# *The anti-diabetic and insulin sensitizing potential of a watery extract of Agathosma tested in rat models of type 1 and type 2 diabetes.*

***Vereneque Jansen***

Thesis presented in fulfilment of the requirements for the degree
Master of Science in Medical Sciences
in the Faculty of Medicine and Health Sciences at Stellenbosch University

Supervisor: Prof. Barbara Huisamen
Faculty of Medicine and Health Sciences
Department of Biomedical Sciences
Division of Medical Physiology
Stellenbosch University

March 2015

Stellenbosch University https://scholar.sun.ac.za

# DECLARATION

---

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously in its entirety or part submitted it at any university for a degree.

Signature: .....................................

Date: ...........................................

Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á
Á

Ô[ ] ˆ¦ã @Á ÁG€FÍ ÁÙc^||^} à[ • &@ÁW} ãç^¦• ãc
Œ||Áã @• Á^•¦ç^åÁ

Stellenbosch University https://scholar.sun.ac.za

# Acknowledgements

- Firstly, I would like to thank the Lord for reminding me that with a little bit of faith anything is possible, especially when things got rough in my personal life. “Time does not necessarily heal all wounds BUT it does make living bearable!”

- Secondly, I want to thank my supervisor Prof. Barbara Huisamen for her undivided attention and support! I know there were many times I probably drove her insane with stress and worry, but she still remained by my side. I would not have been able to make it this far without her guidance, love and compassion. For these reasons and so much more, “THANK YOU!”

- Thirdly, I wish to thank my parents (Priscilla and Mervyn Jansen) for always believing in me.

- A special thanks to my fiancé, Keegan Naidoo, for always encouraging me and being a pillar of strength. “Love truly conquers all!”

- For financial support I would like to thank the University of Stellenbosch, the National Research Foundation and the Department of Medical Physiology.

Stellenbosch University https://scholar.sun.ac.za

- To all my colleagues in the Department of Medical Physiology and staff from the Animal Unit, thank you all for your input and support.

- I wish to acknowledge Cape Kingdom for granting me the opportunity to research their product.

Stellenbosch University https://scholar.sun.ac.za

# ABSTRACT

**Introduction**: Epidemiological data highlights that South Africa is currently facing a quadruple burden of disease of which non-communicable diseases (NCDs) are estimated to account for 29% of all deaths. These NCDs include, amongst others, cardiovascular disease, type 2 diabetes, hypertension, cancer, chronic lung disease and depression. Diabetes has become a major problem worldwide and in South Africa and is currently rated as the 5$^{th}$ largest cause of death by Statistics South Africa in 2011. This creates an enormous burden of disease on populations and the utilization of herbal remedies has escalated in popularity because of this. Buchu water is one of these herbal remedies advertised as having anti-diabetic properties. This water is a by-product of the extraction of the oil from the leaves of Agathosma and is already freely available to the public.

The **aim** of this study was therefore to use animal models of type 1 diabetes, and obesity and insulin resistance to scientifically verify or refute these claims.

**Methods**: We utilized male Wistar rats. Two different rat models were used: (i) a type 1 diabetic model; induced via a once-off intraperitoneal Streptozotocin (40mg/kg) injection ablating ~50% of pancreatic beta cells (T1D); (ii) A diet-induced obese model, rendering rats insulin resistant after receiving a high caloric diet for 16 weeks. Half of each experimental group was treated with diluted Buchu water for a period of 14 weeks and 16 weeks, respectively, while the rest consumed normal water. Water and food consumption were monitored, body weight and intraperitoneal fat measured, blood was collected to determine serum glucose and insulin levels, skeletal muscle was removed

Stellenbosch University https://scholar.sun.ac.za

to test insulin sensitivity using radiolabelled deoxyglucose, pancreas and skeletal muscle harvested and stored in liquid nitrogen for further biochemical analysis.

**Results**: One of the main findings of this study was that ingestion of Buchu water results in weight loss despite no decrease in food consumption. This occurred in both the pathological models and control animals. In the obese animals, this weight loss was due to a decrease in intra-peritoneal fat.

A second important finding was that the ingestion of Buchu water in all instances, whether given as treatment (treated with Buchu water 3 weeks after the start of the experiment) or as prophylactic (treated with Buchu water from the start of the start of the experiment), resulted in normalization of glucose levels in a type-1 diabetic model with residual beta-cell mass.

An insulin-sensitizing effect was not clearly established in skeletal muscle but this may be because of a large variation in values obtained, as well as the use of a slow-twitch muscle.

A definite effect on pancreatic insulin secretion has been demonstrated by raised C-peptide levels in the diet-induced obese model.

**Conclusion**: This study, with regard to the type 1 diabetic model, has confirmed the anti-diabetic effect of Buchu water by significantly lowering blood glucose levels of fasted and non-fasted blood and normalizing Intraperitoneal glucose tolerance test (IPGTT) curves. This was however not so evident in the obese model utilized. Despite this, animals lost weight which was mainly intra-peritoneal fat.

Stellenbosch University https://scholar.sun.ac.za

# ABSTRAK

**Inleiding:** Epidemiologiese data dui aan dat daar tans ‘n viervoudige siektelading Suid Afrika in die gesig staar. Hiervan is nie-oordraagbare siektes (NCDs) vir ongeveer 29% van van sterftes verantwoordelik. Hierdie siektetoestande sluit kardiovaskulêre siektes, tipe 2 diabetes, hipertensie, kanker, kroniese longsiektes en depressie in. Diabetes is tans ook in Suid Afrika een van die groot gesondheidsprobleme en word deur die MNR 10de op die ranglys van oorsake van sterftes geplaas. Dit veroorsaak ‘n groot siektelas op die populasie in geheel. As gevolg hiervan het die gebruik van natuurlike produkte om siektes te behandel, geweldig toegeneem. Buchuwater is een so ‘n natuurlike produk wat geadverteer word om anti-diabetiese effekte te hê. Hierdie water is ‘n afvalproduk van die ekstraksie van Buchu-olie uit die blare van *Agathosma* en is alreeds vrylik aan die publiek beskikbaar.

**Metodes:** Manlike Wistarrotte is vir die studie gebruik. Twee verskillende modelle is gebruik naamlik (i) ‘n tipe 1 diabetiese model daargestel deur ‘n enkele intraperitoneale Streptozotocin (40mg/kg) inspuiting waarmee ~50% van die betaselle van die pankreas vernietig word en (ii) ‘n dieet-geïnduseerde vetsugtige model waar rotte vir 16 weke op ‘n hoë kalorie dieet geplaas word. Helfte van elke eksperimentele groep diere se drinkwater is deur verdunde Buchuwater vervang vir die volle 16 weke. Water- en kosinname is gemonitor, liggaamsgewig en intraperitoneale vetgewig bepaal. Bloedglukose is gemonitor en intraperitoneale glukosetoleransie kurwes opgestel. Bloed is gekollekteer om serum insulienvlakke te bepaal en skeletspier

Stellenbosch University https://scholar.sun.ac.za

insulienweerstandigheid is bepaal deur die opname van isotoopgemerkte deoksiglukose te meet. Pankreata is geoes en in vloeibare stikstof vir biochemiese analise gestoor.

**Resultate:** Een van die hoofbevindinge van hierdie studie was dat die inname van Buchuwater gewigsverlies in diere teweeggebring het sonder dat hulle kosinname verminder het. Dit het in beide modelle, sowel in kontrol diere gebeur. In die vetsugtige diere was hierdie verlies as gevolg van verlies van intraperitoneale vet.

'N tweede belangrike bevinding was dat die inname van Buchuwater 'n normalisering van bloedglukosevlakke in die tipe 1 diabetiese diere met 'n betasel reserwe teweeggebring het, beide wanneer dit as voorkomend of as behandeling gegee is.

Sensitisering teenoor die effekte van insulin in die skeletspiere van die vetsugtige model rotte kon nie waargeneem word met die inname van Buchuwater nie, deels as gevolg van 'n baie groot variasie in die resultate.

Effekte van die Buchuwaterinname op die betaselle van die pankreas en insuliensekresie is gevind.

**Gevolgtrekking:** Met betrekking tot die tipe 1 diabetiese model het die studie die antidiabetiese effekte van Buchuwater bevestig deur 'n beduidende verlaging van vastende sowel as nie-vastende bloedglukosevlakke en normalisering van IPGTT kurwes teweeg te bring. Hierdie effekte was nie so voor die hand liggend in die vetsugtige model nie maar hierdie diere het gewig, hoofsaaklik intraperitoneale vet, verloof.

Stellenbosch University https://scholar.sun.ac.za

# TABLE OF CONTENTS


| | |
|---|---|
| **Declaration** | **ii** |
| **Acknowledgements** | **iii** |
| **Abstract/Opsomming** | **v** |
| **Table of contents** | **ix** |
| **List of Abbreviations** | **xv** |
| **List of Figures** | **xxiii** |
| **List of Tables** | **xxvi** |
| **Motivation for research** | **xxvii** |
| **Disclosure of interest** | **xxx** |
| **Chapter 1: Literature Review** | **1** |
| **1.1. The Digestive system** | **1** |
| **1.2. Glucose transport across the intestinal epithelium** | **4** |
| **1.3. The Pancreas** | **5** |
| 1.3.1. Exocrine pancreas | 6 |
| 1.3.2. Endocrine pancreas | 6 |
| **1.4. Pancreatic response to hyperglycemia** | **7** |
| **1.5. The link between insulin action and diabetes mellitus** | **9** |
| 1.5.1. Insulin biosynthesis | 9 |

Stellenbosch University https://scholar.sun.ac.za

**1.5.2.** Insulin action in regulating glucose homeostasis 11

**1.5.3.** Insulin signalling pathway in skeletal muscle 13

1.5.3.1. Glycolysis and Glycogenesis 14

1.5.3.2. GLUT 4 translocation to the plasma membrane 14

1.5.3.2.1. PI3K dependent pathway 15

1.5.3.2.2. PI3K Independent pathway (CAP-Cbl-TC10 pathway) 16

1.5.3.2.3. PI3K Independent pathway (AMPK pathway) 17

**1.5.4. Regulators of the insulin pathway 19**

1.5.4.1 PTEN 20

1.5.4.2. SHIP 23

**1.5.5. Role of insulin in the development of diabetes 24**

**1.6. Diabetes Mellitus 25**

**1.6.1. Type 1 Diabetes 26**

1.6.1.1. Pancreatic β-cell dysfunction 27

1.6.1.2. β-cell mass 28

1.6.1.3. β-cell neogenesis 29

1.6.1.4. Transcription factors promoting β-cell neogenesis 30

1.6.1.4.1 Pdx-1 30

Stellenbosch University https://scholar.sun.ac.za


1.6.1.4.2 Maf A 31

1.6.1.5. Treatment: Type 1 diabetes 32

1.6.1.6. The Streptozotocin model of type 1 diabetes. 33

**1.6.2. Type 2 Diabetes 34**

1.6.2.1. The role of obesity in the development of insulin resistance 35

1.6.2.1.1 The link between obesity and adipose dysfunction 36

1.6.2.1.2 Fatty acid induced insulin resistance 37

1.6.2.2. The link between adipocyte secretions and insulin resistance 39

1.6.2.2.1 Adipokines 39

1.6.2.2.2 Cytokines 42

1.6.2.3 Treatment: Type 2 diabetes 44

**1.7. Skeletal muscle metabolism 46**

1.7.1. Fatty acid metabolism 47

1.7.2. Glucose metabolism 49

1.7.3. Oxidation of Acetyl CoA 52

1.7.3.1. Krebs cycle 52

1.7.3.2. Electron transport chain 53

Stellenbosch University https://scholar.sun.ac.za

**Chapter 2: Materials and Methods** **55**

**2.1. Animal care** **55**

**2.2. The administration procedure of Buchu (*Agathosma*)** **55**

**2.3. Animal models** **56**

2.3.1. Streptozotocin-induced type 1 diabetes rat model 56

2.3.2. Diet-induced obese insulin resistant rat model 59

**2.4. Western blot analysis** **61**

2.4.1. Sample Preparation 61

2.4.2. Bradford protein determination assay 63

2.4.3. Protein separation by gel electrophoresis 65

2.4.4. Transfer of proteins to a membrane 65

2.4.5. Blocking non-specific sites with milk 65

2.4.6. Immunodetection of proteins 66

**2.5. 2-Deoxy-D-3[H] glucose (2DG) uptake by soleus muscle** **67**

**2.6. Biochemical analysis** **70**

2.6.1. Blood sample collection 70

2.6.2. Serum insulin determination 70

2.6.3. Enzyme Linked Immunosorbent Assay (ELISA) 73

**2.7. Statistical Analysis** **75**

Stellenbosch University https://scholar.sun.ac.za

**Chapter 3: Results** **76**

**3.1. Type 1 Diabetes** **76**

**3.1.1. Biometric parameters** **76**

3.1.1.1. Food and water/Buchu consumption 76

3.1.1.2. Body weight and intra-peritoneal fat mass 78

**3.1.2. Metabolic parameters** **81**

3.1.2.1. Blood Glucose Levels 81

3.1.2.2. Intraperitoneal glucose tolerance test (IPGTT) 84

3.1.2.3. Serum Insulin levels 89

**3.2. Type 2 Diabetes** **90**

**3.2.1. Biometric parameters** **91**

3.2.1.1. Food and water/Buchu consumption 91

3.2.1.2. Body weight and intra-peritoneal fat mass 93

**3.2.2. Metabolic parameters** **95**

3.2.2.1. Intraperitoneal glucose tolerance test (IPGTT) 95

3.2.2.2. 2-Deoxy-D-3[H] glucose (2DG) uptake by soleus muscle 98

3.2.2.3. Serum insulin levels 99

3.2.2.4. C-peptide level 102

3.2.2.5. Pancreatic Transcription Factors 103

3.2.2.5.1. Pdx-1 103

Stellenbosch University https://scholar.sun.ac.za

3.2.2.5.2. MafA 104

**Chapter 4: Discussion 106**

**4. Diabetic models 108**

**4.1. Streptozotocin-induced type 1 diabetic model 108**

4.1.1. The effect of Buchu water on biometric parameters 109

4.1.2. The effect of Buchu water on metabolic parameters 110

**4.2. Diet-induced obese type 2 diabetic model 112**

4.2.1. The effect of Buchu water on biometric parameters 112

4.2.2. Anti-diabetic and insulin sensitizing effects of buchu water 114

4.2.2.1 The effect of Buchu water on insulin secretion and glucose homeostasis 114

4.2.3 Buchu water as an insulin sensitizing agent/ promoter 115

4.2.4. Buchu water promotes β-cell neogenic activity 116

**4.3. Adverse effects of Buchu water 117**

**Chapter 5: Conclusion 118**

**5.1 Conclusion 118**

**5.2 Limitations of this study 118**

**5.3 Future perspectives 119**

**Chapter 6: References 120**

Stellenbosch University https://scholar.sun.ac.za

# List of Abbreviations

---

## Units

| | |
|---|---|
| µl: | Microlitres |
| µM: | Micromolar |
| °C: | degrees Celsius |
| mg/kg: | milligrams per kilogram |
| rpm: | revolutions per minute |
| V: | Volts |
| mm: | millimeters |
| pM: | Picomolar |
| nM: | Nanomolar |
| mM: | Millimolar |
| %: | Percentage |
| g: | Grams |
| ml: | Millilitres |
| mmol/L: | Millimole per litre |
| pmol/L: | Picomole per litre |

Stellenbosch University  https://scholar.sun.ac.za

## Greek symbols

α: Alpha

β: Beta

δ: Delta

ε: Epsilon

γ: Gamma

## Other abbreviations

2DG: 2-Deoxy-D-3[H] glucose

$^{125}$I insulin: Radiolabelled Iodinated insulin

ACD: Acyl-CoA dehydrogenase

ADA: American Diabetes Association

AdipoR1: Adiponectin receptor1

AdipoR2: Adiponectin receptor2

AMPK: AMP-activated protein kinase

ANS: Autonomic system

aPKC: atypical protein kinase c

APS Adapter containing pleckstrin homology domain and Src homology 2 domain

ATP: Adenosine triphosphate

Stellenbosch University https://scholar.sun.ac.za

BMI: Body mass index

BSA: Bovine serum albumin

Bzip: Basic leucine zipper

BW: Body Weight

CAP: cb1 associated protein

CCK: Cholecystokinin

CK2: Casein kinase 2

CPK: Creatine phosphokinase

CNS: Central nervous system

Cys: Cysteine

DIO: Diet induced obese

DHAP: Dihydroxyacetone phosphate

DPP4: Dipeptidyl peptidase 4

ECL: Enhanced chemiluminescence

eIF4E: elongation initiation factor 4E

ELISA: Enzyme Linked Immunosorbent Assay

ER: Endoplasmic reticulum

ETC: Electron transport chain

Stellenbosch University https://scholar.sun.ac.za

| | |
|---|---|
| FDA: | Food and Drug Administration |
| GAP: | Glyceraldehyde 3-phosphate |
| GCK: | Glucokinase |
| GLP-1: | Glucagon-like peptide-1 |
| GLUTs: | Glucose transporters |
| Glut2: | Glucose transporter 2 |
| GLUT 4: | Glucose transporter 4 |
| GS: | Glycogen synthase |
| GSK-3: | Glycogen synthase kinase 3 |
| $H_2O$: | Water molecule |
| HCl: | Hydrochloric acid |
| HOMA: | Homeostasis model assessment |
| IFN-γR: | Interferon-gamma receptor |
| IKK: | IκB kinase |
| IL-1βR: | Interleukin-1β receptor |
| IP: | Intraperitoneal |
| IPGTT: | Intraperitoneal glucose tolerance tests |

Stellenbosch University https://scholar.sun.ac.za

IRS: Insulin receptor substrate

JNK1: c-Jun N-terminal kinase 1

LCAD: Long-chain ACD

Maf A: Musculoaponeurotic fibrosarcoma homolog A

MAREs: Maf responsive elements

MCAD: Medium-chain ACD

mTOR: Mammalian target of rapamycin

$Na^+$: Sodium

$Na^+/K^+$ Sodium-potassium

$NAD^+$: Nicotinamide adenine dinucleotide

NCDs: Non-communicable diseases

NF-κB: Nuclear factor-κB

NSB: Non-specific binding

ob/ob: Leptin deficient mice

p70s6k: p70 ribosomal s6 kinase

Stellenbosch University https://scholar.sun.ac.za

PDK1: Phosphoinositide dependent protein kinase 1

PDK2: Phosphoinositide dependent protein kinase 2

PDZ: a peptide sequence that is rich in Threonine, Lysine and Valine

Pdx-1: Pancreatic duodenal homeobox 1

PEST: a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T)

PH: Pleckstrin homology

PI-3K: Phosphatidylinositol 3-kinase

PIP2: PtdIns 4, 5-bisphosphate

PIP3: PtdIns 3, 4, 5-trisphosphate

PKB: serine/threonine protein kinase B

PKC-θ: Protein kinase C θ

PPAR-α: Peroxisome proliferator-activated receptor-α

PTB: Phosphotyrosine binding

PTEN: Phosphate and tensin homolog deleted on chromosome 10

PtdIns: Phosphatidylinositol

PVDF: Polyvinylidene fluoride

QC: Quality control

RIA: Radioimmunoassay

Stellenbosch University https://scholar.sun.ac.za

ROS: Reactive oxygen species

SAM: Sterile alpha motif

SCAD: Short chain ACD

SEM: Standard error of the mean

Ser: Serine

SGLT: Sodium-glucose co-transporters

SGLT-1: Sodium-glucose co-transporter 1

SH: Src homology

SH2: Src homology domain 2

SHIP: Src homology 2 containing inositol-5-phosphatase

SHIP 1: Src homology 2 containing inositol-5-phosphatase 1

SHIP 2: Src homology 2 containing inositol-5-phosphatase 2

SOCS-3: Suppressor of cytokine signalling 3

SREs: Sterol-regulatory elements

SREBP-1: Sterol regulating element binding protein 1

STAT-1: Signal transducer and activator of transcription-1

STZ: Streptozotocin

T: Total counts

T1D: Type 1 Diabetes

T2D: Type 2 diabetes

Stellenbosch University https://scholar.sun.ac.za

TAD: Transactivation domain

TAG: Triacylglycerol

Thr : Threonine

TNFR: Tumor necrosis factor receptor

Tyr: Tyrosine

VLCAD: Very long chain ACD

WHO: World Health Organisation

Stellenbosch University  https://scholar.sun.ac.za

# List of Figures


| | | |
|---|---|---|
| Figure 1: | Breakdown of complex polysaccharides | 3 |
| Figure 2: | Glucose transport across the enterocyte | 5 |
| Figure 3: | Insulin release by pancreatic β-cells | 8 |
| Figure 4: | Insulin synthesis and secretion | 10 |
| Figure 5: | PKB/Akt activation pathway | 13 |
| Figure 6: | Regulation and activation of AMPK | 19 |
| Figure 7: | PTEN structure | 22 |
| Figure 8: | The difference between the SHIP structure | 24 |
| Figure 9: | The pathogenesis progression of type 1 diabetes | 27 |
| Figure 10: | Type 2 diabetic treatment system | 45 |
| Figure 11: | Glycolytic pathway | 51 |
| Figure 12: | Electron transport chain | 54 |
| Figure 13: | Schematic representation of the division of type 1 diabetic animals | 57 |
| Figure 14: | The time frame of the type 1 diabetic model | 59 |
| Figure 15: | Schematic representation of the division of type 2 diabetic animals | 60 |
| Figure 16: | Tube arrangement for the Bradford protein assay | 63 |
| Figure 17: | Standard curve generated from the optical density | |

Stellenbosch University https://scholar.sun.ac.za


readings 64

Figure 18: Diagrammatic layout of tube labelling and preparation 71

Figure 19: Standard curve generated by the gamma counter 72

Figure 20: A graph of a typical standard curve 75

Figure 21: Food consumed by control and STZ animals, from week 3 77

Figure 22: Water/Buchu water consumed by control and STZ animals, from week 3. 78

