 ± 0.06	0.54 ± 0.18
Day 20 of gestation	0.31 ± 0.09	0.40 ± 0.07	0.44 ± 0.05	0.38 ± 0.18
Day of delivery	0.08 ± 0.01	0.15 ± 0.06	0.11 ± 0.02	0.18 ± 0.10
Behenic acid (22:0)				
Before pregnancy	1.11 ± 0.70	0.41 ± 0.03	0.37 ± 0.02	0.32 ± 0.05
Day 7 of gestation	0.28 ± 0.01	0.26 ± 0.05	0.41 ± 0.03†	0.36 ± 0.04
Day 14 of gestation	0.23 ± 0.01	0.18 ± 0.02	0.24 ± 0.02	0.18 ± 0.003‡
Day 20 of gestation	0.23 ± 0.02	0.22 ± 0.02	0.33 ± 0.08	0.19 ± 0.01
Day of delivery	0.31 ± 0.02	0.29 ± 0.02	0.35 ± 0.04	0.21 ± 0.003‡

Data are as means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{\dagger}p < 0.05$ vs. 20F, $^{\ddagger}p < 0.05$ vs. 30F. All groups were compared with One-way ANOVA.

MUFA

On day 14 of gestation, the 30F and 40F mothers had reduced palmitoleic acid (16:1 *n*-7) compared to control mothers (Table 4.4). On the day of delivery, 40F mothers had increased oleic acid (18:1 *n*-9) compared to 20F and 30F mothers. On day 7 of gestation, vaccenic acid (18:1 *n*-7) was reduced in 30F and 40F mothers compared to control and 20F mothers. Further, on the day of delivery, vaccenic acid (18:0 *n*-7) was reduced in 20F, 30F and 40F mothers compared control mothers and in 40F mothers compared to control mothers. On day 7 of gestation, nervonic acid (24:1 *n*-9) was increased in 30F mothers compared to control mothers.

Table 4.4 Maternal serum monounsaturated fatty acid composition (%)

	Control	20F	30F	40F
Palmitoleic acid (16:1 n-7)				
Before pregnancy	1.86 ± 0.29	1.01 ± 0.09	0.84 ± 0.16	0.89 ± 0.26
Day 7 of gestation	2.96 ± 0.29	1.62 ± 0.66	1.41 ± 0.08	1.41 ± 0.14
Day 14 of gestation	3.30 ± 0.08	2.06 ± 0.46	1.10 ± 0.21*	1.27 ± 0.16*
Day 20 of gestation	3.76 ± 0.94	2.55 ± 0.23	1.63 ± 0.41	1.51 ± 0.26
Day of delivery	1.01 ± 0.12	0.66 ± 0.12	0.64 ± 0.07	0.84 ± 0.07
Oleic acid (18:1 n-9)				
Before pregnancy	15.65 ± 1.23	12.27 ± 1.02	8.89 ± 1.14	11.51 ± 3.80
Day 7 of gestation	19.94 ± 0.90	18.53 ± 2.54	15.50 ± 0.71	16.96 ± 1.15
Day 14 of gestation	20.30 ± 1.26	20.60 ± 1.31	17.92 ± 1.83	21.87 ± 0.82
Day 20 of gestation	21.69 ± 0.06	21.73 ± 0.74	19.48 ± 3.30	22.97 ± 0.47
Day of delivery	8.72 ± 0.63	7.79 ± 0.31	8.47 ± 1.02	12.67 ± 0.84†‡
Vaccenic acid (18:1 n-7)				
Before pregnancy	2.79 ± 0.18	2.28 ± 0.30	1.76 ± 0.37	1.76 ± 0.37
Day 7 of gestation	3.08 ± 0.16	2.09 ± 0.44	1.02 ± 0.07*†	0.97 ± 0.02*†
Day 14 of gestation	2.92 ± 0.15	2.10 ± 0.58	1.38 ± 0.50	1.48 ± 0.42
Day 20 of gestation	3.88 ± 0.68	2.89 ± 0.13	2.92 ± 0.08	1.93 ± 1.01
Day of delivery	1.63 ± 0.06	1.26 ± 0.04*	1.04 ± 0.09*	0.85 ± 0.05*†
Nervonic acid (24:1 n-9)				
Before pregnancy	0.40 ± 0.11	0.99 ± 0.26	0.73 ± 0.30	1.13 ± 0.10
Day 7 of gestation	0.59 ± 0.04	0.74 ± 0.10	0.94 ± 0.08*	0.76 ± 0.08
Day 14 of gestation	0.28 ± 0.11	0.36 ± 0.01	0.49 ± 0.09	0.36 ± 0.02
Day 20 of gestation	0.34 ± 0.10	0.39 ± 0.05	0.91 ± 0.46	0.33 ± 0.00
Day of delivery	0.65 ± 0.04	0.59 ± 0.07	0.47 ± 0.09	0.52 ± 0.07

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. 20F, $^{\ddagger}p < 0.05$ vs. 30F. All groups were compared with One-way ANOVA.

4.1.1.4 Individual n-3 PUFA and n-6 PUFA composition (%)

n-3 PUFA

On day 7 of gestation, linolenic acid (18:3 *n*-3) was reduced in 30F and 40F mothers compared to control mothers (Table 4.5). On day 7 of gestation, eicosatrienoic acid (20:3 *n*-3) was reduced in 30F mothers compared to the other groups and in 20F mothers compared to control mothers (Table 4.5). On day 14 of gestation, eicosapentaenoic acid (20:5 *n*-3) was reduced in 40F mothers compared to control mothers whereas on the day delivery, the 40F mothers had increased eicosapentaenoic acid (20:5 *n*-3) compared to the other groups (Table 4.5). On day 14 of gestation, docosapentaenoic acid (22:5 *n*-3) was reduced in 30F and 40F mothers compared to control mothers and remained reduced on the day of delivery in 40F mothers compared control mothers (Table 4.5).

Table 4.5 Maternal serum n-3 polyunsaturated fatty acids compositions (%)

	Control	20F	30F	40F
Linolenic acid (18:3 n-3)				
Before pregnancy	0.41 ± 0.02	0.55 ± 0.08	0.51 ± 0.13	0.80 ± 0.18
Day 7 of gestation	0.77 ± 0.04	0.63 ± 0.10	0.48 ± 0.04*	0.49 ± 0.03*
Day 14 of gestation	0.96 ± 0.14	0.53 ± 0.02	0.47 ± 0.13	0.67 ± 0.09
Day 20 of gestation	1.05 ± 0.49	0.75 ± 0.20	0.49 ± 0.10	1.04 ± 0.32
Day of delivery	0.31 ± 0.02	0.24 ± 0.02	0.28 ± 0.06	0.30 ± 0.00
Eicosatrienoic acid (20:3 n-3)				
Before pregnancy	-	0.05 ± 0.01	0.07 ± 0.00	0.10 ± 0.03
Day 7 of gestation	0.07 ± 0.003	0.05 ± 0.003*	0.04 ± 0.00*†§	0.06 ± 0.03
Day 14 of gestation	0.07 ± 0.01	0.05 ± 0.003	0.04 ± 0.00	0.05 ± 0.01
Day 20 of gestation	0.06 ± 0.01	0.05 ± 0.01	0.03 ± 0.00	0.05 ± 0.01
Day of delivery	-	0.09± 0.001	0.05 ± 0.00	0.07 ± 0.00

Table 4.5 continued on page 41

Control	20F	30F	40F	Control
Eicosapentaenoic acid (20:5 n-3)				
Before pregnancy	0.27 ± 0.02	0.25 ± 0.03	0.23 ± 0.02	0.24 ± 0.05
Day 7 of gestation	0.79 ± 0.17	0.61 ± 0.20	0.25 ± 0.03	0.29 ± 0.05
Day 14 of gestation	0.88 ± 0.13	0.59 ± 0.24	0.22 ± 0.02	0.22 ± 0.05*
Day 20 of gestation	0.57 ± 0.03	0.35 ± 0.04	0.23 ± 0.07	0.80 ± 0.25
Day of delivery	0.27 ± 0.04	0.25 ± 0.01	0.25 ± 0.04	0.62 ± 0.10*†‡
Docosapentaenoic acid (22:5 n-3)				
Before pregnancy	0.42 ± 0.07	0.79 ± 0.32	0.49 ± 0.09	0.56 ± 0.15
Day 7 of gestation	0.90 ± 0.04	0.71 ± 0.05	0.49 ± 0.02	0.55 ± 0.04
Day 14 of gestation	1.16 ± 0.25	0.83 ± 0.03	0.41 ± 0.11*	0.49 ± 0.10*
Day 20 of gestation	1.15 ± 0.15	0.92 ± 0.18	0.40 ± 0.05	0.82 ± 0.18
Day of delivery	1.20 ± 0.09	1.12 ± 0.07	0.98 ± 0.08	0.82 ± 0.02*

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. 20F, $^{\ddagger}p < 0.05$ vs. 30F, $^{\S}p < 0.05$ vs. 40F; -, insufficient sample volumes. All groups were compared with One-way ANOVA.

n-6 PUFA

On day 7 of gestation, linoleic acid (18:2 *n*-6) was increased in 30F mothers compared to the other groups (Table 4.6). On day 14 of gestation, the 30F mothers had a reduced dihomo-γ linolenic acid (20:3 *n*-6) compared to control mothers; on day 20 of gestation, dihomo-γ linolenic acid (20:3 *n*-6) was reduced in 30F mothers compared to the other groups (Table 4.6). On the day of delivery, docosapentaenoic acid (20:5 *n*-6) was reduced in 40F mothers compared to control and 20F mothers (Table 4.6).

Table 4.6 Maternal serum n-6 polyunsaturated fatty acids compositions (%)

	Control	20F	30F	40F
Linoleic acid (18:2 n-6)				
Before pregnancy	16.15 ± 1.93	19.97 ± 1.02	13.67 ± 1.80	14.72 ± 1.36
Day 7 of gestation	16.71 ± 0.47	18.63 ± 0.41	22.47 ± 0.97*†§	17.58 ± 0.76
Day 14 of gestation	15.39 ± 0.53	18.18 ± 0.11	14.86 ± 3.04	16.32 ± 1.77
Day 20 of gestation	18.49 ± 2.46	17.91 ± 0.57	19.44 ± 1.05	16.87 ± 0.28
Day of delivery	20.65 ± 1.23	16.02 ± 1.26	21.22 ± 0.91	16.95 ± 1.84
Dihomo-γ linolenic acid (20:3 n-6)				
Before pregnancy	0.50 ± 0.04	0.48 ± 0.05	0.46 ± 0.07	0.48 ± 0.11
Day 7 of gestation	1.06 ± 0.10	0.86 ± 0.12	0.77 ± 0.06	1.00 ± 0.14
Day 14 of gestation	0.92 ± 0.08	0.76 ± 0.09	0.49 ± 0.07*	0.65 ± 0.07
Day 20 of gestation	0.84 ± 0.05	0.68 ± 0.06	0.37 ± 0.07*†§	0.77 ± 0.04
Day of delivery	0.94 ± 0.05	0.54 ± 0.03	0.79 ± 0.12	0.78 ± 0.18
Docosapentaenoic acid (22:5 n-6)				
Before pregnancy	-	0.10 ± 0.00	-	0.90 ± 0.57
Day 7 of gestation	0.22 ± 0.03	0.58 ± 0.35	0.08 ± 0.04	0.36 ± 0.03
Day 14 of gestation	0.25 ± 0.05	0.27 ± 0.05	0.34 ± 0.23	0.66 ± 0.19
Day 20 of gestation	0.46 ± 0.02	0.90 ± 0.27	1.66 ± 1.19	2.78 ± 0.04
Day of delivery	0.66 ± 0.06	1.11 ± 0.15	1.60 ± 0.16	2.59 ± 0.48*†

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. 20F, -, insufficient sample volumes. All groups were compared with One-way ANOVA.

