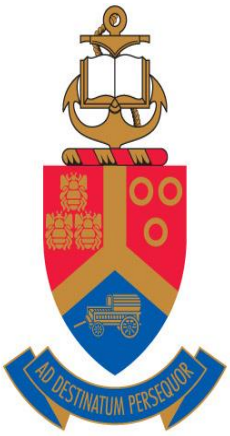
The Effects of Diet Varying in Fat Content on the Pathogenesis of Diabetes Mellitus in Wistar Rat Offspring



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Denkleiers • Leading Minds • Dikgopolo tša Dihlalefi

Nhlanhla Simphiwe Christa Mthembu

Dissertation submitted in fulfillment of the requirements for the degree of Master of Science in Human Physiology
Department of Physiology, Faculty of Health Sciences, University of Pretoria

Supervisor: Dr Craig W. Grobbelaar
Co- Supervisor: Dr Marlon E. Cerf

November 2015

The Effects of Diet Varying in Fat Content on the Pathogenesis of Diabetes Mellitus in Wistar Rat Offspring

Nhlanhla Simphiwe Christa Mthembu

ABSTRACT

Aims/hypothesis: The incidence of type 2 diabetes (T2D) is increasing globally. T2D is characterised by progressive deterioration of glycaemic control. These changes in glucose homeostasis are primarily due to β-cell secretory dysfunction and/or peripheral insulin resistance. Studies show that maternal health directly influences foetal development and birth outcomes. Malnutrition of the growing foetus may lead to development of T2D and other metabolic diseases later in life. Consequently, several studies have reported that maternal diet programmes the foetus leading to altered physiology and metabolism in the offspring. Foetal programming refers to the exposure to a stimulus and/or insult during the critical periods of development i.e. foetal and early neonatal life. We therefore sought to ascertain how a dietary fat content in maternal diet affects foetal programming and its contribution to the pathogenesis of T2D of the offspring. ***Methods****:* Pregnant rats were randomly grouped and maintained on diets varying in fat content: 10% (Control), 20% (20F), 30% (30F) and 40% (40F) fat throughout their pregnancy. Pancreata were collected and quantitative polymerase chain reaction tests were performed to determine the mRNA expression profiles of the insulin signaling and transcription factors including Pdx1, MafB, IRα, insulin and glucagon. Other pancreata were immunostained followed by image analysis of these factors. ***Results****:* In 40F neonates, Ins1, Ins2, glucagon, MafB and IRS2 mRNA expression was reduced. Further, in 30F neonates, Ins1, Pdx1 and MafB mRNA expression was reduced. There were no changes in immunoreactivity for the factors studied. However, when separating the offspring according to gender, IRα immunoreactivity was reduced in 40F females compared to 40F males. ***Conclusion****:* Continuous exposure of pregnant rats to an excessively high fat diet impairs gene expression of key factors involved in insulin signaling and islet development in their neonatal offspring. This reflects foetal programming of metabolic pathways in insulin signaling and β-cell development and function which potentially renders these offspring susceptible to metabolic disease and the development of T2D.

Keywords:

Pancreatic transcription factors

Foetal programming

Type 2 diabetes,

β-cell

High fat diet

DECLARATION

By submitting this dissertation, I declare that the work contained therein is my own, and that all the sources that I have used, have been indicated and acknowledged as complete references, that reproduction and publication thereof by the University of Pretoria will not infringe any third party rights.

November 2015

ACKNOWLEDGEMENTS

I would like to express my deepest and sincerest gratitude to my supervisors Dr Craig Grobbelaar and Dr Marlon Cerf, for your guidance, scientific knowledge, support, patience and encouragement. Without both your guidance, I would not have completed by MSc degree and have the knowledge and skills that I have gained. I acknowledge your challenges to ensure the completion of this project. Because you did not give up, I could not give up. Thank you for your sacrifices to help me, may God bless you and show you favour in all that you do.

Dr Andre Stander, your assistance with the lab work and understanding of concepts is greatly appreciated. Your patience was amazing and your kind words and positivity helped me more than you can imagine.

I also wish to extend my gratitude to the following:

Carlo Jackson from the Department of Immunology of the University of Pretoria

Helena Taute from Cell Biology University of Pretoria for her assistance with microscopy

Lerato Mpye from the Witwatersrand University for assisting me with the image analysis.

Department of Physiology staff of the University of Pretoria.

Dr Steve Olorunju of the Medical Research of South Africa, Pretoria for your assistance with the statistics.

Mrs Charna Chapman and Mr Desmond Linden for their assistance with the immunohistochemical analysis.

Diabetes Discovery Platform (DDP), Medical Research Council: Dr. Johan Louw, Dr. Christo JF. Muller and the entire DDP staff for their continued support during this project.

JB Marks Education Trust Fund

The National Research Foundation for all their support.

Finally, my family and friends, especially my mother, I do not have the words to express my appreciation for you, for the love and support you continued to give me throughout the duration of this project. Your prayers helped me, gave me the strength and motivation that I needed to want to continue. I love you and could not have asked for a better mother to help guide me in this life.

Where would I be without my Heavenly Father? There are not enough words to thank Him and glorify Him. Could never have have come this far without His grace and favor.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

β-cell	Beta cell
α-cell	Alpha cell
δ- cell	Delta cell
ε-cell	Epsilon cells
DM	Diabetes Mellitus
DAB	Diaminobenzidine tetrachloride
DNA	Deoxyribonucleic acid
E-	Embryonic day
ADP	Adenosine diphosphate
Arx	Aristaless related homeobox
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
FFA	Free fatty acids
Foxa2	Forkhead box protein A2
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GSK3	Glycogen synthase kinase 3
HDL	High-density lipoprotein
HFD	High fat diet
HNF-	Hepatocyte nuclear factor-

HOMA-IR	Homeostasis Model of Assessment - Insulin Resistance
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IGF	Insulin-like growth factor
IgG	Immunoglobulin
IHC	Immunohistochemistry
IHF-1α	Insulin promoter factor- 1α
IL-6	Interleukin 6
INS1	Insulin- 1
INS2	Insulin- 2
INSR	Insulin receptor
IR	Insulin Resistance
IRS	Insulin receptor substrate
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
IRα	Insulin receptor alpha
IRβ	Insulin receptor beta
LDL	Low-density lipoprotein
MafA	Musculoaponeurotic fibrosarcoma oncogene family A
MafB	Musculoaponeurotic fibrosarcoma oncogene family A
MetS	Metabolic syndrome
MODY	Maturity-onset diabetes of the young
NeuroD1	Neurogenic differentiation 1
Nkx2.2	NK2 related transcription factor related, locus 2
NGS	Normal Goat Serum

NHS	Normal Hoarse Serum
NIDDM	Non- Insulin dependent diabetes mellitus
NPY	Neuropeptide Y
Pax 4	Paired box gene 4
Pax 6	Paired box gene 6
Pdx1	Pancreatic duodenal homeobox 1
PBS	Phosphate Buffered Saline
pH	Protons of hydrogen
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
PTB	Phosphotyrosine binding
PP	Pancreatic polypeptide
Prox1	Prospero homeobox protein 1
PtdIns(3,4,5)P_3	Phosphatidylinositol (3,4,5)-triphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real-time RT-PCR
RNA	Ribonucleic acid
SEM	Standard error of the mean
SH2	Src Homology 2
SOP	Standard operating procedure
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus

TBS	Tris-Buffered Saline
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

AND

LITERATURE REVIEW

1.1 INTRODUCTION

The incidence of chronic diseases has reached epidemic proportions globally with Diabetes Mellitus (DM) becoming a global health burden since the early 1980s, and one of the leading causes of morbidity and mortality. According to the International Diabetes Federation (IDF), in 2013, 381.8 million people globally were estimated to have diabetes. This number is expected to increase to 591.9 million by 2035. However, an estimated 174.8 million (45.8%) individuals remain undiagnosed. About 90-95% of diabetes is attributed to type 2 diabetes mellitus (T2D) (1-3).

Non-modifiable risk factors e.g. age, ethnicity and genetics, are no longer the sole cause of the high prevalence of T2D. Research has established that modifiable factors, most notably environmental factors contribute appreciably to this epidemic (4). Initially, T2D was a condition believed to occur primarily in adults. However, recent evidence indicates that both children and adolescents are also largely affected by this metabolic disorder (4).

The increase in the prevalence of T2D in children may result from a combination of factors that are intrinsically linked to maternal nutrition during foetal development (5). Maternal nutrition not only provides nutrients to the foetus but also affects the capacity of maternal metabolic regulation of the hormones secreted by the placenta which influences the metabolism of all nutrients. Nutrition during early development is important for foetal growth, organ development, body composition and body system functions (5).

Both under-nutrition and over-nutrition of the growing foetus are associated with an increased risk of developing T2D. The consistency of these findings has led to evidence that the maternal diet programmes the foetus by altering the physiology and metabolism of the offspring (5-7).

A close relationship exists between obesity, the development of metabolic syndrome and T2D. A westernized lifestyle and a diet rich in saturated fat content have been shown to be the main contributing factors to increasing obesity, insulin resistance and T2D (4-6).

In order to maintain normoglycaemia, the body compensates by increasing synthesis and secretion of insulin. This results in an increase in β-cell mass in individuals with a higher body mass. This greater demand for insulin eventually results in β-cell dysfunction and loss of β-cells due to apoptosis (4-8).

1.2 THE PANCREAS

1.2.1 Pancreas development

The pancreas is a mixed, multifunctional gland essential for digestion and glucose homeostasis (1, 2). It is comprised of the endocrine and exocrine components (3,4). The exocrine segment contains the acinar cells and ductal cells. The acinar cells secrete and deliver digestive enzymes into the gastrointestinal tract (4). The endocrine portion secretes hormones into the bloodstream to regulate plasma glucose concentrations (1,4). The functional unit of the endocrine pancreas is the islets of Langerhans. A normal endocrine pancreas comprises of ±1 million islets with each islet consisting of five cell types, viz. beta (β)-cells comprising majority of the islet endocrine cells (60-80%); followed by alpha (α)-cells which are the glucagon producing cells (20-30%); delta (δ)-cells (5-15%), pancreatic polypeptide (PP)-cells and epsilon (ε)-cells and which produce the hormones somatostatin, pancreatic polypeptide and ghrelin respectively (5,6,15).

Pancreas development involves a cascade of events concomitant with the initiation of transcription factors (1). During embryogenesis, pancreas development originates from the evagination of the endoderm (germ layer), first dorsally and then at a later stage ventrally. Both pancreatic buds proliferate and later fuse to form a functional organ (1-3). In humans, pancreas organogenesis is seen as early as 10 weeks of gestation and the principal stage of isletogenesis ensues in the second trimester (7,63).

In rodents, early morphological signs of pancreas development are detected around embryonic day e8.5 to e10.5, when the first pancreatic tissue, the dorsal pancreatic bud, arises from the endoderm of the foregut which will later fuse to form a functional organ (1,6,7,63). Parallel to this, endocrine cells, specifically the α-cells, are the first

cells to be observed at e9.5. At e10.5, a few β-cells are detected that co-express insulin and glucagon. Only at mid-gestation, from e13.5-e14 when the endocrine cells reach their peak, do fully differentiated α and β cells arise. It is during this stage of gestation when the first population of exocrine cells is detected and at e18, just prior to birth, the PP producing cells start to differentiate and thus the endocrine cells arrange as well organized islets. The pancreas goes through rapid development following birth. The islets continue to grow faster than the rest of the pancreas. This is followed by weight augmentation of the pancreas and the rest of the other cell types. This process continues until weaning (3,4,6).

1.2.2 Transcription factors

Transcription factors are gene regulatory proteins that play an integral role in islet cell development and direct cell fates by regulating the transcription of genes involved in specification and ultimately mature function of the pancreas (12). Furthermore, transcription factors are key targets of nutritional programming through epigenetic mechanisms (12,13) as they bind to a specific DNA sequence in the promoter region of other genes and regulate the expression of their proteins (14). Transcription factors are significant constituents of signaling cascades that regulate various physiological cellular processes. The development and maturation of the pancreas and its cells requires the specific sequential expression of transcription factors (12-14).

The development and differentiation of α-cells is dependent on several transcription factors that include Isl1, NeuroD1, Nkx2.2, Sox 4, and Prox1, with Pax6 and Foxa2 considered the most critical as displayed in Figure 1 (3-5). Pax 6 is a critical component of α-cell differentiation, primarily through glucagon production by directly and indirectly controlling glucagon gene transcription and processing (6). Arx plays an essential role in α-cell formation and Isl1 is a key activator of Arx transcription during α-cell formation. Isl1 is only necessary for the maintenance of transcription in forming α-cells (8). Pax4 is transcription factor that plays an essential role in β-cell differentiation. Pax4-deficient mice do not develop β-cells and die shortly after birth from impaired insulin production (5). Thus, the Pax4 gene could represent a possible susceptibility gene for diabetes (5). Pdx1, Pax6, NeuroD (Beta2) and Nkx2.2 represent core components of a transcription factor complex of an islet-enriched

gene that contributes to regulating expression of genes selectively expressed in β-cells during development (9). Pax6 functions in parallel to Pdx1, Nkx2.2, and Nkx6.1 in some of the β-cells during the late stages of pancreatic development (10). MafA has recently been hypothesized to be important for the differentiation of β-cells, with Nkx6.1 occurring upstream to MafA during pancreatic development (11). The transcription factors, Pdx1, MafA, Nkx2.2, Pax6 and NeuroD1 (Beta2) all regulate the insulin gene (5,11).