Figure 23: Body weight (BW) of control and STZ animals, with and without Buchu treatment, after 14 weeks. 79

Figure 24: Intra-peritoneal (IP) fat of control and STZ animals, with and without Buchu treatment, after 14 weeks. 80

Figure 25: Weekly blood glucose levels of control and STZ animals, with and without Buchu treatment, for 12 weeks. 83

Figure 26: Intraperitoneal glucose tolerance test of control and STZ animals, with and without Buchu treatment, at 13 weeks. 88

Figure 27: Fasting serum insulin levels in control and STZ animals, with and without Buchu treatment, after 13 weeks. 89

Figure 28: Food consumed by control and DIO animals, from week 8. 91

Figure 29: Water/Buchu consumed by control and DIO animals, from week 8. 92

Figure 30: Body weight (BW) of control and DIO animals, with and without Buchu treatment, after 16 weeks. 93

Stellenbosch University https://scholar.sun.ac.za

Figure 31: Intra-peritoneal (IP) fat of control and DIO animals, with and without Buchu treatment, after 16 weeks. 94

Figure 32: Intraperitoneal glucose tolerance test of control and DIO animals, with and without Buchu treatment, after 15 weeks. 98

Figure 33: Glucose uptake by the soleus muscle of control and DIO animals, with and without Buchu treatment, after 16 weeks. 99

Figure 34: Fasting insulin levels in control and DIO animals after 16 weeks. 101

Figure 35: C-peptide levels in control and DIO animals after 16 weeks. 102

Figure 36: Total Pdx-1 expression in control and DIO groups after 16 weeks 104

Figure 37: Total MafA expression in control and DIO groups after 16 weeks. 105

Stellenbosch University https://scholar.sun.ac.za

# List of Tables

| | | |
|---|---|---|
| Table 1: | Digestive juices secreted by exocrine cells | 6 |
| Table 2: | Hormones secreted by the islets of Langerhans | 7 |
| Table 3: | List of anti-diabetic drugs | 46 |
| Table 4: | List of reagents for the lysis buffer | 62 |
| Table 5: | Western blot analysis | 67 |
| Table 6: | Composition of each tube in the glucose uptake protocol | 69 |
| Table 7: | A representation of calibrators | 71 |
| Table 8: | Microtiter plate arrangement | 73 |
| Table 9: | Diet composition of controls versus DIO animals | 90 |

Stellenbosch University https://scholar.sun.ac.za

# *Motivation for research*

South Africa is a country rich in floral biodiversity and cultural diversity [*Light et al., 2005*]. The Cape region of South Africa has veldt types with arguably the richest composition of indigenous aromatic plant species [*Moolla et al., 2007*]. Amongst these aromatic plants is the genus *Agathosma* [*Moolla et al., 2007*]*.* There are approximately 150 different species within the genus, which branches from a greater family called *Rutaceae [Moolla et al., 2007].* These rutacious shrubs are typical of the fynbos (vegetation found in the Western Cape province of South Africa) and are particularly abundant in the mountainous areas of the Cape [*Moolla et al., 2007*]. The term “Buchu” primarily originated from the Khoisan (indigenous people of the western region of South Africa) and was given to any fragrant plant that could be dried and powdered [*Moolla et al., 2007*]. This name includes a wide variety of aromatic plant species, not singling out a specific genus [*Moolla et al., 2007*]. The Khoisan tribes relied on these plant materials for many centuries to maintain and/or promote good health. In this era, herbal remedies formed the backbone of healthcare and was the central source of medication [*Light et al., 2005*]. Today, the term “Buchu” refers to *Agathosma Betulina* (round-leaf buchu; ‘bergboegoe’; short buchu) and *Agathosma Crenulata* (oval-leaf buchu; ‘anysboegoe’; long-leaf buchu) [*Moolla and Viljoen, 2008*].

Europeans were first introduced to “Buchu” in the 1650’s, upon their arrival in the Cape [*Moolla and Viljoen, 2008*]. The use of these plants then spread to Europe and America

Stellenbosch University https://scholar.sun.ac.za

where it was extensively used for medicinal purposes [*Moolla and Viljoen, 2008*]. Recently, more attention has been placed on the organoleptic properties of “Buchu” rather than on its impact on health, shifting interest towards oil extraction. Oils from these plants are obtained by means of hydro diffusion, a method called hydrodistillation. These oils are typically used in the perfume industry and as flavouring agents to enhance fruit flavours [*Moolla and Viljoen, 2008*]. Currently, Buchu products are virtually found in every store, ranging from teabags, water, lotions, tonics, ointments and even brandy. These products have been approved by the American Food and Drug Administration (FDA), rendering Buchu safe for consumption.

The people of South Africa are faced with many challenges on a daily basis, especially where healthcare is concerned. Illnesses such as diabetes and cardiovascular diseases is a major source of morbidity and mortality in populations that cannot afford western medication [*Light et al., 2005*]. Such people are left with no other option but to consult traditional healers and make use of herbal remedies [*Light et al., 2005*]. There are many concerns associated with the use of indigenous plants in medicine. One of these issues stem from the fact that a large portion of knowledge based on herbal usage is anecdotal, posing great concern [*Light et al., 2005*]. The claims that Buchu exerts anti-microbial, anti- inflammatory, anti-oxidant and diuretic effects have been successfully validated by studies reviewed by Moolla and Viljoen [*Moolla and Viljoen, 2008*]. Claims that Buchu has anti-diabetic effects are anecdotal and needs to be validated scientifically. To date, only a few studies have been published on the pharmacological actions of buchu, underscoring the urgency for research in this regard.

Stellenbosch University https://scholar.sun.ac.za

Our aim was therefore, on request of the producing company, to determine the anti-diabetic and insulin sensitizing potential of a watery extract of Agathosma tested in rat models of type 1 and type 2 diabetes.

Stellenbosch University https://scholar.sun.ac.za

## DISCLOSURE OF INTEREST

We hereby declare, as per contractual agreement between the University of Stellenbosch and Cape Kingdom Nutraceuticals (Pty) Ltd, (Contract number: S003211) the company licenced to distribute Buchu water, that there was no personal financial gain for the researchers in this work. The researchers only retained the right to publish these findings in peer reviewed scientific journals of their choice.

Signed on the …………1……………….. day of …………December…………. 2014

at………………Gordons Bay…………………………….

……………………………  …..………………………………………...

(Prof. B. Huisamen)  (Me V Jansen)

Stellenbosch University https://scholar.sun.ac.za

# *CHAPTER 1*
# *Literature review*

---

## *1.1. The Digestive System*

To maintain an optimal nutritional status, consumption of proteins, fats and carbohydrates are required.

Food entering the mouth is physically digested by the continuous grinding and chewing action of the teeth [*Raven and Johnson, 2001*]. In the oral cavity, salivary glands secrete mucus which aid in lubrication, provides protection against foreign invaders; bacteria, and moisten ingested food [*Fabian et al., 2012*]. Mucus is a liquid-like substance rich in amylase and immune cells [*Fabian et al., 2012*]. Amylase is an enzyme with a catalytic function to hydrolyse carbohydrates, converting starch to maltose [*Collins et al., 1993*]. After mastication, the digestive material in the mouth, known as the bolus, makes it way down the oesophagus towards the abdomen [*Matsuo and Palmer, 2008*]. This action is achieved via peristaltic wave-like movements of the oesophageal wall [*Matsuo and Palmer, 2008*]. Glands lining the abdominal wall secrete hydrochloric acid (HCl) and proteolytic enzymes, ensuring an acidic environment for proper protein denaturation. The material present in the abdomen after absorption is called chyme. Chyme makes its way to the intestine through the pyloric sphincter where it is further digested. The small intestine, specifically the duodenum, is the main site of

Stellenbosch University https://scholar.sun.ac.za

absorption. In the small intestine an enzyme called cholecystokinin (CCK) is secreted, preventing gastric emptying into the intestine and stimulating the release of digestive juices [*Powers and Pappas, 1989*]. This enzyme stimulates organs such as the liver and pancreas to assist with digestion by secreting bile and pancreatic juice, respectively. Bile travels from the liver to the duodenum via a common hepatic duct, where it emulsifies and degrades fat molecules. Pancreatic juice contains bicarbonate, in addition to other digestive enzymes which neutralizes the acidic chyme [*Fieker et al., 2011*]. Amylase secreted by the pancreas and intestine hydrolyzes the last traces of starch in chyme. Maltose gets broken down into glucose with the aid of the enzyme maltase. These glucose molecules are transported by the blood to the rest of the body, where they are used as energy. In the large intestine, no digestion takes place but water and ions are absorbed. Waste material exits the gastrointestinal tract via the rectum and is expelled by the anus. Figure 1 illustrates the digestion of starch only, as our main focus for this study is glucose metabolism.

Stellenbosch University https://scholar.sun.ac.za

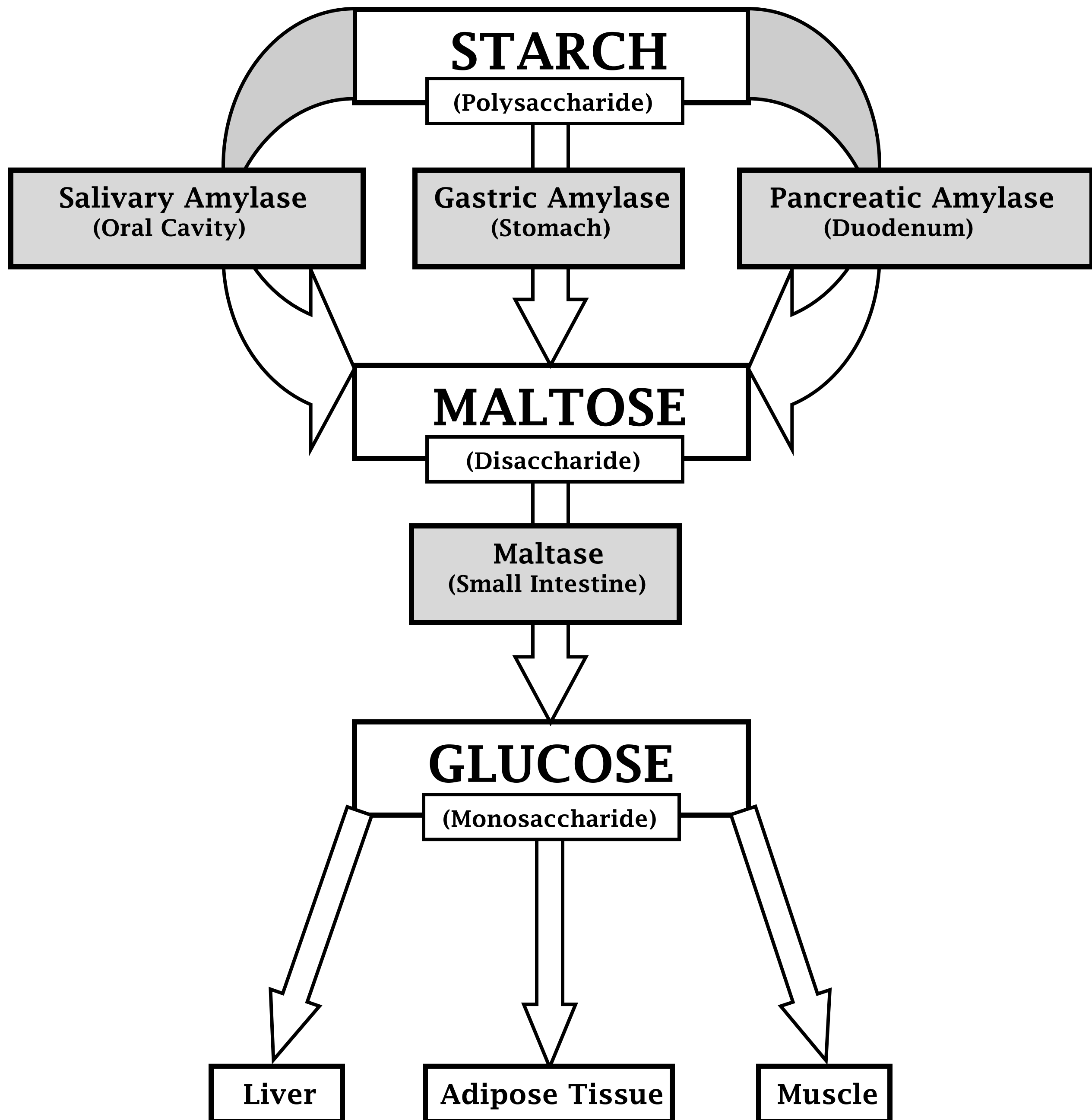



***Figure 1**: Breakdown of complex polysaccharides*

***Starch entering various compartments of the digestive system is broken down by specific enzymes, forming maltose. In the small intestine, maltase catalyzes the production of glucose from maltose. These glucose molecules are released into circulation and transported to a whole range of tissue to sustain life.***

Stellenbosch University https://scholar.sun.ac.za

### *1.2. Glucose transport across the intestinal epithelium*

Glucose is an important nutrient, playing a vital role in many biological processes within mammalian cells [*Shah et al., 2012*]. These processes include cellular homeostasis and metabolism. In order for glucose to execute its function it has to make its way across the lipid bilayer of the cell. This mode of transportation is achieved with the help of carrier proteins, namely, glucose transporters (GLUTs) and sodium-glucose cotransporters (SGLT) [*Bucci et al., 2011*]

After a meal, dietary glucose and sodium ($Na^+$) concentrations increase within the intestinal lumen. Epithelial cells lining the walls of the small intestine maintain homeostasis by transporting molecules and/or ions out of the cell. A low cytoplasmic $Na^+$ concentration is established in the enterocyte via active transport. The sodium-potassium ($Na^+/K^+$) ATPase pump, located on the basolateral membrane, lowers the cytoplasmic sodium concentration by actively transporting three sodium ions out of the enterocyte [*Srichamroen, 2007*]. This results in an ionic imbalance. To restore balance, two potassium ions found in blood vessels have to move against its concentration gradient into the cell with the help of adenosine triphosphate (ATP). The sodium-glucose co-transporter 1 (SGLT-1) allows $Na^+$ in the lumen to be transported into the enterocyte. Energy produced by sodium ions moving down their concentration gradient makes it possible for glucose to move against its concentration gradient [*Steel and Hediger, 1998; Srichamroen, 2007*]. This results in high levels of glucose accumulation within the cytosol. These glucose molecules move into circulation via facilitated diffusion to the necessary cells [*Steel and Hediger, 1998, Srichamroen, 2007*]. Below is a

Stellenbosch University https://scholar.sun.ac.za

diagrammatic figure depicting the movement of glucose from the intestine to its specific targets (figure 2).

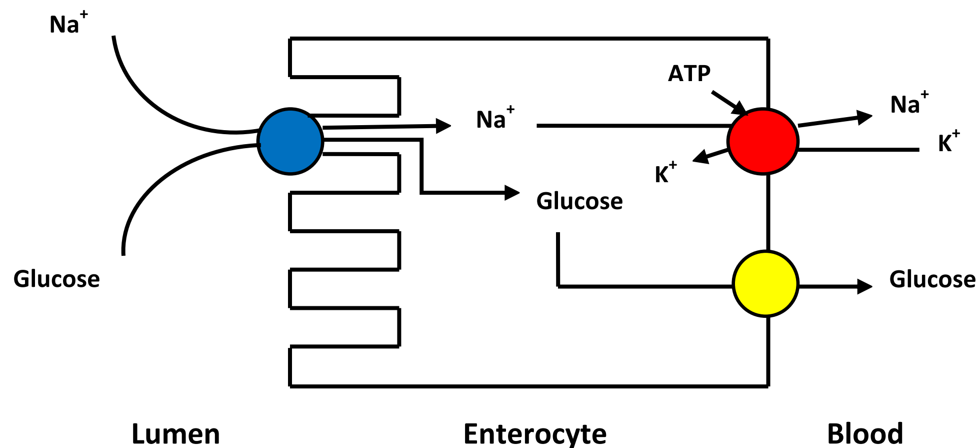



***Figure 2: Glucose transport across the enterocyte [Wright, 1998].***

*sodium-glucose co-transporter* *sodium-potassium ($Na^+/K^+$) ATPase pump*

*glucose transporter 2*

***The diagram is a reconstruction of Wright, 1998. The sodium-glucose co-transporter and the $Na^+/K^+$ ATPase pump allow sodium ions to be transported from the lumen across the enterocyte into circulation. The movement of sodium ions provide enough energy enabling the transport of glucose into the enterocyte which enter circulation via facilitated diffusion.***

## *1.3. The Pancreas*

The pancreas is a gland situated in the abdominal cavity, partially connected to the duodenum [*Torpy et al., 2012*]. This gland functions both as an exocrine organ, secreting digestive juice into the small intestine, and an endocrine organ, producing hormones which regulate metabolism [*Novak, 2008*].

Stellenbosch University https://scholar.sun.ac.za

### *1.3.1. Exocrine pancreas*

The exocrine portion of the pancreas is a major contributor to pancreatic mass. In these regions acinar cells are present [*Jiang et al., 2008*]. Acinar cells form glands which secrete various enzymes necessary for digestion [*Jiang et al., 2008, Logsdon and Ji, 2013*]. The journey of these enzymes to the duodenum is facilitated by pancreatic ducts [*Logsdon and Ji, 2013*]. Cells lining the duct secrete a watery, bicarbonate-rich fluid which flushes digestive enzymes into the small intestine [*Afroze et al., 2013*]. Intestinal enzymes break down proteins, carbohydrates and fats into their respective monomers; allowing effortless absorption.

***Table 1. Digestive juices secreted by exocrine cells***

| Digestive Enzyme | Function |
|---|---|
| Amylase | Digest starch |
| Chymotrypsin, Trypsin and Carboxypeptidase | Digest protein |
| Lipase | Digest Triglycerides |
| Cholesterol esterase | Digest cholesterol |
| Ribonuclease and deoxyribonuclease | Digest nucleic acid |

### *1.3.2. Endocrine pancreas*

Interacinar cells are irregularly shaped regions within the pancreas, known as the islets of Langerhans [*Steiner et al., 2010*]. The presence of these islets were first documented in 1869 by a German physician, Paul Langerhans [*Islam, 2009*]. A healthy adult pancreas contains 1 million islets which accommodate approximately 2% of the pancreas [*Leoni and Roman, 2010*]. The islets of Langerhans are composed of many cells, playing a pivotal role in regulating glucose metabolism.

Stellenbosch University https://scholar.sun.ac.za

***Table 2. Hormones secreted by the islets of Langerhans [Chen et al.,2012]***

| Endocrine cell | Hormone | Total islet cells (%) |
|---|---|---|
| Beta cell (β) | Insulin and Amylin | 65-80 |
| Alpha cell (α) | Glucagon | 15-20 |
| Delta cell (δ) | Somatostatin | 3-10 |
| Gamma cell (γ) | Pancreatic polypeptide | 3-5 |
| Epsilon cell (ε) | Ghrelin | <1 |

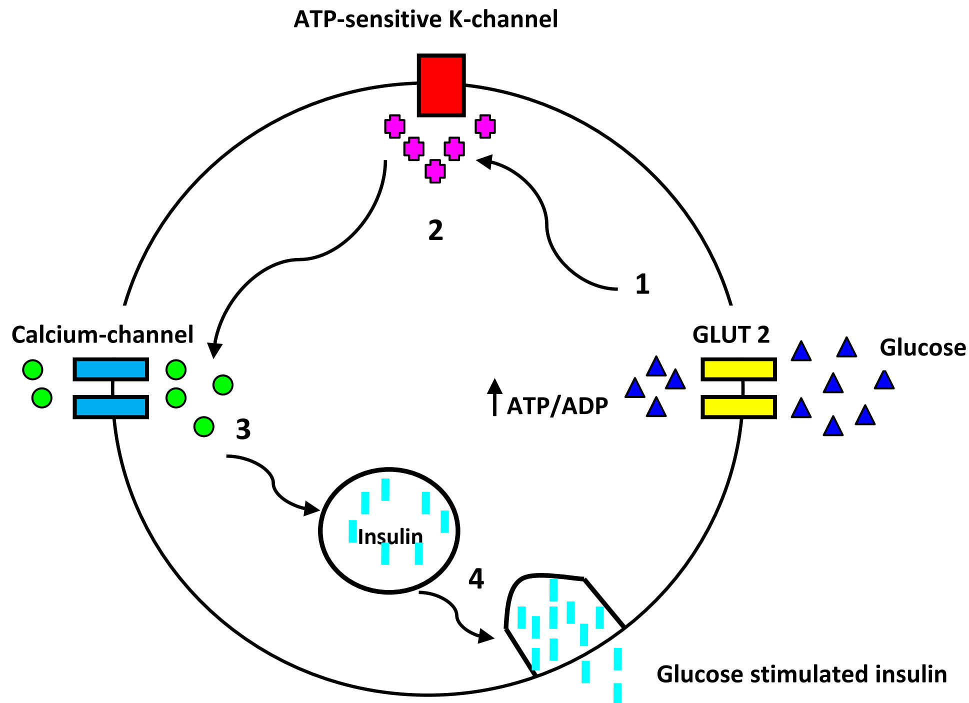
The α- and β- cells regulate blood glucose levels by secreting glucagon and insulin respectively [*Soria et al., 2010*]. These hormones have opposite effects, thus ensuring that fasting blood glucose levels remain within a normal range (4 to 8 mmol/L) [*Andrews et al., 1998*]. Glucagon, secreted by α-cells, increases the concentration of plasma glucose by stimulating glycogenolysis and gluconeogenesis by the liver. β-cells operate inversely, secreting insulin which stimulates glucose uptake by peripheral cells and enhances glycogenesis. Pancreatic β-cells will be discussed in more detail below.