4.1.1.5 Maternal serum fatty acids ratios on the day of delivery

The 30F mothers had increased total PUFA/total SFA ratios compared to control mothers (Table 4.7). The ratio of total MUFA/total SFA was elevated in 40F mothers compared to 20F and 30F mothers (Table 4.7).

Table 4.7 Maternal serum fatty acids ratios

Ratio	Control	20F	30F	40F
16:1 n-7/16:0	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.005	0.043 ± 0.01
Total PUFA/Total SFA	1.03 ± 0.06	1.23 ± 0.03	1.28 ± 0.03*	1.23 ± 0.07
Total MUFA/Total SFA	0.28 ± 0.01	0.26 ± 0.01	0.27 ± 0.03	0.38 ± 0.04†‡
Total PUFA/Total MUFA	3.79 ± 0.25	4.75 ± 0.26	4.83 ± 0.50	3.20 ± 0.21
Total n-6 PUFA/Total n-3 PUFA	6.28 ± 0.42	5.97 ± 0.24	7.85 ± 0.51	7.17 ± 0.83

Data are means ± SEM (n = 5). Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. 20F, $^{\ddagger}p < 0.05$ vs. 30F. All groups were compared with One-way ANOVA.

4.1.2.1 Neonatal hepatic and skeletal muscle IR, IRS2 and PI3K mRNA expression

Foetal programming, with maternal diets varying in fat content, did not affect neonatal hepatic IR (Fig. 4.1A) and IRS2 (Fig. 4.1B) mRNA expression. However, hepatic PI3K mRNA expression was elevated in 30F neonates compared to 20F neonates (Fig. 4.1C). Gel electrophoresis for total RNA is shown in Appendix C (Fig C.1A).

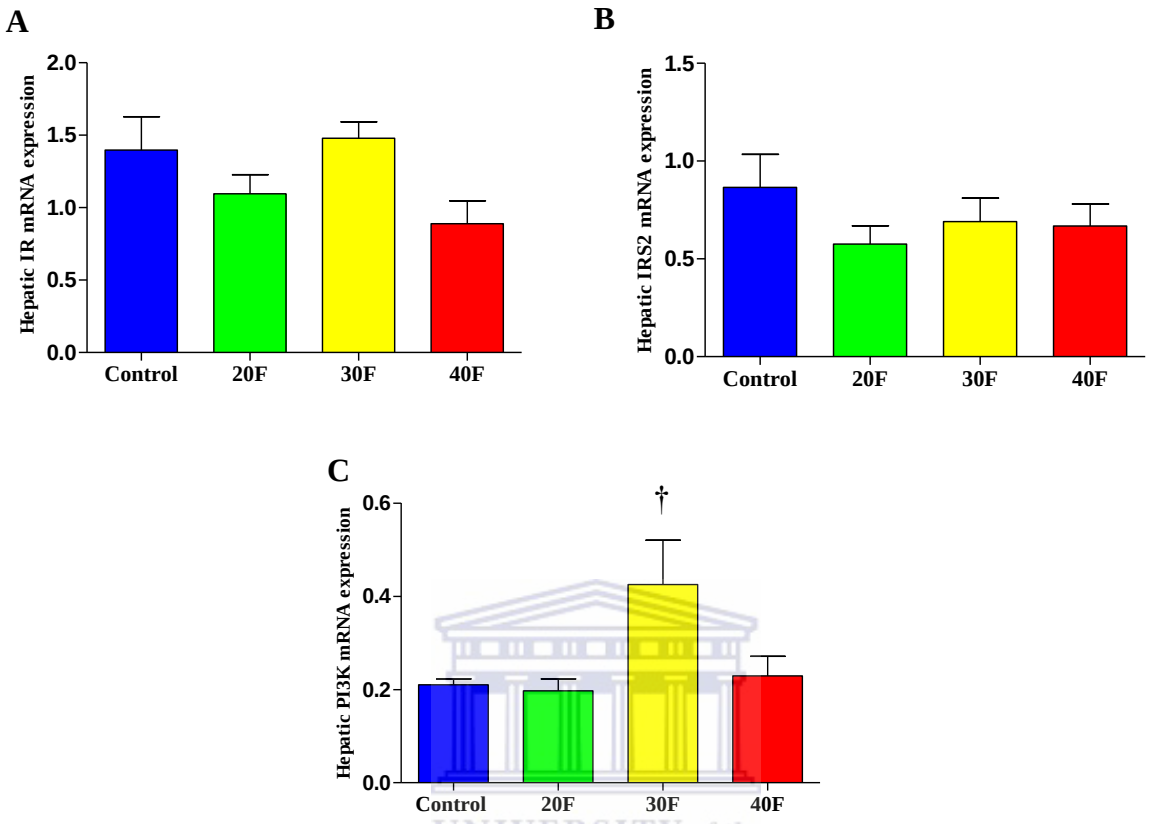


Figure 4.1 Neonatal hepatic IR, IRS2 and PI3K mRNA expression. (A), Neonatal hepatic IR mRNA expression; (B), Neonatal hepatic IRS2 mRNA expression; (C), Neonatal hepatic PI3K mRNA expression. Data are means ± SEM. n = 3-10 for hepatic mRNA expression. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{\dagger}p<0.05$ vs. 20F.

Skeletal muscle IR mRNA was reduced in the 30F and 40F neonates compared to 20F neonates (Fig. 4.2A). There were no significant differences in neonatal skeletal muscle IRS2 mRNA expression among the groups (Fig. 4.2B). However, neonatal skeletal muscle PI3K mRNA expression was reduced in 30F and 40F neonates compared to 20F neonates (Fig. 4.2C). Gel electrophoresis for total RNA is shown in Appendix C (Fig C.1B).

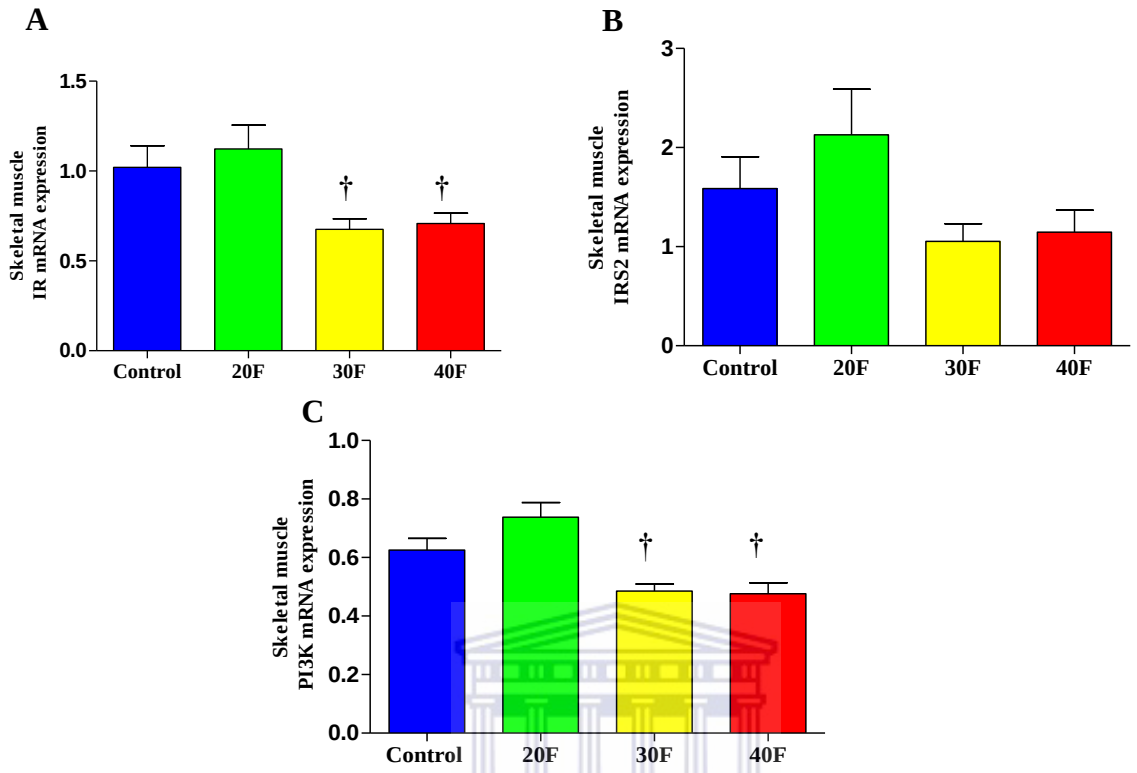


Figure 4.2 Skeletal muscle IR, IRS2 and PI3K mRNA expression. (A), Neonatal skeletal muscle IR mRNA expression; (B), Neonatal skeletal muscle IRS2 mRNA expression; (C), Neonatal skeletal muscle PI3K mRNA expression. Data are means ± SEM. n = 10 for skeletal muscle mRNA expression. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. †$p<0.05$ vs. 20F.

4.1.2.2 Neonatal hepatic and skeletal muscle IRα, IRS2 and PI3K-p110α immunoreactivity

Hepatic IRα immunoreactivity was reduced in 40F neonates compared to control and 20F neonates (Fig. 4.3B). Further, skeletal muscle IRα immunoreactivity was reduced in 30F and 40F neonates compared to control neonates (Fig. 4.4B). There were however no significant differences in hepatic (4.5B) and skeletal muscle IRS2 (Fig. 4.6B) and p110α immunoreactivity (Fig. 4.7A and B). Neonatal liver and skeletal muscle H and E staining are shown in Appendix A (Fig A.1). IRβ and IRS1 were also immunostained but not analysed as it was outside of the scope of this study. The representative immunoreactivity images for IRβ (Fig. B.1) and IRS1 (Fig. B.2) are shown in Appendix B.