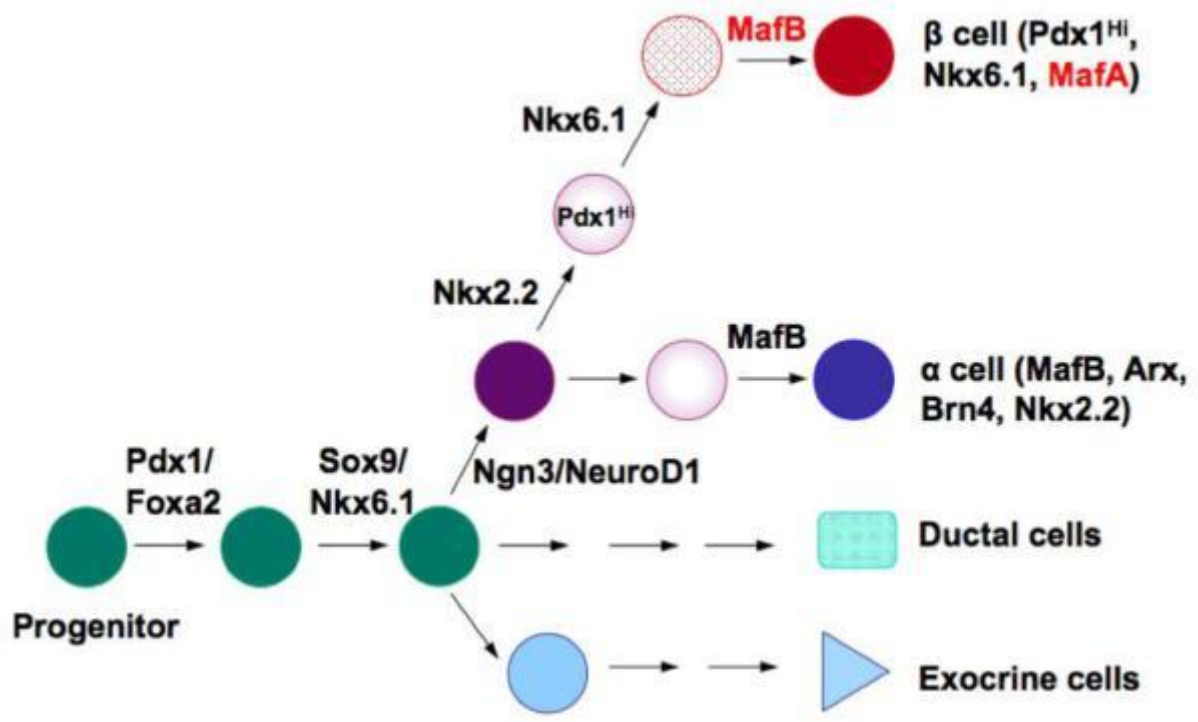


Figure 1. Transcription factor regulation of rodent pancreatic development. Adapted from (18).

Pdx1 is regarded as one of the most critical transcription factors in the regulation of β-cell development and function. The importance of Pdx1 in the pancreas is emphasized by the near-absence of pancreas formation in Pdx1 null mice. This important transcription factor is also required for maintaining function in mature β-cells. The downregulation of Pdx1 expression in β-cells may underlie the pathogenesis of β-cell failure and T2D. Thus, Pdx1 plays both a broad role in pancreas development and a more specific role in β-cell function in adulthood (16,17). Pdx1 is broadly expressed at around 4 weeks with a high level of expression

later restricted in adult human β-cells (10, 11). Elevated Pdx1 expression is specific to rodent β-cells at e15.5, where it regulates the expression of Ins1 and MafA (12).

Table 1: Pdx1 expression and function of in the developing and mature mouse pancreas. Adapted from (16).

Developmental period	Pdx1 localisation	Pdx1 functional role
Early pancreatic development (e8.5-e12)	All cells of the early pancreatic endoderm, and portions of the stomach and duodenum	Required for: • Branching and morphogenesis of the pancreatic buds • Brunner's gland formation in the stomach • Enteroendocrine cell differentiation in the stomach and duodenum
Mid to late pancreatic development (e12.5-e18)	Endocrine and acinar cells	Important for acinar and islet cell formation
Adult pancreas	Primarily β- (insulin) and δ- (somatostatin) cells but occasionally in duct and acinar	Essential for: • Maintenance of mature islet function • Regulation of β-cell mass • Regeneration of β cells

MafA is another essential activator of the insulin gene. MafA is involved in processes important for islet cell formation and function, is first produced in insulin-producing cells of the secondary transition, and can independently induce insulin expression in non-β cells. Members of the large Maf transcription family are associated with processes necessary for cell formation, including the brain, cartilage and immune system (18). In addition to MafA, MafB is capable of activating insulin enhancer-driven expression in non-islet cell transcription assays, even though MafB is primarily found in α-cells. MafB is an important regulator of glucagon gene expression (18). MafB has been reported to specifically activate endogenous glucagon expression when overexpressed in a β-cell line. MafB is present in both insulin and glucagon producing cells during development, with expression only restricted to α-cells shortly after birth (18).

The functional roles of many transcription factors were revealed in genome-wide analyses of various forms of diabetes, e.g. maturity-onset diabetes of the young (MODY) genes are responsible for rare forms of diabetes that are caused by single gene mutations. Although MODY accounts for only 1-2% of diagnosed cases, their monogenic nature links these factors to roles in β-cell identity which implicates them in the pathogenesis of T2D and T1D (19).

MODY is characterised by a high phenotypic penetrance rate, early disease onset (±20-30 years of age), the absence of obesity as well as biochemical and clinical features of impairment in insulin secretion. Six MODY genes, five of which code for transcription factors, have been identified, viz. hepatocytes nuclear factor-4α, -1α, -1β (HNF-4α, -1α, -1β), glucokinase, insulin promoter factor-1α (IPF-1α or Pdx 1) and NeuroD1 (Beta2). The exception is glucokinase which is an enzyme that acts as a pancreatic glucose sensor and facilitates phosphorylation of glucose to glucose-6-phosphate (14).

1.2.3 β- and α-cells

β-cells are highly specialised cells central to fuel regulation and are juxtaposed with α-cells and other counter regulatory hormones mainly involved in glucose homeostasis (20). β-cells have the unique ability to produce and secrete substantial amounts of insulin in a regulated pulsatile modus in response to elevated circulating blood glucose concentrations thereby stimulating glucose uptake in peripheral tissues (e.g. liver, skeletal muscle, adipose tissue and brain) (20,21). β-cells work in conjunction with α-cells (which secrete glucagon) to promote glucose release from stores in response to decreasing blood glucose concentrations. At birth, β-cells are functionally immature, lacking the ability to respond to a changing glucose concentration, only acquiring glucose responsiveness over the first 3-4 weeks postnatally (19-21).

The regulation of β-cell mass is important for understanding the pathogenesis of diabetes. β-cell mass is defined as the overall balance of β-cell replenishment and death which is dependent on several mechanisms. These mechanisms include replication, neogenesis and size variations (β-cell replenishment) and apoptosis, necrosis and autophagy (β-cell death). After birth, there is a transient surge of β-cell replication, followed by a transitory rise in neogenesis. During childhood and

adolescence, the rates of β-cell replication, neogenesis and apoptosis adjust to reach a balance for maintaining sufficient β-cell mass throughout adulthood (23). With aging, β-cell mass decreases as the rate of apoptosis slightly outweighs replication and neogenesis (24).

The half life of β-cells in rodents is estimated to be around 50-60 days with approximately 0.5% of the β-cell population undergoing self-replication and a corresponding number entering apoptosis. Thus, the β-cell mass remains relatively constant under physiological conditions during the animal's adult life (5,23). However, any reduction in β-cell mass may be the result of either an impairment of β-cell replication and/or neogenesis or increased β-cell apoptosis or both combined, induced by genetic or acquired factors acting prenatally or postnatally. Among the acquired factors, glucotoxicity and lipotoxicity have been proposed as major contributors and have thus been investigated thoroughly (5,23). Glucotoxicity is the chronic elevation of blood glucose concentration that impairs β-cell function and insulin sensitivity and lipotoxicity is the injurious effects that accumulated fatty acids and their metabolic products have on β-cells (5,23)

Endocrine pancreas plasticity is defined as the ability of the organ to adapt its β-cell mass to variations in insulin demand for optimal control of glucose homeostasis. This property is essential and can be considered to be long-term regulation of insulin secretion (25). Maternal high fat diet maintenance during gestation significantly reduces β-cell number and volume leading to impaired insulin release (26). In contrast, the volume of α-cells, which are responsible for glucagon secretion, is expanded due to increased cell number and size. Thus at birth these neonates present with hyperglycaemia. However, three weeks after birth, the volume, number and size of both α- and β-cells are similar to controls, although these animals still have reduced fasting insulin concentrations and glucose intolerance. A high fat diet during early development modifies pancreatic development leading to decreased insulin production and hyperglycaemia during early postnatal growth (26).

1.3 INSULIN SIGNALING

The elevation of glucose concentrations triggers the insulin signaling cascade in the major glucose recipient tissues i.e. muscle, liver and adipose tissue, and other glucose-sensitive tissues. The insulin receptor (INSR) is a member of the large class of tyrosine kinase receptors. It is a transmembrane receptor that is activated primarily by insulin. The insulin receptor is a heterotetrameric protein composed of two extracellular α subunits and two transmembrane β subunits connected by disulphide bonds (27,28). Binding of insulin to the extracellular α subunit induces phosphorylation of several endogenous tyrosine residues present in the β subunit (27). The first substrate to be tyrosine-phosphorylated is the β subunit through a mechanism of autophosphorylation. Autophosphorylation of the tyrosine residues on the INSR establishes sites for the conscription of proteins each of which initiates a distinct signaling cascade leading up to a protein phosphorylation cascade. The tyrosine residues are recognised by phosphotyrosine-binding (PTB) domains of adaptor proteins of the insulin receptor substrate family (IRS). Receptor activation leads to the intra-cytoplasmic binding of IRS1 through the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphatidylinositide 3-kinase (PI3K, a lipid kinase). Several tyrosine residues of IRS1 are then phosphorylated by the receptor. Phosphorylated IRS1 and IRS2 then serve as the main docking proteins for several other proteins with the similar SH2 domain, allowing IRS1 to activate additional protein kinase signal systems, the most dominant one being the signaling of PI3K. p110 is the catalytic subunit of PI3K and it phosphorylates phosphatidylinositol [4, 5] biphosphate [PtdIns (4, 5) P_2] leading to the formation of phosphatidylinositol [3, 4, 5] triphosphate [PtdIns (3, 4, 5) P_3]. The above mentioned nucleotides function to bind and activate the downstream kinase proteins. A key downstream effector of [PtdIns (3, 4, 5) P_3] is the kinase Akt (also known as protein kinase B, PKB), which is central to the actions of insulin. Phosphorylated Akt enters the cytoplasm where it leads to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3). Glycogen synthase, a key substrate of GSK3, catalyses the final step in glycogen synthesis. Therefore, inactivation of GSK3 by Akt has the opposite effect and promotes glucose storage as glycogen (27,28). Akt is essential for most insulin dependent processes such as glucose transporter type 4 (GLUT4) translocation and glucose transport from intracellular storage to the plasma membrane, inhibition and

release of glucose, lipogenesis and protein synthesis. PI3K also plays a role in GLUT4 translocation (27,28).

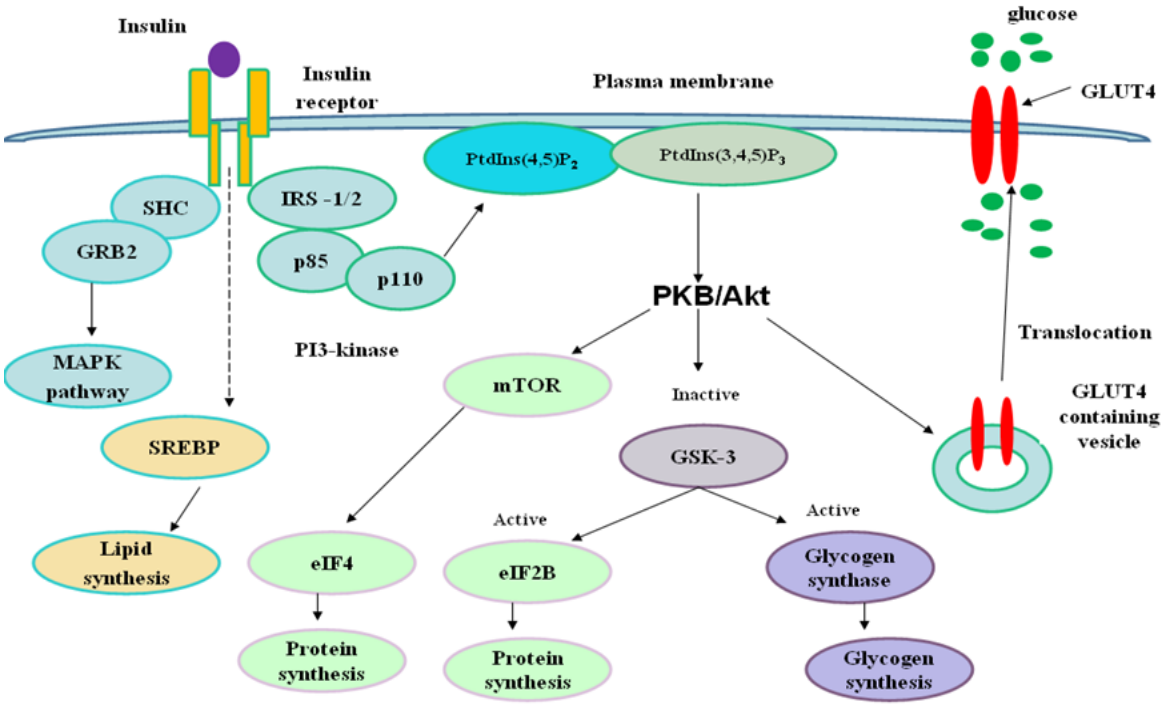


Figure 2. The insulin signaling pathway Adapted from http://www.cellsignalling.com

Unlike tyrosine residues, serine residue phosphorylation of IRS1 has negative feedback and halts the signal for INSR leading to the dissociation of IRS1 from INSR, thus leading to degradation in the proteasome system (28).

The two major IRS isoforms, IRS1 and IRS2 are highly expressed in the liver but down regulated to various extents in diabetic individuals (29). Despite many physiological and molecular studies, it is unclear whether the downregulation of IRS proteins is causative, or merely correlative with pathophysiology and whether IRS1 or IRS2 play unique roles in hepatic insulin action. Taniguchi *et.al* showed that by knocking down IRS1 and IRS2 separately and together in the liver, IRS1 and IRS2 work together via mutual compensation to maintain total PI3K activity and also have unique roles in gene regulation. IRS1 signaling may be more closely linked to the regulation of genes involved in glucose homeostasis, whereas IRS2 signaling may have specific roles in the regulation of hepatic lipid metabolism (29). Further, studies by Cantley *et.al.* suggest that IRS2 in the islets is required for both the maintenance of alpha and beta cells and the regulation of insulin secretion (30).

1.4 DIABETES

Diabetes mellitus is a complex metabolic disease that encompasses a diverse set of maladies characterised by chronic hyperglycaemia due to defects in insulin secretion, action or both (1,2,62). Diabetes is diagnosed when the fasting plasma glucose concentations are ≥7.0 mmol/L (126 mg/dL) or non-fasting glucose concentrations are >11.1 mmol/L (200 mg/dL) (62) (Table 1).