### *1.4. Pancreatic response to hyperglycemia*

Pancreatic β-cells respond to hyperglycemia by increasing glucose metabolism and ATP production [*Layden et al., 2010*]. This, in turn, increases the cytoplasmic ATP/ADP ratio, resulting in the closure of potassium channels [*Layden et al., 2010*]. These cells undergo depolarization causing voltage-gated calcium channels to open, allowing movement of extracellular calcium into the cell [*Layden et al., 2010*]. Calcium acts as an intracellular signal, stimulating exocytosis of insulin-containing vesicles and insulin

Stellenbosch University https://scholar.sun.ac.za

secretion into circulation [*Li et al., 2007*]. The secreted insulin is stored in mature vesicles that are readily available due to prior synthesis.




***<u>Figure 3: Insulin release by pancreatic β-cells [Layden et al., 2010]</u>***

***The diagram is a reconstruction of Layden, 2010. Step 1, when β-cells are exposed to hyperglycemia they adapt by increasing metabolism. Step 2, an increase in metabolism causes ATP levels to rise, triggering the closure of potassium channels. Step 3, β-cells depolarize and initiate the opening of calcium channels. Step 4, calcium accumulates in the cell, stimulating insulin secretion.***

Stellenbosch University https://scholar.sun.ac.za

## *1.5. The link between insulin action and diabetes mellitus*

### *1.5.1. Insulin biosynthesis*

When β-cells are exposed to hyperglycemia, certain pathways such as the IRS-2/PI-3 kinase/p70 s6k and CaM kinase pathway are activated. These kinases phosphorylate transcription factors involved in insulin transcription. Transcription factors interact with the promoter region of the insulin gene, transcribing mRNA. Insulin mRNA is translated to preproinsulin in the rough endoplasmic reticulum (ER) [*Hartley et al., 2009*]. A signalling peptide, attached to preproinsulin, directs movement into the lumen of the rough ER. Enzymes inside the lumen cleave the signalling peptide, converting preproinsulin to proinsulin. Three disulfide bonds hold proinsulin intact after folding [*Yang et al, 2010*]. Shuttle vesicles transport proinsulin from the ER to the golgi apparatus [*Hartley et al., 2009*]. In the golgi, proinsulin is packaged into immature secretory vesicles containing endoprotease and carboxypeptidase E [*Robinson, 2013*]. These enzymes cleave proinsulin, producing insulin and c peptides which are released during exocytosis [*Halban and Irminger, 2003*]. Insulin entering circulation binds to its target cell, activating an entire host of signalling cascades which stimulate glucose uptake, lipogenesis, protein synthesis and glycogenesis. All these processes will be discussed within the section “insulin signalling pathway in skeletal muscle” of this review.

Stellenbosch University  https://scholar.sun.ac.za

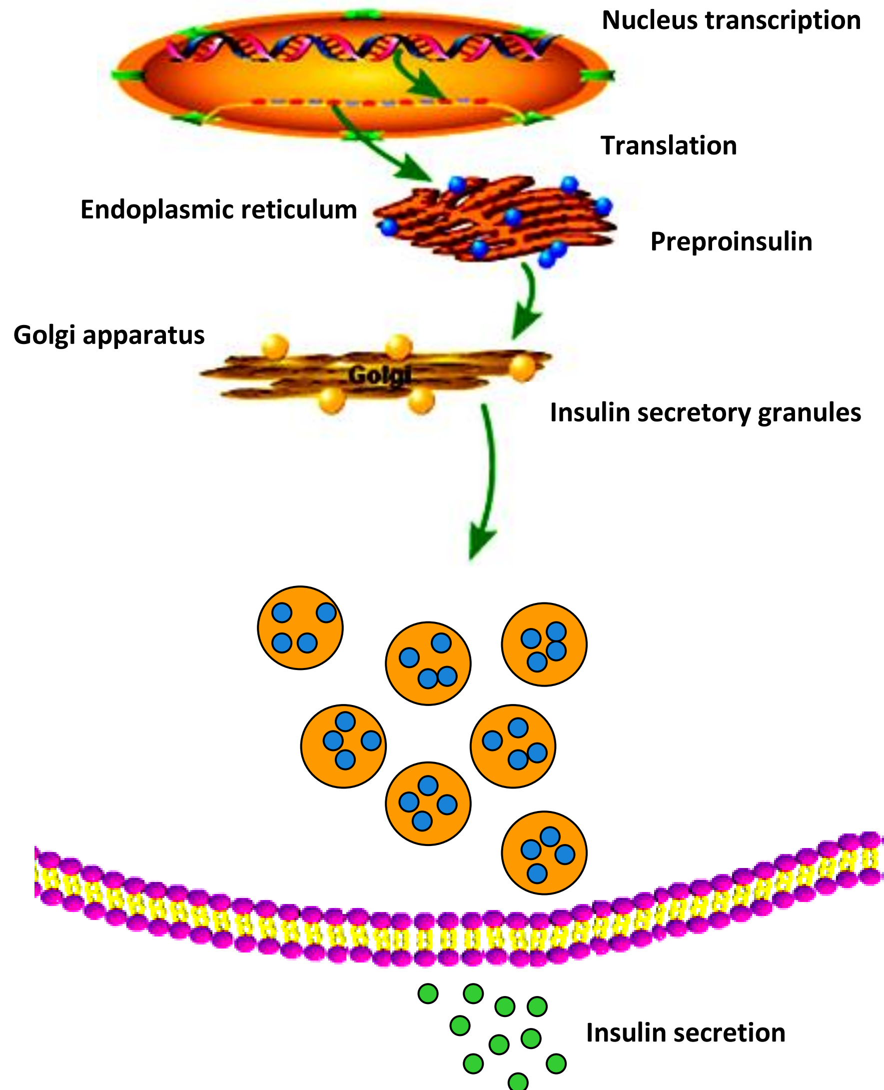



***Figure 4: Insulin synthesis and secretion [Ren et al., 2007]***

***The diagram is a reconstruction of Ren, 2007. Hyperglycemia stimulates the transcription of insulin mRNA which is translated to preproinsulin on the endoplasmic reticulum. Preproinsulin undergoes various cleavage reactions ultimately producing and releasing insulin via exocytosis***

Stellenbosch University https://scholar.sun.ac.za

### *1.5.2. Insulin action in regulating glucose homeostasis*

Insulin is the most potent anabolic hormone, secreted primarily in response to hyperglycemia [*Satiel and Pessin, 2002*]. This hormone signals a cascade of events which promote glucose uptake, glycogenesis, lipogenesis, and protein synthesis; while inhibiting pathways and proteins involved in glucose production. Binding of insulin to the insulin receptor initiates a conformational change that elicits autophoshorylation and activates enzyme activity. The insulin receptor is a tetrameric protein which consists of two α subunits that protrude from the cell membrane and two transmembrane β subunits connected by disulphide bonds [*Dominici et al., 2005*]. Each β-subunit comprises a carboxyl terminus with three tyrosine residues located in the cytoplasmic region. Once phosphorylated, tyrosine motifs recruit src homology 2 (SH2) or phosphotyrosine binding (PTB) domain containing adapter proteins [*Cipok et al., 2006; Kim and Sang, 2007*].

Insulin receptor substrate (IRS), a PTB protein, binds to the insulin receptor and provides docking sites for downstream SH2 proteins. IRS plays a central role in all cells, linking receptor tyrosine kinases to, amongst others, the PI-3K/PKB pathway [*Guo et al., 2006*]. Tyrosine phosphorylated IRS recruits, binds and activates the regulatory subunit (p85) of the heterodimeric phosphatidylinositol 3-kinase (PI-3K) protein, relieving the inhibitory effect of the p110 catalytic subunit [*Fröjdö et al., 2009; Das et al, 2013*]. PI-3K can also bind directly to a phosphorylated tyrosine kinase receptor. P1-3K activation results in the migration of the protein to the cell membrane. At the cell membrane PI-3K interacts with phosphatidylinositol (PtdIns) 4, 5-bisphosphate (PIP2), catalyzing the

Stellenbosch University https://scholar.sun.ac.za

phosphorylation of PIP2 to PtdIns 3, 4, 5-trisphosphate (PIP3) [*Satiel and Pessin, 2002*]. PIP3 has a high affinity for intracellular proteins containing a pleckstrin homology (PH) domain [*Das et al, 2013*]. These phospholipids recruit phosphoinositide dependent protein kinase 1 (PDK1), PDK2 and serine/threonine protein kinase B (PKB) to the intracellular membrane where they interact and activate one another [*Fröjdö et al., 2009*]. PIP3 is able to prime PKB for phosphorylation by initiating a conformational change which allows easy accessibility to threonine 308 (Thr 308) residues. At the cytoplasmic membrane PDK2 phosphorylates PKB on its serine 473 (Ser 473) site subsequently increasing its affinity for PDK1, phosphorylating the Thr 308 residue of PKB. Full activation of PKB requires dual phosphorylation on both Thr 308 and Ser 473 sites. Once the PI3K/PKB pathway is activated, varied downstream kinases and genes that regulate glucose homeostasis are modified.

Stellenbosch University https://scholar.sun.ac.za

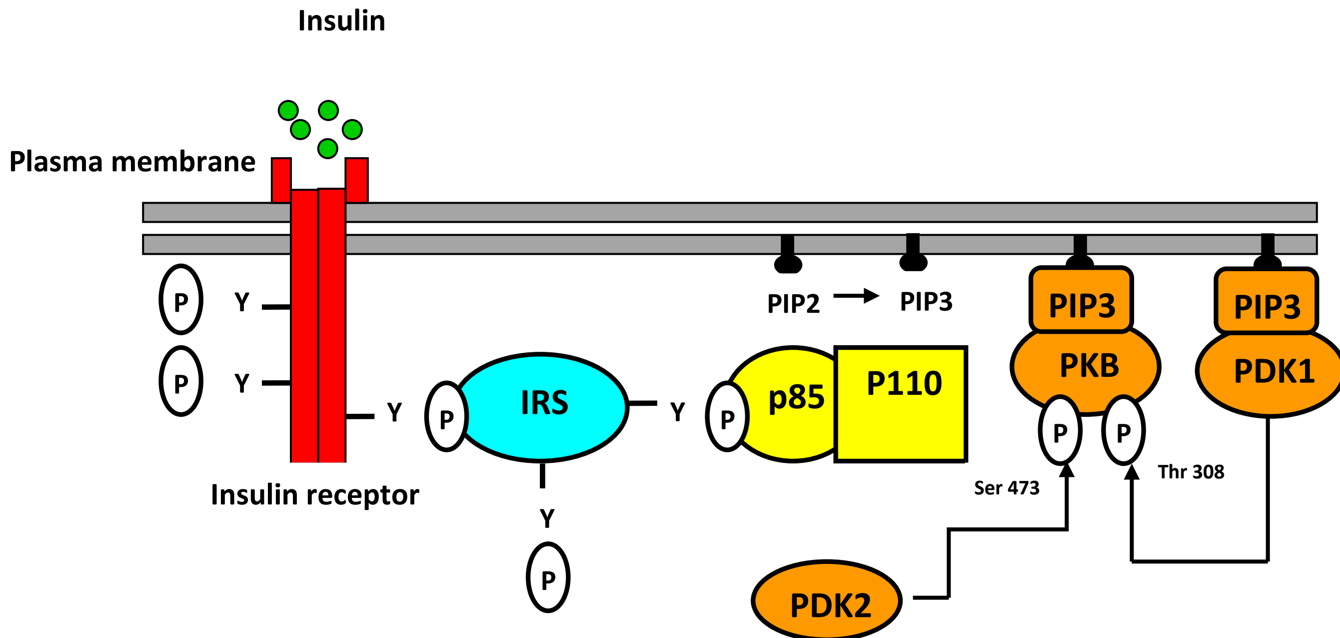



***Figure 5: PKB/Akt activation pathway [Turban and Hajduch, 2011]***

***The diagram is a reconstruction of Turban and Haiduch, 2011. Binding of insulin to the insulin receptor triggers autophosphorylation, resulting in the phosphorylation and activation of an insulin receptor substrate protein, and phosphatidylinositol 3-kinase (PI-3K). The conversion of phosphatidylinositol (PtdIns) 4, 5-bisphosphate (PIP2) to PtdIns 3, 4, 5-trisphosphate (PIP3) is catalyzed by PI-3K. PIP3 recruits protein kinase B (PKB) to the plasma membrane where it is activated by phosphoinositide dependent protein kinase 1 (PDK1) and phosphoinositide dependent protein kinase 2 (PDK2).***

### *1.5.3. Insulin signalling pathway in skeletal muscle*

In skeletal muscle, activated PKB/Akt phosphorylates various proteins capable of lowering blood glucose levels by increasing glucose uptake, glucose oxidation and glucose storage. These cells combat hyperglycemia via the activation of numerous processes which will be discussed below.

Stellenbosch University https://scholar.sun.ac.za

#### *1.5.3.1. Glycolysis and Glycogenesis*

Active PKB/Akt promotes the translocation of glucose transporter 4 (GLUT 4) to the plasma membrane, stimulating glucose uptake. The mechanical procedure of GLUT 4 translocation will be discussed in the section "GLUT 4 translocation to the plasma membrane". Glucose is a monosaccharide with many fates which are essential for sustaining life, e.g. it can either undergo glycolysis, for adenosine triphosphate (ATP) production, or be stored as a reserve fuel. The term glycolysis is derived from two Greek words, 'Glykys' meaning sweet and 'lysis' referring to the ability to split or break. Glycolysis is a series of metabolic processes which provides a quick burst of energy by degrading one molecule of glucose [*Pelicano et al., 2006*]. An in depth review on the glycolytic pathway, will be given in the section "Skeletal muscle metabolism". Excess glucose in skeletal muscle is stored as glycogen. PKB/Akt phosphorylates glycogen synthase kinase 3 (GSK-3), alleviating its inhibitory effect on glycogen synthase (GS) thus promoting glycogen synthesis.

#### *1.5.3.2 GLUT 4 translocation to the plasma membrane*

In the presence of insulin, glucose enters the cell by facilitated diffusion, a passive process which requires no energy [*McCarthy and Elmendorf, 2007*]. The proteins responsible for glucose movement into the skeletal muscle and adipose tissue are glucose transporters, GLUT 1 and GLUT 4 [*McCarthy and Elmendorf, 2007*]. GLUT 1 is responsible for basal glucose uptake, and GLUT 4 for insulin-stimulated glucose uptake. These 12 membrane spanning transporters play a crucial role in maintaining blood glucose homeostasis [*McCarthy and Elmendorf, 2007*]. In the absence of insulin

Stellenbosch University https://scholar.sun.ac.za

stimulation, GLUT 4 continuously circulates between the plasma membrane and intracellular storage vesicles [*McCarthy and Elmendorf, 2007*]. More than 90% of GLUT 4 is localized within endosomal recycling compartments, while only a few are found at the cell surface [*Thong et al., 2005*]. Insulin shifts the movement of GLUT 4 containing vesicles towards the plasma membrane by increasing the rate of exocytosis [*Thong et al., 2005*]. The translocation of GLUT 4 is governed by two pathways, PI-3K dependent pathway and PI-3K independent pathway, which will be discussed in more detail.

#### 1.5.3.2.1. ***PI-3K dependent pathway***

Signalling events that set this pathway into motion are initiated by the binding of insulin to its receptor triggering a cascade of phosphorylation events [*Chang et al., 2002; McCarthy and Elmendorf, 2007*]. Tyrosine phosphorylated IRS catalyzes the activation of PI-3K, which in turn, leads to PIP3 generation [*McCarthy and Elmendorf, 2007*]. These phospholipids recruit pleckstrin homology (PH) domain containing proteins to the membrane where they are activated [*Smith et al., 2011*]. Both PKB/Akt and atypical protein kinase c (aPKC) are phosphorylated by PDK1 on Thr 308 and Thr 410 residues, respectively [*Thong et al., 2005*]. The precise mechanistic action of aPKC is not clear, however, it appears to have effects on both intracellular and surface target events of GLUT 4 trafficking [*Thong et al., 2005*]. There are many other proteins involved in GLUT 4 trafficking, docking and fusion such as Rab GTPase activity proteins, e.g. As160. Basally, As160 associates with the GLUT 4 vesicles, hydrolyzing Rab GTP to RabGDP [*McCarthy and Elmendorf, 2007*]. This inactive form of Rab prevents GLUT 4 translocation to the plasma membrane [*McCarthy and Elmendorf, 2007*]. Upon insulin

stimulation, PKB/Akt phosphorylates As160 on specific serine (Ser 588) and tyrosine (Tyr 642) residues, causing it to dissociate from the vesicle [*Smith et al., 2011*]. Phosphorylated As160 recruits and binds to an accessory protein, 14-3-3, which inhibits GAP activity towards Rab [*McCarthy and Elmendorf, 2007*]. The equilibrium shifts towards a more active GTP loaded Rab, promoting GLUT 4 trafficking [*Smith et al., 2011*]. Several studies have shown that PI-3K activation is necessary but not sufficient for insulin stimulated GLUT 4 translocation, thus shifting attention toward PI-3K independent pathways [*Chiang et al., 2001*].

#### 1.5.3.2.2. *PI-3K Independent pathway (CAP-Cbl-TC10 pathway)*

Activation of one PI-3K independent pathway depends on tyrosine phosphorylation of a proto-oncogene. Upon insulin stimulation, insulin receptors are autophosphorylated recruiting adaptor proteins, APS (adapter containing pleckstrin homology domain and Src homology (SH) 2 domain) and CAP (Cbl associated protein), to the plasma membrane [*Chang et al., 2002*]. CAP phosphorylates tyrosine residues on Cbl by interacting with proline-rich domains [*Chang et al., 2002*]. This triggers the migration of the dimeric CAP-Cbl complex towards caveolin enriched lipid rafts [*Liu et al., 2002*]. The process of migration is facilitated through the association of an amino-terminal SH2 domain on CAP with the lipid raft protein, flotillin [*Chang et al., 2002; Chiang et al., 2001*]. Tyrosine phosphorylated Cbl recruits an adaptor protein, CrkII, along with C3G towards the lipid raft domain on the plasma membrane [*McCarthy and Elmendorf, 2007*]. This occurs in two stages; firstly Cbl binds to the SH2 domain of CrkII and secondly the SH3 domain of CrkII interacts with the proline domain of C3G, forming the

Stellenbosch University https://scholar.sun.ac.za

CrkII-C3G complex [*Liu et al., 2002; Chiang et al., 2001*]. Once translocated, the CrkII-C3G complex brings C3G into close proximity with the Rho family protein, TC10, residing in the raft domains of the membrane [*Chang et al., 2002; Chiang et al., 2001*]. This interaction catalyzes the hydrolysis of GTP to GDP, rendering TC10 active [*Chiang et al., 2001*]. The activation of TC10 plays a critical role in the regulation of GLUT 4 translocation, docking and fusion with the plasma membrane [*Chiang et al., 2001*]. Varies studies have showed that TC10 interferes with actin dynamics and cytoskeletal arrangement, facilitating exocytosis of GLUT 4 [*Thong et al., 2005; Chiang et al., 2001*].

#### 1.5.3.2.3 *PI-3K Independent pathway (AMPK pathway)*

For many years living organisms persevered by mechanistically adapting to nutritional and environmental stressors. In this section of the review, compensatory adjustments in response to contraction/exercise, will be discussed. When skeletal muscles are exposed to exercise, an intramuscular energy change occurs initiating numerous metabolic responses to restore energy balance [*Winder, 2001*]. One of the ways in which these cells counteract high-energy phosphate depletion as a result of exercise, is by alerting the AMP-activated protein kinase (AMPK) low fuel warning system [*Winder, 2001; Nielsen et al., 2003*] . This system operates by switching off ATP utilizing processes, such as biosynthetic pathways while switching on ATP-generating pathways that include fatty acid oxidation and glucose uptake [*Hardie, 2004; Russell et al., 1999; Luiken et al., 2003; Kudo et al., 1995; Fryer et al., 2000*]. AMPK is a heterotrimer that is ubiquitously expressed in cells [*Nielsen et al., 2003*]. These proteins consist of a catalytic alpha subunit containing the kinase domain and two regulatory subunits termed

Stellenbosch University https://scholar.sun.ac.za

beta and gamma [*Sakamoto et al., 2005*]. All these subunits are necessary for the complete activation of AMPK [*Winder, 2001*]. The degree of activation depends on muscle glycogen concentration, pre-exercise diet, exercise duration, exercise intensity and training status [*Nielsen et al., 2003; Winder, 2001; Wojtaszewski et al, 2003*]. During muscle contraction, AMPK becomes allosterically activated by an increase in the AMP:ATP and creatine:phosphocreatine ratio [*Wojtaszewski et al, 2003; Hayashi et al., 1998; Ponticos et al., 1998; Hardie and Carling,1997*]. Once activated, AMPK stimulates GLUT 4 translocation to the plasma membrane by phosphorylating As160, similar to the pathway activated by PKB/Akt, thereby increasing glucose transport [*Nielsen et al., 2003*]. An increase in the level of glucose molecules within the cell and the activation of catabolic processes result in ATP regeneration [*Sakamoto et al., 2005; Hayashi et al., 1998; Fryer et al., 2000; Hardie and Carling,1997*]. Figure 6 summaries the regulation and activation of AMPK.