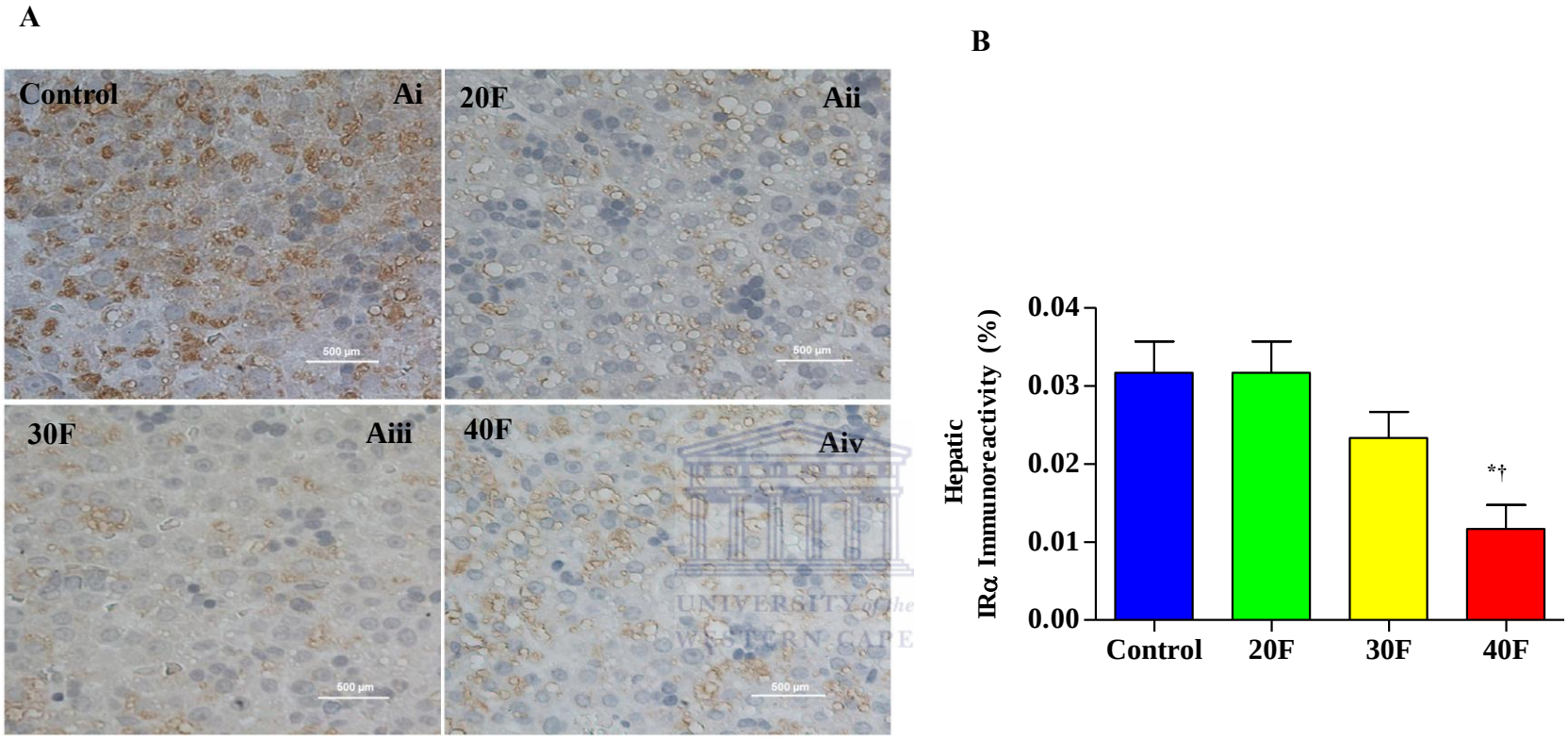
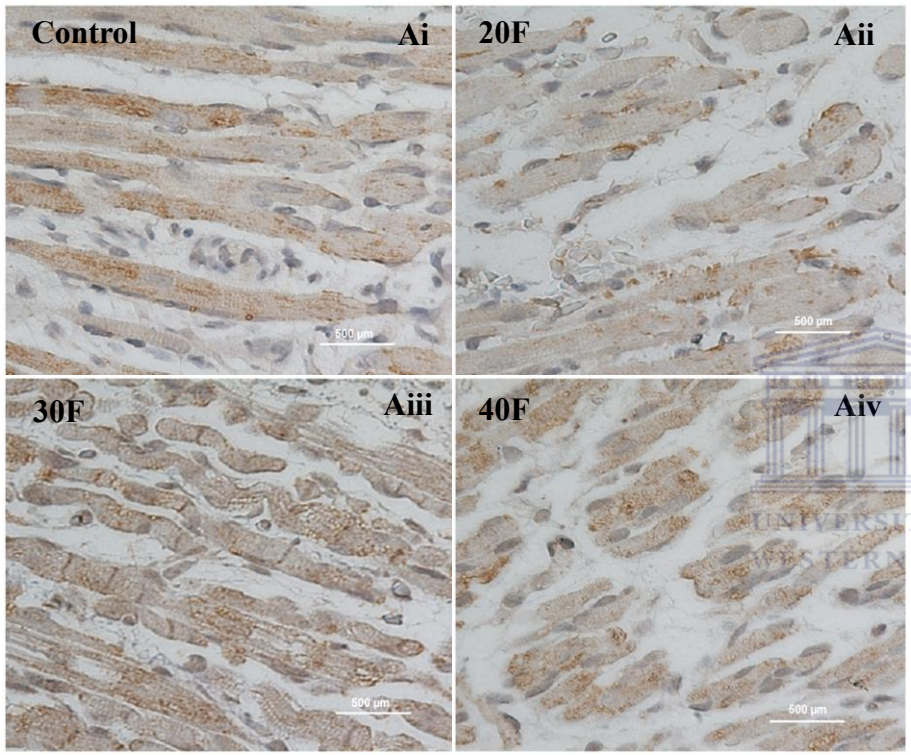


Figure 4.3 Neonatal hepatic IRα immunoreactivity. (A), Neonatal liver immunostained with anti-IRα in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B), Neonatal hepatic IRα immunoreactivity. Data are means ± SEM. n = 6 for immunoreactivity. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p<0.05$ vs. control; $^{\dagger}p<0.05$ vs. 20F.



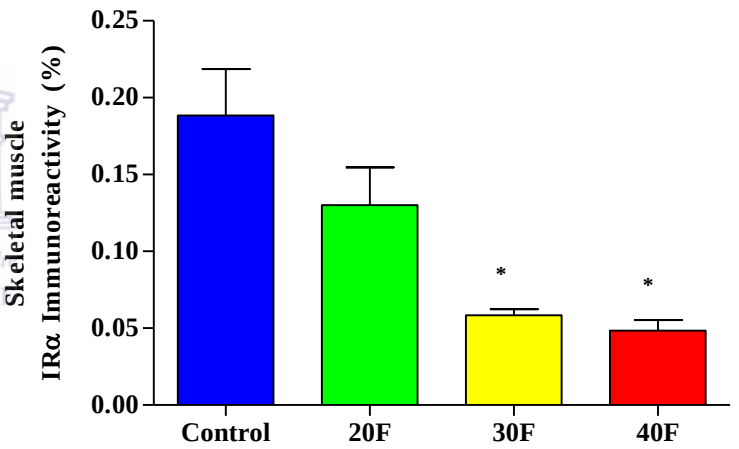


Figure 4.4 Neonatal skeletal muscle IRα immunoreactivity. (A), Neonatal skeletal muscle immunostained with anti-IRα in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B), Neonatal hepatic IRα immunoreactivity. Data are means ± SEM. n = 6 for immunoreactivity. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. $^{*}p<0.05$ vs. control.

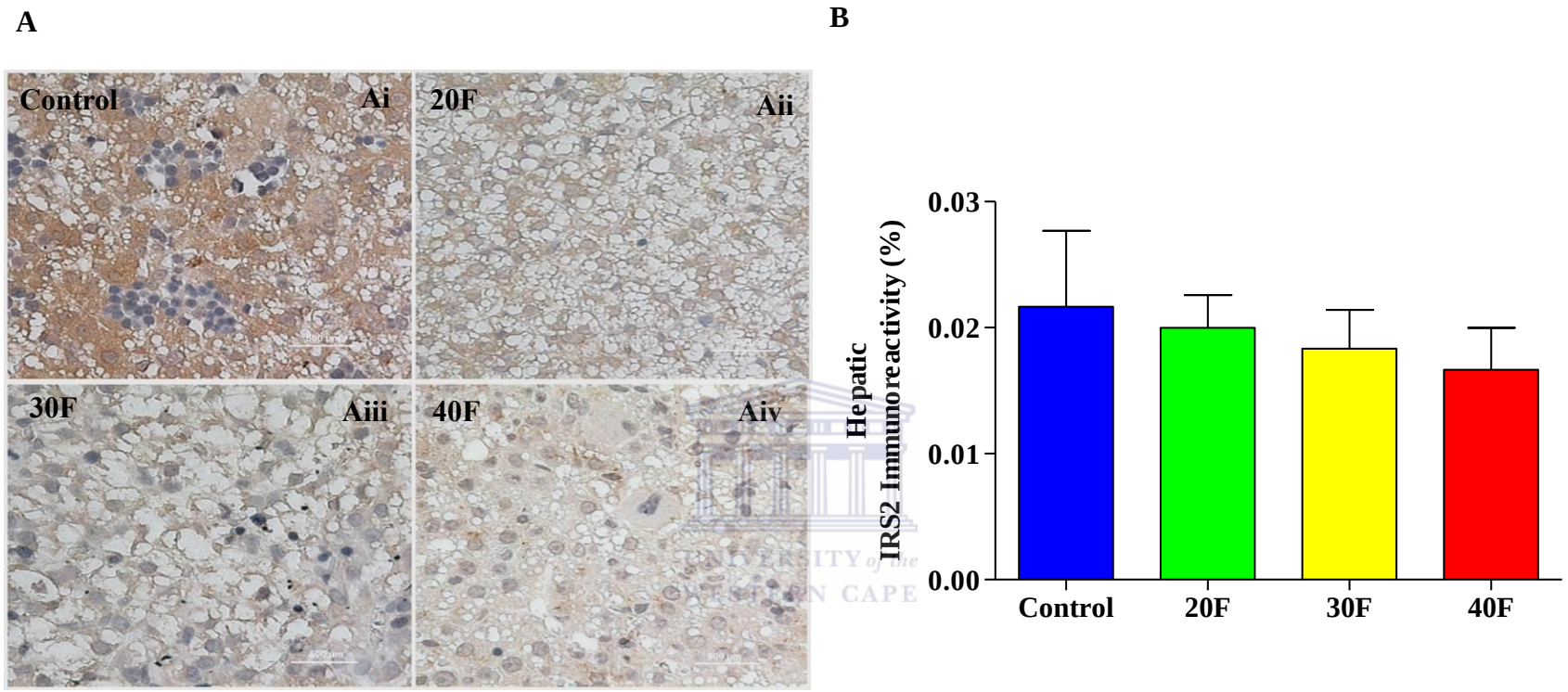


Figure 4.5 Neonatal hepatic IRS2 immunoreactivity. (A), Neonatal liver immunostained with anti-IRS2 in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B), Neonatal hepatic IRS2 immunoreactivity. Data are means ± SEM. n = 6 per group. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