Table 2: Diabetes Mellitus diagnostic criteria. Adapted from (31).

Fasting plasma glucose (mmol/L)	<6.1	Normal
	6.1-6.9 ≥7.0	Impaired fasting glucose Diabetes
2-hour plasma glucose (mmol/L)	<7.8	Normal
	7.8-11.0 ≥ 11.1	Impaired glucose tolerance (IGT)

There are three main types of diabetes. Type 1 diabetes (T1D), also known as juvenile or insulin dependent diabetes, which accounts for 5-10% of all diabetes cases. T1D is characterised by the complete lack of insulin due to autoimmune pancreatic islet β-cell obliteration (2,37). Gestational diabetes mellitus (GDM) refers to glucose intolerance that first emerges during pregnancy. GDM affects about 2-10% of pregnant women and is a risk factor for future health problems for both the mother and offspring (35,38). Lastly, type 2 diabetes mellitus (T2D) is characterised by the β-cell's inability to secrete insulin and accounts for >90% of diabetes cases (1,35-36). Other less common forms of diabetes exist and are attributed to causes such as hyperglycaemia due to endocrinopathies and genetic defects. MODY forms another subset of diabetes.

1.4.1 Type 2 diabetes

Risk factors associated with T2D are considered modifiable and non-modifiable. Modifiable risk factors include obesity, poor nutrition, physical inactivity and malnutrition during pregnancy. Age, ethnicity and hereditary factors are considered non-modifiable risk factors. Non-modifiable factors cannot fully explain the increasing prevalence of T2D, thus it has been established that modifiable factors, specifically environmental and lifestyle factors contribute largely to this epidemic and continue to be the best discriminators for T2D (1,42-44).

1.4.1.1 Prevalence of type 2 diabetes in Sub-Saharan Africa

The prevalence of T2D in sub-Saharan Africa was moderately low in 2013. Even though the prevalence was relatively low, T2D in Africa is expected to increase with the number of those diagnosed with the condition rising from 19.8 million in 2013 to 41.5 million in 2035, signifying a 110% total increase. South Africa was ranked amongst the top ten countries in Africa with the highest prevalence of diabetes at 9.8%. Furthermore, South Africa was also ranked second in countries in Africa with the highest number of people with diabetes in the 20-79 year old age group at 2646.05 (in 1000s) (32,33).

1.4.1.2 Pathophysiology of type 2 diabetes

T2D is a disease characterised by progressive deterioration of glycaemic control and often starts with minor alterations in postprandial glucose homeostasis. These changes in glucose homeostasis are largely attributed to β-cell secretory dysfunction and peripheral insulin resistance. There is no consensus on whether insulin resistance or impaired insulin secretion is the primary event in the pathogenesis of T2D; both compromised metabolic states are clearly precusors in the progression to overt diabetes (43,47).

β-cell dysfunction and insulin resistance contribute differently to the progression to overt diabetes. Insulin resistance is most probably at its optimum level in the initial stages of the disease (37). At this stage glucose tolerance is maintained at the cost of increased insulin secretion such that insulin resistant individuals are characterised by compensatory hyperinsulinaemia. When this compensatory effect dissipates, glucose intolerance and diabetes ensues (37). Hypoinsulinaemia and failed

metabolic control may aggravate insulin insensitivity. Continuous β-cell dysfunction sets the pace for demand of treatment and increase in plasma glucose concentrations. Studies have indicated that individuals with T2D, whether obese or lean, have a reduced β-cell mass. A reduction in β-cell mass and number was reported to be due to increased apoptosis and expression of caspases 3 and 8 which are apoptotic mediators (37).

1.4.2 Insulin resistance and the metabolic syndrome

Metabolic syndrome (MetS) encompasses various disorders including obesity, cardiovascular disease and T2D. Reaven hypothesized that MetS was a central feature in the development of T2D, primarily through target organ resistance to insulin action (34,39). MetS is clinically associated with insulin resistance, dyslipidaemia (decreased high-density lipoprotein (HDL), and elevated triglycerides), central obesity, hypertension, impaired glucose tolerance and high rates of atherosclerotic disease. Most individuals with MetS exhibit resistance to the cellular actions of insulin to modify biochemical responses in a way that predisposes to metabolic risk factors. The presence of insulin resistance appears to be due to complex interplay of genetic factors with environmental factors, such as obesity and physical inactivity (34,39). Although MetS is associated with insulin resistance, it is however not a consequence of insulin resistance alone, nor is it a direct consequence of the lack of insulin action (34,39).

Table 3: Diagnostic criteria for metabolic syndrome. Adapted from (40).

- Abdominal obesity

  Men: waist circumference >102 cm

  Women: waist circumference >89cm

- Fasting glucose ≥ 6.1 - ≤7.0 mmol/L (≥110 - <126 mg/dL)
- Blood pressure ≥130/80 Hg
- Triglycerides ≥1.7 mmol/L (≥150 g/dL)
- HDL-C

  Men: <1.04 mmol/L) (<40 mg/dL)

  Women: <1.3 mmol/L (<50 mg/dL)

*when 3 or more of the 5 criteria are met = MetS

Insulin resistance (IR) refers to depressed cellular sensitivity to insulin and is a central feature of MetS. The primary cause of IR is the increase in circulating fatty acids and obesity (45). Although several metabolic derangements emerge due to the overabundance of fat accumulation, insulin resistance appears to be the most significant as it may lead to overt T2D. Insulin resistance is defined as an inadequate response by insulin target organs (e.g. liver, muscle and adipose tissue) to the physiological effects of insulin (45). Abnormal fat excess significantly affects lipid metabolism and inflammation. The release of adipokines (i.e. proinflammatory markers) contributes to insulin resistance. Increased cholesterol synthesis and decreased absorption of cholesterol occurs as a result of insulin resistance, resulting in a diminished plasma lipid response to diet (41). Insulin resistance may also exert an action directly on small blood vessels, leading to high blood pressure. In addition, by its stimulatory effect on the β-cells in the pancreas, insulin resistance may accelerate the age-related decrease in insulin secretion and thereby hasten the onset of glucose intolerance (41).

The relationship between insulin resistance and β-cell dysfunction is dynamic and largely dependent on the metabolic state that is primarily determined by glycaemic

status and consequently insulinaemic status. In the aetiolgy of β-cell dysfunction, β-cell physiology is maintained in healthy individuals. However, glucolipotoxicity and proinflammatory cytokines induce oxidative stress leading to β-cell death. β-cell compensation occurs when β-cell integrity is diminished. If β-cell compensation is successful, β-cell physiology is maintained. However, if β-cell compensation is exhausted, β-cell dysfunction ensues. Insulin resistance impairs β-cell physiology and compensation, thereby inducing β-cell death and dysfunction (24).

1.5 PROGRAMMING

The growth and maturation of a developing foetus is decidedly reliant on the immediate milieu and nutritional status of the mother (48). Foetal development particularly body weight and energy homeostasis can be adversely affected by modest disparities in maternal nutrition. This would therefore affect the progression of *in utero* growth as development is somewhat multifaceted and requires intermingling machineries from both mother and foetus to withstand survival and optimal growth throughout the course of gestation (49-54). Unfavourable environments or any stimuli during a precarious phase of development which may prompt metabolic disease in the progeny is termed developmental programming (46,48,52). In contrast, the same environmental stimulus outside that critical period has been found to only prompt reversible modifications (52). During the preliminary events of development, there is a period during which the organism can still advance in different directions; an occurrence labeled the window of plasticity (52). For the duration of this plasticity period, the organism has the potential to adapt to its immediate milieu; however, once this window of plasticity has closed, many of these adaptations will become irreparable (52). It has been observed that health in the later stages of life may be compromised as a result of maternal over-nutrition during pregnancy and more importantly, these significances were associated with the timing in the course of gestation (53). On account of this enhanced nutrient availability, new-borns suffered from elevated risk for insulin resistance. In later life, this population is programmed for developing metabolic syndrome which jointly encompasses a combination of increased risk factors for cardiovascular disease, hypertension and insulin resistance (54). Nevertheless, data still remain questionable

with regards to whether the obesity that forms part of the metabolic syndrome is also in fact programmed (52). Cerf *et al.* explored the effect of varying gestational fat diets in neonatal Wistar rats and reported marked deviations in head dimensions in the experimental groups (8). Throughout gestation, pregnant mothers were maintained on intakes of 10% (control), 20%, 30% or 40% fat as energy and anthropometric measurements were then conducted on one-day-old neonatal offspring (8). Compared to the controls, the offspring maintained on the 40% fat diet (HFD) during foetal life presented stunted head lengths i.e. decreases in head length, independent of brain weight. The study therefore showed that the body weight of the neonates was influenced by maternal dietary fat intake (8). The data concluded that exposure to a gestational diet with 30% or 40% fat as energy resulted in augmented immunoreactivity of both GLUT2 and neuropeptide Y (NPY), proposing that these proportions of dietary fat content may essentially represent a 'programming effect' and reflect an early event in the pathogenesis of obesity (8). Many studies have also reported that an increased risk for the development of obesity in later life was directly correlated with both low and high birth weights (55-59). Elevated birth weight could possibly be attributed to either maternal obesity or diabetes (11-15). It has been proposed that due to abundant nutrient availability in neonates born to obese mothers, foetal adipogenesis is permitted and concurrently, the systems that regulate energy balance whilst they are still plastic are modified (60,61).

.

CHAPTER TWO

STUDY AIMS

2.1 Study aim

The purpose of this study is to ascertain how foetal programming via maternal nutrition (nutritional programming) may contribute to the pathogenesis of T2D and also how nutritional programming affects islet cell transcription and insulin signaling factors in the pancreas of the offspring.

2.2 Specific objectives

To determine the effect of diets, varying in fat content, on transcription and insulin signaling factors in neonatal Wistar rat offspring.

Specific objective 1: Determine the effect of 10% fat on transcription and insulin signaling factors on the pancreata of neonatal Wistar rat offspring.

Specific objective 2: Determine the effect of 20% fat on transcription and insulin signaling factors on the pancreata of neonatal Wistar rat offspring.

Specific objective 3: Determine the effect of 30% fat on transcription and insulin signaling factors on the pancreata of neonatal Wistar rat offspring.

Specific objective 4: Determine the effect of 40% fat on transcription and insulin signaling factors on the pancreata of neonatal Wistar rat offspring.

2.3 Expected outcomes

- Exposure to varying dietary fat content, *in utero*, may have differential effects on the expression profiles of transcription and insulin signaling factors in neonatal pancreata.
- A high fat diet (40% fat) may also programme neonatal insulin resistance which will be reflected by reduced expression of proximal insulin signaling factors.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animal model and husbandry

Wistar rats were housed at the South African Medical Research Council (SAMRC), Primate Unit, Tygerberg, South Africa, and treated in accordance with their standard operating procedures (SOPs) and in agreement with the MRC Guidelines for the Use of Animals in Research and Training and the National Code for Animal Use in Research. Ethical approval for the study conducted was obtained from the ethical committees of the University of Pretoria and the South African Medical Research Council of South Africa (ECRA # 10/09;H002-13).

Three-month-old virgin Wistar female rats were housed and mated overnight with pregnancy confirmed by the presence of vaginal plug(s). After confirmation of pregnancy, rats were removed, individually housed and randomly assigned to groups (n = 6 per group). The rats had free access to drinking water and food and were maintained in a temperature controlled room at 22-25°C, humidity of 45-55%, 15-20 air changes per hour and a 12 hour light/dark cycle (light daily from 06:00 am-18:00 pm).

3.2 Experimental diet composition

Before pregnancy was confirmed, the rats were fed a standard laboratory diet (2.6 kcal/g). The pregnant rats were randomly assigned to groups (n = 6 rats per group) and maintained on either a 10% fat (as energy) diet (control); a 20% fat (as energy) diet (20F); a 30% fat (as energy) diet (30F); and a 40% fat (as energy) diet (40F and HFD).

Table 4: Dietary composition

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

All the rat diets were in patty form. The HFD was designed by registered dieticians to mimic human HFDs. The fat in the HFD was mainly from saturated animal fat. The protein content was kept at a constant of 15% for all the diets to prevent any adverse effects of protein deficiency which would alter and compromise β-cell architecture and function; thus only the fat and carbohydrate content varied (10).

3.3 Experimental design

The dams were maintained on their respective diets throughout gestation. Upon delivery, their one-day-old neonatal offspring were then studied.