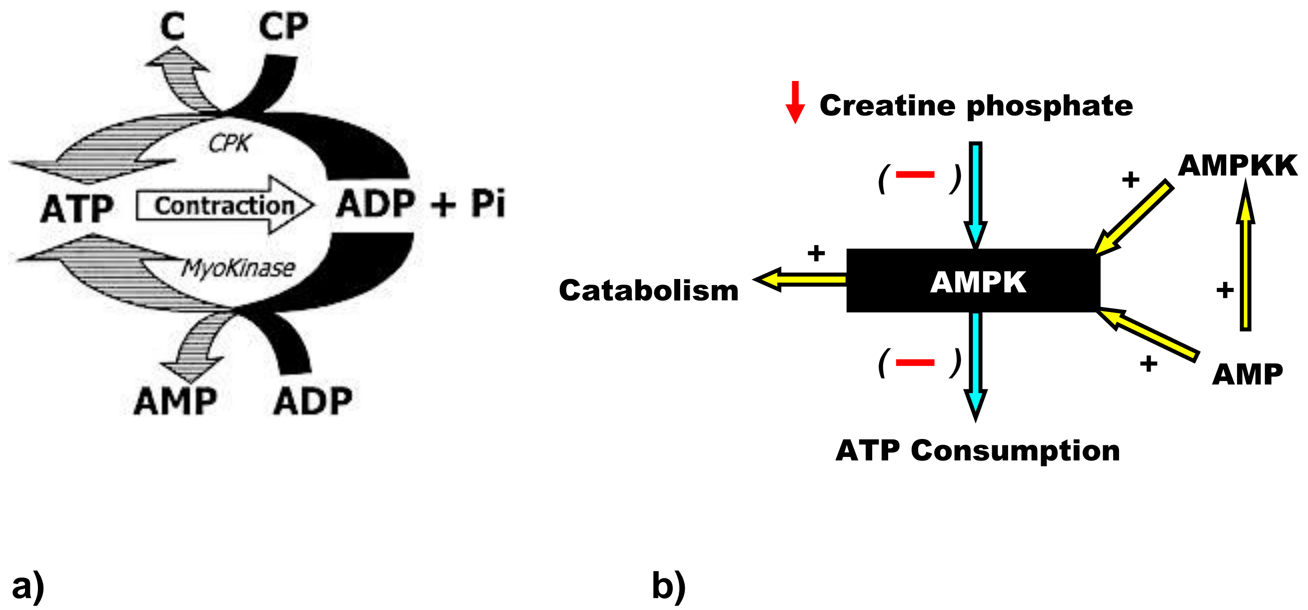



***Figure 6: Regulation and activation of AMPK [Winder, 2001]***

***(— ): Inhibit   +: Stimulate   ↓: Decrease***

***(a) Myokinase and creatine phosphokinase (CPK) are two enzymes that maintain physiologically normal ATP levels in skeletal muscle in response to contraction. During exercise, Myokinase increases the AMP concentration within the muscle by transferring a phosphate from one ADP to another ADP molecule. CPK stimulates the transfer of a phosphate from CP to ADP, forming ATP. (b)[a graphical modification of Winder, 2001] As a result, high levels of AMP can either stimulate AMPK activity directly or indirectly via AMPKK. A decrease in the concentration of creatine phosphate removes/relieves its inhibitory effects on AMPK activation.***

### 1.5.4. *Regulators of the insulin pathway*

The P1-3K pathway plays a critical role in regulating a wide variety of biological processes through the generation of a potent second messenger, PIP3 [*Sly et al., 2003*]. These processes include cell proliferation, growth, differentiation and survival [*Kalesnikoff et al., 2002*]. PIP3 recruits and activates many PH domain containing

Stellenbosch University  https://scholar.sun.ac.za

proteins, initiating a cascade of events necessary for cell survival [*Conďe et al., 2011*]. Cellular PIP3 levels need to be tightly regulated to prevent abnormal cell proliferation and impaired glucose metabolism [*Conďe et al., 2011; Kennah et al., 2009*]. These levels are kept under control by lipid phosphatases, namely phosphate and tensin homolog deleted on chromosome 10 (PTEN) and Src homology 2 containing inositol-5-phosphatase (SHIP) [*Chow and Baker, 2006*]. Both of these phosphatases modulate the PI-3K pathway by dephosphorylating PIP3, thus downregulating PKB/Akt activation.

#### 1.5.4.1. *PTEN*

PTEN was first identified in 1997; and has a large region of homology to chicken tensin, bovine auxilin, and a protein tyrosine phosphatase domain [*Waite and Eng, 2002*]. This 54 KDa lipid phosphatase consists of three structural domains: a C2, amino-terminal and a tail domain [*Parsons, 2004*]. The amino-terminal domain contains a phosphatase catalytic region composed of β sheets surrounded by alpha helices [*Waite and Eng, 2002].* This catalytic region has an enlarged active site that allows phosphoinositide substrates to enter the site with ease [*Simpson and Parsons, 2001*]. The width and depth of the active site is consistent with PTENs ability to dephosphorylate lipid substrates [*He et al, 2010*]. The C2 domain is required for efficient binding of PTEN to the membrane [*Parsons, 2004; Waite and Eng, 2002*].  Studies have shown PTEN to have a higher affinity for membrane bound phospholipids, 'in vitro'. The tail domain contains a PDZ (Threonine, Lysine, Valine) -binding motif and a PEST (Proline, Glutamic acid, Serine, and Threonine) motif responsible for protein-protein interactions and PTEN stability, respectively [*Waite and Eng, 2002*]. PTEN appears to be

Stellenbosch University https://scholar.sun.ac.za

ubiquitously expressed within cells and its activity depends on PIP3 levels and/or post-translational modification [*Gericke et al., 2006; Chow and Baker, 2006*]. The phosphatase activity of PTEN is down regulated by modifying it in two ways, either via phosphorylation of the tail domain or oxidation of the catalytic domain [*Wang and Jiang, 2008*]. Casein kinase 2 (CK2) is considered to be responsible for a large portion of PTEN phosphorylation [*Gericke et al., 2006*]. Torres and Pulido (2001) demonstrated that CK2 phosphorylates PTEN on serine (Ser370, Ser380 and Ser 385) and threonine (Thr383) residues, 'in vivo' [*Waite and Eng, 2002*]. Phosphorylation of the tail domain causes a conformational change which masks the PDZ domain and enhances PTEN stability [*Waite and Eng, 2002; Gericke et al., 2006*]. It is widely accepted that phosphorylation of PTEN is required for its inactivation to maintain PTEN cytosolic levels, prevent proteasomal degradation and slow PTENs interaction with phospholipids [*Wang and Jiang, 2008; Waite and Eng, 2002*]. GSK-3 has been reported to phosphorylate the tail domain of PTEN on Ser 362 and Thr 366 residues [*Wang and Jiang, 2008*].

PTEN can be inactivated through oxidation which is induced by reactive oxygen species (ROS). Insulin triggers a complex signalling cascade which enhances the production of ROS [*Espinosa et al., 2009*]. Insulin stimulated ROS, catalyzed by NADPH oxidase, oxidizes cysteine (Cys 71 and Cys 124) residues in the catalytic domain of PTEN [*Wang and Jiang, 2008; Espinosa et al., 2009*]. This oxidative process inhibits PTEN and allows PIP3 levels to rise, rendering PKB/Akt active. PTEN knockout mice are embryonically lethal whereas heterozygotes develop a variety of cancers [*Sly et al.,*

Stellenbosch University https://scholar.sun.ac.za

*2003*]. Overexpression of PTEN induces cell cycle arrest and results in a metabolic switch from aerobic glycolysis to anaerobic pathways. This protects cells from oncogenic transformation, thus promoting a healthier metabolism [*Simpson and Parsons, 2001; Braccini et al., 2012*].

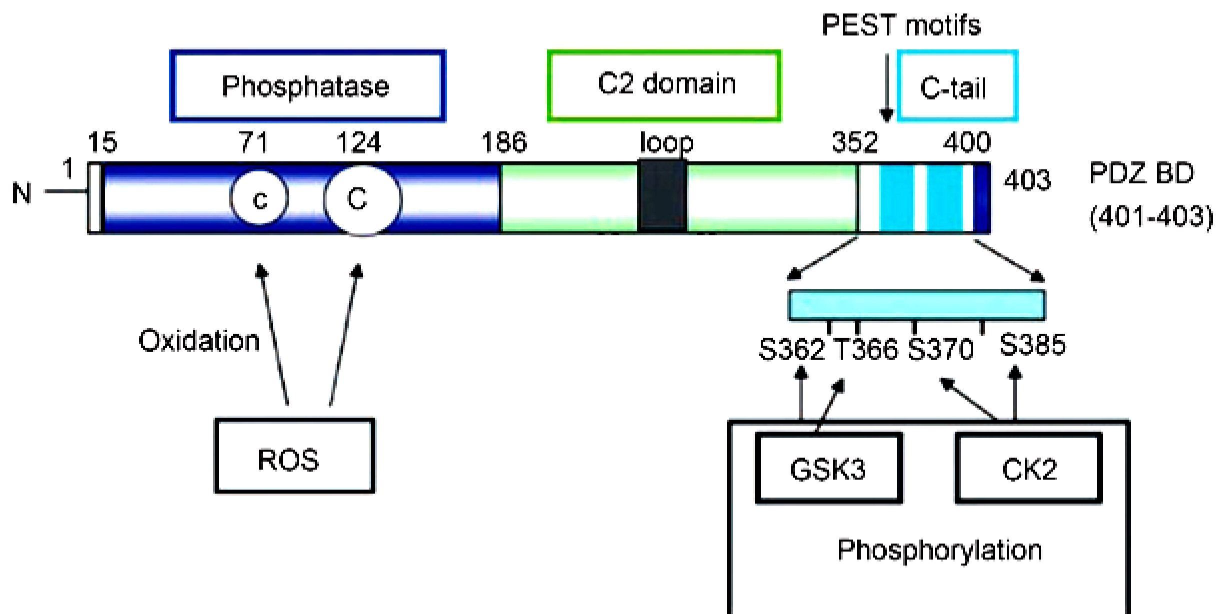



***Figure 7: PTEN structure [Wang and Jiang, 2008]***

***Phosphate and tensin homolog deleted on chromosome 10 (PTEN) consists of three major structural domains: an amino-terminal (phosphatase domain), a C2 domain and a tail domain. PTEN is regulated by glycogen synthase kinase 3 (GSK3) and casein kinase 2 (CK2) via the phosphorylation of specific serine and threonine residues. Other factors such as reactive oxygen species can also regulate PTEN activity by oxidizing cysteine residues.***

Stellenbosch University https://scholar.sun.ac.za

#### 1.5.4.2. *SHIP*

The SHIP family of phosphatases includes two members, SHIP-1 and SHIP-2, which are isoforms of each other [*Braccini et al., 2012*]. The expression of SHIP-1 phosphatase is restricted to hematopoietic cells while SHIP-2 is ubiquitously expressed in the brain and skeletal muscle [*Braccini et al., 2012; Kennah et al., 2009; Conďe et al., 2011*]. The SHIP-2 isoform contains an amino terminal, a central catalytic domain and a C-terminus [*Conďe et al., 2011*]. The amino-terminal SH2 domain and the catalytic domain is essential for binding proteins which consist of phosphorylated tyrosine residues and molecules exerting phosphatase activity on PIP3, respectively. The C-terminus is made up of a proline rich region and a SAM (sterile alpha motif) domain, which are important for SHIPs translocation to the plasma membrane [*Sly et al., 2003*]. SHIP-2 is an important regulator of the PI-3K pathway especially in β-cells.

A study done by Laura and colleagues showed a complete lack of SHIP-2 in mice to be linked to prenatal death and hypoglycaemia [*Sly et al., 2003*]. Various studies on SHIP-2 heterozygous mice have demonstrated enhanced insulin sensitivity, upon insulin stimulation [*Conďe et al., 2011*]. These mice exhibited higher levels of GLUT 4 translocation and glycogen synthesis when compared to normal control mice [*Conďe et al., 2011*].

A balance between the activation and inhibition of lipid phosphatases plays a critical role in glucose homeostasis and cancer development [*Conďe et al., 2011*]. It has been reported that phosphatases have a negative impact on insulin resistance associated with obesity [*Gupta and Dey, 2009*]. Insulin resistance appears to be linked to an

Stellenbosch University https://scholar.sun.ac.za

increase in phosphatase expression and/or activity in skeletal muscle and adipose tissue [*Sly et al., 2003*]. Phosphatases are involved in many biological processes, thus phosphatase inhibition offers many challenges.

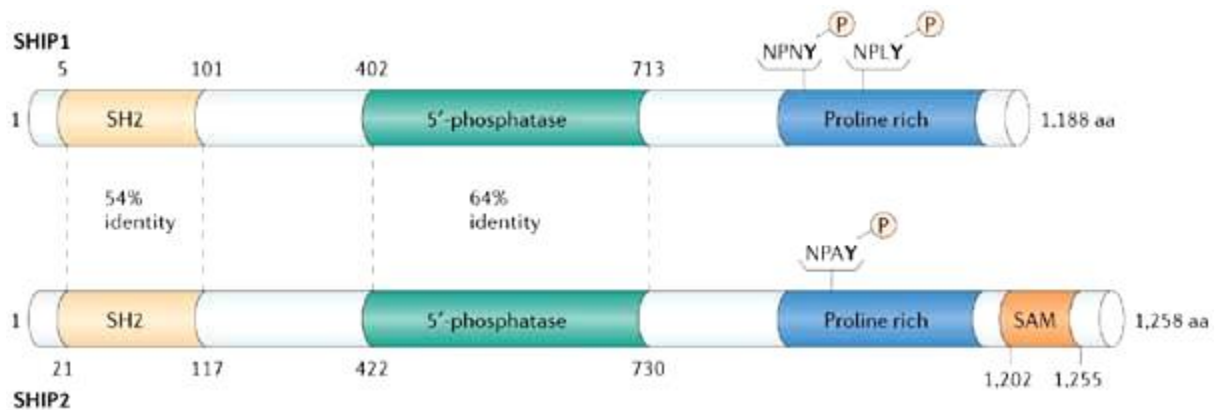



***Figure 8: The difference between the SHIP structure [Lazar and Saltiel, 2006]***

***The Src homology 2 containing inositol-5-phosphatase 1 (SHIP 1) and Src homology 2 containing inositol-5-phosphatase 2 (SHIP 2) is the presence of a SAM domain. SHIP 2 consists of an amino-terminal SH2 domain, a central catalytic domain and a C-terminus. The C-terminus is made up of a proline rich region and a SAM domain, which are important for SHIPs translocation to the plasma membrane***

### *1.5.5. Role of insulin in the development of diabetes*

The presence of plasma insulin in response to a meal or high blood glucose levels is crucial in maintaining glucose homeostasis. A defect in insulin secretion or insulin action would have adverse effects on metabolism as well as on an individual's quality of life.

Stellenbosch University https://scholar.sun.ac.za

Diabetes mellitus is a disease which manifests as a result of an impairment in insulin response by peripheral tissue or an impairment of pancreatic insulin secretion.

### 1.6. *Diabetes Mellitus*

The World Health Organization estimated that 347 million people worldwide have diabetes mellitus, with approximately 80% of diabetic deaths occurring in low and middle income countries [*Mathers and Loncar, 2006; WHO, 2014*]. The earliest descriptive report of diabetes dates back to 1550 BC [*Moitra and Meiler, 2006*]. In this era, a physician, Mesy Ra, documented frequent urination (polyuria) on Egyptian papyrus, a characteristic symptom of the diabetic condition [*Moitra and Meiler, 2006*]. According to World Health Organisation (WHO), diabetes will be the 7$^{th}$ leading cause of death by the year 2030. Diabetes mellitus is a chronic disease associated with many complications and occur in individuals' of all ages. Diabetes is characterized by hyperglycemia which originates as a result of pancreatic β-cells not being able to produce sufficient insulin or the inability of target tissues to respond to insulin action. There are three main types of diabetes: Type 1, Type 2 and gestational diabetes. Diabetic individuals require lifelong management as there is as yet no cure for diabetes, only treatment options.

Stellenbosch University https://scholar.sun.ac.za

### 1.6.1 ***Type 1 Diabetes***

Type 1 Diabetes (T1D) is an organ specific autoimmune disease caused by the immunological destruction of insulin producing pancreatic β-cells [*Atkinson and Elsenbarth, 2001; Narendran et al., 2003; Atkinson and Elsenbarth, 2001*]. T1D was formerly known as juvenile onset or insulin dependent diabetes mellitus, a term which is no longer used [*Atkinson and Elsenbarth, 2001*]. The American Diabetes Association (ADA) eliminated these terms based on the fact that not all T1D individuals are children or insulin dependent at onset [*Atkinson and Elsenbarth, 2001*]. T1D is a disorder of all ages with peak expression in children between the ages of 10-14 years [*Atkinson and Elsenbarth, 2001*]. It should be emphasized that patients with T1D are at an increased risk for developing other autoimmune diseases such as Graves' disease, Addison's disease and hypothyroidism [*Atkinson and Elsenbarth, 2001*]. An interplay between genetic susceptibility and environmental factors trigger the initial stages of pre-diabetes, followed by insulitus [*Barker, 2008*]. The progression of pre-diabetes to overt diabetes is characterised by a loss of the first phase insulin response to intravenous glucose, glucose intolerance and loss of serum C-peptides [*Thrower and Bingley, 2010*]. Upon the development of overt diabetes the total mass of working β-cells is approximately 10-20%, corresponding with the reduced ability of the pancreas to produce sufficient insulin to maintain normoglycemia [*Barker, 2008*]. Once this point has been reached, insulin administration should be adhered to for life to reinforce a patient's survival [*Thrower and Bingley, 2010*]. Insulin treatment serves to be very beneficial by lowering blood glucose levels and stimulating residual β-cells, to regain functionality [*Barker, 2008*].

Stellenbosch University https://scholar.sun.ac.za

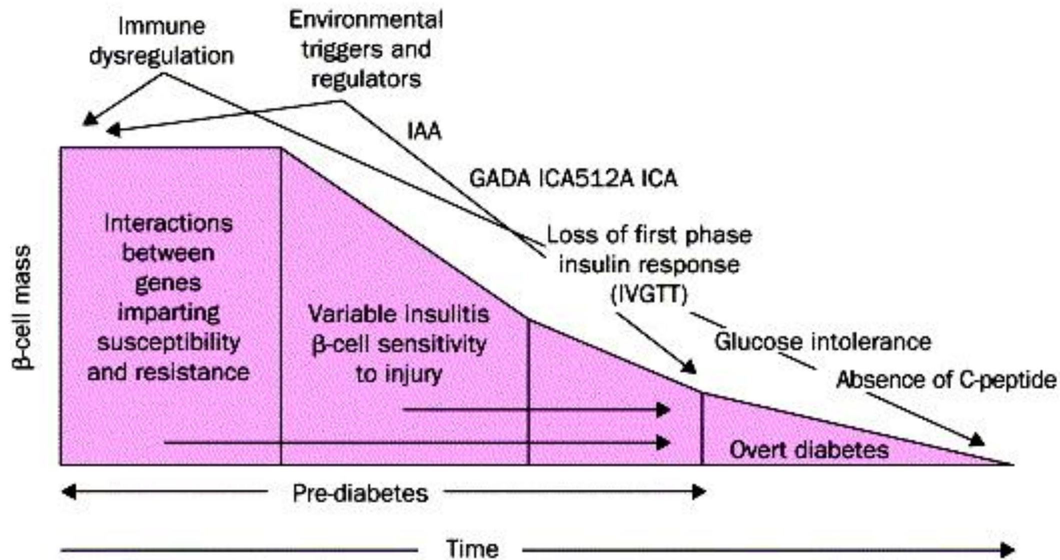



***Figure 9: The pathogenesis progression of type 1 diabetes [Atkinson and Elsenbarth, 2001].***

***β-cell dysfunction is triggered by genetic and environmental factors, subsequently followed by insulitis. Infiltration of inflammatory cells or proteins impairs β-cell function, ultimately leading to inflammation and β-cell death. The release of β-cell debri as a consequence of apoptotic death further exacerbates the autoimmune response, promoting overt diabetes. IAA, insulin autoantibodies; ICA, islet cell autoantibodies; GADA, autoantibodies to glutamic acid decarboxylase.***

### 1.6.1.1 *Pancreatic β-cell dysfunction*

Inflammatory infiltration of β lymphocytes, macrophages, CD8 and CD4 T cells, initiates the destruction of β-cells via the activation of specific apoptotic pathways [*Atkinson and Elsenbarth, 2001].* Potential mechanisms of β-cell destruction include pro-inflammatory cytokines, Fas/Fas ligand and reactive oxygen species [*Atkinson and Elsenbarth, 2001*]. These inflammatory destructive factors interact with β-cell cytokine receptors (tumor necrosis factor receptor (TNFR), interferon-gamma receptor (IFN-γR) and Interleukin-1β receptor (IL-1βR)), activating the MAP kinase pathway and a diverse group of

Stellenbosch University https://scholar.sun.ac.za

transcription factors, STAT-1 (signal transducer and activator of transcription-1) and NF-κβ. This ultimately results in functional impairment and apoptotic β-cell death [*Atkinson and Elsenbarth, 2001*]. Damaged β-cells release cell debris and auto-antigens which are recognized by auto-reactive effector T-cells, exacerbating the autoimmune response, thus promoting T1D [*Thrower and Bingley, 2010*].

The autoimmune destruction of β-cells varies widely between individuals and can occur over months and even years [*Thrower and Bingley, 2010*]. The rate at which destruction proceeds depends on an individual's tolerance to regulate the immune response [*Ziegler and Nepom, 2010*]. Therefore, prolonging complete eradication of β-cells would serve to be favourable in a clinical setup.

#### 1.6.1.2 *β-cell mass*

As previously stated, β-cells secrete insulin which regulates glucose homeostasis by targeting various tissues. For this reason, β-cell mass is vital in the manifestation of diabetes mellitus and should be closely monitored. β-cell mass are dynamic components which readily adapt to changes in insulin demand or glucose concentrations. To maintain an optimal glucose level, an exquisite balance exists between insulin biosynthesis, stimulation and secretion.

The quantitative amount of β-cells present can be calculated based on the interplay between β-cell growth and loss. There are three mechanisms involved in β-cell expansion which include replication of existing differentiated β-cells, neogenesis of β-cells from undifferentiated precursors and hypertrophy [*Masiello, 2006*]. A reduction in

Stellenbosch University https://scholar.sun.ac.za

β-cell mass is associated with hypotrophy and β-cell death as a result of apoptosis, and/or necrosis. Altering any of these mechanisms would result in a change in β-cell mass.