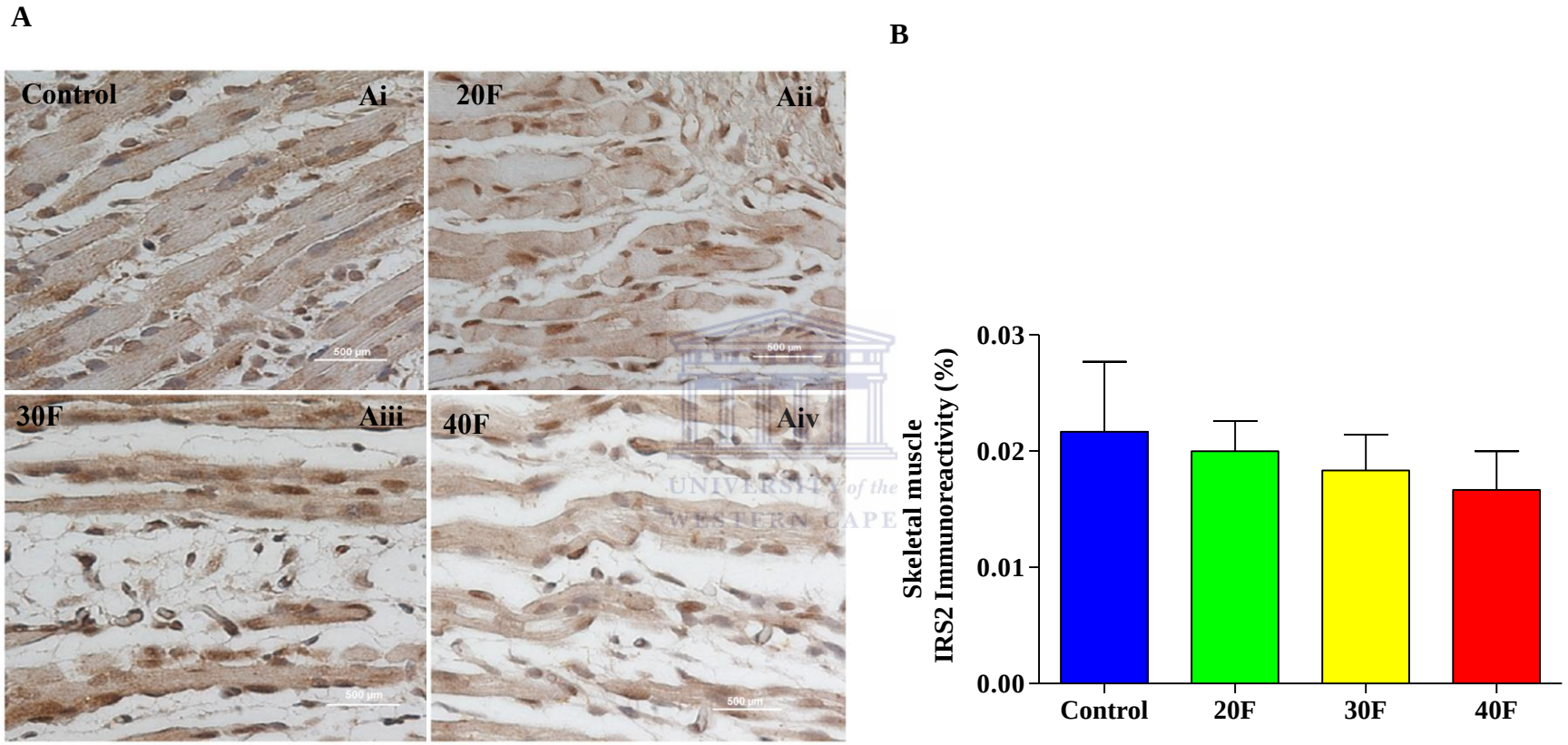


Figure 4.6 Neonatal skeletal muscle IRS2 immunoreactivity. (A), Neonatal skeletal muscle immunostained with ant-IRS2 in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B), Neonatal hepatic IRS2 immunoreactivity. Data are means ± SEM. n = 6 per group. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

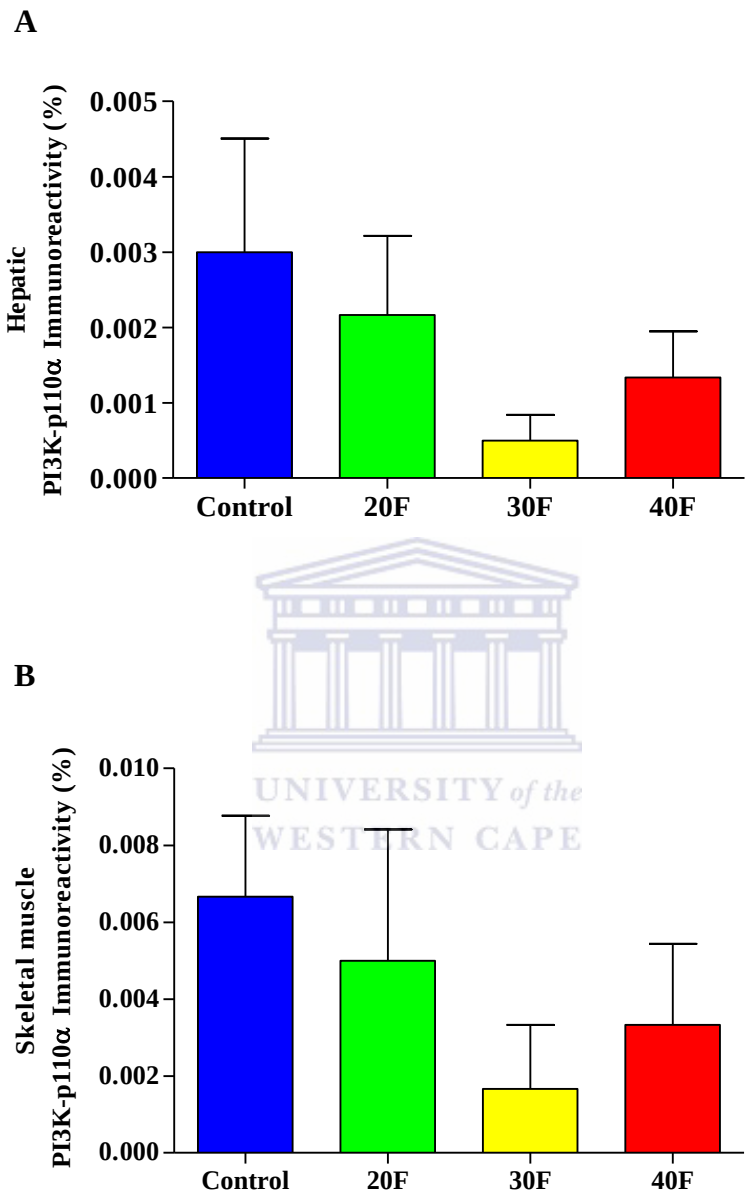


Figure 4.7 Neonatal hepatic and skeletal muscle PI3K-p110α immunoreactivity. (A), Neonatal hepatic PI3K-p110α immunoreactivity; (B), Neonatal skeletal muscle PI3K-p110α immunoreactivity. Data are means ± SEM. n = 6 per group. Control; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

4.2 PILOT STUDY

4.2.1. Maternal food intake and body weight and litter size

There were no differences in maternal calorie intake and body weights throughout gestation and on the day of delivery or in litter size amongst the groups (Table 4.8). Further, there were no differences in maternal liver, heart, brain and pancreas weights amongst the groups (Table 4.8).

Table 4.8 Maternal food intake and body weight during gestation, organ weights and litter size

Parameter	Control	Control-Al	HFD	HFD-Al
Calorie intake (Kcal/g)				
Week 1 (e0-e7)	1691 ± 57.33	1671 ± 63.54	1723 ± 90.58	1900 ± 137.3
Week 2 (e8-e14	1570 ± 42.58	1688 ± 35.79	1715 ± 44.25	1611 ± 63.81
Week 3 (e15-e21)	1510 ± 52.05	1467 ± 50.98	1538 ± 58.09	1640 ± 85.32
Overall (Kcal/g)	4771 ± 151.96	4826 ± 150.31	4976 ± 192.92	5151 ± 286.43
Body weight (g)				
Week 1 (e7)	261 ± 6.35	2720 ± 6.99	282 ± 2.34	285 ± 12.71
Week 2 (e0)	295 ± 7.35	303 ± 6.72	315 ± 6.72	318 ± 12.53
Week 3 (e20)	356 ± 12.86	369 ± 5.69	382 ± 20.84	395 ± 16.20
Day of delivery	267 ± 12.74	300 ± 18.42	304 ± 6.43	302 ± 14.11
Organ weights (g)				
Liver	10.01 ± 0.38	10.65 ± 0.45	11.18 ± 0.47	11.32 ± 0.47
Heart	0.89 ± 0.08	0.91 ± 0.06	0.97 ± 0.02	1.12 ± 0.07
Brain	1.75 ± 0.05	1.82 ± 0.08	1.87 ± 0.02	1.79 ± 0.03
Pancreas	1.03 ± 0.10	1.34 ± 0.20	1.47 ± 0.12	1.25 ± 0.13
Litter size	12.0 ± 0.95	11.2 ± 1.39	12.0 ± 2.04	12.8 ± 1.56

Data are means ± SEM; n = 5 per group. Control; Control-Al, control-*Aspalathus linearis*; HFD, high fat diet; HFD-Al, HFD-*Aspalathus linearis*. All groups were compared with One-way ANOVA.

4.2.2. Maternal blood glucose, serum insulin and glucagon concentrations and homeostasis model assessment for insulin resistance (HOMA-IR)

The HFD-Al mothers presented elevated blood glucose concentrations on day 20 of gestation compared to control mothers (Fig. 4.8A). There were no other differences in blood glucose concentrations throughout gestation (Fig. 4.8A) and on the day of delivery (Fig.4.8B). Further, on the day of delivery, there were no differences in serum insulin (Fig. 4.8C) and serum glucagon (Fig. 4.8D) concentrations and HOMA-IR (Fig. 4.8E).