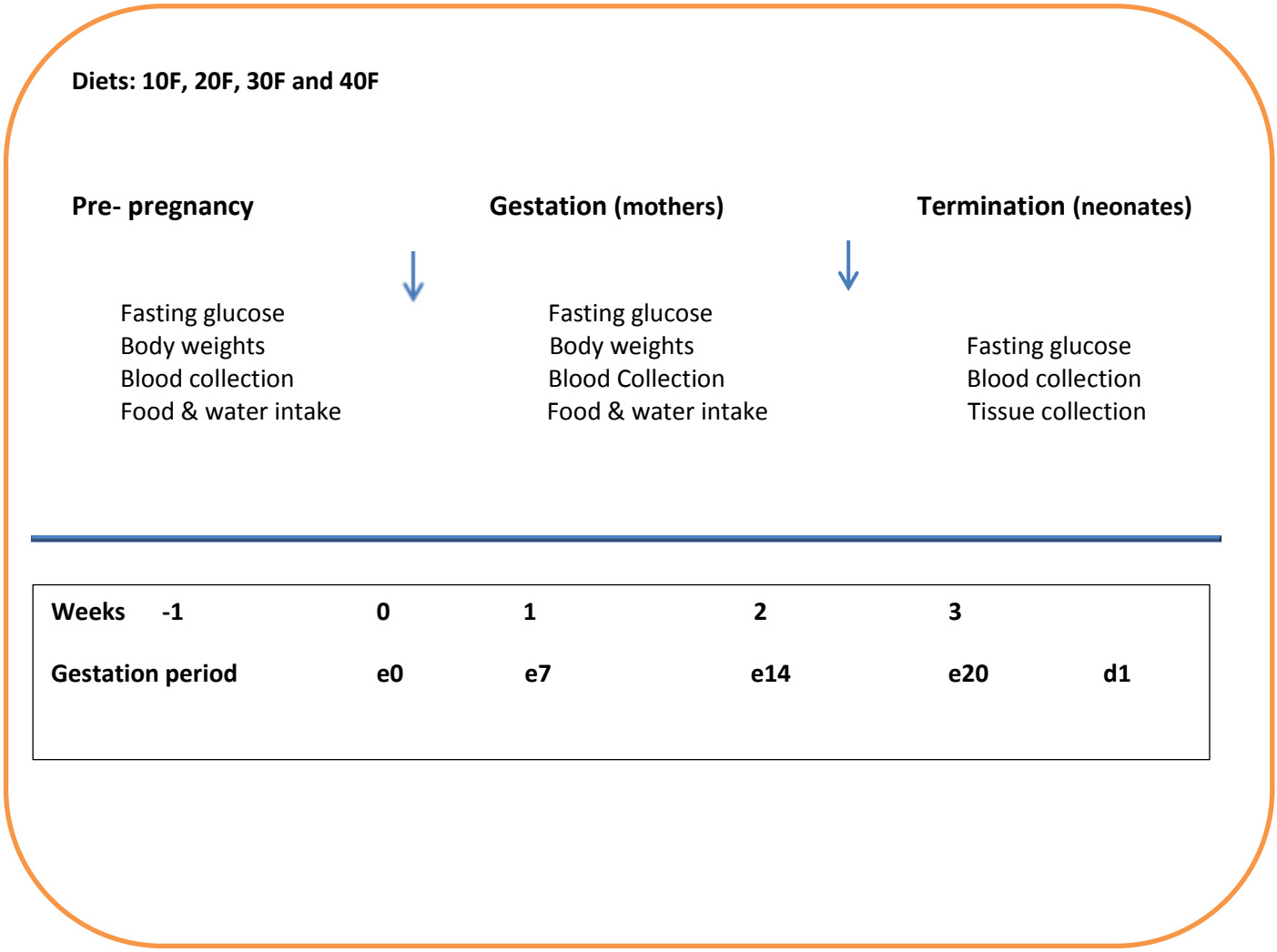


Figure 3. Experimental timeline

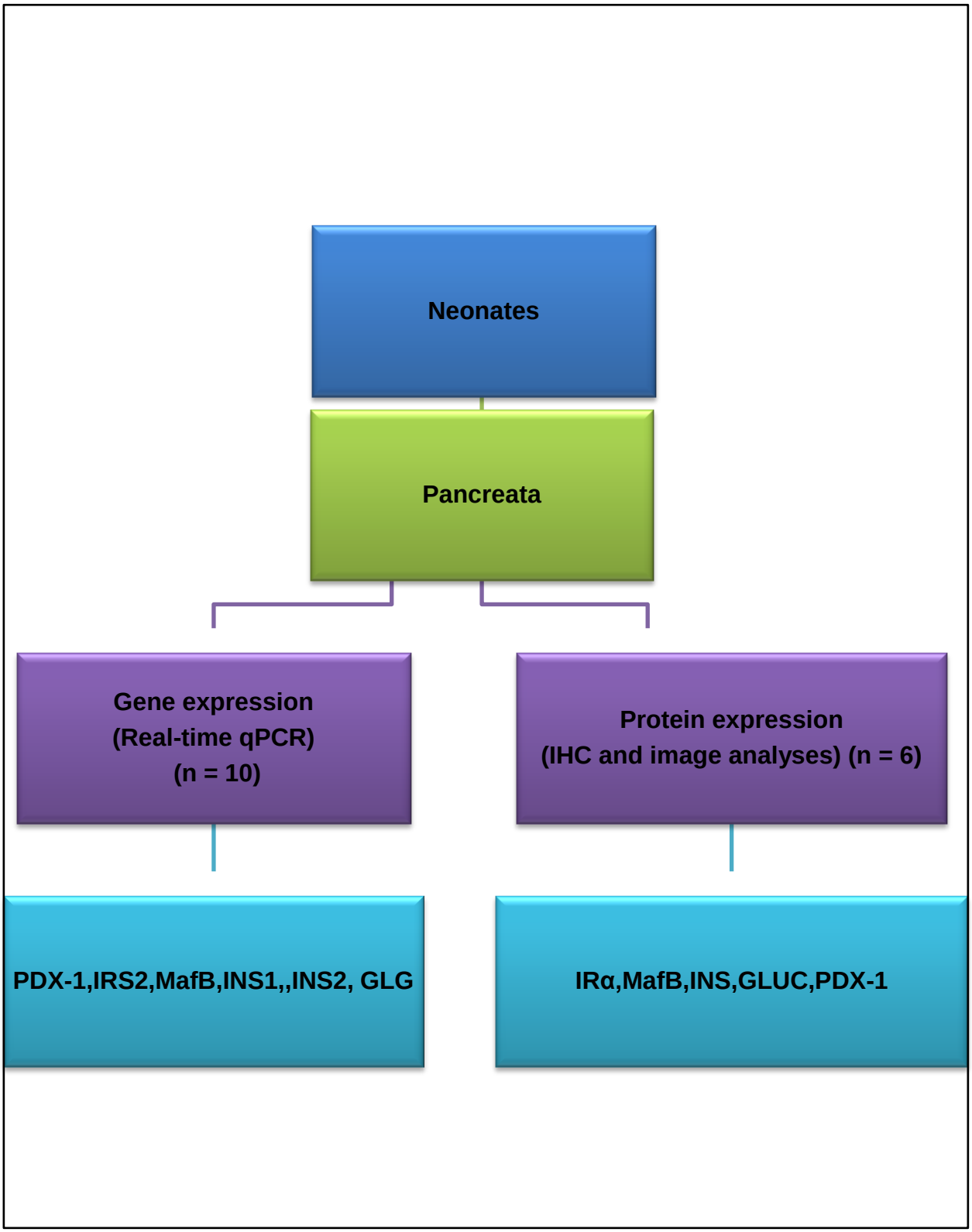


Figure 4. Experimental framework

3.4 Relative PCR quantification

Gene expression analysis is relevant for many fields of biological research. Quantitative reverse transcription polymerase chain reaction (qRT-PCR or real time RT-PCR) has become the most popular technique for gene expression studies due to its ability to efficiently amplify small quantities of RNA in a relatively short period in many different samples for a limited number of genes. qRT-PCR also enables the detection and quantification of sequences in a DNA sample. Data are collected throughout the PCR process, not just at the end of PCR. Reactions are characterised by the point in time during cycling when amplification of the target is first detected (71).

Notwithstanding its advantages, qRT-PCR has a some limitations that may influence the interpretation of the data, viz. reliable extraction of equal amounts of non-degraded RNA from each sample; constant reverse transcriptase efficacy resulting in equal amounts of cDNA in all samples; sufficient primer specificity; presence of inhibitors in samples (71).

Overcoming these drawbacks requires data to be normalised to the expression of reference genes (also known as housekeeping genes) as they maintain constant expression levels across sample groups 71).

Different sequence detection chemistries are found, viz. SYBR Green I dye and Taqman (fluorogenic 5’ nuclease chemistry) (71).

For our study, we used Taqman chemistry as it uses a fluorogenic probe to enable the detection of a specific PCR product as it collects during PCR. Probes may be labeled with different reporter dyes which then allow amplification of two distinct sequences in one reaction tube. Post-PCR processing is eliminated, which reduces labour and material costs (71).

3.4.1 Housekeeping gene selection

In the pancreas, HPRT and β-actin were reported as stable rat housekeeping genes (Yuzbasioglu et. al, 2010). We used β-actin as our housekeeping gene for qRT-PCR analysis.

3.4.2 RNA preparation and qRT-PCR

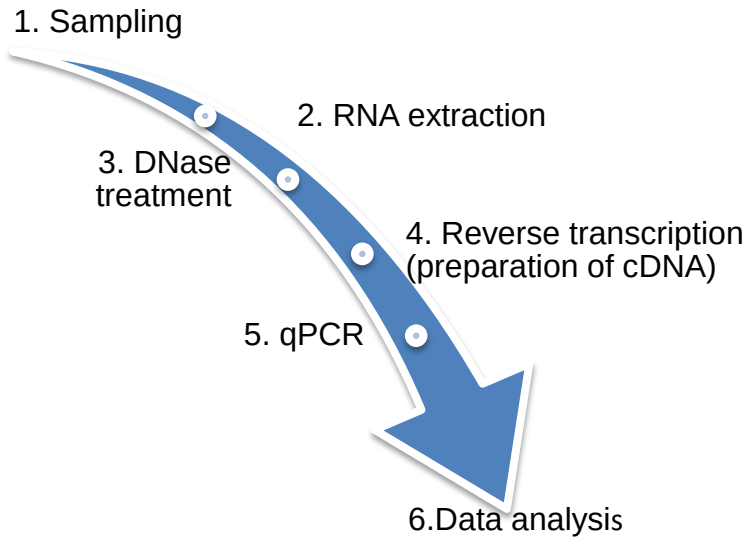


Figure 5. qRT-PCR technical workflow

Pancreata were harvested and snap frozen at -80°C. Total RNA was isolated from 100 mg pancreata 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 6000 nano kit (Agilent technologies, Santa Clara, CA, USA). A total of 1 µg RNA per sample constituted to 10 µl with sterile water was reverse transcribed into first strand complementary DNA (cDNA) using high capacity reverse transcription kit (Biosystems). Taqman probes for MafB, Pdx1, Ins1, Ins2, Gluc, IRS2 were used together with the housekeeping genes β-actin and HPRT. qPCR was performed on the LightCycler Nano Instrument using a cycler programme consisting of an activation step of 10 min at 95°C, 40 cycles with a 15 second denaturing step at 95°C and 60 s at 60°C for annealing and extension and cooling step at 40°C for 30 s.

3.5 Preparation of tissue for histology

Pancreas samples were fixed in buffered formalin (pH 7.4) for 12 hours, labelled and placed in tissue cassettes. Cassettes were then placed in an automated histology tissue processor (Leica, Wetzar, Germany).

Table 5: Processing schedule

Carousel position	Reagent	Time (mins)
1	10% formalin	12 hours fixation
2	70% alcohol	60
3	80% alcohol	60
4	95% alcohol	120
5	95% alcohol	60
6	100% alcohol	120
7	100% alcohol	120
8	Xylene	60
9	Xylene	60
10	Xylene	60
11	Wax	120
12	Wax	180

The total running time for a complete cycle was 17 hours.

3.5.1 Tissue embedding and sectioning

The tissue cassettes were removed from the processor and embedded in paraffin wax. Sections, ranging between 5 – 7 µm in thickness, were then cut using a rotary microtome floated onto warm (30°C) water to remove wrinkles and placed onto 3-aminopropyltriethoxy-Silane (APES) coated slides.

3.5.2 Coating of APES slides

The sections were placed in metal slide staining racks and immersed in acetone for 2 min to remove any dirt from the slides. Sections were air dried for 5 min. A 2% APES solution was prepared in acetone (98 ml acetone + 2 ml APES). Sections

were immersed in 2% APES solution for 2 min. Sections were then washed in acetone by dipping sections ~10 times and this step was repeated twice. Finally, sections were allowed to dry at 60°C in an incubator for ~30 min.

3.6 Immunohistochemistry

Immunohistochemistry (IHC) is a process used to detect proteins in cells of tissues by utilising the principle of binding immunoglobulins to specific antigens. It is mostly used to diagnose abnormal cells in biological tissue. IHC is also used to understand the dissemination and localisation of biomarkers and differentially expressed proteins in different parts of a biological tissue. There are two different target antigen detection methods that can be used, the direct and indirect method. For our experiment, we used the indirect method using a primary antibody and a secondary antibody. The primary antibody binds to the target antigen in the tissue and the secondary antibody then reacts with the primary antibody. The indirect method was specifically used because of its higher sensitivity due to signal amplification owing to the binding of several secondary antibodies to each primary antibody. The IHC technique indicates where a specified protein is located within the tissue being studied (69).

3.6.1 Double immunostaining for glucagon and insulin in the rat pancreas

The double immunostaining procedure was performed over two days. Sections were dewaxed in an oven at 60°C for 30 min, thereafter removed and placed in xylene for 20 min, hydrated in 95% ethanol for 4 min and rinsed in distilled water. The sections were then incubated for 5 min in 3% hydrogen peroxide (H_2O_2) to block for endogenous peroxidases. The sections were rinsed in 50 mM-tri (hydroxymethyl)-aminomethane (Tris) buffered saline (TBS) for 5 min in a staining jar on a magnetic stirrer at pH 7.2. To block non-specific binding, sections were incubated for 20 min in 1:20 normal goat serum (NGS) (MRC Animal facility, Delft, South Africa). This prevents the secondary antibody from cross-reacting with endogenous immunoglobulins in the tissue and eliminates the non-specific fragment crystallisable region (Fc region) binding of both the primary and secondary antibody. After blotting the excess serum, a 1:50 dilution of the primary antibody, anti-glucagon (Dako,

Carpinteria, CA, USA), was added, and the sections were incubated for 30 min at room temperature. Thereafter, the sections were jet washed with TBS and rinsed in TBS for 5 min. After rinsing, sections were incubated in a dilution of 1:1000 biotinylated anti-rabbit IgG (Vector laboratories, Burlingame, CA, USA) for 30 min in a moisture chamber. To remove unbound antibody sections were jet washed and rinsed in TBS buffer for 10 min. A volume of 5 ml of TBS**,** at pH 7.2, with 1 drop (20 µl) of solution A and 1 drop (20 µl) of solution B of Vectastain (Vector laboratories, Burlingame, CA, USA) was applied to the sections and incubated for 60 min at room temperature. Sections were then washed in TBS buffer for 10 min at pH 7.2. Immunostaining was visualized using liquid diaminobenzidine tetrachloride (DAB) Plus Substrate Chromagen System (Dako Corporation, Carpinteria, CA, USA) using 1 drop (20 µl) DAB chromagen per 1 ml of substrate buffer provided. An insoluble brown reaction DAB precipitate developed at the glucagon antibody/antigen binding site. Before applying the second primary antibody, sections were washed and rinsed with distilled water for 5 min. After rinsing and drying the slides, 1:20 normal horse serum (NHS) (MRC Animal facility, Delft, South Africa) was applied to slides in a moisture chamber and incubated for 20 min at room temperature. Excess serum was blotted and a dilution of 1:10000 anti-insulin (Sigma Immunochemicals St. Louis, MO, USA) was applied to sections in a moisture chamber and incubated overnight (16 hours) at 4°C.