Under normal conditions, β-cell growth and remodeling occurs prenatally, and continues for a short period of time after birth [*Masiello, 2006*]. During this time, β-cell replication is enhanced followed by a surge in β-cell neogenesis. This results in β-cell growth as a consequence of apoptotic activity being kept at a low level. The rate at which β-cell replication, neogenesis and apoptosis occur soon stabilizes as individuals enter childhood and adulthood, thus maintaining a relative constant β-cell mass [*Masiello, 2006*]. Various factors contribute to a reduction in β-cell mass, such as old age and the exposure of β-cells to cytotoxic factors, stimulating apoptosis.

#### 1.6.1.3 *β-cell neogenesis*

As a result of β-cell dysfunction or death, research has shifted its focus to β-cell neogenesis. β-cell neogenesis is a mechanism that triggers the generation of new β-cells from precursor cells [*Paris, 2004*]. These precursor cells originate from (i) ductal cells (budding from duct epithelium), (ii) differentiated pancreatic cells through a process called transdifferentiation and (iii) intra islet precursors [*Paris, 2004*]. β-cell neogenesis is a well documented process that is involved in β-cell mass expansion and maintenance, when exposed to increased insulin demand. For this reason, β-cell neogenesis would serve to be beneficial in treating diabetes mellitus. There are various transcription factors promoting β-cell neogenesis which will be discussed in more depth.

Stellenbosch University https://scholar.sun.ac.za

#### 1.6.1.4 ***Transcription factors promoting β-cell neogenesis***

Transcription factors control several biological processes such as β-cell differentiation, proliferation, apoptosis, and pancreatic development [*Rocques et al, 2007*]. The regulation of transcription factors is of utmost importance due to the link between β-cell dysfunction and diabetes. In this study we will be focusing on pancreatic duodenal homeobox 1 (Pdx-1) and musculoaponeurotic fibrosarcoma homolog A (Maf A).

##### 1.6.1.4.1 ***Pdx-1***

The pancreatic duodenal homeobox 1 protein is a homeodomain containing transcription factor that is essential for early pancreatic development and β-cell survival [*Yang et al., 2012*]. During early prenatal development, Pdx-1 is mainly expressed in pancreatic progenitor cells, later giving rise to endocrine and exocrine cells [*Yang et al., 2012*]. As the pancreas matures, Pdx-1 expression becomes restricted to β-cells. Pdx-1 maintains normal pancreatic function by regulating insulin, glucose transporter 2 (Glut2) and glucokinase (GCK) gene expression in the promoter region of β-cells. In the last decade, researchers have invested a lot of time in determining the importance of Pdx-1 expression. Genetic studies on homozygous and heterozygous Pdx-1 mutated mice have linked Pdx-1 to pancreatic agenesis and impaired glucose tolerance, respectively [*Fujimoto et al., 2010; Claiborn et al., 2010*]. There are many stimuli which stimulate Pdx-1 expression in β-cells such as glucose, oxidative stress and free fatty acids [*Ardestani et al., 2011*]. Acute exposure of β-cells to glucose translocates Pdx-1 to the nucleus, inducing β-cell proliferation and insulin secretion [*Ardestani et al., 2011*]. Prolonged exposure of β-cells to hyperglycemia exhibited increased DNA methylation

Stellenbosch University https://scholar.sun.ac.za

followed by a reduction in Pdx-1 expression [*Yang et al., 2012*]. This impairs β-cell function and insulin secretion ultimately leading to β-cell death via apoptosis [*Ardestani et al., 2011; Yang et al., 2012*]. Pdx-1 expression is regulated by post-translational modification and cellular localization [*Ardestani et al., 2011*].

Insulin resistance has detrimental effects on Pdx-1 expression and activity. During insulin resistance, the inhibitory effect of insulin on GSK-3β and FoxO1 is lifted, reducing β-cell proliferation. GSK-3β negatively regulates β-cell mass and function by phosphorylating Pdx-1 on specific serine (Ser61 and Ser66) residues, marking them for proteasomal degradation, thus, reducing its half life [*Fujimoto and Polonsky, 2009*]. FoxO1 blocks Pdx-1 expression via translocation, moving Pdx-1 from the nucleus to the cytosol [*Fujimoto and Polonsky, 2009*]. GSK-3β and FoxO1 enhances β-cell death at a protein and transcriptional level, respectively [*Fujimoto and Polonsky, 2009*].

#### 1.6.1.4.2 *Maf A*

Musculoaponeurotic fibrosarcoma homolog A is a transcription factor of the basic leucine zipper (bzip) family [*Kannan et al., 2012*]. Members of the MAF family are divided up into two classes, “large” (Maf A, Maf B and c-Maf) and “small” (Maf F, Maf G and Maf K) MAF proteins, depending on their molecular size [*Nishimura et al., 2009*]. Large Maf proteins consist of an additional amino-terminal transactivation domain (TAD) and binds to two palindromic sequences on the insulin promoter region called Maf responsive elements (MAREs), stimulating the transcription of target genes [*Nishimura et al., 2009; Rocques et al., 2007*]. Maf A is a unique transcription factor expressed

Stellenbosch University https://scholar.sun.ac.za

during late pancreatic development and restricted to pancreatic β-cells [*Hunter et al., 2011*]. The main role of Maf A is to maintain normal blood glucose levels in the body by regulating insulin gene expression [*Jorgensen et al., 2007; Kataoka et al., 2002*].

A study done by Nishimura and colleagues on Maf A deficient mice have shown a gradual reduction in the β to α cell ratio with age even though postnatal β-cell levels were normal [*Nishimura et al., 2009*]. These animals were glucose intolerant and developed diabetes; emphasizing the importance of Maf A as a regulator of β-cell maturation [*Nishimura et al., 2009*]. Zhao reported the transcriptional rate of insulin to be moderate when Maf A was expressed in the β-cell alone, but in accordance with Pdx-1, the synergistic action enhanced insulin promoter activity [*Zhao et al., 2005*].

#### 1.6.1.5 ***Treatment: type 1 diabetes***

T1D occurs as a result of autoimmunity against pancreatic β-cells, ultimately leading to β-cell destruction. Therefore, therapeutic strategies prolonging or preventing β-cell dysfunction would serve to be beneficial in treating T1D patients. Annihilation of β-cells by the immune system reduces the amount of endogenous insulin secreted into circulation. As a result of the body not producing sufficient insulin to sustain normal glucose homeostasis, these patients are required to take alternative sources of insulin for life. Patients are primarily treated with insulin injections taken on a regular basis. There are several other modes of administrating insulin which include external insulin pumps, insulin inhalers and internal insulin pumps. These patients could also undergo more invasive treatments such as gene therapy, pancreatic islet transplantation and whole pancreas transplantation. Other treatment options available are β-cell replication,

Stellenbosch University https://scholar.sun.ac.za

reprogramming of mature β-cells and stem cell transplantation. These procedures are very expensive and is therefore not a common option in developing countries.

**1.6.1.6 *The Streptozotocin-model of type 1 diabetes.***

Streptozotocin (STZ) is a glucosamine compound which contains a very reactive nitrosourea group [*Wei et al., 2003; Arora et al., 2009*]. This compound, derived from a soil microbe called *Streptomyces achromogenes,* was initially used as an antibiotic and later added to medication for the treatment of pancreatic cancer, specifically cancer of the islets of Langerhans [*Motyl and McCabe, 2009*]. Streptozotocin has a glucose moiety which is easily transported into pancreatic β-cells via glucose transporter 2, where it exerts its toxic effects by damaging DNA [*Wei et al., 2003; Motyl and McCabe, 2009*]. This leads to β-cell dysfunction, hypoinsulinemia and hyperglycemia; all characteristics associated with diabetes [*Motyl and McCabe, 2009*]. When inducing a T1D model, one should keep in mind that STZ performs at its best when stabilized [*Motyl and McCabe, 2009*]. STZ is stable at a pH of approximately 4 and it is for this reason that STZ is prepared in a citrate buffer with a pH of 4.5; thus enhancing stability and inducing diabetes [*Motyl and McCabe, 2009*]. The severity of type 1 diabetes depends on the species of the animal, age and the amount of STZ administrated intravenously or intraperitoneally [*Arora et al., 2009*]. For example, a single dose of STZ is effective in inducing T1D in rat models while multiple low doses of STZ serves to be most effective in inducing T1D in mice models [*Motyl and McCabe, 2009*].

Stellenbosch University https://scholar.sun.ac.za

### *1.6.2 Type 2 Diabetes*

Type 2 Diabetes, formerly accepted as adult onset diabetes, is a multi-factorial disorder that accounts for 90-95% of all diabetic cases worldwide, making it the most common type of diabetes known [*Stumvoll et al., 2005*]. The pathological sequence for T2D is complex and entails many risk factors promoting its onset and development [*Leahy, 2005*]. T2D occurs as a result of insufficient insulin production by pancreatic β-cells or reduced insulin action at target tissues. Reports have shown insulin resistance to be present prior to β-cell dysfunction in type 2 diabetic individuals maintaining normal blood glucose levels [*Leahy, 2005*]. The progression from normoglycemia to glucose intolerance occurs as a result of reduced β-cell function rather than a spike in insulin resistance [*Leahy, 2005*]. Beta cell function varies and is dependent on the degree of insulin sensitivity [*Leahy, 2005*]. A reduction in insulin action stimulates the glucose homeostatic system to increase β-cell function by increasing β-cell mass [*Stumvoll et al., 2005*]. This compensatory mechanism gradually loses its ability to produce the additional insulin needed to overcome insulin resistance, impairing the responsiveness of β-cells to glucose [*Leahy, 2005*]. As blood glucose levels rise, hyperglycemia promotes β-cell dysfunction by altering various metabolic pathways, enzymes and genes (impairing proinsulin transcription) important for proper functioning. Hyperglycemia deteriorates β-cells even further by stimulating the production of malonyl CoA (in skeletal muscle and the liver) that inhibits fatty acid oxidation, thus, favouring lipotoxicity [*Leahy, 2005*]. An excess of free fatty acids promote β-cell dysfunction and death by producing fatty acid metabolites such as ceramides and oxidative stress

Stellenbosch University  https://scholar.sun.ac.za

precursors [*Leahy, 2005*]. In addition, the generation of amyloids contribute to defective β-cell function [*Leahy, 2005*].

#### *1.6.2.1 The role of obesity in the development of insulin resistance*

Obesity is a disease which affects individuals of all ages and both sexes, globally [*Aballay et al., 2013*]. This disease occurs as a result of excessive fat accumulation due to a chronic caloric imbalance, impairing an individual's health status [*Pulgarón, 2013*]. Obesity is assessed and defined by the body mass index (BMI) as being ≥ 30 $kg/m^2$ according to the World Health Organization [*2013*]. The BMI is calculated based on the weight in kilograms divided by the square of the height in meters [*WHO, 2013*].

Obesity has become a major public health problem prevailing in both developing and developed countries [*Aballay, 2009*]. This problem is further exacerbated by an increase in urbanization, changes in eating habits and a reduction in physical activity. According to WHO, more than 1,4 billion adults worldwide are overweight and of these individuals, over 200 million men and nearly 300 million women were obese [*WHO, 2013*]. This organization also predicted that these numbers would double by the year 2015 emphasizing the importance of treatment [*Nguyen et al., 2010*].

Obesity has a tremendous impact on society, drawing attention towards the economic and health sector.  In terms of the economy, obesity exerts an enormous financial strain on the economic budget as more finances are pushed into treating obese related morbidities and secondary diseases [*Dodd, 2008*]. With regards to the health sector, obesity affects an individual's quality of life as it is associated with a variety of co-

Stellenbosch University  https://scholar.sun.ac.za

morbidities such as type 2 diabetes (T2D), cardiovascular disease, sleep apnea and an array of cancers [*Stumbo et al., 2005; Sahani et al., 2010*]. These individuals exhibit a strong correlation between obesity and the development of the metabolic syndrome which encompasses dyslipidemia, hypertension, insulin resistance and proinflammation [*Chen and Morris, 2007*].

The section that follows will explain the link between obesity and insulin resistance as well as the manifestation of T2D. Current therapeutic strategies will also be discussed.

##### *1.6.2.1.1 The link between obesity and adipose dysfunction*

As previously stated, obesity stems from a caloric imbalance which arises when energy intake exceeds energy expenditure. The human body makes use of two main types of energy substrates to maintain survival, namely, glucose (carbohydrates) and free fatty acids (lipids). In this section, we will be focusing on triacylglycerol stores, circulating free fatty acids and the origin of insulin resistance.

Adipose tissues are the largest and main lipid storage depots in our body, playing a vitally important role in energy metabolism [*Goossens, 2008*]. These tissues are made up of many adipocytes which produce and secrete numerous adipokines, and cytokines in response to various stimuli. During the postprandial state, adipocytes regulate the influx of dietary fats via a buffering action which suppresses the release of free fatty acids into circulation and increases the activity of lipoprotein lipase, thus enhancing triacylglycerol clearance [*Goossens, 2008*]. Adipocytes of obese individuals are enlarged as a result of adipose tissue being overloaded with triacylglycerol (TAG)

Stellenbosch University https://scholar.sun.ac.za

[*Goossens, 2008*]. During the fed state these individuals are exposed to an additional amount of fatty acids that cannot be cleared effectively due to adipocytes reaching its optimum storage capacity [*Goossens, 2008*]. As a result, the number of fatty acids and triacylglycerol entering circulation increases. Non-adipose tissue such as the liver and muscle would try and mop up excess substrates in circulation by oxidizing fats, thus preventing any adverse metabolic effects [*Goossens, 2008*]. Over time these tissues lose the ability to oxidize fats efficiently leading to the accumulation of lipids which are associated with conditions (glucose intolerance, hyperlipidemia and hyperinsulinemia) related to insulin resistance. Insulin resistance is defined as a state of reduced responsiveness of target tissues to normal circulating levels of insulin [*Sesti, 2006*].

It should be noted that adipose tissue not only serve as the main TAG store in the human body but also secrete numerous adipokines and cytokines which affect insulin sensitivity [*Kahn and Flier, 2000*]. The role free fatty acids, adipokines and cytokines play in insulin resistance will be further discussed below.

#### ***1.6.2.1.2 Fatty acid induced insulin resistance***

Free fatty acids play an extremely important role in insulin resistance of obese or overweight individuals by inducing hyperinsulinemia; impairing insulin action and impairing glucose metabolism [*Golay and Ybarra, 2005*]. Two mechanisms concerning the induction of insulin resistance, via the abundance of free fatty acids in circulation, has been postulated by Randle and Shulman [*Randle et al., 1963; Shulman, 2000*].

Stellenbosch University https://scholar.sun.ac.za

In the 1960's, Randle and colleagues proposed that elevated plasma FFA levels inhibit glucose oxidation by negatively influencing key enzymes in the glycolytic pathway of isolated rat heart muscles, and diaphragms [*Randle et al., 1963*]. The theory behind Randle's hypothesis states that an increase in lipid oxidation raises the mitochondrial acetyl-CoA/CoA and NADH/$NAD^+$ ratios to a point where these factors are capable of inhibiting the enzyme pyruvate dehydrogenase [*Kovacs and Stumvoll, 2005*]. The cell would try and get rid of excess acetyl CoA by diverting it into the Krebs cycle, increasing the concentration of citrate. As citrate levels increase, phosphofructokinase activity is inhibited to prevent the accumulation of acetyl CoA by blocking the production of pyruvate [*Kovacs and Stumvoll, 2005*]. The inactivation of phosphofructokinase causes the level of glucose-6-phosphate to rise to an extent where it inhibits hexokinase II activity, thus reducing glucose uptake and ultimately impairing glucose homeostasis [*Kovacs and Stumvoll, 2005*]. There have been many conflicts with Randle's theory, which has been rectified by several authors investigating the link between lipid accumulation and the inhibition of insulin stimulated glucose uptake at a cellular level [*Golay and Ybarra, 2005*]. These studies have shown an influx of lipids to be associated with insulin resistance by inhibiting glucose transport and/or phosphorylation [*Kovacs and Stumvoll, 2005*]. This subsequently decreases the rate of glucose oxidation and storage by inhibiting glycogen synthase.

An investigation done by Shulman confirmed that high plasma fatty acid levels induce insulin resistance via the inhibition of glucose transport [*Shulman, 2000*]. Under normal physiological conditions, insulin mediates tyrosine phosphorylation of IRS which

Stellenbosch University https://scholar.sun.ac.za

stimulates PI-3K activation [*Kovacs and Stumvoll, 2005; Shulman, 2000*]. As discussed in section 1.5.2, active PI-3K triggers a cascade of events, promoting the translocation of GLUT to the membrane. Shulman hypothesized that the skeletal muscle of obese individuals are exposed to an abundance of lipid metabolites, such as fatty acyl CoA, ceramides and diacylglycerol, which negatively affect glucose uptake [*Shulman, 2000*]. A concentration hike in lipid metabolites sets off a range of adverse effects, triggering the serine/threonine kinase cascade by increasing the activity of protein kinase C θ (PKC-θ) [*Kovacs and Stumvoll, 2005; Shulman, 2000* ]. Serine phosphorylation by PKC-θ sterically hinders IRS-1 tyrosine phosphorylation, reducing the activity of PI-3K by 50 % ultimately reducing the translocation of GLUT 4 to the membrane [*Petersen and Shulman, 2006*].

#### *1.6.2.2 The link between adipocyte secretions and insulin resistance*

Adipocytes secrete several bioactive compounds which play a crucial role in regulating blood pressure, lipid metabolism, inflammation and glucose metabolism [*Rabe et al., 2008*]. These secretory compounds include adipokines and cytokines. In this section, the influence of certain adipokines and cytokines on insulin resistance, will be discussed.

##### *1.6.2.2.1 Adipokines*

Leptin is a 16 kD hormone produced by the *ob* gene, playing an essential role in nutrient homeostasis [*Fantuzzi, 2005; Elmquist, 2001*]. Principally, leptin exerts its metabolic action in the hypothalamic region of the central nervous system (CNS) [*Kahn and Flier, 2000*]. Once leptin signals the CNS, the autonomic system (ANS) and the

neuroendocrine pathway are immediately alerted. In response, these pathways regulate food intake, energy storage, blood pressure and energy expenditure by modulating various metabolic processes in peripheral tissue, thus improving insulin sensitivity [*Rabe et al., 2008; Elmquist, 2001*]. Aside from the ability of leptin to act via the CNS, it is capable of directly stimulating the liver, skeletal muscle and pancreatic β-cells [*Kahn and Flier, 2000].*

The adipo-insular axis is a feedback loop established between the adipose tissue and the pancreatic β-cell [*Rabe et al., 2008; Kieffer and Habener, 2000*]. This axis is fully operational when body weight increases, which correlates positively with leptin levels. Leptin protects β-cells from detrimental effects associated with lipid accumulation by reducing lipid levels through fatty acid oxidation, thus improving β-cell function [*Friedman, 2002*]. In turn, viable β-cells secrete insulin which promotes both leptin biosynthesis and secretion by adipocytes. In summary, leptin promotes insulin sensitivity in all peripheral tissues. The theory behind this axis is that an accumulation of fatty acids in adipose tissue causes an increase in leptin levels, which protects β-cells from deleterious effects associated with lipid accumulation [*Rabe et al., 2008; Kieffer and Habener, 2000* ].

The link between leptin and insulin resistance was made evident in studies done on leptin deficient (*ob/ob*) mice [*Friedman, 2002*]. These *ob/ob* mice exhibited immune defects, decreased body temperature, infertility, hyperphagia, obesity, decreased energy expenditure, diabetes and hypercortisolemia which were associated with abnormalities in metabolic and/or endocrine pathways. Leptin treatment reversed all

Stellenbosch University https://scholar.sun.ac.za

adverse effects by decreasing the intracellular lipid content in all tissues [*Friedman, 2002*].

The concept of leptin resistance arose from obese individuals who portrayed high leptin levels without suppressing appetite or inducing weight loss [*Kahn and Flier, 2000*]. This occurs as a result of lipid accumulation within target tissues limiting insulin action, thus causing insulin resistance [*Friedman, 2002*]. As a result pancreatic β-cells would produce more insulin to compensate for the resistance leading to hyperinsulinemia. Hyperinsulinemia is thought to promote leptin synthesis and secretion via adipose tissue, further enhancing leptin resistance.

Adiponectin is an anti-inflammatory cytokine abundantly secreted by adipose tissue. As a result of adipocytes being the main source of adiponectin, one would expect serum adiponectin levels to rise with an increase in body weight but this is not the case [*Fantuzzi, 2005*]. Studies have shown adiponectin levels to be reduced in obese and insulin resistant subjects, while individuals suffering from anorexia expressed high levels of adiponectin [*Fantuzzi, 2005*]. In obese subjects, the administration of adiponectin improved whole body insulin sensitivity by targeting various tissues. In the skeletal muscle, adiponectin increases glucose uptake and fatty acid oxidation, while reducing hepatic glucose production [*Rabe et al., 2008*].

These insulin sensitizing effects are initiated when adiponectin binds to specific adiponectin receptors, adiponectin receptor1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). The expression of AdipoR1 and AdipoR2 has been shown to be linked to the

activation of AMPK and peroxisome proliferator-activated receptor-α (PPAR-α), respectively [*Rabe et al., 2008*]. Yamauchi *et al*. reported that a disruption in AdipoR1 and AdipoR2 correlated with an increase in insulin resistance as a result of a reduction in adiponectin binding and action [*Yamauchi et al., 2007*]. Many human studies have shown adiponectin levels to be negatively related to type 2 diabetes, obesity and insulin resistance [*Rabe et al., 2008*]. Therefore, boosting adiponectin levels or adiponectin receptor function would be beneficial in treating all negative components associated with the establishment of T2D.