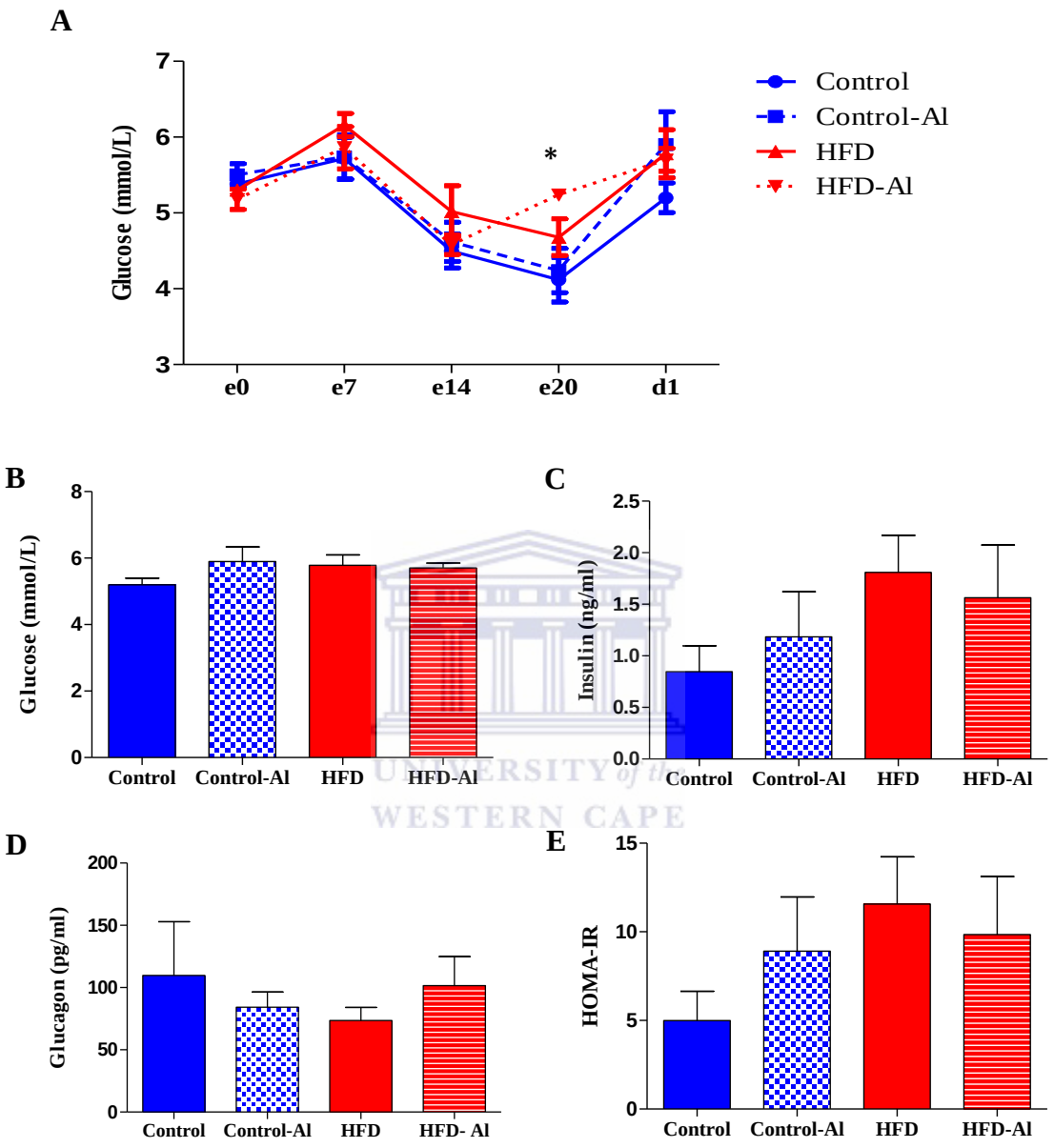


Figure 4.8 Maternal blood glucose, serum insulin and serum glucagon concentrations, and HOMA-IR. (A), Maternal blood glucose concentrations during pregnancy; (B), Maternal blood glucose concentrations on the day of delivery; (C), Maternal serum insulin concentrations on the day of delivery; (D), Maternal serum glucagon concentrations on the day of delivery; (E), Maternal HOMA-IR on the day of delivery. Data are means ± SEM; n = 5 per group. Control; Control-Al, control-*Aspalathus linearis*; HFD, high fat diet; HFD-Al, HFD-*Aspalathus linearis*. *p<0.05 vs. control.

4.2.3 Neonatal anthropometric measurements

For each group of neonates, head length, width, circumference, crown-rump and crown-tail lengths were measured in millimetres (mm) (Table 4.9). The HFD-Al neonates had increased head length and width compared to control neonates. The head circumference of HFD-Al neonates were increased compared to control and control-Al neonates also increased in HFD neonates compared to control neonates (Table 4.9). The crown-rump length and crown-tail length of Control-Al neonates were increased compared to control and HFD-Al neonates; the crown-tail length of the HFD-Al neonates was reduced compared to HFD neonates (Table 4.9).

Table 4.9 Neonatal anthropometry (head length, width, circumference, crown-rump and crown-tail length)

	Control	Control-Al	HFD	HFD-Al
Head length (mm)	15.4 ± 0.12	15.69 ± 0.15	15.7 ± 0.14	16.24 ± 0.22*
Head width (mm)	12.47 ± 0.32	12.52 ± 0.17	12.74 ± 0.19	13.46 ± 0.32*
Head circumference (mm)	32.53 ± 0.32	33.04 ± 0.25	34.33 ± 0.29*	35.19 ± 0.49*†
Crown-rump (mm)	41.33 ± 0.42	43.66 ± 0.69*§	41.93 ± 0.46	40.83 ± 0.23
Crown-tail (mm)	58.59 ± 0.97	62.77 ± 0.88*§	61.5 ± 1.01	56.35 ± 0.70‡

Data are means ± SEM; n = 24-31. Control; HFD, Control-Al, control-*Aspalathus linearis*; high fat diet; HFD-Al, HFD-*Aspalathus linearis*. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. Control-Al, $^{\ddagger}p < 0.05$ vs. HFD, $^{\S}p < 0.05$ vs. HFD-Al. All groups were compared with One-way ANOVA.

4.2.4 Neonatal gender distribution, body weight and organ weights

There was no difference in the distribution of male and female neonates between the groups (Table 4.10). Control-Al, HFD and HFD-Al neonates were all heavier than control neonates (Table 4.10). Brain weight was increased in the HFD neonates compared to control and Control-Al neonates (Table 4.10). There were no significant differences in liver and pancreas weights amongst the groups.

Table 4.10 Neonatal gender distribution, body weight, and liver, brain and pancreas weights

	Control	Control-Al	HFD	HFD-Al
Male (%)	59.46 ± 4.94	54.8 ± 7.64	40.63 ± 4.86	50.34 ± 2.16
Female (%)	40.58 ± 4.95	45.2 ± 7.64	59.18 ± 4.88	49.66 ± 2.16
Body weights (g)	6.052 ± 0.10	6.595 ± 0.11*	6.831 ± 0.12*	6.665 ± 0.11*
Liver (g)	0.223 ± 0.01	0.237 ± 0.01	0.243 ± 0.004	0.238 ± 0.01
Brain (g)	0.244 ± 0.01	0.241 ± 0.01	0.274 ± 0.004*†	0.252 ± 0.01
Pancreas (g)	0.025 ± 0.003	0.02 ± 0.001	0.024 ± 0.003	0.026 ± 0.01
Heart (g)	0.89 ± 0.08	0.91 ± 0.06	0.97 ± 0.02	1.12 ± 0.07

Data are means ± SEM; n = 24-31). Control; Control-Al, control-*Aspalathus linearis*; HFD, high fat diet; HFD-Al, HFD-*Aspalathus linearis*. $^{*}p < 0.05$ vs. Control, $^{\dagger}p < 0.05$ vs. Control-Al. All groups were compared with One-way ANOVA.

4.2.5 Neonatal blood glucose and serum insulin concentrations and HOMA-IR

HFD and HFD-Al neonates had reduced glucose concentrations compared to control and Control-Al neonates (Fig. 4.9A). There were no significant differences in serum insulin concentrations (Fig. 4.9B) or HOMA-IR (Fig. 4.9C) amongst the groups. There were insufficient sera volumes for the determination of glucagon concentrations.

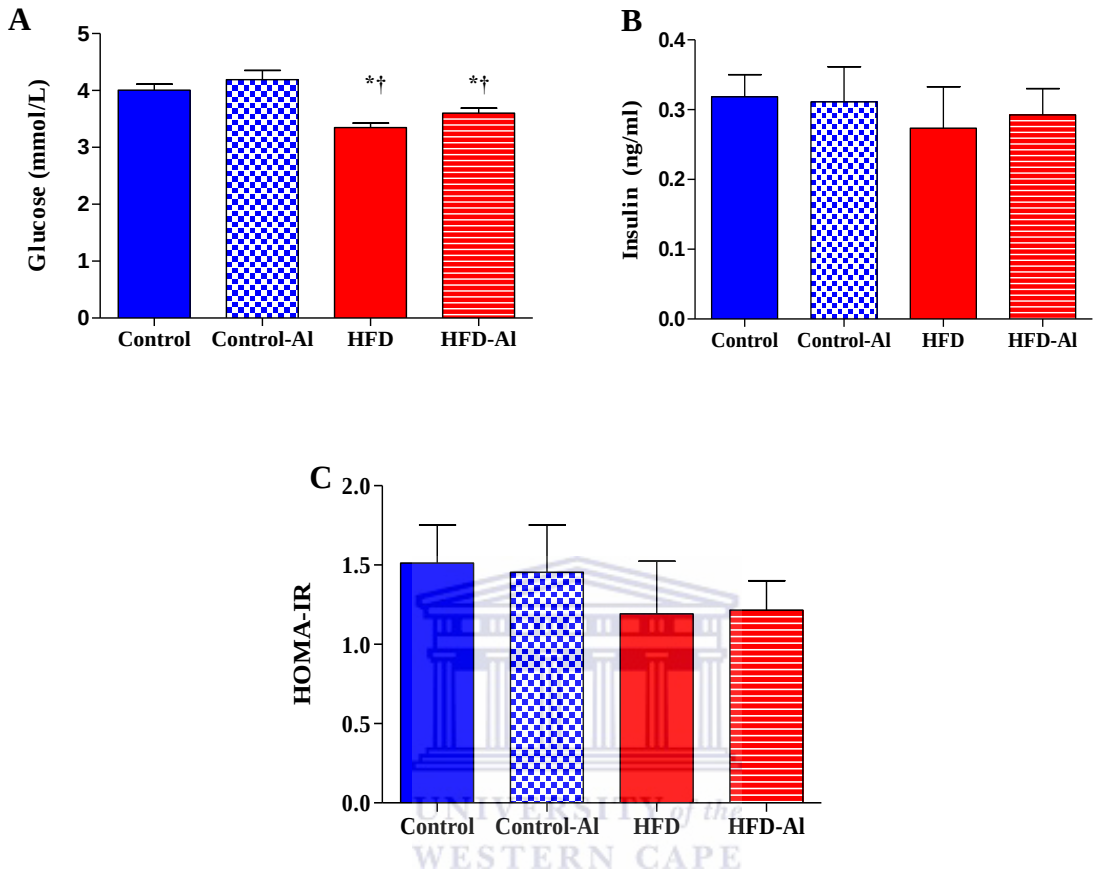


Figure 4.9 Neonatal blood glucose and serum insulin concentrations and HOMA-IR. (A), Neonatal blood glucose concentrations; (B), Neonatal serum insulin concentrations; (C), HOMA-IR in neonates. Data are means ± SEM. Control; Control-Al, control-*Aspalathus linearis*; HFD, high fat diet; HFD-Al, HFD-*Aspalathus linearis*. $^{*}p<0.05$ vs. control; $^{\dagger}p<0.05$ vs. Control-Al.