Following overnight incubation, sections were jet washed and rinsed with 0.05 M TBS for 5 min. A volume of 100 µl of rabbit/mouse link (Envision G/2 System/AP, Rabbit/Mouse Kit, Dako, Denmark) was then added to slides and incubated for 30 min. After incubation and washing in TBS for 10 min, AP Enzyme Enhancer (Envision G/2 System/AP, Rabbit/Mouse Kit, Dako, Denmark) was applied to sections and incubated in a moisture chamber for 30 min at room temperature. Sections were washed with 0.5 M TBS. A volume of 100 µl of substrate working solution (Envision G/2 System/AP, Rabbit/Mouse Kit) was applied to each section for 3 min. An insoluble red reaction of the permanent red precipitate developed at the insulin antibody/antigen binding site. Sections were washed and rinsed with distilled water for 5 min before counterstaining with Mayers Haemotoxylin for 2 min. Sections were then left to “blue” in running tap water for 30 min and blotted on paper towel to

remove water. After air drying, the sections were mounted with Entellan (Merck, Darmstadt, Germany) and cover slipped.

To verify the specificity of the immunohistochemistry, three negative method controls were included. To demonstrate non-specific binding of link antibodies and endogenous enzyme activity, both the primary antibodies (glucagon and insulin) were omitted. A second method control was included where the insulin antibody was omitted thereby demonstrating the specificity of glucagon staining. A third method control was included where the glucagon antibody was omitted thereby demonstrating the specificity of insulin staining.

3.6.2 Immunolabeling for transcription factors

Sections immunostained for insulin receptor alpha (IR-α; 1:500; Abcam, Cambridge, UK) were blocked with normal goat serum for 20 min, incubated with IR-α overnight at 4°C then incubated in 1:200 biotinylated anti-rabbit IgG (Vector, Laboratories, Burlingame, CA, USA) for 30 min. Sections immunostained for IRS2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were blocked with normal goat serum for 20 min, incubated with IRS2 overnight at 4°C then incubated in 1:200 biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min. Sections immunostained for MafB (1:10; Cell Signalling Technology, Danvers, MA, USA) were blocked with normal horse serum for 20 min, labeled with MafB overnight at 4°C, then incubated in 1:200 biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min. Sections immunostained for Pdx1 (1:10; Cell Signalling Technology, Danvers, MA, USA) were blocked with normal horse serum for 20 min, labeled with Pdx1 overnight at 4°C, then incubated in 1:200 biotinylated anti-rabbit IgG (Vector Laboratories) for 30 min. All sections were then washed with 50 mM Tris buffer (pH 7.2) for 5 min and incubated with ABC complex (Vector Laboratories) for 60 min at room temperature, washed in 50 mM tris buffer (pH 7.2) for 5 min and stained with 0.05% diaminobenzadine containing 0.01% H_2O_2 for 5-10 min at room temperature. All sections were counterstained with haematoxylin for 2 min, left to dry and mounted with Entellan™ (Merck Millipore).

3.7 Image analysis

Images were captured with a ZEISS AxioCam ERc 5 mounted on ZEISS Axio light microscope (Oberkochen, Germany). All images were captured at X10, X20 and X40 and stored in tiff format at a final resolution of 1024 X 796. Immunoreactivity was expressed as the area of immunostained target protein, i.e. IRα, Pdx1, MafB, insulin, and glucagon in the pancreas per total tissue area. CellProfiler was used for image analysis.

3.7.1 CellProfiler

CellProfiler is a free, open-source software, designed and maintained by scientists at the Broad Institute at the Massachusets Institute of Technology (MIT). The software was designed to allow researchers to extract quantitative measurements of cellular events and phenotypes from microscopy images without specialist training. CellProfiler contains predefined modules that each perform a specific image processing or analysis function. These modules, when linked together, create what is called a CellProfiler pipeline, which consists of a series of instructions to automatically convert images to quantitative data for the features of interest in the images (70).

CHAPTER FOUR

RESULTS

4.1 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To quantify our results, we used relative quantification as it allows the quantification of the differences in the expression levels of a target gene between different samples and also requires two different genes viz. a target and housekeeping (or reference) gene. The reference gene serves as a normaliser of the quantification of targets for differences in the amount of total nucleic acid added to each reaction. The output is expressed as a fold difference/change of expression levels. To calculate the fold difference/change value, we used the delta delta Cq method as described by Livak and Schmittgen (68).

After analysis of results, samples with a Ct value >35 were discarded as this revealed that the target gene was approaching a single copy; thus the study would have been compromised with a low fold difference, which cannot be accurately quantified.

Table 6. Gene expression profiles for Ins1, Ins2, Glucagon, Pdx1, MafB and IRS2

Target gene	Diet	Result	Fold difference
Ins1	20F	No change	0.98
	30F	Decrease	2.17
	40F	Decrease	2.71
Ins2	20F	No change	0.78
	30F	No change	0.82
	40F	Decrease	3.19
Glucagon	20F	No detection	-
	30F	No change	0.72
	40F	Decrease	2.17
Pdx1	20F	No change	1.21
	30F	Decrease	1.65
	40F	No change	0.92
MafB	20F	No change	0.84
	30F	Decrease	2.41
	40F	Decrease	3.59
IRS2	20F	Increase	3.8
	30F	No change	0.75
	40F	Decrease	1.53

*Decrease = Under-expressed; Increase = Over-expressed. (-)= No detection. The target genes expressed >1.5 increase or decrease are up or downregulated respectively (or under- and over-rexpressed respectively).

The target factors investigated straddled key factors involved in the pathogenesis of T2D. These included islet cell hormones (insulin i.e. Ins1 and Ins2 from β-cells; glucagon from α-cells); the islet cell transcription factors, Pdx1 (β-cell) and MafB (α-cell); and an insulin signaling factor, IRS2. In 20F neonates, there was over-expression of glucagon and IRS2 mRNA expression whereas the expression of the other target genes were unaltered (Table 6). In 30F neonates, there was under-expression of Ins1, Pdx1 and MafB mRNA expression (Table 6). In 40F neonates,

there was under-expression of Ins1, Ins2, glucagon, MafB and IRS2 mRNA expession (Table 6).

Note: Gender-based fold differences could not be calculated because we did not have a representative sample for male and female offspring.

4.2 Immunohistochemistry (IHC) staining of pancreatic sections

4.2.1 Double immunolabeling for insulin and glucagon

Double immunolabeling for insulin and glucagon revealed the typical endocrine arrangement of α-cells at the periphery of the islets (brown stain) and the more abundant β-cells in the centre of the islets (red stain) in the control group (Figure 10). Normal islet architecture was demonstrated in control (Figure 10A) and 20F neonates (Figure 10B). Some disruption in the islet organisation was reflected in 30F (Figure 10C) and 40F neonates (Figure 10D).

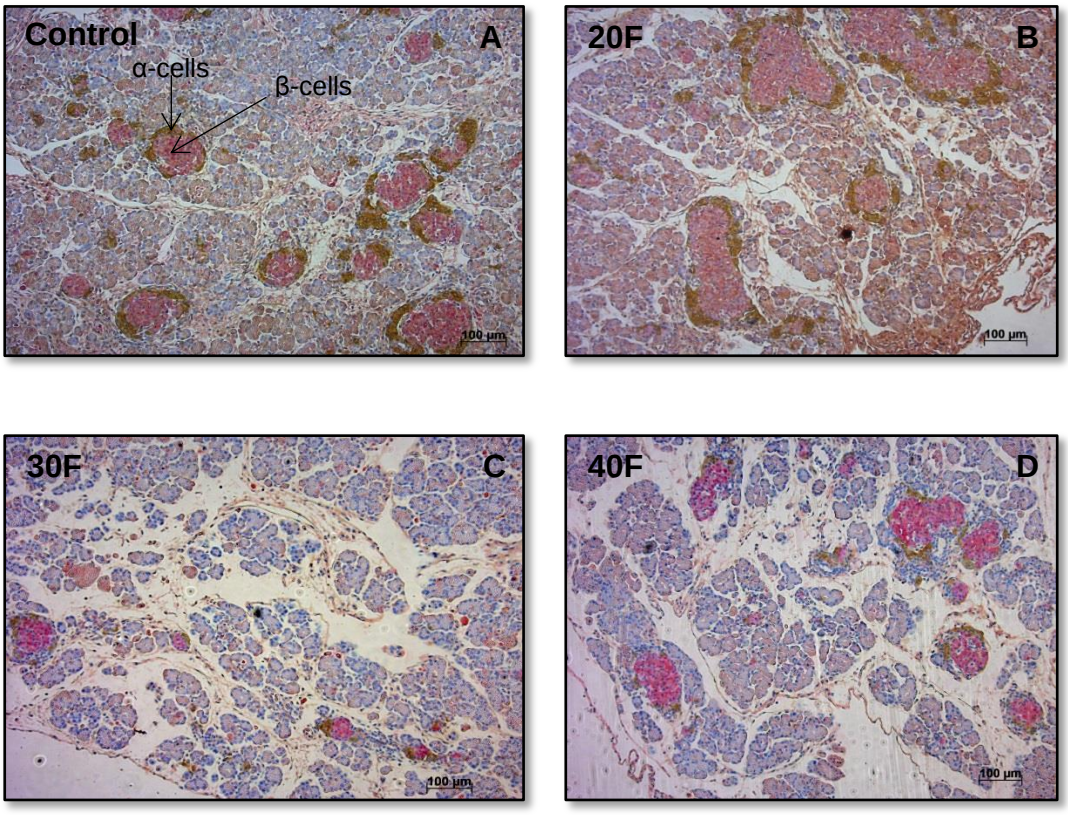


Figure 6. Immunostaining of the β-cells with insulin (stained red) and the α-cells with glucagon (stained brown). Islets were double immunostained for insulin and glucagon in neonatal offspring maintained on either a control (10F; A), 20% fat (20F; B), 30% fat (30F; C) or 40% fat (40F or high fat diet; D) diet. Scale bar: 100µm

4.2.2 Immunolabeling for Pdx1

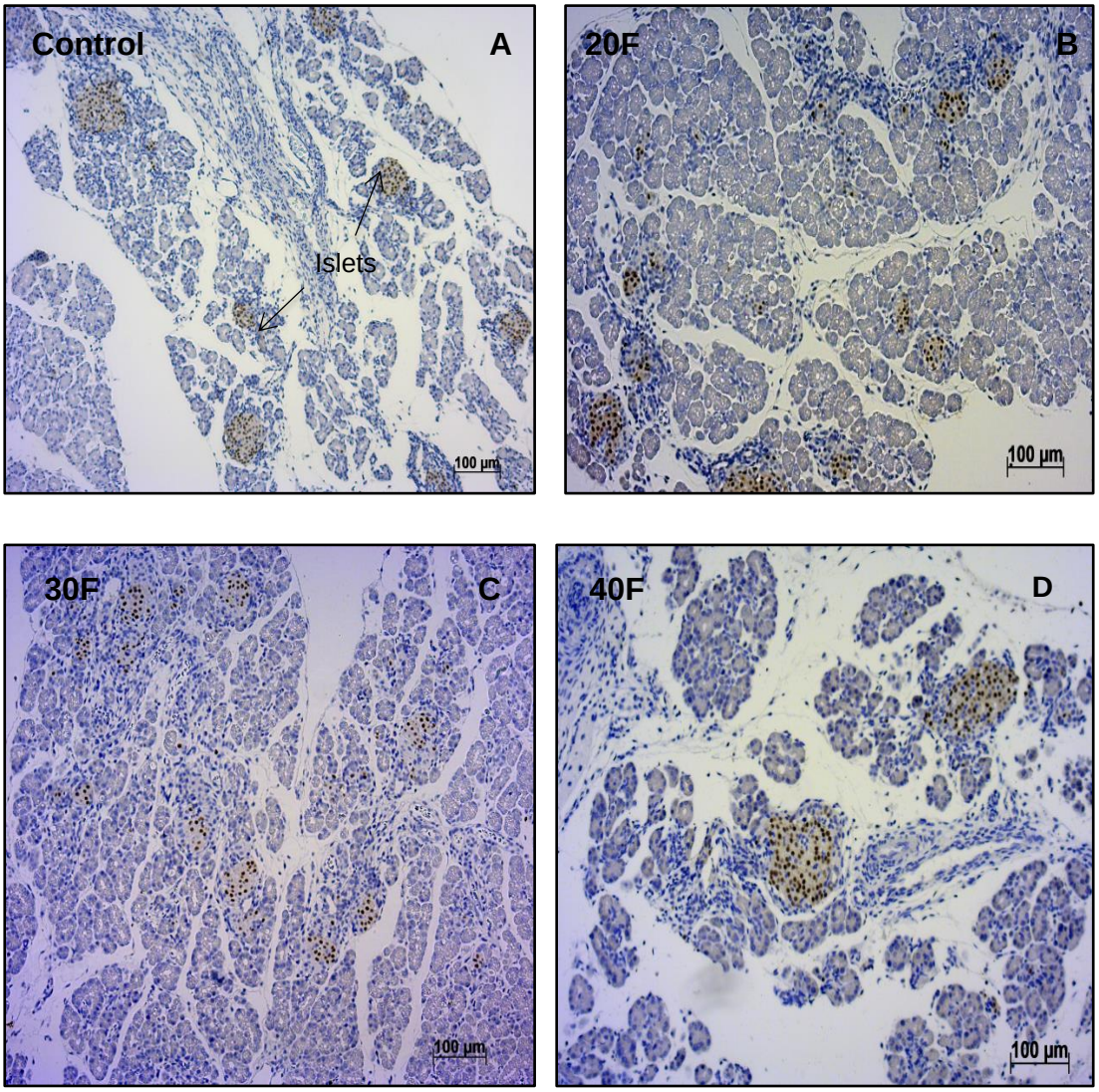


Figure 7. Immunostaining for Pdx1 (β-cells) in neonatal offspring maintained on either a control (10F; A), 20% fat (20F; B), 30% fat (30F; C) or 40% fat (40F or high fat diet; D) diet. Scale bar: 100µm