#### 1.6.2.2.2 *Cytokines*

Obesity is a chronic low-grade inflammatory state that is linked to the expression of various pro-inflammatory cytokines [*Fantuzzi, 2005*]. Numerous studies have reported a correlation between pro-inflammatory cytokines and clinical features associated with the metabolic syndrome such as BMI and insulin resistance [*Sesti, 2006*]. There are many other factors which promote the expression of pro-inflammatory cytokines involved in the manifestation of obesity-induced insulin resistance. These factors include oxidative stress that is generated as a result of lipid accumulation [*Styskal et al., 2012*]. Oxidative stress can modulate insulin action either by targeting the insulin pathway directly or by stimulating the activation of proteins involved in inflammation. In this section we will be focusing on TNF-α and IL-6.

A study done by Hotamisligil and colleagues (1993) reported that TNF-α mediates insulin resistance in obese rodent models [*Hotamisligil et al., 1993*]. This notion was

Stellenbosch University https://scholar.sun.ac.za

deduced when they observed high levels of TNF-α messenger RNA being expressed by adipose tissues which were further enhanced in adipose tissue of obese and diabetic rodent models. In addition, several other studies on TNF-α-null and TNF-α receptor-null mice support this hypothesis [*Chen, 2006*]. TNF-α signaling can directly alter insulin action by phosphorylating specific serine residues on the insulin receptor or insulin receptor substrate 1, thus inhibiting insulin signaling, giving rise to insulin resistance [*Fantuzzi, 2005*]. Other mechanisms by which TNF-α induces insulin resistance is by downregulating adiponectin expression, increasing IL-6 production, stimulating lipolysis and increasing the release of free fatty acids from adipocytes [*Goossens, 2008*]. Despite the correlation between TNF-α and insulin resistance, TNF-α neutralizing treatments have not shown improved insulin sensitivity in diabetic patients [*Fantuzzi, 2005].* Instead, these patients were exposed to an increased risk of contracting opportunistic infections.

Interleukin 6 is a multifunctional cytokine which plays an important role in a wide range of processes [*Simpson et al., 1997*]. For this review, we will only be focusing on the link which exists between IL-6 and insulin resistance. In the presence of obesity, an overload of nutrients in adipose tissue upregulates the release of free fatty acids and pro-inflammatory cytokines; activating resident macrophages [*Chen, 2006*]. Activated macrophages secrete TNF-α and IL-6 which recruit additional macrophages into adipocytes [*Chen, 2006*]. Increased levels of circulating TNF-α and IL-6 cause insulin resistance in adipocytes through several distinct mechanisms which include c-Jun N-terminal kinase 1 (JNK1) mediated serine phosphorylation and inactivation of IRS-1, IκB

Stellenbosch University https://scholar.sun.ac.za

kinase (IKK)-mediated nuclear factor-κB (NF-κB) activation, and induction of suppressor of cytokine signaling 3 (SOCS-3) [*Chen, 2006; Rabe et al., 2008*]. Studies have shown IL-6 levels to be high in obese and insulin resistant subjects with an increase in hepatic glucose production [*Rabe et al., 2008*]. A reduction in weight has positive effects on insulin sensitivity amongst others, by lowering IL-6 expression.

#### 1.6.2.3 ***Treatment: Type 2 diabetes***

Type 2 diabetes is a disease which attracts a lot of attention and is constantly researched for the establishment of new anti-diabetic drugs which are safe and effective. Interest in the treatment of T2D patients arose as a result of these patients not being able to control glycaemia sufficiently in the presence of anti-diabetic drugs [*Bailey, 1999*]. Below is a flow chart indicating various treatment options available as well as a table containing a list of anti-diabetic drugs with their specific functions [*Bailey, 1999; Shah et al., 2013; Doyle and Egan, 2007*].

Stellenbosch University https://scholar.sun.ac.za

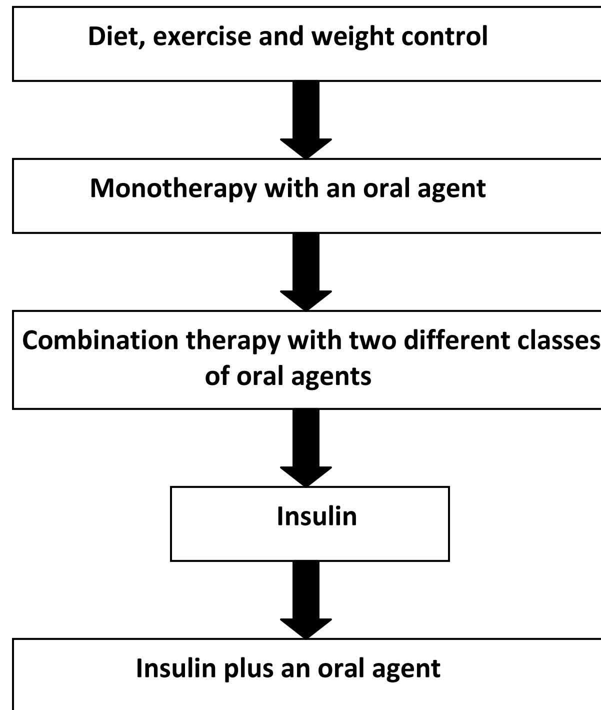



***Figure 10: Type 2 diabetic treatment system [Bailey, 1999]***

***Initially, T2D patients undergo lifestyle changes (increased physical activity, healthier eating patterns and a reduction in weight). This option serves to be ineffective in approximately four-fifths of newly diagnosed patients causing them to move to the next treatment level [Bailey, 1999]. If a patient cannot maintain glycaemic control after being treated with an oral agent, be that in a combination of two oral agents or a single oral agent, insulin therapy would be required.***

Stellenbosch University https://scholar.sun.ac.za

***Table 3: List of anti-diabetic drugs [Bailey, 1999; Shah et al, 2013; Doyle and Egan, 2007].***

| Agent | Action |
|---|---|
| Sulfonylureas eg. Glipizide | Increase insulin secretion by stimulating β-cells |
| Biguanides eg. Metformin | Improve insulin sensitivity in peripheral tissue |
| Alpha-glucoside inhibitors eg. Acarbose | Slow carbohydrate digestion |
| Thiazolidinediones eg. Avandia | Improve insulin sensitivity in peripheral tissue |
| Glucagon-like peptide-1 (GLP-1) analogues | Increase insulin synthesis and secretion by stimulating β-cells |
| Dipeptidyl peptidase 4 (DPP4) inhibitors | Inhibit the breakdown of GLP-1 |
| Insulin | Decrease hepatic glucose output and increase peripheral glucose utilization |
| Bariatric surgery (Weight loss surgery) | Alters the digestive system by restricting food intake. Mainly focusing on the stomach and/or intestine. |

Thiazolidinedione is one of the anti-diabetic drugs removed from the market as a result of this drug promoting liver toxicity [*Tolman and Chandramouli, 2003*]

## 1.7 *Skeletal muscle metabolism*

Skeletal muscle is the most abundant tissue within the human body and is accountable for 40 percent of body weight [*Andres et al., 1956*]. This tissue demands energy in the form of adenosine triphosphate (ATP) for contractile purposes and to sustain proper cellular function. Skeletal muscle utilize numerous substrates depending on their exposure to specific stressors. At rest, skeletal muscle prefers using fatty acids as their main energy source which is followed by glucose [*El-Bacha et al., 2010*]. During hypoxia or intense physical activity for a long duration of time, skeletal muscle switches from fatty acid metabolism to glucose metabolism [*Kessler and Friedman, 1998; El-Bacha et al., 2010*]. A switch in metabolism stems from the fact that glucose oxidation results in a higher production of energy (in the form of high energy phosphate bonds) when oxygen

Stellenbosch University https://scholar.sun.ac.za

is scarce compared to fatty acid oxidation [*Kessler and Friedman, 1998*]. In this section of the review we will focus on fatty acid and glucose metabolism.

As stated above, fatty acids are the main source of energy in skeletal muscle during rest and mild-intensity exercise. As exercise intensity increases, glucose oxidation suppresses fatty acid oxidation [*El-Bacha et al., 2010*].

### 1.7.1 *Fatty acid metabolism*

The amount of fatty acids taken up by myocytes depends on the rate of lipolysis within adipose tissue. The lipolytic activity of adipocytes is enhanced when the body is exposed to metabolic stresses such as fasting or diabetes. These states ultimately lead to the accumulation of free fatty acids in blood, thus increasing fatty acid uptake and oxidation in peripheral tissue. Under normal conditions, insulin stimulates the clearance of circulating free fatty acids by inhibiting lipolysis and fatty acid β-oxidation.

Circulating free fatty acids enter the cell via fatty acid transporters or diffusion [*Koonen et al., 2005*]. Once inside the cell, fatty acids are surrounded by acyl-CoA synthetase, a cytosolic enzyme which adds an acyl-CoA group to fatty acids [*Finn and Dice, 2006*]. Long fatty acyl-CoA molecules are able to cross the outer mitochondrial membrane but not the inner membrane, therefore, requiring a transportation or shuttling mechanism [*Jaswal et al., 2011*]. Carnitine palmitoyl transferase I mediates the movement of fatty acids into the mitochondrial matrix by replacing the CoA group with carnitine. Inside the matrix, fatty acyl carnitine is converted back into fatty acyl CoA, with the help of carnitine palmitoyl transferase II, which is localized on the inner mitochondrial

Stellenbosch University https://scholar.sun.ac.za

membrane [*Jaswal et al., 2011*]. Carnitine released from the fatty acyl molecule moves back to the outer mitochondrial membrane where it is reused.

In the mitochondrial matrix fatty acyl CoA undergoes β-oxidation. Mitochondrial β-oxidation is a step-wise reaction that repetitively breaks down long chain fatty acyl CoA to form acetyl CoA molecules. Acetyl CoA undergoes a whole range of reactions eventually producing ATP in the Krebs cycle. The number of acetyl-CoA molecules produced depends on the fatty acid carbon length being oxidized. The first step of fatty acid oxidation is the dehydrogenation of fatty acyl CoA [*Kim and Battaile, 2002*]. In this reaction, acyl-CoA dehydrogenase (ACD) catalyzes the conversion of fatty acyl CoA to trans 2 enoyl CoA and removes protons from fatty acyl CoA which are transferred to a FAD cofactor, yielding $FADH_2$ [*Schultz, 2008*]. There are four ACDs involved in fatty acid β-oxidation, namely, short chain (SCAD), medium-chain (MCAD), long-chain (LCAD) and very long chain (VLCAD) ACD [*Kim and Battaile, 2002*]. The acyl chain length of each ACD reflects their preferred substrate specificity, ensuring complete degradation of all fatty acids [*Lea et al., 2000; Schultz, 2008*]. The second step in the β-oxidation cycle is a hydration reaction [*Kim and Battaile, 2002*]. The enzyme enoyl CoA hydratase together with a water molecule ($H_2O$) hydrates 2-trans-enoyl CoA to form L-3-hydroxy acyl COA [*Schultz, 2008*]. The third step is a dehydrogenation reaction catalyzed by L-3-hydroxy acyl COA dehydrogenase, with nicotinamide adenine dinucleotide ($NAD^+$) as the cofactor [*Kim and Battaile, 2002*]. The hydroxyl group of L-hydroxy acyl CoA is oxidized to a keto group, producing 3- ketoacyl CoA and NADH [*Kim and Battaile, 2002*]. In the final step of β-oxidation, 3-ketoacyl CoA is cleaved by

Stellenbosch University https://scholar.sun.ac.za

thiolase to yield acetyl CoA and an acyl CoA molecule [*Schultz, 2008*]. Every cycle of β-oxidation sequentially shortens fatty acyl CoA molecules by two carbon atoms, producing one acetyl CoA as well as NADH and $FADH_2$ [*Jaswal et al., 2011; Fillmore and Lopaschuk, 2013*]. Acetyl CoA can either enter the Krebs cycle or be used in the production of ketone bodies [*Frick et al., 2008*].

### 1.7.2 *Glucose metabolism*

Insulin stimulated GLUT 4 translocation to the plasma membrane promotes glucose uptake, leading to the accumulation of glucose molecules within the cell [*Huang et al., 2005*]. Glucose transporter 4 is the insulin-responsive protein responsible for the transport of glucose across the plasma membrane into myocytes. Glucose uptake is triggered by insulin which is stimulated by high blood glucose levels. Insulin promotes GLUT 4 translocation to the membrane by activating PKB/Akt [*Wang et al., 1999*]. In diabetic patients, glucose uptake in skeletal muscle is drastically reduced as a result of insulin resistance or insufficient insulin secretion [*Wilcox, 2005*].

Glucose accumulation in skeletal muscle can either be stored as glycogen or enter glycolysis. The glycolytic pathway consists of three stages: the priming, splitting and energy producing stage (figure 11) [*Berg et al., 2002; Pelicano et al., 2006*]. During the priming stage, glucose is converted to fructose 1,6 bisphosphate via three reactions, catalyzed by various enzymes [*Berg et al., 2002; Pelicano et al., 2006*]. These reactions trap and destabilize glucose by the addition of two phosphoryl groups from ATP molecules [*Berg et al., 2002*]. In the splitting stage, fructose 1,6 bisphosphate is readily cleaved into two three-carbon fragments by aldolase, producing glyceraldehyde 3-

Stellenbosch University https://scholar.sun.ac.za

phosphate (GAP) and dihydroxyacetone phosphate (DHAP) [*Berg et al., 2002*]. GAP and DHAP are isomers, which are easily converted to the one or other with the help of triosephosphate isomerase [*Berg et al., 2002; Pelicano et al., 2006*]. In the presence of glycolysis, DHAP would be converted to GAP, rendering two molecules of GAP for every glucose molecule broken down [*Berg et al., 2002*]. The energy producing stage is a five step reaction converting two GAP molecules to pyruvate [*Berg et al., 2002; Pelicano et al., 2006*]. This stage produces four ATPs from a series of oxidative phosphorylation reactions [*Berg et al., 2002*]. The final glycolytic product, pyruvate, can either be converted to lactate or acetyl CoA via oxidative decarboxylation [*Berg et al., 2002*].

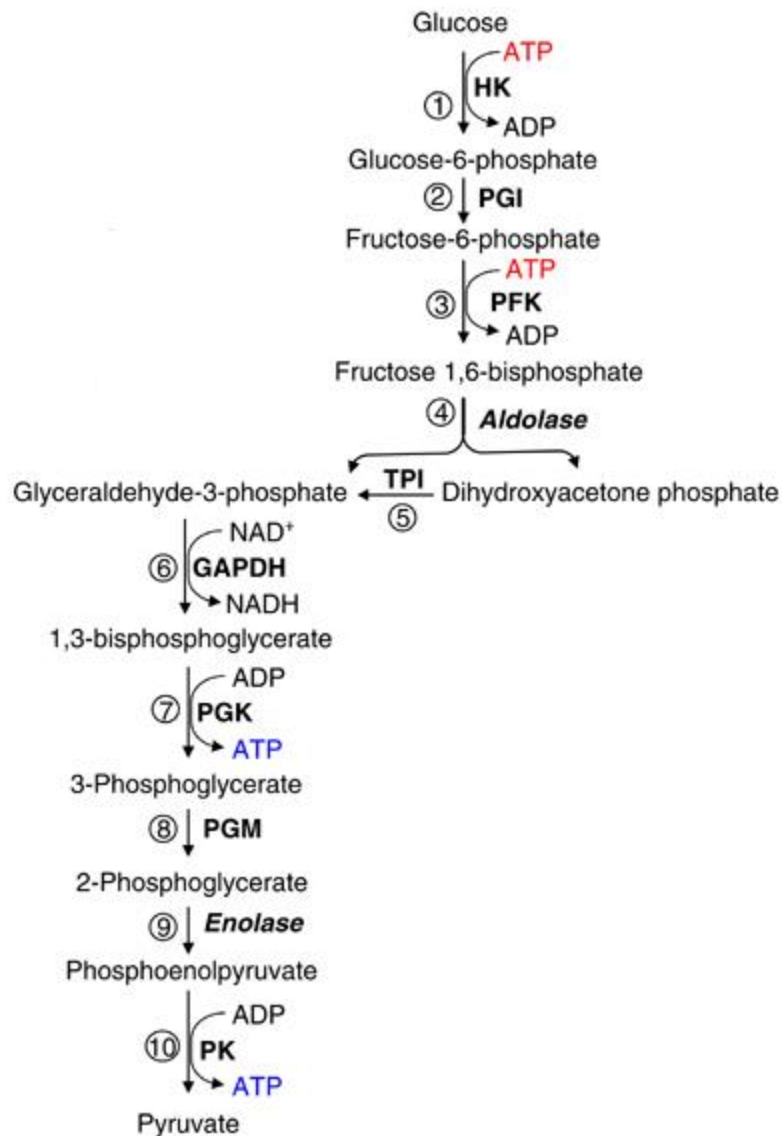



***Figure 11: glycolytic pathway [Pelicano et al., 2006]***

***Each glucose molecule produces two pyruvate molecules via the stimulation of several enzymes. In step 1, glucose is phosphorylated to glucose-6-phosphate with the help of hexokinase. Glycogen can serve as a supplementary source of glucose-6-phosphate in the presence of metabolic stress, such as fasting. Every enzyme in the glycolytic pathway is tightly regulated. ATP, adenosine triphosphate; ADP, adenosine diphosphate; PK, Pyruvate kinase; PGM, phospho glycerate mutase; PGK, phospho glycerate kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; TPI, triose phosphate; PFK, phosphofructokinase; PGI, phosphohexose isomerase; HK, hexokinase.***

Stellenbosch University https://scholar.sun.ac.za

### 1.7.3 *Oxidation of Acetyl CoA*

The production of acetyl CoA from the oxidative cleavage of fatty acids or the decarboxylation of pyruvate, made available by glycolysis, is completely oxidized by the Krebs cycle and electron transport chain [*Zubay, 2000*].

#### 1.7.3.1 *Krebs cycle*

The Krebs cycle, also known as the tricarboxylic acid or citric acid cycle, is the final catabolic pathway for the oxidation of fuel molecules and occupies a central position in metabolism [*Koubaa et al, 2013; Hagopian et al., 2004*]. The cycle is composed of a variety of enzymes and metabolites, which are important for the progression of many other pathways; therefore, its position is of utmost significance [*Hagopian et al., 2004*]. Acetyl CoA is the key substrate initiating the cyclic process. The Krebs cycle is an eight step reaction which generates carbon dioxide molecules and reducing equivalents, NADH and $FADH_2$ [*Hagopian et al., 2004*]. The cycle begins when a two carbon acetyl CoA molecule binds to oxaloacetate to form a six carbon molecule, citrate. The addition of a single water molecule is essential for the release of the CoA subunit from acetyl CoA. Citrate undergoes two consecutive decarboxylation events and several oxidative reactions, ultimately generating the four carbon compound, oxaloacetate [*Zubay, 2000*]. Each molecule of acetyl CoA produces two carbon dioxide molecules, one $FADH_2$ molecule and three molecules of NADH ($+H^+$).

Stellenbosch University https://scholar.sun.ac.za

#### 1.7.3.2 *Electron transport chain*

Reducing equivalents produced by β-oxidation and the Krebs cycle transport energy to the mitochondrial electron transport chain (ETC) [*Rossignol and Frye, 2012*]. The ETC is the final respiratory stage, consisting of a series of oxidative phosphorylation reactions for ATP generation [*Rossignol and Frye, 2012*]. This energy is produced by five multimeric complexes located on the inner mitochondrial membrane [*Bayir and Kagan, 2008*]. Once NADH and $FADH_2$ molecules release hydrogen atoms to complexes I and II, respectively, an electrochemical gradient is established. Electrons discharged by reducing equivalents moves down the chain of protein complexes with the help of two electron carriers, co-enzyme Q10 and cytochrome C [*Rossignol and Frye, 2012*]. In complexes I, III and IV, electrons give up some of their energy to extrude protons out of the mitochondrial matrix into the intermembrane space via active transport [*Bayir and Kagan, 2008*]. The movement of electrons generates an alkaline matrix and an acidic intermembrane space [*Bayir and Kagan, 2008*]. Electrons finally reach complex IV, where they bind to protons and an oxygen molecule to form water [*Swerdlow, 2012*]. Protons re-enter the matrix through complex V, a non-ETC enzyme, providing enough energy to synthesize ATP from ADP, which is utilized as energy by the rest of the body [*Bayir and Kagan, 2008; Swerdlow, 2012*].