CHAPTER 5

DISCUSSION

5.1 Introduction to the main study

In our previous studies, the 20F neonates had elevated blood glucose concentrations compared to control neonates but this did not reflect hyperglycaemia as it was within the physiological range (Cerf *et al*., 2011). The serum insulin and glucagon concentrations remained unaltered (Cerf *et al*., 2011). We further investigated neonatal brain glucose transporter 2 (GLUT2) and neuropeptide Y expression. Both the 30F and 40F neonates displayed increased brain GLUT2 and neuropeptide Y immunoreactivity (Cerf *et al*., 2010) which may represent early events in the development of obesity and T2D. Hepatic GLUT2 and glucokinase (GCK) mRNA expression and immunoreactivity showed no significant differences amongst the groups (Cerf *et al*., 2011). This prompted further studies on foetal programming of metabolic disease. The present study therefore aimed to investigate the effect of gestational maternal diets varying fat (as energy) on proximal hepatic and skeletal muscle insulin signalling in neonatal Wistar rat offspring specifically focusing on IRα, IRS2 and PI3K-p110α.

5.1.1. Skeletal muscle

Skeletal muscle is the major site for insulin stimulated glucose uptake (Lin and Sun, 2010). The development of insulin resistance in skeletal muscle is critical in the pathogenesis of T2D (Yan *et al*., 2011). Foetal life is important for skeletal muscle development; after birth there is no net increase in the number of muscle fibers (Nissen *et al*., 2003). Late foetal life is also important for adipogenesis which may increase intracellular adipogenesis in skeletal muscle leading to insulin resistance (Du *et al*., 2010). Abnormal skeletal muscle development has been reported in several studies; offspring from mothers fed a cafeteria during gestation and lactation had reduced myocyte proliferation and insulin receptor mRNA expression (Bayol *et al*., 2005). In this study, neonates from mothers fed a 30% fat (30F) or 40% fat (40F) fat diet during gestation had a reduced IR and PI3K mRNA expression compared to neonates from mothers fed a 20% fat (20F) diet. These findings suggest compromised proximal insulin signalling, at the gene level, in 30F

and 40F neonates relative to 20F neonates. In skeletal muscle, reduced PI3K-p110β mRNA expression was reported in female offspring from obese mice (Shelley *et al.,* 2009).

In the present study, the 30F and 40F neonates had a reduced IRα immunoreactivity compared to control neonates with no significant changes in both IRS2 and PI3K-p110α immunoreactivity. The reduced IRα immunoreactivity may reflect impaired skeletal muscle proximal insulin signalling in 30F and 40F neonates. A decline in IR autophosphorylation of IRS1 and PI3K-p110 phosphorylation suggests impairment in early events of the insulin signalling pathway in high fat fed animals (Hansen *et al*., 1998).

The present study did not investigate IRS1 mRNA and protein expression but instead focused on IRS2. IRS2 was initially identified as an alternative in animals with IRS1 defects (Sesti *et al.*, 2001). Both IRS1 and IRS2 may regulate unique signalling pathways in different tissues but both mediate metabolic pathways (Sesti *et al.*, 2001). Based on the results obtained, further investigation on the phosphorylation of these insulin signalling cascades is warranted. In addition, the programming effects of maternal fat diets on offspring physiology and metabolism should be gender specific.

5.1.2. Liver

Offspring from mothers fed a "junk" diet had reduced hepatic IR and IGF mRNA expression with reduced hepatic IRS2 and GLUT2 mRNA expression compared to control offspring (Bayol *et al*., 2010), which was indicative of hepatic insulin resistance. We previously found no significant changes in both hepatic GLUT2 and GK mRNA expression and immunoreactivity (Cerf *et al*., 2011). In the present study, no changes in hepatic IR and IRS2 mRNA expression were found. However, hepatic IRα immunoreactivity was reduced in 40F neonates compared to control and 20F neonates which may reflect impaired hepatic insulin signalling at the receptor level. Therefore proximal insulin signalling may be compromised thereby limiting the effect of insulin's action in the liver. Specifically, reduced hepatic IRα immunoreactivity induced by high fat foetal programming may result in the inability of insulin to suppress hepatic gluconeogenesis thereby stimulating glycogen synthesis leading to hyperglycaemia and insulin resistance. However, there were no changes in glycaemia in 40F neonates (Cerf *et al*., 2011). β cell

compensation may prevent hyperglycaemia at the neonatal life stage but these neonates may be glucose intolerant. Further, with age, the adverse effects of foetal programming may become more profound particularly with additional insults such as prolonged high fat diet consumption. In rodents, offspring from high fat fed mothers showed reduced protein expression of hepatic IRβ and IRS1 but elevated protein expression of PKC-ζ, independent of hyperglycaemia (Buckley *et al.,* 2005; Considine *et al.,* 1995). Based on the results, further investigation on the phosphorylation of insulin signalling cascades, including PKC, PKB, DAG and ceramide is required to link the proximal and distal factors of insulin signalling.

The RNA integrity number for all the liver samples was less than 6 (the quality standard) therefore the RNA integrity of the samples was degraded. New samples should be analysed by qPCR to validate the hepatic gene expression findings. Further, there was disparity between the mRNA and protein expression (immunoreactivity) in liver samples which may be due to stability, degradation or contamination of mRNA samples. The IR mRNA and IRα protein expression (immunoreactivity) were reduced in 30F and 40F neonates compared to 20F neonates reflecting some changes in gene expression. The IRS2 mRNA and protein expression were not altered in both liver and skeletal muscle.

5.2. Maternal serum lipid profiles

The current evidence shows that FFA are implicated in obesity, insulin resistance and type 2 diabetes (Eckel *et al*., 2005). Endogenous fatty acids are the best biomarkers for FFA intake since they cannot be synthesised from carbohydrates (Riserus *et al*.,2009). The various FFA consumed by mothers during gestation play crucial roles in the growth and development of their foetuses. Maternal circulating FFA are correlated to the foetal circulating FFA with reduced total concentrations in foetuses relative to mothers (Cetin *et al.,* 2002). Changes in maternal and foetal plasma FFA concentrations may lead to the development of metabolic disease including type 2 diabetes. Animal studies demonstrated that insulin sensitivity is impaired by SFA and improved by PUFA (omega 3) (Siri-Tarino *et al*., 2010). In rats, SFA have shown to increase intramuscular palmitic acid (16:0) accumulation that may lead to insulin resistance in (Reynoso *et al*., 2003).

The present study investigated the changes in serum lipids of pregnant mothers fed diets varying in fat (as energy) throughout the gestation period. Previous studies have demonstrated that high fat diet fed rats exhibited a significant increase in total triglyceride and cholesterol concentrations compared to control rats (Srinivasan *et al.*, 2008). In contrast, in our study, there were no significant changes in serum total triglyceride, cholesterol and FFA concentrations. Therefore we further investigated serum total and individual FFA composition.

Previous studies in humans reported a positive association between serum FFA composition and diabetes, characterised by higher proportions of palmitic acid (16:0), dihomo-γ-linolenic (20:3 n-6) and palmitoleic acid (16:1 n-7) in cholesterol esters with low proportions of linoleic acid (18:2 n-6) (Coelho *et al*., 2011; Vessby *et al*., 1994). In this study, we found a reduction in total SFA composition in 30F and 40F mothers compared to control mothers. Further, circulating palmitic acid (16:0) and stearic acid (18:0) were not affected by diet varying in fat (as energy) in these mothers. We further investigated the palmitoleic (16:1 n-7)/palmitic (16:0) ratio which is a better marker for palmitic acid (16:0) intake via activity of desaturate enzyme (Warensjo *et al*., 2008). The serum palmitoleic (16:1 n-7)/palmitic (16:0) ratio was unaltered. However, circulating palmitoleic acid was reduced in both 30F and 40F mothers on day 14 of gestation. Therefore, a reduction in SFA and circulating palmitoleic acid may reflect transient improved insulin sensitivity in both 30F and 40F mothers at this specific time points in gestation.

MUFA have been reported to improve insulin sensitivity and SFA reduced insulin sensitivity by 24% compared to MUFA in overweight subjects (Riserus *et al*., 2009). The total MUFA and oleic acid (18:1 n-9) composition was increased in 40F mothers compared to both 20F and 30F mothers on the day of delivery with no detected changes in 40F mothers compared to control mothers. This may reflect improved insulin sensitivity at delivery in 40F mothers relative to 20F and 30F mothers.

PUFA, particularity the omega-3 fatty acids docosahexaeonic acid (22:6 n-3) and eicosapentaenoic acid (20:5 n-3), have been reported to improve insulin sensitivity compared to SFA (Riserus *et al*., 2009). In high fat diet fed rats, dietary intake of medium and long-chain triglycerols ameliorated insulin resistance (Terada *et al*., 2012). This may be due to their greater oxidative rates compared to SFA (Coelho *et al*., 2011). Other studies have shown that FFA

oxidation increases proportionately with PUFA/SFA ratio (Bradshaw *et al*., 2009; Coelho *et al*., 2011). In this study, the total n-3 PUFA was reduced in both 30F and 40F mothers on day 7 of gestation which may reflect insulin insensitivity in early gestation. On the day of delivery, the total n-3 PUFA remained reduced (albeit not significant) in 30F and 40F mothers. Linoleic acid (18:3 n-3) was also reduced in both 30F and 40F mothers on day 7 of gestation. On day 14 of gestation, docosapentaenoic acid (22:5 n-3) was reduced in 30F and 40F mothers. This reduction in total and specific n-3 PUFA may reduce insulin sensitivity of the mothers and developing foetus. However, the n-6 PUFA/n-3 PUFA ratio was not affected.