4.2.3 Immunolabeling for MafB

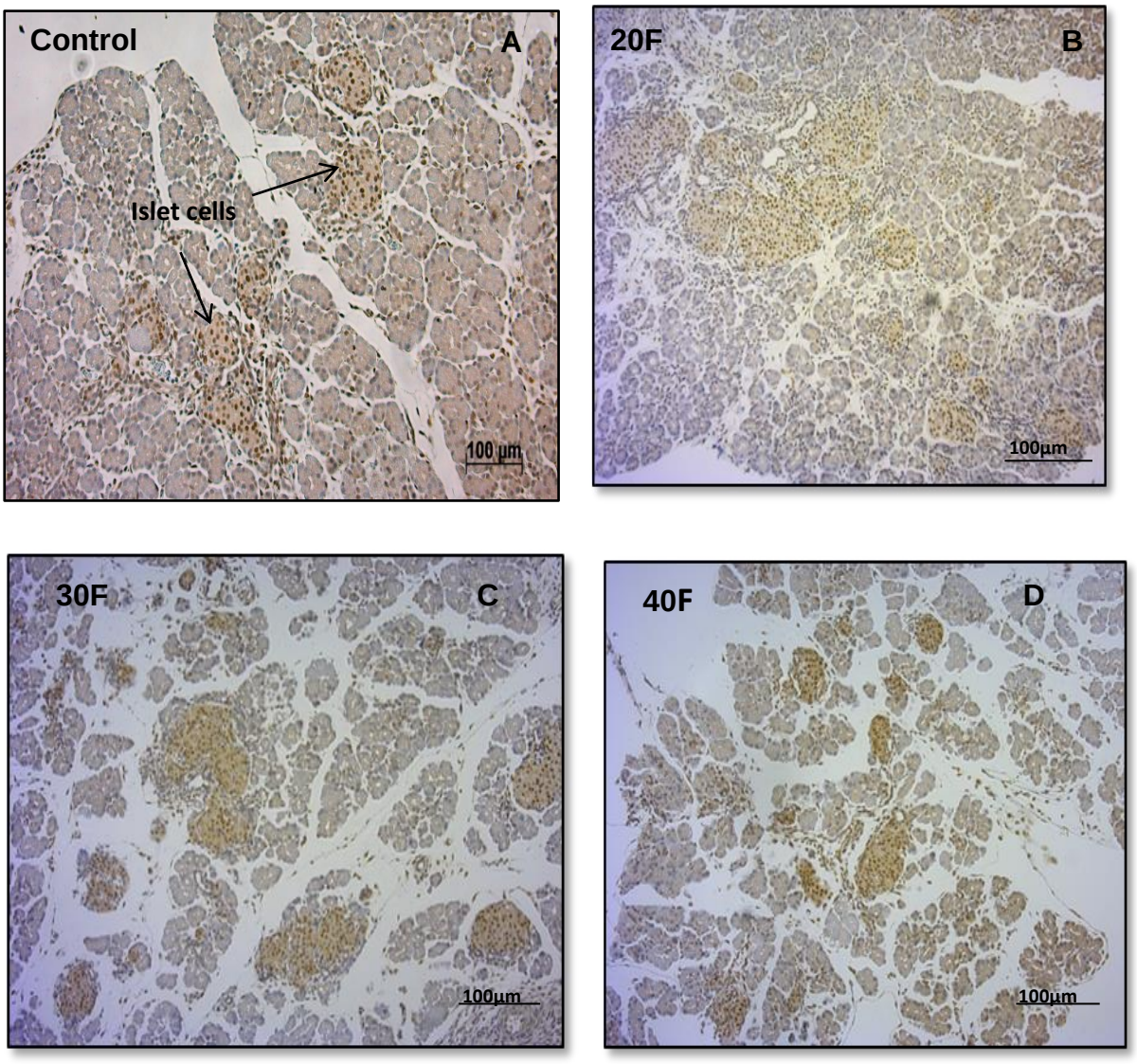


Figure 8. Immunostaining for MafB (α-cells) in neonatal offspring maintained on either a control (10F; A), 20% fat (20F; B), 30% fat (30F; C) or 40% fat (40F or high fat diet; D) diet. Scale bar: 100µm

4.2.4 Immunolabelling for IRα

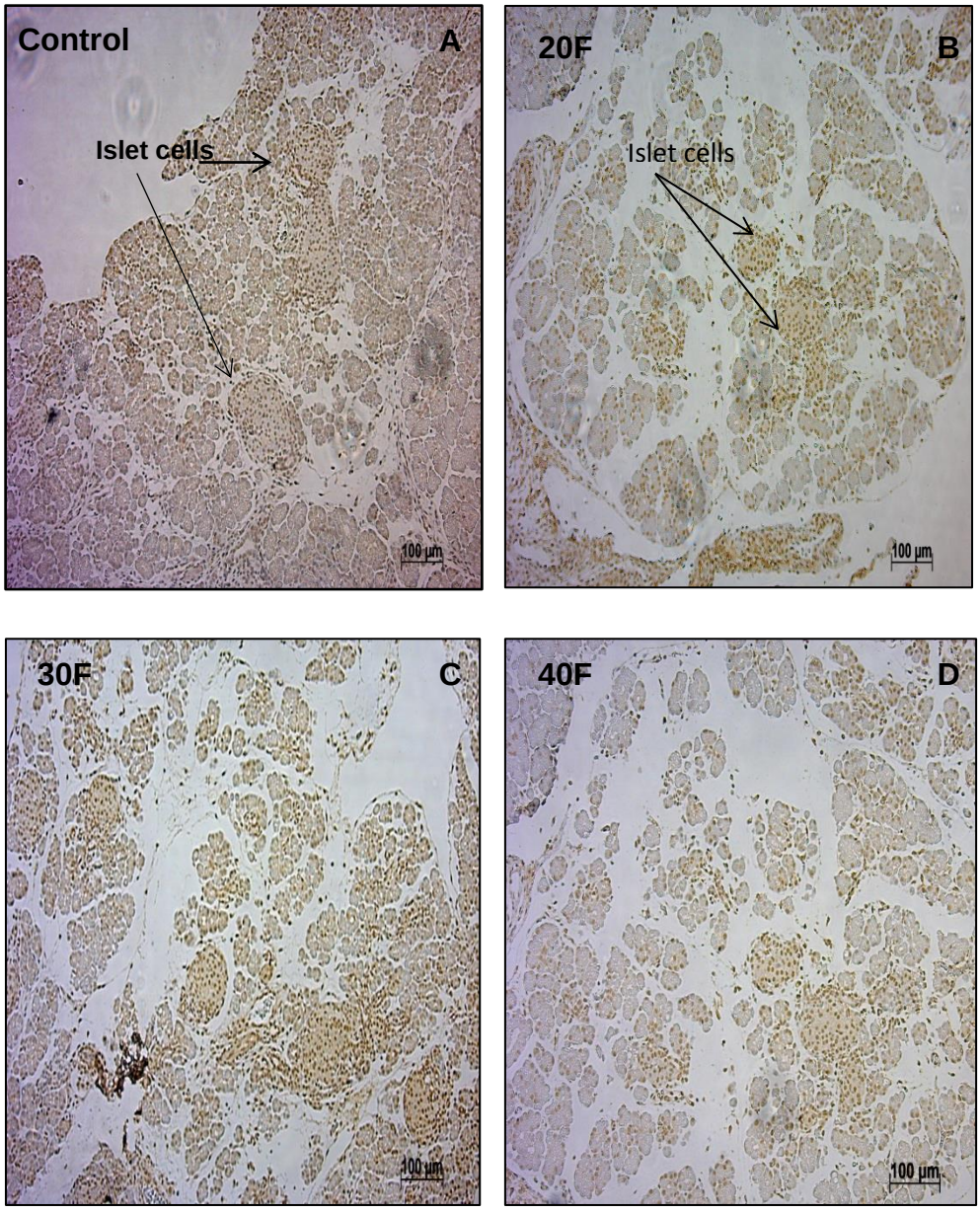


Figure 9. Immunostaining for IRα (insulin signaling) in neonatal offspring maintained on either a control (10F; A), 20% fat (20F; B), 30% fat (30F; C) or 40% fat (40F or high fat diet; D) diet. Scale bar: 100µm

4.3 Image analysis

In male neonates, there were no changes in immunoreactivity for insulin/glucagon, Pdx1, IRα and MafB (Figure 6). Similarly, in female neonates, immunoreactivity for the target factors remained unaltered (Figure 7). However, IRα immunoreactivity was reduced in 40F female neonates compared to 40F male neonates (Figure 9).

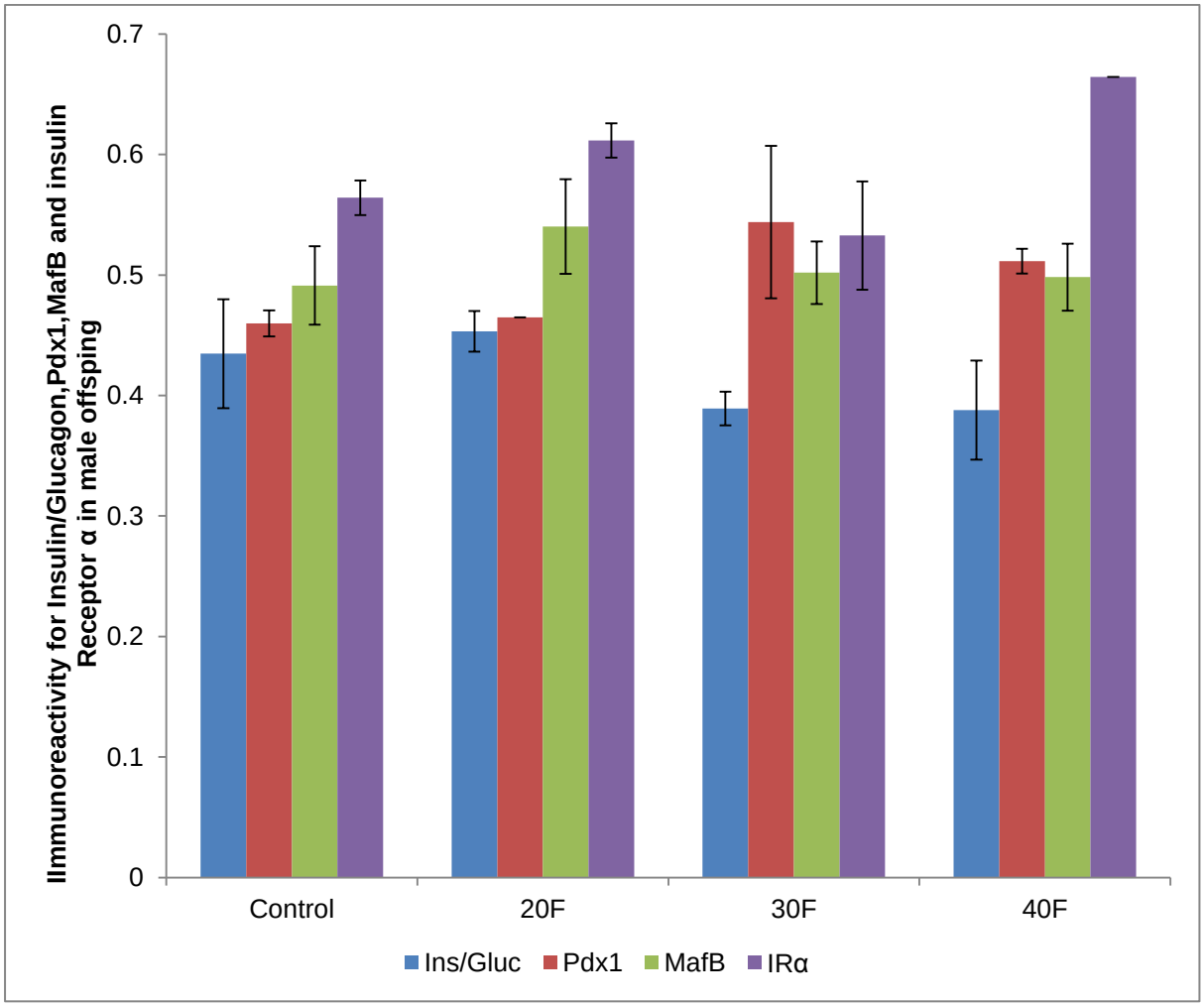


Figure 10. Immunoreactivity for insulin/glucagon, Pdx1, MafB and insulin receptor α in neonatal male offspring. 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet; Pdx1, duodenal box 1. Data are means ± SEM. P>0.05.

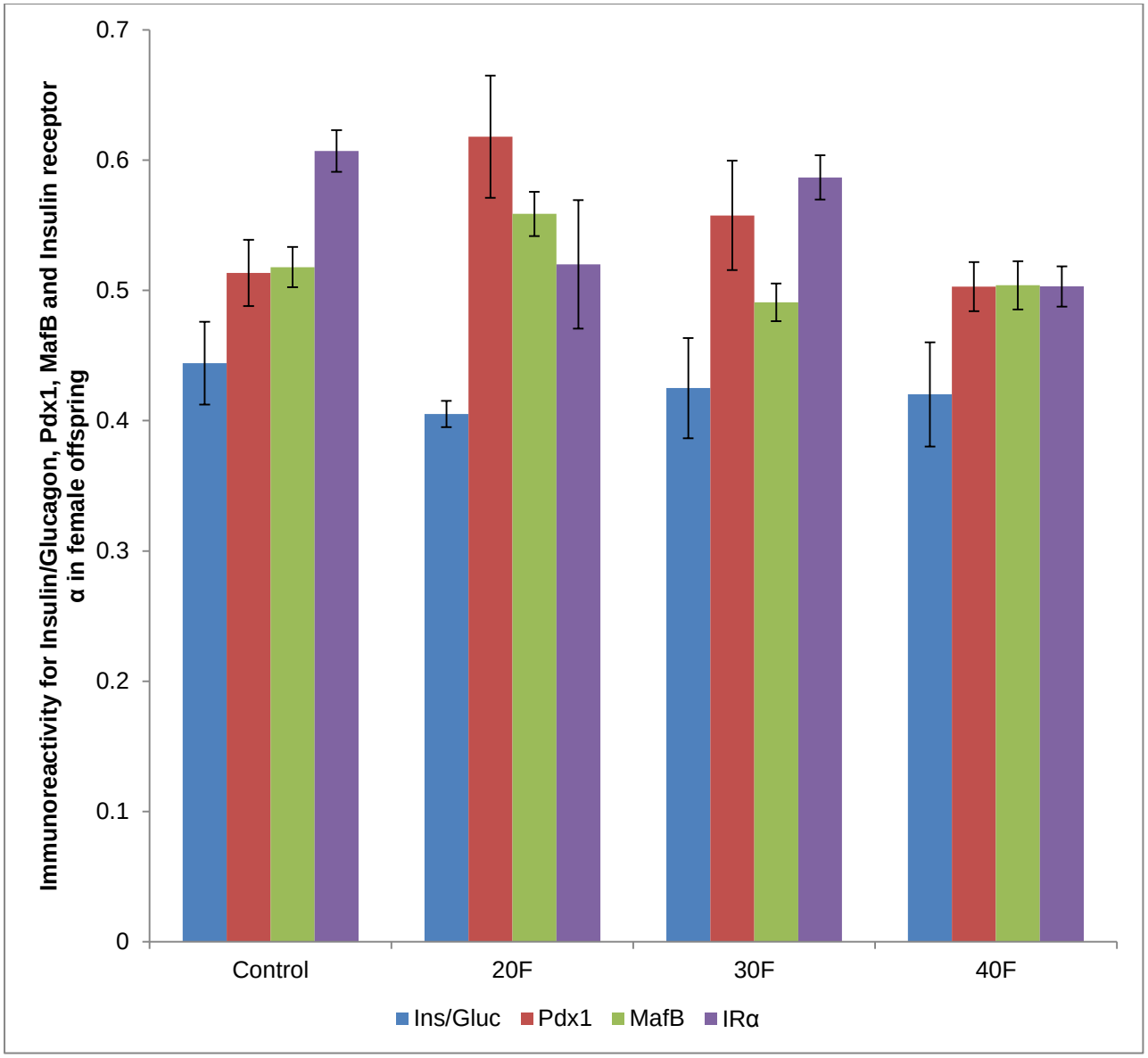


Figure 11. Immunoreactivity of insulin/glucagon, Pdx1, MafB and insulin receptor α in neonatal female offspring. 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet; Pdx1, duodenal box 1. Data are means ± SEM. P>0.05.