Stellenbosch University https://scholar.sun.ac.za

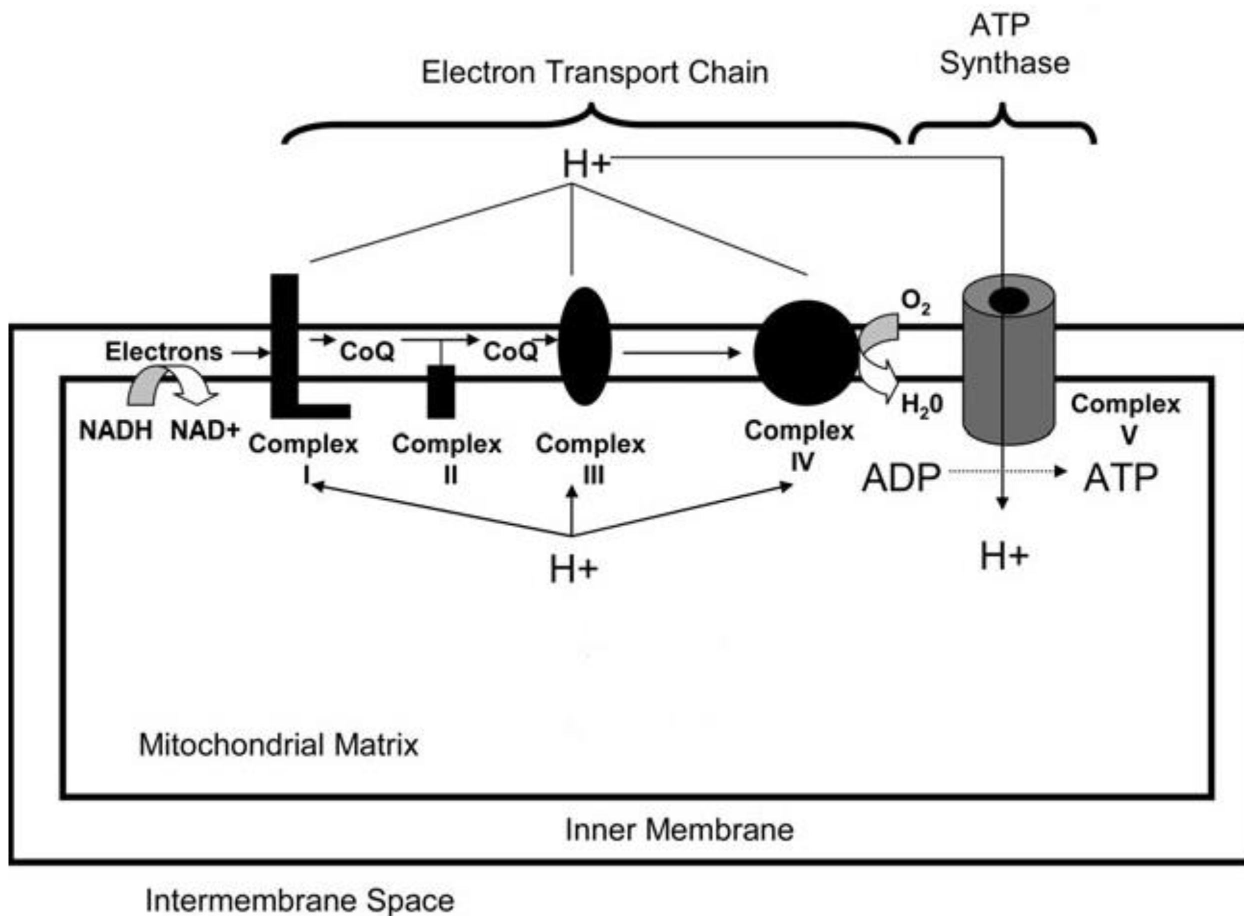



***Figure 12: Electron transport chain [Swerdlow, 2012]***

***NADH and $FADH_2$ molecules donate electrons to the electron transport chain. The movement of electrons down the chain produces enough energy to transport protons across the mitochondrial membrane into the intermembrane space. Electrons bind to oxygen, the final electron receptor, and forms water. Protons move back into the mitochondrial matrix through ATP synthase, producing ATP. FADH2, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; CoQ, Coenzyme Q (ubiquinone); ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; $H^+$, proton.***

Stellenbosch University https://scholar.sun.ac.za

# CHAPTER 2

# Materials and Methods

---

## 2.1. *Animal Care*

Male Wistar rats were used in this study. These rats were age and weight matched at the onset of the experiment. All animals were housed at the University of Stellenbosch Central Research Facility, which provided a simulated environment of the animals' natural habitat, exposing them to a temperature controlled room (22 °C - 24 °C) and a 12-hour light/dark cycle. Animals had free access to water and food for the duration of the experiment. All animals were fed normal rat chow, unless otherwise stated/ specified, and specific groups in both diabetic models were treated with Buchu water. The project was approved by the Ethics committee of the University of Stellenbosch (Faculty of Health Sciences – protocol number SU-ACUM11-00003) and conforms to the principals revised in the South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

## 2.2. *The administration procedure of Buchu (Agathosma)*

1.3L of the watery extract of Buchu, provided by the company Cape Kingdom, was mixed with tap water to make up a volume of 5000 ml. This mixture replaced water given to specific animal groups. The dosage of Buchu was calculated based on the

Stellenbosch University https://scholar.sun.ac.za

average weight of a 70 kg individual ingesting 350 ml of undiluted Buchu water/day. Equivalent surface area dose expressed as mg/kg was used for calculation of the dosage using the assumptions and constants of the paper by Freireich et al. 1996. For the initial estimations, it was assumed that a rat will drink 30ml of water per day.

### 2.3. *Animal models*

#### 2.3.1. *Streptozotocin-induced type 1 diabetes rat model*

In this model, a total of 50 animals were used and the experiment lasted for a period of 14 weeks. Animals weighing approximately 250 g were randomly divided into two main groups: a control (n=20) and diabetic (n=30) group. All animals allocated to the diabetic group were subjected to a once off intra-peritoneal STZ injection of 40 mg/kg (dissolved in a citrate buffer; pH 4.5) and blood glucose levels were determined after 24 hours, and at 1 week. After 1 week, animals that have been injected with STZ whose blood glucose levels normalized spontaneously, were excluded from the experimental group. The main purpose for using STZ at this concentration was to induce a type 1 diabetic model, ensuring ablation of approximately 50% of pancreatic beta cells. This brings about high blood glucose levels ranging between 10 and 20 mmol/L but the animals still produce endogenous insulin. A commercial glucometer was used to monitor blood glucose levels by collecting blood from a single tail prick. For a diagrammatic interpretation of the divisional state of all animals involved in the STZ- induced type 1 diabetic model refer to figure 13. The food and water intake of the animals were monitored for a period of a week and 20 days, respectively, 3 weeks after the start of the experiment. The amount

Stellenbosch University https://scholar.sun.ac.za

of food consumed was measured by the following equation: Food consumed per day (g) = food given (g) – food remaining in cage (g).

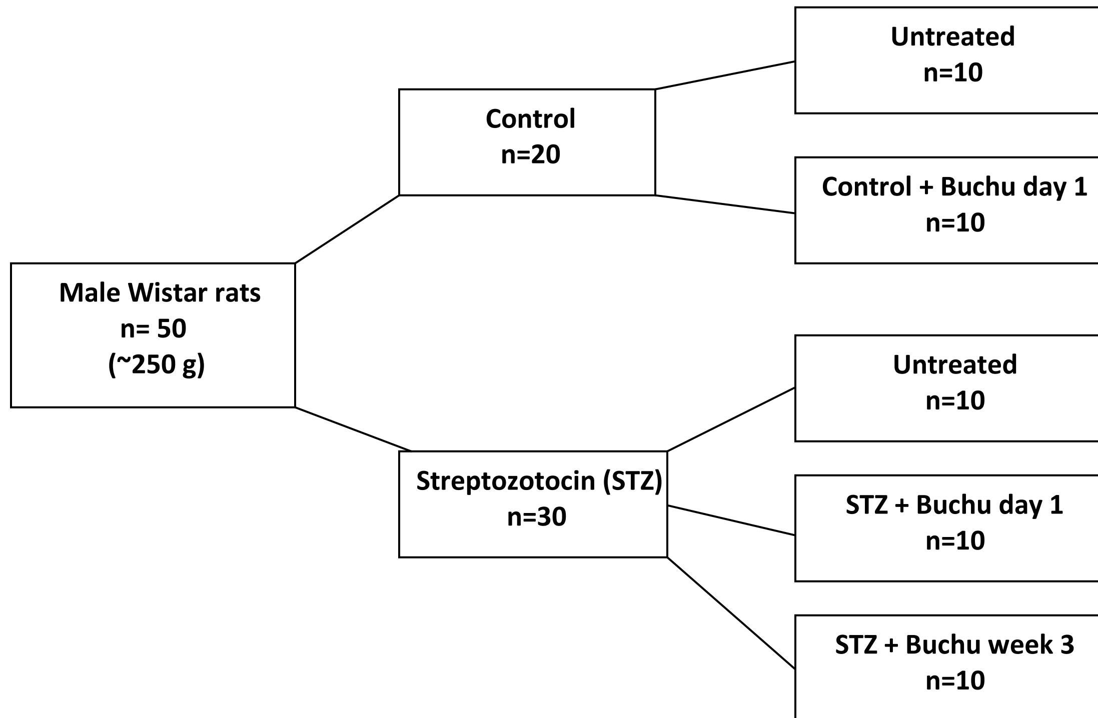



***Figure 13: Schematic representation of the division of type 1 diabetic animals***

***Buchu day 1:*** *The administration of Buchu water from the start of the experiment*

***Buchu week 3****: The administration of Buchu water 3 weeks after the initiation of the experiment*

Intraperitoneal glucose tolerance tests (IPGTT) were performed on all animals to determine the effect of Buchu water on whole-body insulin sensitivity. This was done by fasting animals overnight a week (week 13) prior to the time of sacrifice. Animals were anaesthetized (< 50 mg/kg pentobarbital) and fasting blood (1 ml) was collected from

Stellenbosch University https://scholar.sun.ac.za

the carotid artery for the determination of insulin levels (Coat-A-Count®) Radioimmunoassay (RIA) kit and baseline blood glucose levels determined. The animals were then injected intraperitoneally with a 50% sucrose solution, to give a final concentration 1 mg/kg. Blood glucose levels were monitored over a period of 120 minutes using a glucometer; blood was collected via a tail prick. At week 14, all the animals were anaesthetized with a 160 mg/kg overdose of a sodium pentobarbital intraperitoneal (IP) injection. When all sensation was absent, as indicated by no response on a foot pinch, the animals were sacrificed via exsanguination. Body weight was determined and IP fat dissected out and weighed. Blood samples were collected for biochemical determinations. Figure 14 illustrates on the experimental time frame and biochemical analysis.

Stellenbosch University https://scholar.sun.ac.za

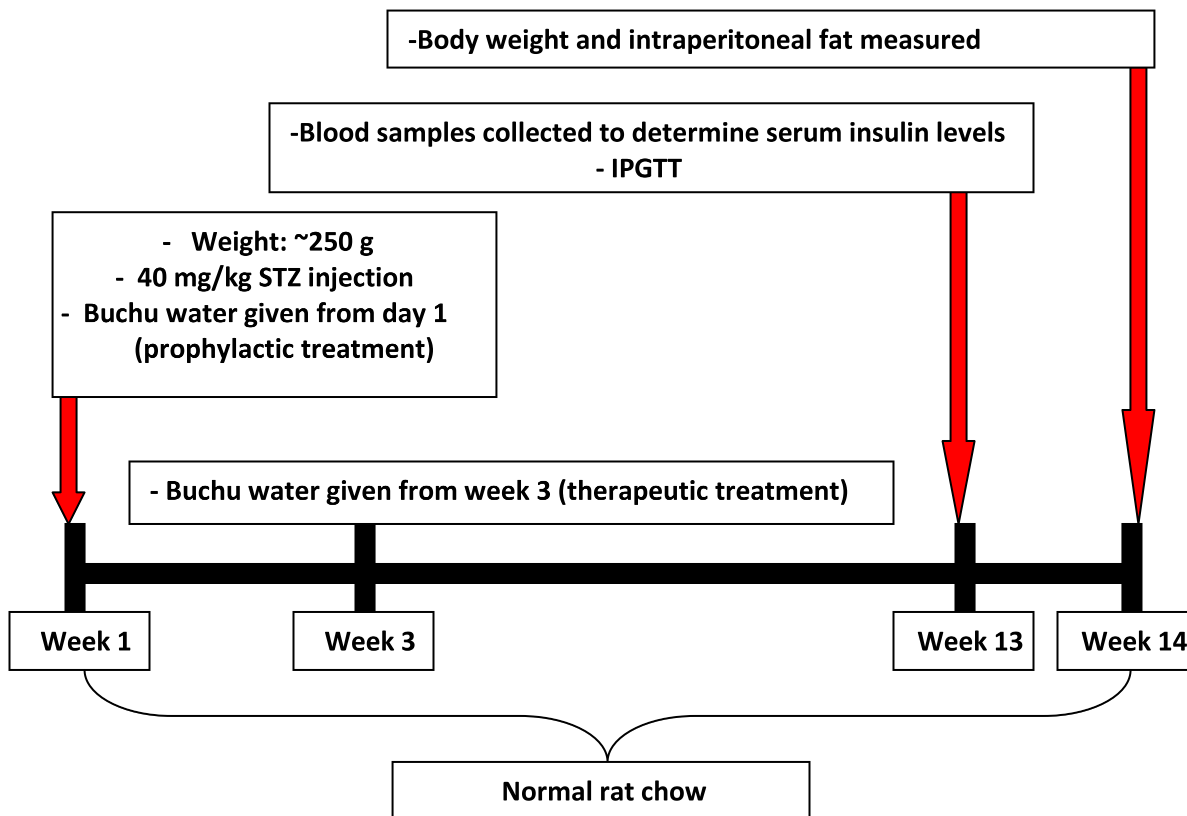
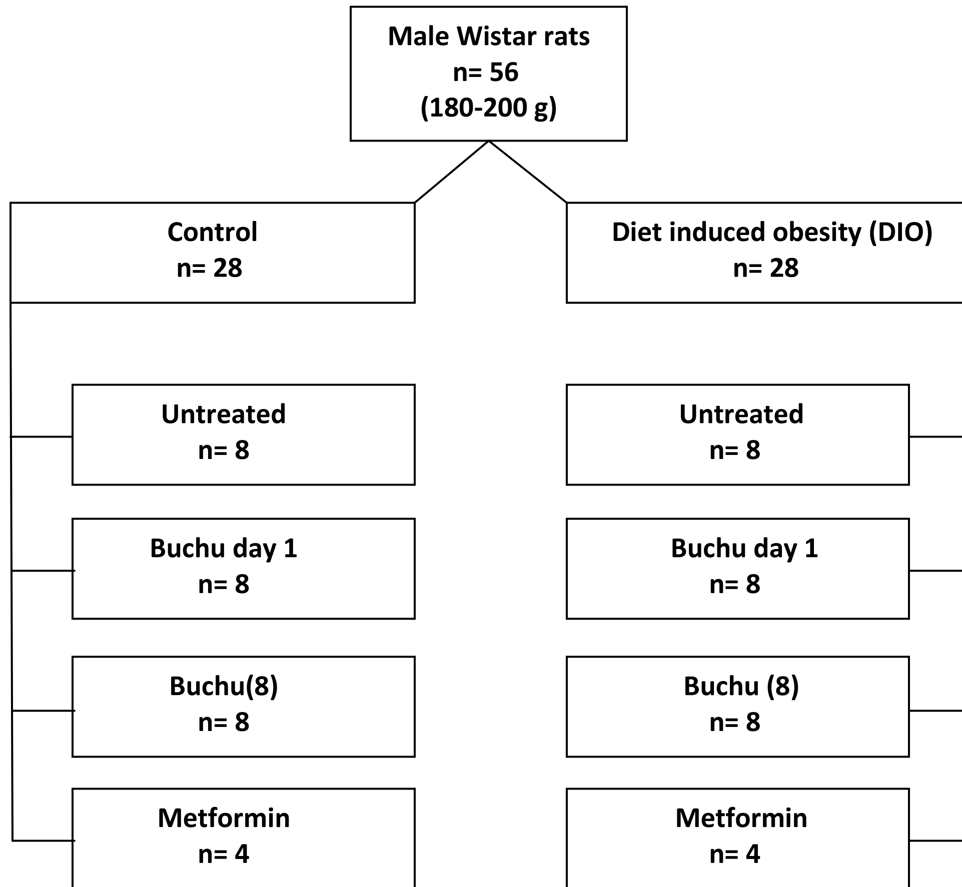



***Figure 14: The time frame of the type 1 diabetic model***

#### 2.3.2. *Diet-induced obese insulin resistant rat model*

In this model a total of 56 animals were used and the experiment was conducted for a period of 16 weeks. Animals weighing between 180 and 200 g were divided into two groups: a control (n=28) and diabetic (n=28) group. The food and water intake of the animals were monitored on a daily basis. Animals were put on a high caloric diet containing normal rat chow supplemented with sucrose and condensed milk for a period of 16 weeks. Treatment with Buchu water commenced either at the onset of the change in diet or from week 8 after onset. A group of animals were treated with metformin as a positive control. Animals in this model were divided according to Figure 15.




***Buchu(1)*** ***: Buchu treatment from day 1***
***Buchu (8)*** ***: Buchu treatment from 8 weeks***
***Metformin*** ***: Metformin treatment from 8 weeks***

***Figure 15: Schematic representation of the division of type 2 diabetic animals***

Stellenbosch University https://scholar.sun.ac.za

Metformin treatment was given at a dose of 250 mg/kg/day by oral gavage. At week 15, IPGTTs' (procedure described above) were performed on all animals to test whole-body insulin sensitivity. A milliliter of fasting blood was collected for insulin determination. After recuperating for one week (week 16), animals were sacrificed via a 160 mg/kg sodium pentobarbital IP injection. Body weight and IP fat were measured and, blood samples were collected for biochemical analysis. Both soleus muscles were removed for determination of insulin sensitivity. The pancreas was removed and stored in liquid nitrogen for further biochemical analysis.

### 2.4. *Western blot analysis*

#### 2.4.1. *Sample preparation*

Total proteins are extracted from pancreatic tissue of diet induced obese insulin resistant animals followed by Western blot analysis used to investigate the expression of certain proteins of interest. The extraction of proteins was made possible by means of a lysis buffer which consisted of a wide variety of reagents which inhibited phosphatases and enzymes involved in proteolysis. Table 4 summarizes the list of the reagents, their stock concentrations and purpose.

Stellenbosch University https://scholar.sun.ac.za

***Table 4: List of reagents for the lysis buffer***

| Stock reagents | Content | Function |
|---|---|---|
| 200 mM Tris-HCL | 20 mM | Buffer with a pH of 7.5 |
| 10 mM EGTA | 1 mM | A chelating agent that acts as a metallo-protease inhibitor |
| 100 mM EDTA | 1 mM | Metallo-protease inhibitor |
| 1M NaCl | 150 mM | Maintain ionic strength of medium |
| 1 mM β-glycerophosphate | 1 mM | Non-specific phosphatase inhibitor |
| 2.5 mM tetrasodiumpyrophosphate | 2.5 mM | Non-specific phosphatase inhibitor |
| 100 mM sodium orthovanadate | 1 mM | An inhibitor of protein tyrosine phosphatases, alkaline phosphatases and various ATPases |
| 100% Triton-X100 | 1% | Non-ionic detergent for solubilizing membrane proteins |
| 10 μg/ml Leupeptin | 10 μg/ml | Protease inhibitor |
| 10 μg/ml Aprotinin | 10 μg/ml | Serine protease inhibitor |
| 100 mM phenylmethyl sulfonyl fluoride | 50 μg/ml | Irreversible serine protease inhibitor |

A Polytron PT10 homogenizer was used to homogenize tissue in 0.9 ml of ice cold lysis buffer. These samples were homogenized twice for 5 seconds on setting 4. The homogenized samples were placed on ice for 15 minutes, allowing the buffer to fully compromise cellular integrity. Once the samples have settled, the homogenates were transferred to specifically marked eppendorf tubes and centrifuged (14 000 rpm for 10 minutes), at 4°C. After centrifugation, the pellet was discarded and the supernatant transferred to a new eppendorf tube.

Stellenbosch University https://scholar.sun.ac.za

### 2.4.2. *Bradford protein determination assay*

Aliquots of the supernatant were used to determine protein concentrations of each sample, using the Bradford assay (Bradford, 1976). Tubes were arranged in a rack according to Figure 16.

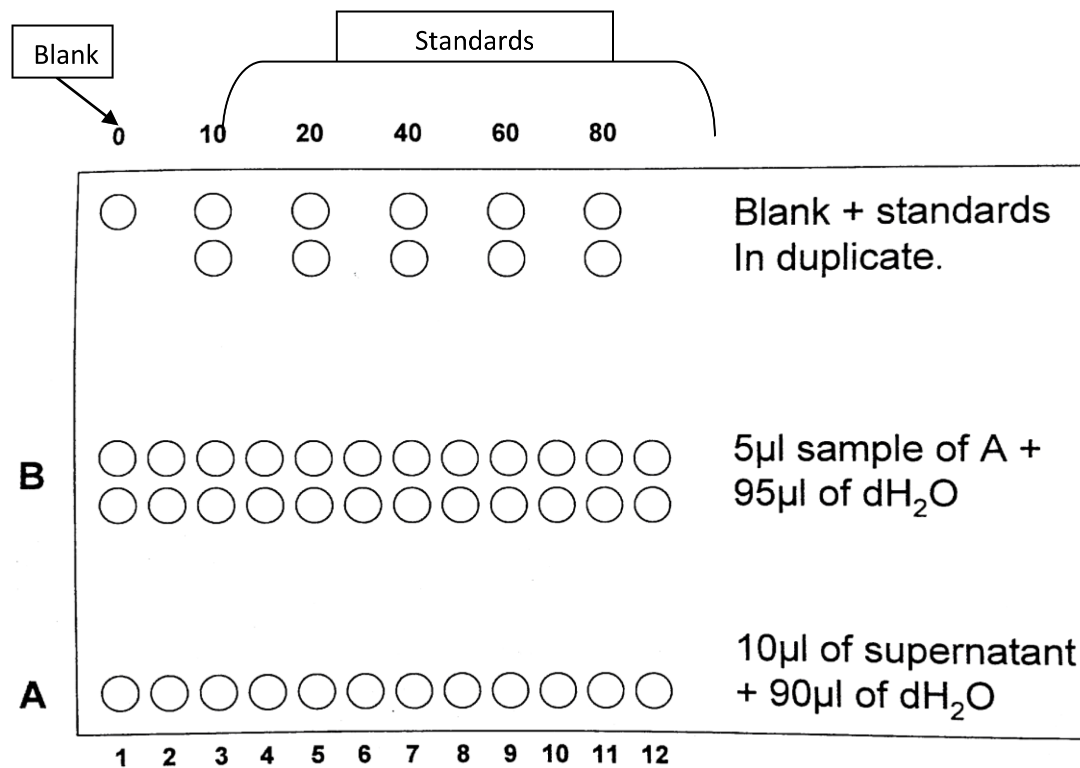



***Figure 16: Tube arrangement for the Bradford protein assay***

The supernatant obtained as described in section 2.4.1. was pipetted into tubes within line up **A**. A 1:10 dilution of the supernatant was made by the addition of $dH_2O$ to dilute the detergent which would otherwise interfere with the assay. In line up **B** 5 µl of each diluted sample was pipetted accordingly, with the addition of 95 µl of $H_2O$**.** The blank tube contained 100 µl of $H_2O$. Diluted bovine serum albumin (BSA) of a known concentration, containing protein concentrations of 1-20 µg, were aliquoted into protein

Stellenbosch University https://scholar.sun.ac.za

determination tubes. The volume pipetted into each was (µl): 10, 20, 40, 60 and 80, followed by the addition of $dH_2O$, making a final volume of 100 µl. A 1:5 dilution of Bradford reagent (900 µl) was added to the blank, standards and tubes in line up **B**. Samples were left for 15 minutes to allow proper colour development then read spectrophotometrically at an absorbance wavelength of 595 nm. The protein content in each sample was calculated from the standard curve established by BSA. Samples were diluted with lysis buffer to render equal protein concentrations, and then diluted with Laemmli sample buffer. Aliquots were boiled for 5 minutes and stored at - 80°C until required.