5.3. Pilot study: *Aspalatus linearis* (green rooibos)

The prevalence of T2D in children continues to increase globally. Maternal over-nutrition is associated with an increased risk of metabolic disease including T2D later in life. Rooibos tea is considered healthy and safe to drink during pregnancy due to the absence of alkaloids and low tannin content (Joubert *et al*., 2008). Rooibos tea contains several active compounds; one of the major active compounds is aspalatin, a flavanoid that is unique to rooibos and believed to be partially responsible for its hypoglycaemic effects (Son *et al*., 2012; Kawano et al., 2009). Aspalatin has been reported to improve glucose tolerance *in vivo* (Kawano *et al*., 2009). In this study, we assessed the therapeutic potential of *Aspalatus linearis* extract (aspalatin-enriched) on the programming effects of a high fat diet. The HFD-Al mothers had elevated blood glucose concentrations on day 20 of gestation; however this did not reflect hyperglycaemia as it was within the physiological range. The rooibos tea extract had no effect on maternal serum insulin and glucagon concentration. In addition, there were no differences in insulin sensitivity as estimated by HOMA-IR. Further, there were no changes in maternal calorie intake, body weights and organ weights (liver, heart, brain and pancreas). We also found no differences in litter size among the groups. These findings suggested that rooibos tea extract had neither harmful nor ameliorative effects in pregnant mothers.

In the present study, HFD neonates had increased head circumference, brain weight and body weight compared to control neonates reflecting more rapid growth and development. Increased body weight in HFD neonates may reflect adiposity which should be investigated further. Offspring from rats fed a high fat diet (35% fat) during gestation displayed elevated FFA

concentrations which could lead to increased adiposity and insulin resistance late in life (McCurdy *et al*., 2009). The HFD-Al neonates were heavier than control neonates with no differences between HFD and HFD-Al neonates. Therefore *Aspalathus linearis* supplementation in offspring maintained on a high fat diet increased body weight and did not reduce body weight in high fat exposed neonatal progeny.

The hypoglycaemic effect of aspalatin has been reported to be via AMPK activation to promote endogenous GLUT4 translocation in L6 myocytes (Son *et al.*, 2012). However, the HFD and HFD-Al neonates displayed reduced blood glucose concentrations compared to control neonates. With the insult of high fat diet consumption, *Aspalathus linearis* treatment may therefore hold some glucose lowering potential. This should be confirmed in further studies. Serum insulin concentrations and HOMA-IR were not affected but these could potentially be altered as glycaemia fluctuates.

ROS triggers insulin resistance (Kawano *et al*., 2009). By reducing oxidative stress, *Aspalathus linearis* may prevent peripheral and tissue-specific insulin resistance. In STZ-induced diabetic rats, rooibos tea partially ameliorated oxidative stress (Ulicna *et al*., 2006). Therefore oxidative stress should be assessed in foetal programmed progeny and their mothers.

CHAPTER 6

CONCLUSION AND FUTURE WORK

6.1 Study limitations

A limitation of the present study was low sera volumes for maternal lipidaemia analyses as only total cholesterol concentrations were determined without measuring HDL and LDL cholesterol.

The study could be strengthened by determining insulin signalling phosphorylation which reflects activity of these insulin signalling cascades and could reinforce some of the findings. Further, Western blot analyses could confirm our immunoreactivity findings.

Gender specific programming effects also present a limitation as the physiological systems and hormones differ in males and females.

6.2 Conclusion and future work

The overall aims of the study was to investigate the effects of maternal diets varying in fat content on neonatal gene and protein expression profiles of proximal insulin signalling factors (IRα, IRS2 and PI3K-p110α) and to assess the therapeutic potential of *Aspalathus linearis* (green rooibos) on the programming effects of a high fat diet.

Foetal high fat programming reduced neonatal IRα immunoreactivity in both the liver and skeletal muscle which may impair proximal insulin signalling in these organs at the receptor level. Further, skeletal muscle IR and PI3K mRNA expression were also reduced by high fat diet in the 30F and 40F neonates compared to 20F neonates. However, hepatic IR and IRS2 mRNA expression was not affected and further studies are needed. High fat diet had no effect of maternal circulating total FFA, triglycerides and cholesterol concentrations. The reduction in total and specific omega 3 fatty acids may have a negative impact on the insulin sensitivity of the mother and foetus.

Aspalathus linearis had no effect of maternal serum insulin and glucagon concentrations. In addition, maternal calorie intake, body weight and organ weights (liver, brain and pancreas) were

not altered amongst the groups, suggesting that rooibos had neither harmful nor ameliorative effects pregnant mothers. The HFD-Al neonates were heavier than control neonates suggesting that rooibos treatment at the dose of 150 mg/kg had no ameliorative effect on the body weight of offspring from mothers fed high fat diet during pregnancy.

Futures studies

New samples should be analysed for qPCR to validate the gene expression findings of the present study. Based on the results obtained, further investigation on the phosphorylation of these insulin signalling cascades is warranted. In addition, the programming effects of maternal fat diet on offspring physiology and metabolism should be gender specific.

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APPENDIX A

HAEMATOXYLIN AND EOSIN STAINING OF NEONATAL LIVER AND SKELETAL MUSCLE

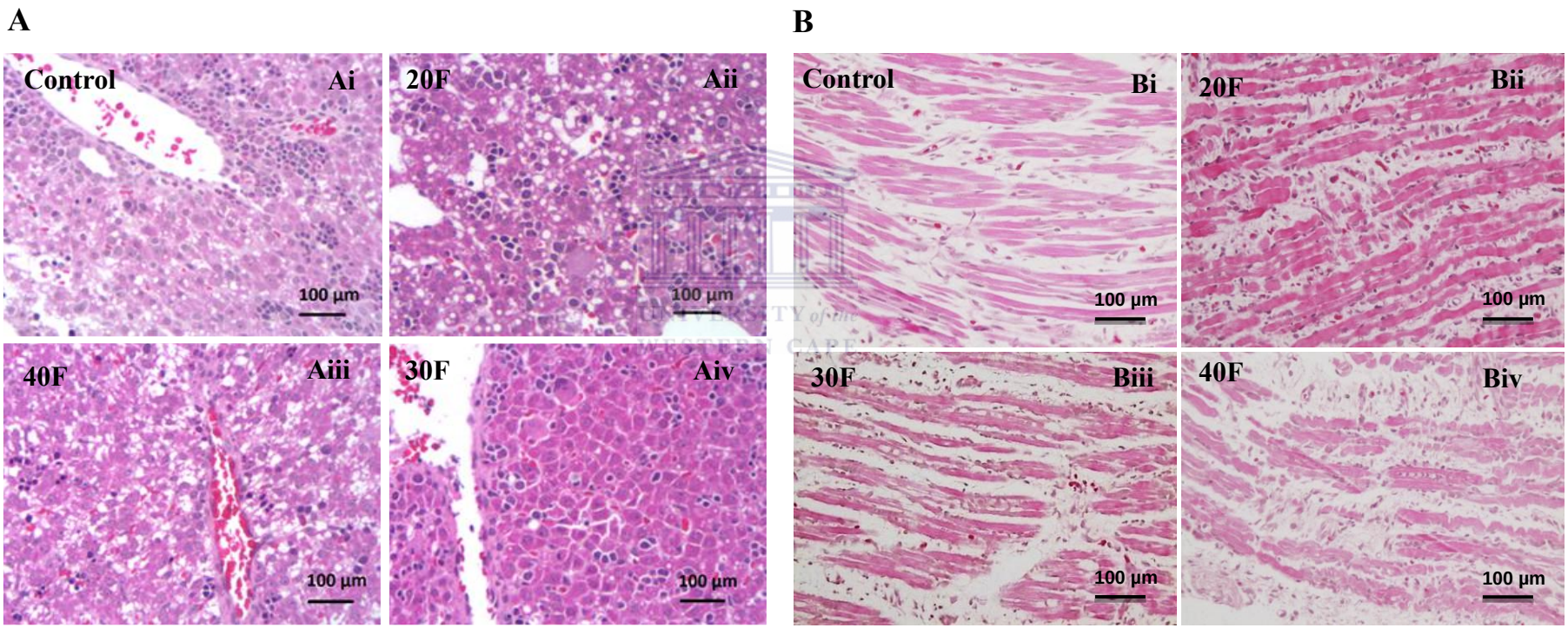

Figure A.1 Morphological analysis of neonatal tissue using haematoxylin and eosin stain. (A), Neonatal liver, Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B), Neonatal skeletal muscle, Control (Bi), 20F (Bii), 30F (Biii) and 40F (Biv). Control; 10F, 10% fat diet; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

APPENDIX B

IMMUNOHISTOCHEMICAL STAINING FOR NEONATAL LIVER AND SKELETAL MUSCLE IRβ AND IRS1

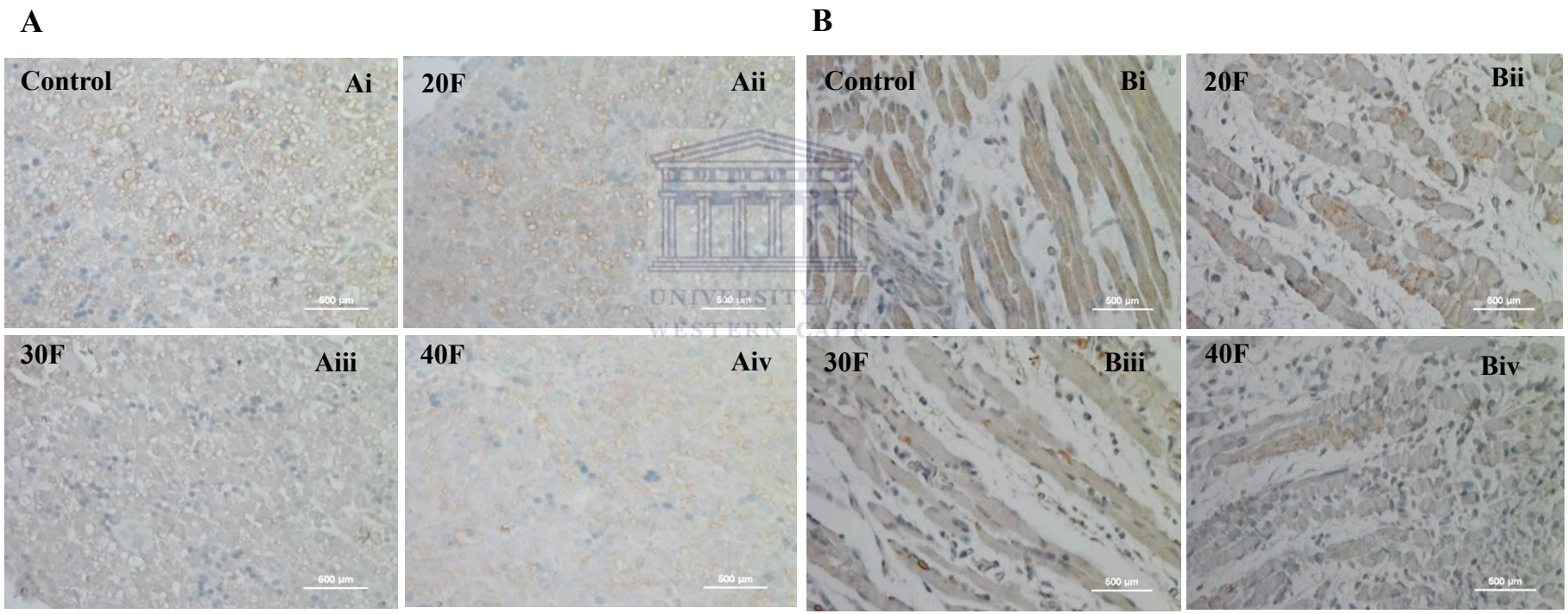

Figure B.1 Neonatal hepatic and skeletal muscle IRβ immunoreactivity. (A), Neonatal liver immunostained with ant-IRβ in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B) Neonatal skeletal muscle immunostained with ant-IRβ in Control (Bi), 20F (Bii), 30F (Biii) and 40F (Biv). Control; 10F, 10% fat diet; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