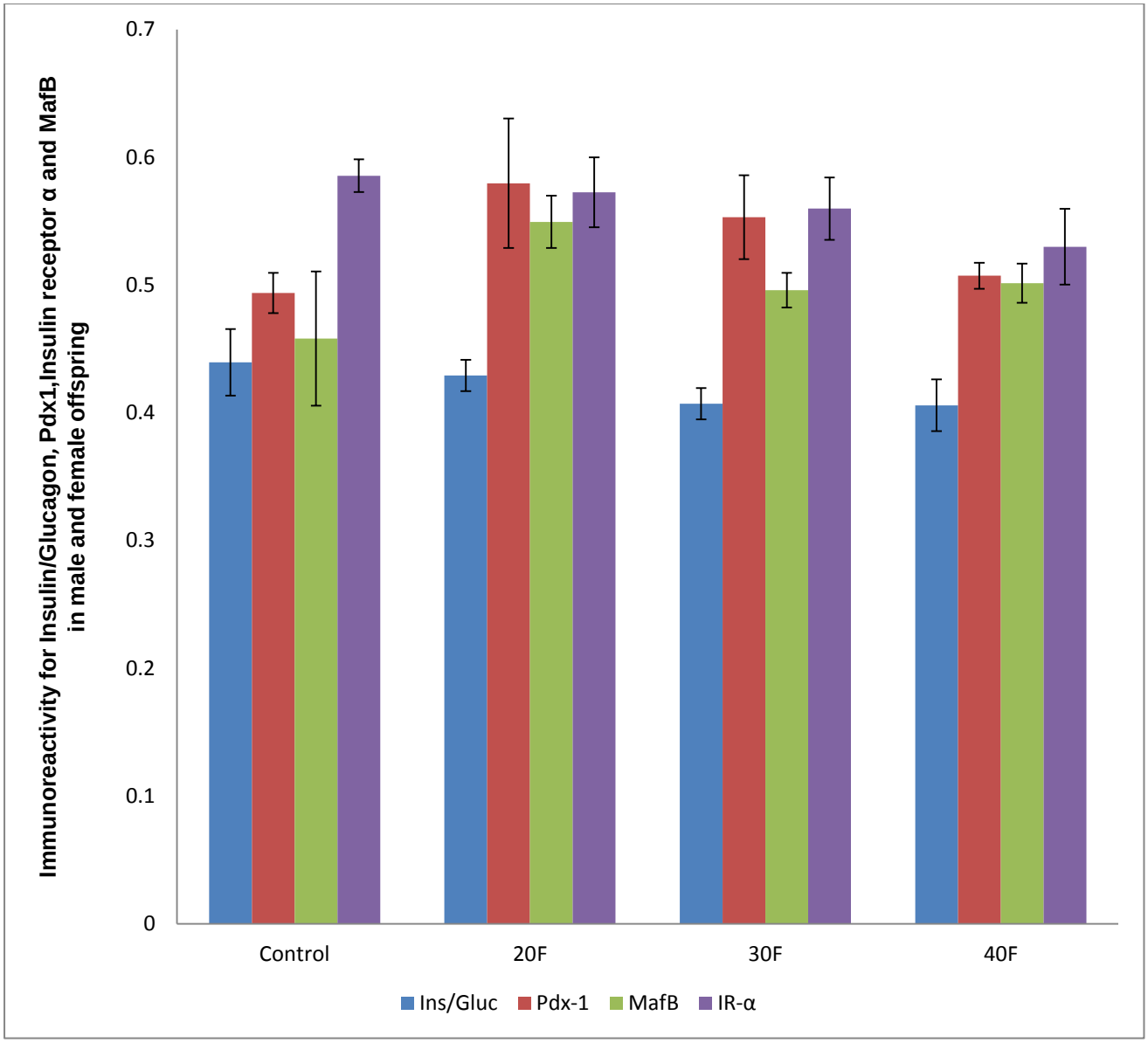


Figure 12. . Immunoreactivity of insulin/glucagon, Pdx1, MafB and insulin receptor α in neonatal male and female offspring. 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet; Pdx1, duodenal box 1. Data are means ± SEM. P>0.05.

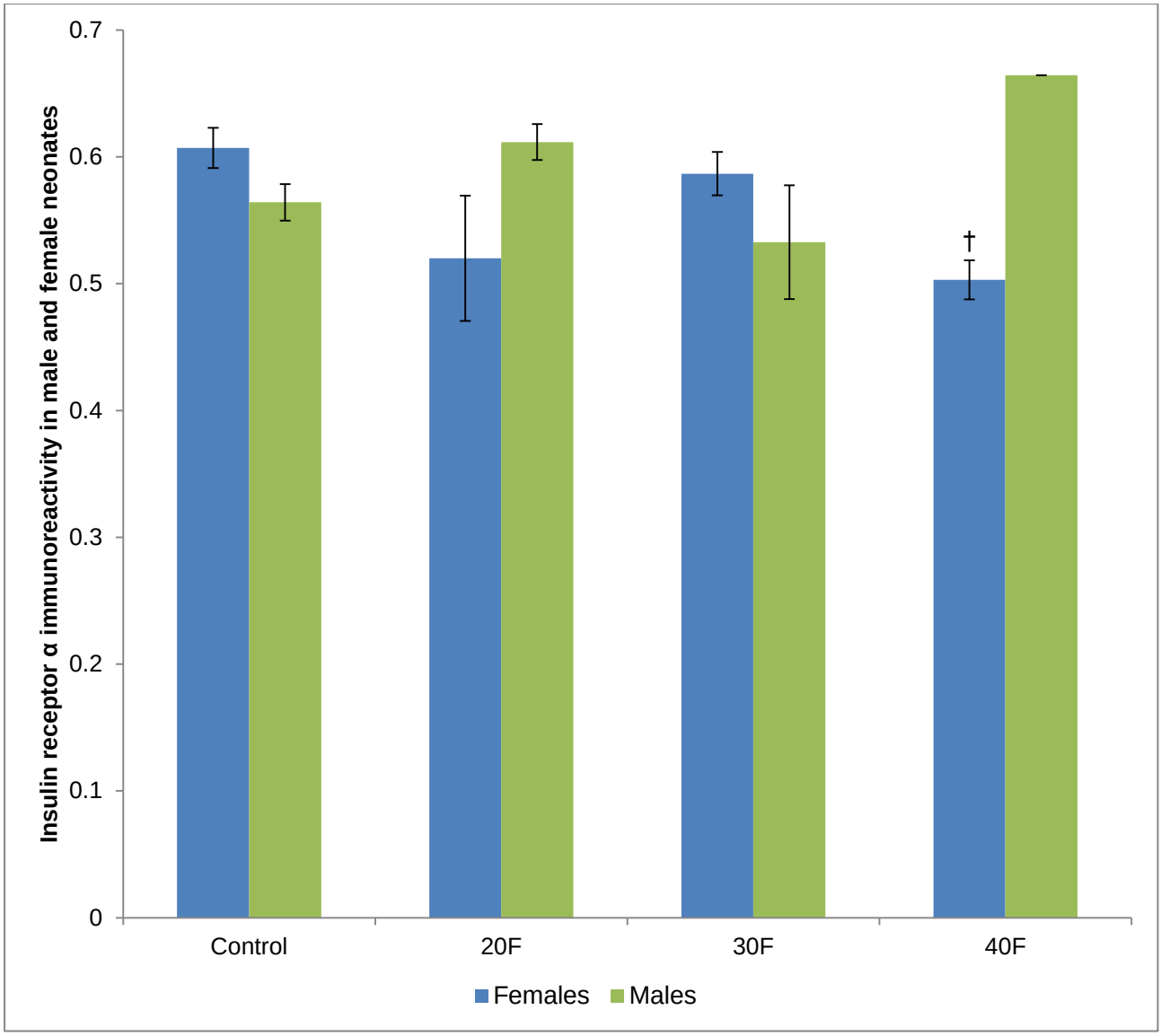


Figure 13. Immunoreactivity for insulin receptor α in neonatal male and female offspring. 20F, 20% fat diet; 30F, 30% fat diet; 40F, 40% fat diet. Data are means ± SEM. $^{\dagger}P < 0.05$ in female 40F offspring compared to male 40F offspring.

Note: There were no individuals graphs for Ins/Glucagon, Pdx1 and MfB as there were no significant differences shown in both female and male offspring.

Chapter Five

DISCUSSION

Developmental programming alters pancreatic structure and function with evidence from several studies. In addition, developmental programming can shape the pancreatic milieu thereby altering hormonal secretion. Similar to nutrient-restriction studies, maintenance on a HFD (throughout foetal life) reduced β-cell number and volume compounding to impaired insulin release in neonatal offspring (26).

Hereditary factors also affect offspring health. However, parental nutrition may also influence the health outcomes of their offspring. A study by Chakravarthy and colleagues reported that low birth weight predisposes to T2D, supporting the hypothesis that impairing growth in early life, programmes metabolic disease in adulthood (66).

Exposure to a detrimental environment in utero can programme the physiology and metabolism of the offspring permanently, with long term consequences for health in adulthood (6). Hyperglycaemic high fat programmed neonates displayed beta cell hypoplasia and reduced beta cell volume (67). Therefore, adequate and healthy nutrition during the early phases of life is required for proper organ development and functional maturation.

Parlee *et al.* reported that when offspring are exposed to a HFD during both gestation and lactation, elevated body weights, hyperleptinaemia, hyperglycaemia and hyperinsulinaemia presented without changes in β-cell or α-cell size or number (26). Furthermore, HFD maintenance during early life modifies pancreatic development leading to decreased insulin production and hyperglycaemia during early postnatal life (26). As the animal continues to grow, the pancreas largely compensates after HFD maintenance during both gestation and lactation. However it may still be incapable of completely regulating blood glucose concentrations which may then result in a compromised metabolic phenotype (26). Birth weight is recognised as an important predictor of the offspring health. This resonates with low birth weight and adult fat mass predisposing offspring to increased fat mass and to the development of MetS (8).

In the present study, rats were fed a maternal HFD during gestation to determine the effects of the diet on the islet hormones, islet transcription factors and insulin signaling factors. Immunoreactivity for insulin, glucagon and Pdx1 were unaltered in offspring maintained on 20%, 30% or 40% fat diets; hence a maternal diet varying in

fat content did not appear to affect these key pancreatic factors at a protein level. However, Western blot analyses should be performed to confirm or refute these findings.

We previously reported elevated brain GLUT2 and neuropeptide (NPY) immunoreactivity in both 30F and 40F neonates demonstrating that programming with a diet of either 30% and 40% fat influences factors associated with glucose sensing and the feeding response, respectively (8). Although Pdx1 mRNA expression in 20F and 40F neonates showed no fold difference, in 30F neonates, Pdx1 was under-expressed. Surpringly, no change in Pdx mRNA expression in 40F neonates was evident. We previously reported a 2.8 fold increase in Pdx mRNA expression in 40F neonates. The discrepancy in these findings may be attributed to biological variability and the differences in control diet formulation. Although both control diets had the same macronutrient profile, the previous diet was in pellet form whereas the diet used in the current study was in patty form.

The most profound effects on key factors implicated in diabetes, at gene level, were found in neonates maintained on a high fat diet i.e. 40F neonates. This was evident by reduced Ins1, Ins2, glucagon, MafB and IRS2 mRNA expression. The reduced mRNA expression after high fat diet maintenance therefore impairs key islet factors at the gene level. Despite no evidence of altered expression at a protein level, further studies using more sensitive protein techniques may provide further insight.

The under-expression of Pdx1 mRNA in 30F neonates may play a role in compromising β-cell integrity. However, Pdx1 immunoreactivity was unaltered in 30F neonates. Although the expression of mRNA and protein for a specific factor may not correlate, e.g. due to post-translational modifications, Western blot analyses, which is more specific for protein quantification, should be conducted for these key pancreatic factors. Immunostaining and image analysis does however have the advantage of demonstrating where the factor is localised, e.g. Pdx1 was localised in the nucleus.

Interestingly, there was an under-expression of MafB mRNA in both 30F and 40F neonates. Therefore, diets with higher fat content, viz. 30% and 40% fat as energy, reduced MafB mRNA expression. MafB is required for insulin and glucagon transcription in developing α- and β-cells and also plays a crucial role in a variety of

other cellular differentiation processes, including the islet α cell-enriched activator differentiation. Thus under-expression in MafB could impair islet cell differention and α-cell integrity in these neonates.

Glucagon and IRS2 mRNA were differentially expressed amongst the groups, viz. over-expressed in 20F neonates but under-expressed in 40F neonates. Since IRS2 dependent signaling in islets is required for the maintenance of α-cell mass and glucagon is the hormone secreted by α-cells, the over-expression of these islet factors may augment α-cell developmet and function in neonates maintained on a 20% fat diet. Conversely, in neonates maintained on a high fact diet, i.e. 40F neonates, α-cell development and function may be impaired.

Any impairment in β-cell development will influence insulin secretion in response to increased circulating glucose concentrations (14). Insulin is the key regulatory hormone in glucose homeostasis and promotes cellular glucose uptake and storage, whereas glucagon promotes glucose release. Rodents have two insulin genes. Insulin 2 is the rodent homologue of the human insulin whereas insulin 1 likely evolved by a gene duplication event (64,65). Neonates maintained on a high fat diet had reduced Ins2 and Ins1 mRNA expression which suggested that foetal high fat programming impaired insulin transcription. To a lesser extent, a 30% fat diet, reduced Ins1 mRNA expression, which is the less dominant insulin homologue (64,65).