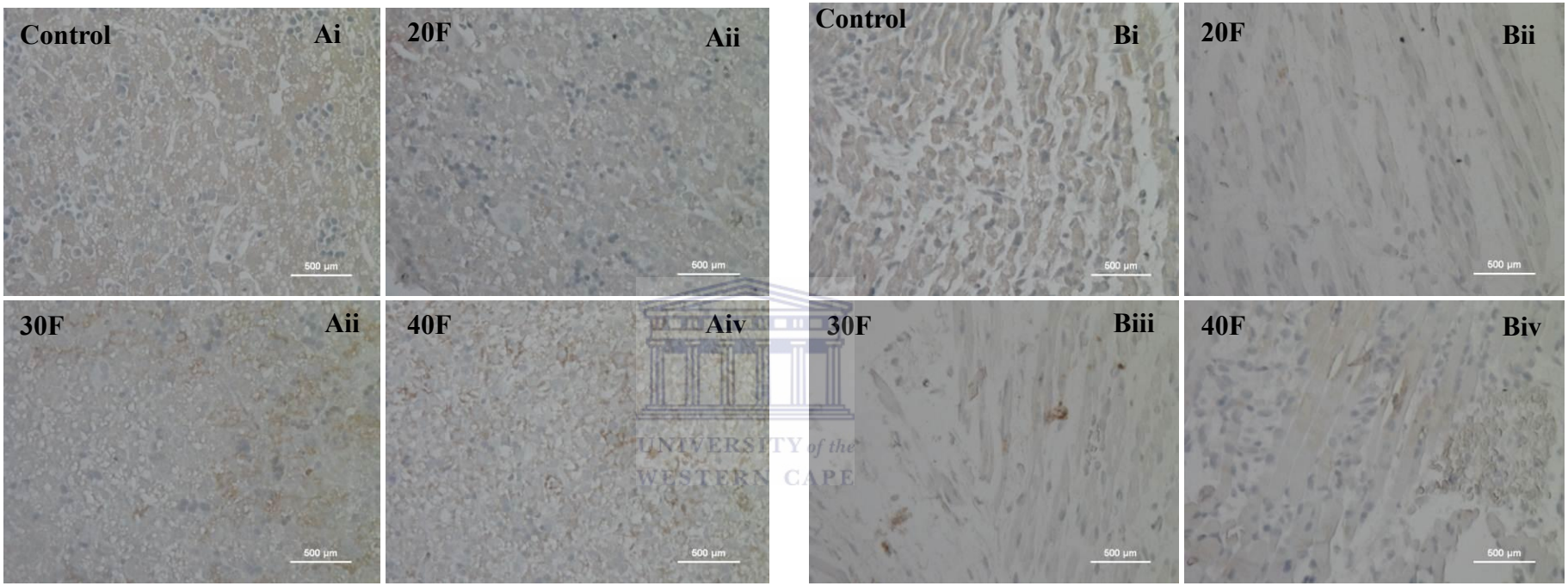

Figure B.2 Neonatal hepatic and skeletal muscle IRS1 immunoreactivity. (A), Neonatal liver immunostained with ant-IRS1 in Control (Ai), 20F (Aii), 30F (Aiii) and 40F (Aiv); (B) Neonatal skeletal muscle immunostained with ant-IRS1 in Control (Bi), 20F (Bii), 30F (Biii) and 40F (Biv).Control; 10F, 10% fat diet; 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet.

APPENDIX C

TOTAL RNA QUALITY CONTROL

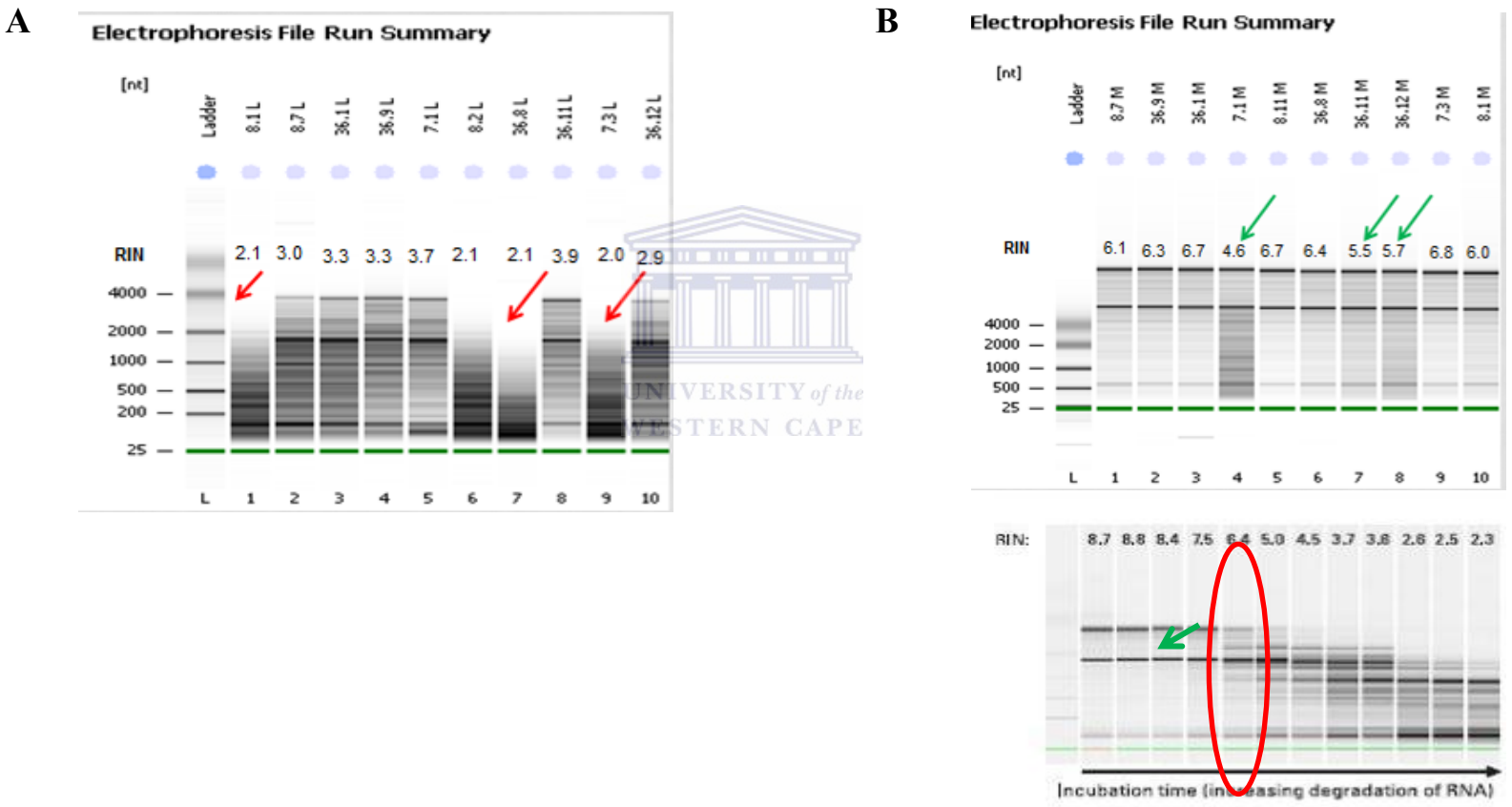

Figure C.1 Total RNA quality control. (A) Liver samples; (B) Skeletal muscle samples

APPENDIX D

Table D.1 Antibodies used in immunehistochemical (IHC) staining

Antibody	*Clonality*	*Company*
IRα (ab78424)	Rabbit polyclonal	Abcam, Cambridge, UK
IRS2 (H-205) : (sc-8299)	Rabbit polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA
PI3K-p110α (C73F8)	Rabbit monoclonal	Cell signalling Technology, Danvers, MA, USA
IRβ (C-19) : (sc-711)	Rabbit polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA
IRS1 (1M92-7)	Mouse monoclonal	Millipore upstate, Singe Oak Drive, Temecula, CA, USA

Table D.2 Buffer and reagents used in IHC

Reagent	Supplier
0.05M Tris-Buffered Saline (TBS) pH 7.2	
Tris (hydroxymethyl aminomethan)	Merck
Sodium chloride	Merck
0.1M Phosphate Buffered Saline (PBS) pH 7.2	
Sodium dihydrogen orthophosphate (NaH_2PO_4)	Merck
Disodium dihydrogen orthophosphate (Na_2HPO_4)	Merck
Sadium chloride	Merck
Bovine serum albumen	Merck
Sodium azide	Merck
0.01M Citrate Buffer	
Citric acid ($C_6H_8O_7.H_2O$)	Merck
Tri-sodium citrate ($C_6H_5Na_3O_7.2H_2O$)	Merck
Liquid DAB + Substrate Chromagen System	DAKO Corporation (Diagnostech)
Normal Horse Serum (NHS) 65µl NHS diluted in 1235µl 0.1M PBS pH 7.2	
Normal Goat Serum (NGS) 65µl NGS diluted in 1235µl 0.1M PBS	
Biotinylated anti-Mouse IgG	Vector Labolatories
Biotinylated anti-Rabbit IgG	Vector Labolatories
Vectastain ABC kit	Vector Labolatories