Chapter Six

CONCLUSION

Conclusion and future Work

6.1 Study limitations

RNA is an easily degraded molecule, thus the quality of RNA differs from one extraction to another. The degradation process is constant and as result the RNA integrity may not always be consistent. This may influence the quality of the data.

Immunostaining followed by image analysis reveals the localization and relative quantification of target proteins. Western blot analyses, however, could provide further insights into our protein expression profiles.

For insulin signaling, IRS2 was studied at gene level whereas IRα was studied at protein level. This was due to difficulty in obtaining robust data for each factor using the alternative methology i.e. qRT-PCR was not reliable for IRα and conversely immunostaining for IRS2 was not achieved. This was despite using different probes for IRα and various antibodies and dilutions for IRS2.

There were insufficient samples to conduct gender-specific qRT-PCR analysis. In future studies, more offspring will be generated to ensure that sufficient sample sizes are realised.

6.2 Conclusion

In this study we investigated how foetal programming by maternal diets, varying in fat content, contributed to the pathogenesis of T2D and also how it affected islet hormones (insulin and glucagon), transcription factors (Pdx1 and MafB) and insulin signaling factors (IRS2 and IRα) in the neonatal pancreas.

The modest effects of a maternal HFD in neonates reflected by unaltered insulin, glucagon, Pdx1 and MafB expression suggest that more studies are warranted with either a higher fat content e.g. 60% or extending the high fat diet challenge and studying offspring over the life course. The reduced Pdx1 mRNA expression in 30F neonates may impair their β-cell development and function. Therefore, investigating β-cell number, size and volume and glucose-stimulated insulin secretion may provide further insight and better characterise this animal model.

A maternal diet varying in fat content alters key factors involved in the pathogenesis of T2D, at the gene level. Furthermore, foetal programming with a 30% and 40% fat diet had the most profound effects on factors implicated in the pathogenesis of T2D. Further studies are required to determine the global effects and whether insulin resistance manifests in glucose recipient organs such as the liver, muscle and adipose tissue.

6.3 Future perspectives

Immunostaining and Western blotting of the other islet hormones viz. somatostatin, pancreatic polypeptide and ghrelin will provide further insight into islet architecture. Other β- and α-cell transcription factors should be studied to determine which are most affected by diets varying in fat content. Several factors along the insulin signaling pathway should be investigated by PCR array to provide a more holistic overview of the foetal programming effects in the pancreas.

References:

1. Beagley J, Guariguata L, Weil C, Motala AA. Global estimates of undiagnosed diabetes mellitus. Diabetes Res Clin Pract 2014;103:150-160

2. Guariguta L, Whiting DR, Hambleton I,Beagley J.Global estimates of diabetes prevalence for 2013 and projections for 2035. Diabetes Res Clin Pract 2014; 103: 137-149

3. Wolden Kirk H, Overbergh L, Christesen HT, Brusgaard K, Mathieu C: Vitamin D and Diabetes: It's importance for the beta cell and immune function. Mol Cell Endocr 2011;347:106-120

4. Dunn GA, Bale TL: Maternal high- fat diet promotes body length increases and insulin insensitivity in second- generation mice. Endocr 2009; 150(11): 4999-5009

5. Tzanetakou IP, Mikhaailidis DP, Perrea DN: Nutrion during pregnancy and the effect of carbohydrates on the offspring's metabolic profile: In search of the "perfect maternal diet". Open Cardiovasc Med J 2011; 5: 103-109

6. Cerf ME, Williams K, Nkomo XI, Muller CJ, Louw J, Wolfe- Coote SA: Islet cell response in the neonatal rat after exposure to a high- fat diet during pregnancy. Am J physiol Regul Integr Comp Physiol 2005; 288: 1122-1128

7. Cerf ME, Williams K, Chapman CS: Compromised β- cell dysfunction in weanling offspring from dams maintained on a high- diet during gestation. Pancreas 2007; 34: 347-353

8. Cerf ME, Williams K, van RJ, Esterhuyse AJ, Muller CJ, Louw J: Gestational 30% and 40% fat diets increase brain GLUT2 and neuropeptide Y immunoreactivity in neonatal Wistar rats. Int J Dev Neurosci 2010; 28: 625-630

9. Cerf ME, Chapman CS, Muller CJ, Louw J: Gestational high- fat programming impairs insulin release and reduces Pdx1 and glucokinase immunoreactivity in neonatal Wistar rats. Metabolism 2009; 58: 1787-1792

10. Wilson ME, Scheel D, German MS: Gene expression cascades in pancreatic development. Mechan Devel 2003;120:65-80

11. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, Lin JK, Farzadfar F, Khang YH, Stevens GA, Rao M, Ali MK, Riley LM, Robinson CA, Ezzati M: National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. Lancet 2011;378:31-40

12. Kaneto H, Miyatsuka T, Kawamori D, Yamamoto K, Kato K, Shiraiwa T et al. Pdx1 and MafA play a crucial Role in pancreatic β-cell differentiation and maintenance of mature β-cell funcyion. Endocrine j 2008;55(2):235-252

13. Duque-Guimaraes DE, Ozanne SE. Nutritional programming of insulin resistance: causes and consequences. Trends in Endocr and Met 2013;

14. Malecki MT. Genetics of type 2 diabetes mellitus. Diabetes Res & Clinical Prac 2005,68SI;S10-S21

15. Gastaldelli A: Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. Diabetes Resear & Clini Pract 2011;93S:S60-S65

16. Babu DA, Deering TG, Mirmira RG. A feat of metabolic proportions: Pdx1 orchestrates islet development and function in the maintenance of glucose homeostasis. Molec Genetic & Metab 2007;92:43-55

17. Kemp DM, Thomas MK, Habener JF. Developmental Aspects of the Endocrine Pancreas. Endocr & Metaboli Disorders 2003;4:5-17

18. Hang Y, Stein R. MafA and MafB activity in pancreatic β cells.Trends in Endocr and Met 2011;22(9):364-373

19. Kavvoura F, Owen KR. Maturity onset diabetes of the young:clinical characteristics, diagnosis and management. Paediatric Endo Reviews 2013;10(2):234-242

20. Bernardo AS, Hay CW, Docherty K: Pancreatic transcription factors and their role in the birth, life and survival of the pancreatic β cell. Molec & Cell Endo 2008: 294;1-9

21. Cerf ME. Parental high-fat programming of offspring development, health and β-cells. Islets 2011;3:118-120

22. Aguayo-Mazzucato C, Koh A, Khattabi EI, Li WC, Toschi E, Jermendy A et al. MafA expression enhances glucose-responsive insulin secretion in neonatal rat beta cells. Diabetologia 2011,54;583-593

23. Marchetti P, Dotta F, Lauro D, Purrello F. An overview of pancreatic beta-cell defects in human type 2 diabetes: Implications for treatment. Reg Peptides 2008;146:411

24. Rhodes CJ. Type 2 diabetes- A matter of beta-cell life and death? Science 2005;307:380-3844

25. Karaca M, Magnan C, Kargar C. Functional pancreatic beta- cell ass: Involvement in type 2 diabetes and therapeutic intervention. Diabetes & Metab 2009;35:77-84

26. Parlee SD, MacDougald AO. Maternal nutrition and risk of obesity in offspring: The Trojan horse of developmental plasticity. Biochim at Biophy Acta 1842 2014:495-506

27. Hooper C. insulin signaling pathways: defects in this hormone pathway and type-2 diabetes.www. abcam.com/pathways/overview-of-insulin-signaling-pathways/7/28/2014

28. Capurso C, Capurso A. From excess adiposity to insulin resistance: The role of free fatty acids. Vascular Pharm 2012;57:91-97

29. Taniguchi CM, Ueka K, Kahn CR. Complementary roles of IRS1 and IRS2 in the hepatic regulation of metabolism. J. Clinical Investigation 2005;115:718-727

30. Cantley J, Choudhury AI, Asare-Anne H, Selman C, Lingard S, Heffron H et al. Pancreatic deletion of insulin receptor substrate 2 reduces beta and alpha

cell mass and impairs glucose homeostasis in mice. Diabetologia 2007;50:1248-1256

31. Nolan J. What is type 2 diabetes? Medicine 2006;34(2):52-56

32. Peer N, Kengne P, Motala AA, Mbanya JC. Diabetes in the Africa region: an update. Diabetes Res and Clin Pract 2014;103:197-205

33. Mbanya JC, Motala AA, Sobngwi E, Assah FK, Enoru ST. diabetes in sub-Saharan Africa. Lancet 2010;375:2254-2266

34. Reaven GM. Why Syndrome X? From Hisworth to the insulin resistance syndrome. Cell Metabolism 2005;1:9-14

35. St-Onge L, Wehr R, Gruss P. Pancreas development and diabetes.Cur Opinions in Genetics & Develop 1999;9:295-300

36. Berends LM, Ozanne SE. early determinants of type-2 diabetes. Best Pract& Res Clini Endo& Metab 2012;26:569-580

37. www.idf.org/diabetesatlas/5e/the-global-burden: available online on the 18 January 2012

38. Blyth NJ. Mechanisms and techniques of reprograming-using Pdx1 homeobox protein as a novel treatment of insulin dependent diabetes mellitus. Diabetes&Metabolic Syndr: Clin Res & Rev 2012;6:113-119

39. Chandler-Laney PC, Bush NC, Granger WM, Rouse DJ, Mancuso MS, Gower BA. Overweight status and intrauterine exposure to gestational diabetes are associated with children's metabolic health. Pediatric Obesity 2011;7:44-52

40. Fisher RE, Steele M, Karrow NA. Fetal programing of the neuroendocrine-Immune system and metabolic disease. J of Pregnancy 2012;2012:1-10

41. Reaven GM. The Metabolic syndrome:Requiescat in pace. Clinic Chem 2005;51(6):931-938

42. Capurso C, capurso A. From excess adiposity to insulin resistance: the role of free fatty acids. Vascular Pharm 2012;57:91-97

43. Alejandro EU, Gregg B, Blandino-Rosano M, Cras- Meneur C, Bernal-Mizrachi E. Mol Aspp of Med 2015;15:19-41

44. Flock MR, green MH, Kris-Etherton M. Effects of adiposity on plasma lipid response to reductions in dietary saturated fatty acids and cholesterol. Adv. Nutr 2011;2:261-274

45. Poston L. Developmental programming and diabetes-the human experience and insight from animal models. Best Pract & Res Clin Endo & Met 2010;24:541-55

46. Stout SA, Espel VE, Sandman CA, Glynn LM, Davis PE. Fetal programming of children's obesity risk. Psychoneuroendocrinology 2015;53:29-39

47. Masiello P. Animal models of type 2 diabetes with reduced pancreatic β-cell mass. Int J. Biochem & Cell Bio 2006;38:873-893

48. Gluckman PD, Hanson MA. Developmental origins of disease paradigm:a mechanistic and evolutionary perspective. Paediatr. Res. 2004;56:311-317

49. Tamashiro KL, Moran TH. Perinatal environment and its influence on metabolic programming of offspring Physiol.Behav.2010;100:560-566

50. Moore VM, Davies MJ, Wilson KJ, Worsely A, Robinson JS. Dietary composition of pregnant women is related to the size of the baby at birth.J.Nutr.2004;134:1820-1826

51. Warner MJ, OzanneSE. Mechanisms nvolved in the developmental programming of adulthood disease. Biochem. J.2010;427:333-347

52. Remmers F, Delemarre-van der Waal HA. Developmental programming of energy balance and its hypothalamic regulation. Endocrine Reviews 2011;32(2):272-311

53. Roseboom T, de Rooij S, Painter R. The Dutch Famine and its long term consequences for adult health. Early Hum Dev 2006;82:485-491

54. Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, et al. The metabolic syndrome. Endocr Rev 2008;29:777-822

55. Martorell R, Stein AD, Schoeder DG. Early nutrition and later adiposity. J Nutr 2001;131:874S-880S

56. Curhan GC, Chertow GM, Willet WC, Spiegelman D, Colditz GA, Mason JE, et al. Birth weight and adult hypertension and obesity in women.Circulation 1996;94:1310-1315

57. Curhan GC, Willet WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ. Birth weight and hypertension, diabetes mellitus, and obesity in US men. Circulation 1996;94:3246-3250.

58. Kahn HS, Narayan KM, Williamson DF, Valdez R, Relation of birth weight to lean and fat thigh tissue in young men. Int J Obes Relat Metab Disord 2000;24:667-672.

59. Ravelli GP, Stein Z, Susser MW. Obesity in young men after famine exposure in utero and early infancy. N Eng J Med 1976;295:349-353

60. Grove KL, Grayson BE, Glavas MM, xizo ZQ, Smith MS. Development of metabolic systems. Physiol Behav 2005;86:646-660

61. Gluckman PD, Hanson MA. Developmental and epigenetics pathways to obesity:an evolutionary-developmental perspective. Int J Obes (Lond) 2008;32 (Suppl 7): S62-S71

62. Smushkin G, Vella A. What is type 2 diabetes? Medicine 2010;38(11):597-600

63. Wilson EW, Scheel D, German MS. Gene expression cascades in pancreatic development. Mechanisms of Dev 2003;120:65-80

64. Shiao M, Liao B, Long M, Yu H. Adaptive evolution of the Insulin two0gene system in mouse. Genetics 2008;178:1683-1691

65. Paronen J, Moriyama H, Abiru N, Sikora K, Melnitou E, Babu S et al. establishing insulin 1 and Insulin 2 knockout congenic strains on NOD genetic background. Ann. NY. Acad. Sci 2003;1005:205-210

66. Chakravarthy MV, Zhu Y, Wice MB, Coleman T, Pappan KL, Marshall CA et al. decreased fetal size is associated with β-cell hyperfunction in early life and failure with age. Diabetes 2008;57(10):2698-2707

67. Cerf ME. High fat programming of beta cell compensation, exhaustion, death and dysfunction. Pediatric Diabetes 2014;10;1-10

68. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-408

69. Boenish T et al.20090Education guide:Immunohostochemical staining methods. 5th ed. Santa Barbara: dako North America Inc.

70. Carpenter AE, Jones TR, Lamprecht MR, Clarke C, kang HI, Friman O. cellprofiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biology 2006;7(10):R100.1-R100.11

71. www.eurogentec.com/uploads/filebrowser/technical%20guides/genomic%20Solutions/qPCR-booklet.pdf: available online on 18 June 2014

.