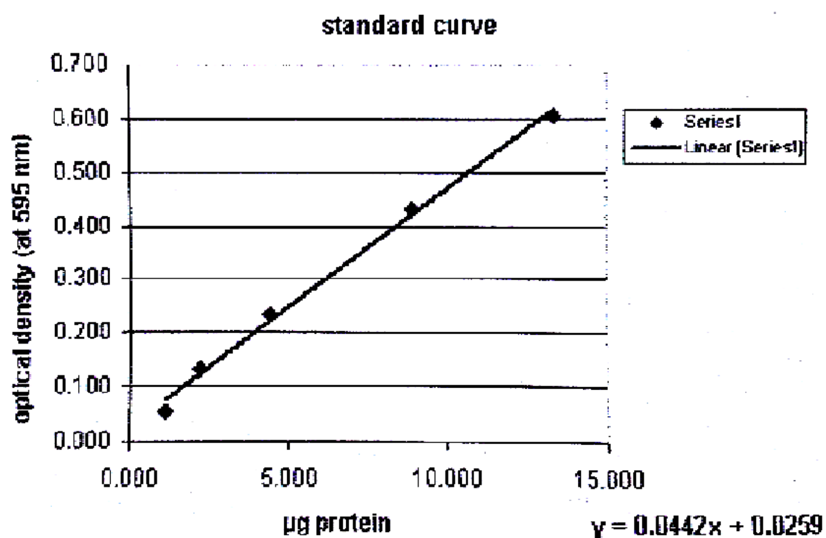



***Figure 17: Standard curve generated from the optical density readings***

Stellenbosch University https://scholar.sun.ac.za

### 2.4.3. *Protein separation by gel electrophoresis*

Stored aliquots were boiled for 5 minutes, then microfuged for 2 minutes at 15 000 rpm to remove particulate matter. The polyacrylamide gel was prepared and samples were loaded (Table 5 shows the proteins used, their concentrations and percentage polyacrylamide gel utilized). A protein ladder was included to serve as a marker to identify the molecular weight of a particular protein. These gels were then exposed to electrophoresis (SDS-PAGE) in running buffer containing 50 mM Tris, 384 mM glycine and 1% SDS, ensuring proper protein separation. A BioRad Mini Protean III system was utilized. Proteins were separated using two different settings: 1$^{st}$ run at 100 V, 200 mA for 10 minutes and 2$^{nd}$ run at 200 V, 200 mA for 50 minutes.

### 2.4.4. *Transfer of proteins to a membrane*

Running buffer was discarded and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon$^{TM}$ P, Millipore). The transfer of proteins to the PVDF membrane was accomplished in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at an electrical current of 200V for 1 hour. At the end of the transfer period, the membrane was soaked in methanol for 30 seconds and allowed to air dry. Membranes were stained with Ponçeau Red reversible stain to visualize the transfer of proteins as well as equal loading.

### 2.4.5. *Blocking non-specific sites with milk*

Ponçeau Red was then rinsed off with TBS-tween (Tris-buffered saline containing 0.1% Tween-20) repeatedly followed by the membranes being blocked, non-specifically, with

Stellenbosch University https://scholar.sun.ac.za

5% TBS-tween fat-free milk (5% fat-free milk in Tris-buffered saline- 0.1% Tween 20) for 2 hours at room temperature. After the blocking period, membranes were rinsed with TBS-tween and then probed with primary antibodies directed against specific proteins (Table 5) overnight at 4°C.

### 2.4.6. *Immunodetection of proteins*

After being incubated overnight at 4°C, the membranes were washed with TBS-tween to remove any unbound primary antibody and incubated with secondary antibody (anti-rabbit immunoglobulin G, Horseradish-peroxidase conjugated secondary antibody) for one hour at room temperature. This enables the secondary antibody to bind to the already bound primary antibody. At the end of the incubation period, membranes were rinsed with TBS-tween and then covered with an enhanced chemiluminescence (ECL) detection reagent for 1 minute. The horseradish-peroxidase coupled to the secondary antibody in conjunction with the ECL chemiluminescent agent facilitates protein visualization by producing luminescence in proportion to the amount of protein present. Light emission was captured on radiography film and analyzed by laser scanning densitometry and suitable software (Silk Scientific Inc, USA).

In all instances, the mean of the total pixels of the bands generated by controls on a specific blot was set as 1 and used to normalize data as a ratio to controls.

Stellenbosch University https://scholar.sun.ac.za

***Table 5: Western blot analysis***

| Protein | Molecular weight | Protein loaded | Polyacrylamide stacking gel | Separation gel | Blocking solution dilutions | Primary antibody dilutions | Secondary antibody dilutions |
|---|---|---|---|---|---|---|---|
| MafA | 36 -47kDA | 60µg | 4% | 10% | 5% milk in TBS/Tween | 20µl/5ml in TBS-Tween | 3µl/20ml in 1% milk |
| Pdx-1 | 42kDA | 60µg | 4% | 10% | 5% milk in TBS/Tween | 5µl/5ml in TBS-Tween | 5µl/20ml in 2.5% milk |

### 2.5. *2-Deoxy-D-3[H] glucose (2DG) uptake by soleus muscle*

The soleus muscles were rapidly isolated and sliced into five thin longitudinal strips. Each strip was placed into a specific flat-bottomed incubation tube containing incubation medium and various other reagents (Table 6). The tubes were gassed with 5% carbon dioxide and 95% oxygen. The incubation medium consisted of: 6 mM KCl, 1 mM $Na_2HPO_4$, 0.2 mM $NaH_2PO_4$, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM HEPES, 1.25 mM $CaCl_2$, 1 mM D-glucose, 2 mM pyruvate and 1% fatty acid free BSA (pH 7.4). The incubation tubes were placed in a shaking waterbath at a temperature of 37°C for 15 minutes. After 15 minutes, insulin was added from a 10 µM stock solution to specific tubes to render 1 nM, 10 nM and 100 nM insulin, and incubated for a further 30 minutes in the waterbath. Soleus muscle strips were incubated with 1.5 µCi/ml [$^3$H]2DG, in a final concentration of 1.8 µM deoxyglucose, for an additional 30 minutes. To stop the glucose uptake reaction, phloretin from a stock solution (final concentration of 400 µM) was added to all tubes which were then removed from the waterbath. Phloretin is a compound which inhibits glucose uptake by inhibiting glucose transporters. Muscle

Stellenbosch University https://scholar.sun.ac.za

strips were washed 5 times with incubation medium, transferred to eppendorf tubes filled with 500 µl of 1M NaOH. The muscle was fully dissolved in the NaOH solution when placed into a 70°C waterbath for 1 hour. Finally, 500 µl of distilled water was added to each sample, resulting in a final NaOH concentration of 0.5 M. At this stage radioactivity within the tissue as well as the protein content could be determined. An aliquot of the homogeneous solution (200 µl) of each sample was mixed with 2 ml of scintillation fluid in counting vials which were stored overnight in the dark before being counted by a beta-scintillation counter.

The protein content of each sample was determined by the Lowry method [Lowry *et al.*, 1951] using a standard curve of known BSA concentrations and a 0.5 M NaOH blank. The protein assay was performed in duplicate with each tube containing 50 µl of a blank, standard or sample. Once the blank, standards and samples were loaded; 1 ml of reaction buffer was pipetted into each tube which was then vortexed, and allowed to react for 10 minutes at room temperature. The reaction buffer consisted of a 2% $Na_2CO_3$, 1% $CuSO_4.5H_2O$ and 2% NaK-tartrate mixture. After 10 minutes, 100 µl Folin Ceiocalteus reagent (Merck) (1:2 dilution with distilled water) was added, vortexed and left to stand for 30 minutes for full colour development. Absorbance was read at 750 nm against the blank. At the end of the assay a standard curve was drawn and unknown concentrations were determined.

Stellenbosch University https://scholar.sun.ac.za

***Table 6: Composition of each tube in the glucose uptake protocol***

| | **Basal (µl)** (Tube 1) | **Phloretin (µl)** (Tube 2) | **1nM Insulin (µl)** (Tube 3) | **10nM Insulin (µl)** (Tube 4) | **100nM Insulin (µl)** (Tube 5) |
|---|---|---|---|---|---|
| **Soleus muscle** | Strip | Strip | Strip | Strip | Strip |
| **Incubation medium** | 675 | 675 | 600 | 600 | 600 |
| **Phloretin** | 0 | 50 | 0 | 0 | 0 |
| **Insulin** | 0 | 0 | 75 | 75 | 75 |
| **2-deoxy-D-[$^3$H] glucose** | 25 | 25 | 25 | 25 | 25 |
| **Phloretin (block)** | 50 | 0 | 50 | 50 | 50 |
| **Total (µl)** | 750 | 750 | 750 | 750 | 750 |

**All samples were analysed in duplicate, a total of 10 tubes per assay.**

Stellenbosch University https://scholar.sun.ac.za

## 2.6. *Biochemical analysis*

### 2.6.1. *Blood sample collection*

Blood samples were allowed to stand on ice for 30 - 60 minutes to permit clotting. Samples were then subjected to centrifugation for 10 minutes at 14 000 rpm at 4°C. The serum was then pipetted into eppendorf tubes and stored at -80°C for further biochemical analysis.

### 2.6.2. *Serum insulin determination*

The Coat-A-Count Insulin assay (Count-A-Count®Insulin Diagnostics Corporation; LA, USA) is a solid phase $^{125}$I radioimmunoassay designed to quantitatively measure insulin in serum. In this assay $^{125}$I-labeled insulin and insulin in the serum samples compete for sites on insulin-specific antibodies. These antibodies are immobilized to the wall of polypropylene tubes and competition can be terminated by simply decanting the supernatant. This step isolates the antibody bound fraction of radio-labelled insulin.

Components of the entire procedure were brought to room temperature (15-28°C), prior to the onset of the assay. All samples were analyzed in duplicate. Plain uncoated polypropylene tubes (12 x 75 mm) were labeled for total counts (T) and non-specific binding (NSB), respectively. Fourteen insulin-antibody coated tubes were labeled for standards and additional tubes were labeled for samples. The addition of insulin calibrators and iodinated insulin ($^{125}$I insulin) are depicted below.

***Table 7: A representation of calibrators***

| Calibrator | Approximate µIU/mL 1st IRP [66/304] |
|---|---|
| A | 0 |
| B | 5 |
| C | 15 |
| D | 50 |
| E | 100 |
| F | 200 |
| G | 350 |

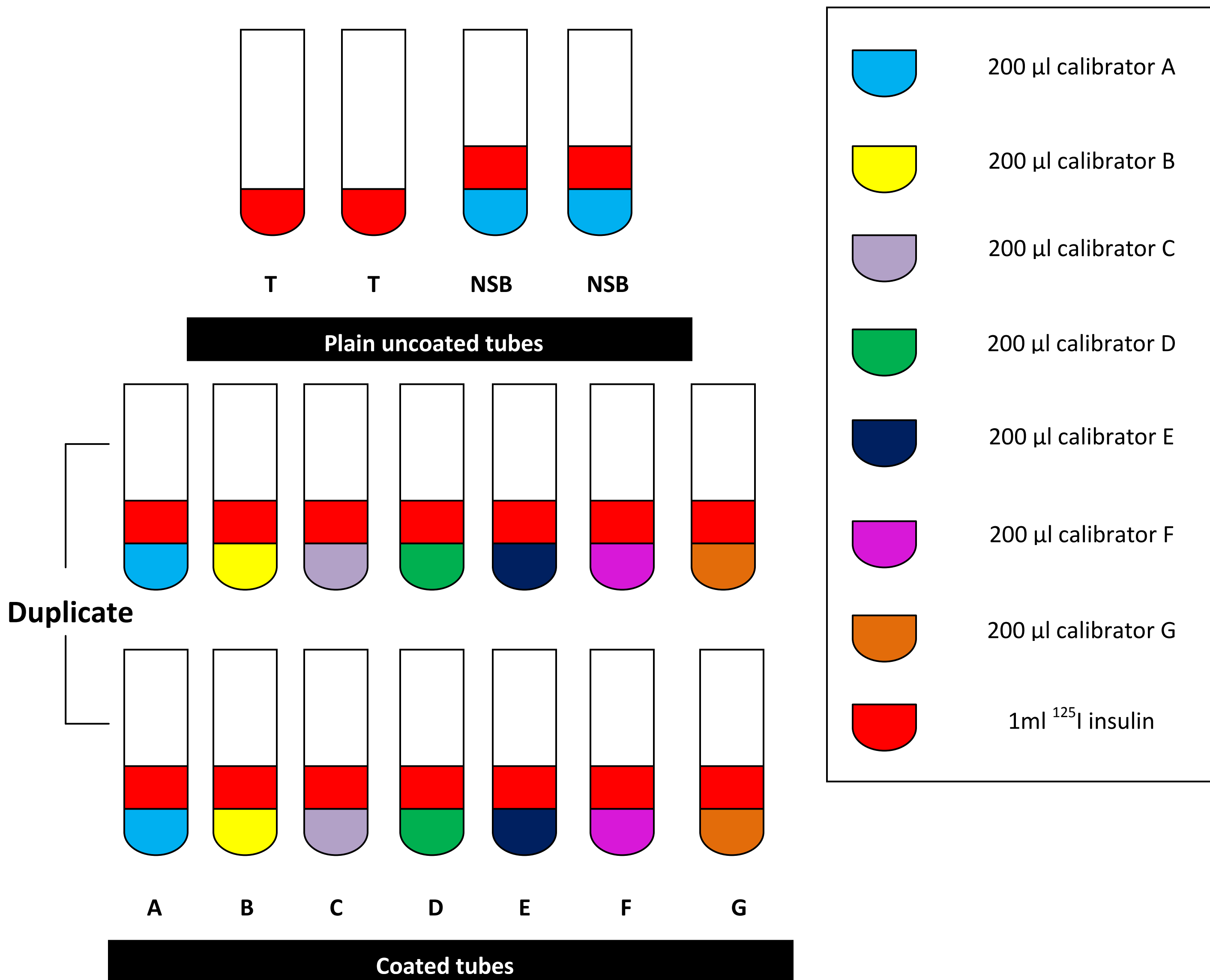
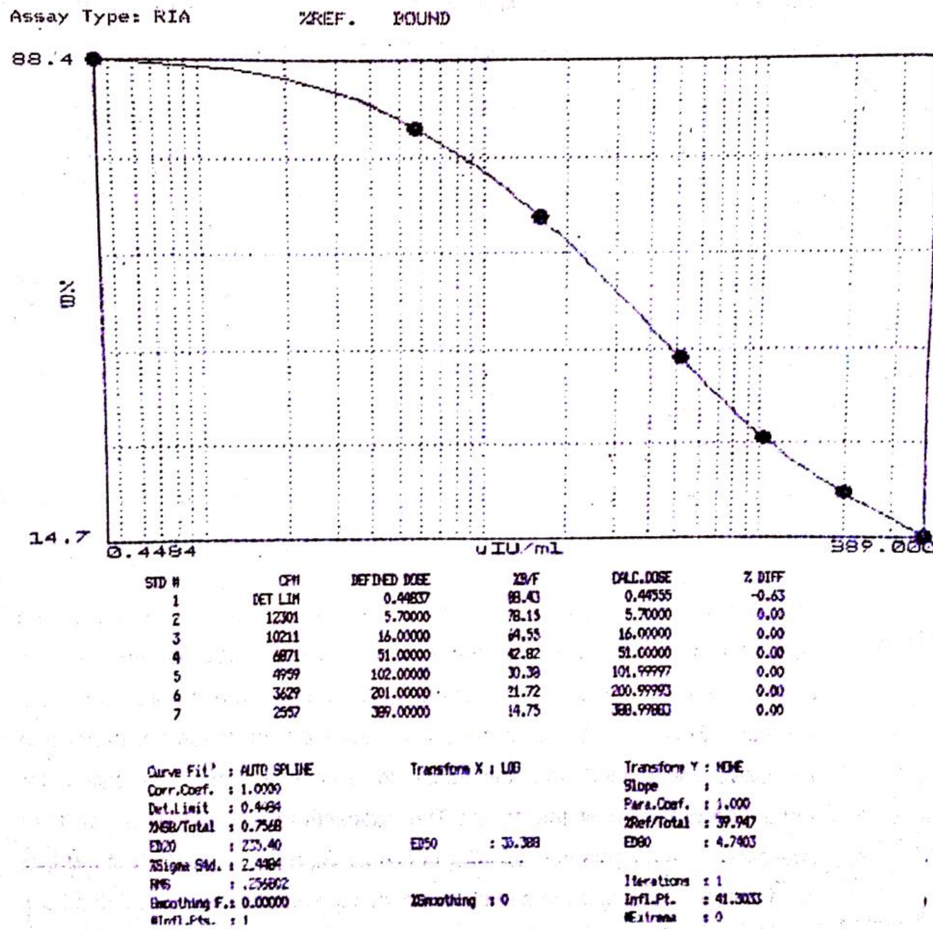



***Figure 18: Diagrammatic layout of tube labelling and preparation***

200 µl of standards or samples in duplicate, were pipetted into the bottom of the reaction tubes. Here after, the radio-labelled insulin was added, and the tubes were incubated for 18-24 hours at room temperature. After the elapsed time, samples were decanted thoroughly using a decanting rack, then struck repeatedly on absorbent paper to remove excess residue. Tubes were carefully dried to remove all traces of unbound radioactive material. Radioactivity of each tube was counted for 1 minute in a gamma counter. The standard curve (Figure 18: an example of a standard curve) formed a displacement curve of radio activity and the sample values were calculated from this.




| STD # | CPM | DEFINED DOSE | %B/F | CALC.DOSE | % DIFF |
|---|---|---|---|---|---|
| 1 | DET LIM | 0.44837 | 88.40 | 0.44555 | -0.63 |
| 2 | 12301 | 5.70000 | 78.15 | 5.70000 | 0.00 |
| 3 | 10211 | 16.00000 | 64.55 | 16.00000 | 0.00 |
| 4 | 6871 | 51.00000 | 42.82 | 51.00000 | 0.00 |
| 5 | 4959 | 102.00000 | 30.38 | 101.99997 | 0.00 |
| 6 | 3629 | 201.00000 | 21.72 | 200.99993 | 0.00 |
| 7 | 2557 | 389.00000 | 14.75 | 388.99883 | 0.00 |

Curve Fit : AUTO SPLINE
Corr.Coef. : 1.0000
Det.Limit : 0.4484
%NSB/Total : 0.7568
ED20 : 235.40
%Sigma Std. : 2.4484
RMS : .256802
Smoothing F. : 0.00000
#Infl.Pts. : 1

Transform X : LOG
ED50 : 35.388
%Smoothing : 0

Transform Y : NONE
Slope :
Para.Coef. : 1.000
%Ref/Total : 39.947
ED80 : 4.7403
Iterations : 1
Infl.Pt. : 41.3033
#Extrema : 0

***Figure 19: Standard curve generated by the gamma counter***

### 2.6.3. *Enzyme Linked Immunosorbent Assay (ELISA)*

Previously stored serum was utilized to determine C-peptide levels of each sample using an Elisa kit (Abcam Ltd, Cambridge, UK). In this assay, C-peptide molecules in the sample bind to the wells of a microtiter plate, coated with a pre-titered amount of anchor antibodies. Binding of a second biotinylated antibody to insulin occur simultaneously. The microtiter plate consists of 96 wells which were divided into blanks, standards, quality control (QC) and samples as seen in Table 8.

***Table 8: Microtiter plate arrangement***

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Blank | 200 pM | QC1 | Etc. | | | | | | | | |
| B | Blank | 200 pM | QC1 | Etc. | | | | | | | | |
| C | 25 pM | 400 pM | QC2 | | | | | | | | | |
| D | 25 pM | 400 pM | QC2 | | | | | | | | | |
| E | 50 pM | 800 pM | Sample 1 | | | | | | | | | |
| F | 50 pM | 800 pM | Sample 1 | | | | | | | | | |
| G | 100 pM | 1,600 pM | Sample 2 | | | | | | | | | |
| H | 100 pM | 1,600 pM | Sample 2 | | | | | | | | | |

**This assay was done in duplicate**

**Washing procedure**: Wells were washed three times with 300 µl of diluted (1x) wash buffer and then decanted. The remaining wash buffer was removed by inverting the plate and tapping it on absorbent towels several times.

Stellenbosch University https://scholar.sun.ac.za

Matrix solution (20 µl) was added to blank, standard and quality control wells. Followed by 30 µl of assay buffer to blank and sample wells. Standard and QC wells were filled with 10 µl of assay buffer. Rat insulin standards (20 µl) were added to specific wells in ascending order while 20 µl of QC1 and 20 µl of QC2 were added to wells labeled QC. The remaining wells were filled with 20 µl of the unknown sample. 50 µl of the antibody solution mixture (a mixture which consists of capture and detection antibody at a 1:1 ratio) were added to each well. The plate was then covered with a plate sealer and incubated for 2 hours at room temperature on an orbital microtiter plate shaker (400-500 rpm). After 2 hours, the plate sealer was removed and the solution decanted from the plate. Residual solution was removed using a tapping motion and wells were washed according to the washing procedure. Enzyme solution (100 µl) was added to each well, the plate covered and incubated on the shaker at room temperature for 30 minutes. Once the time has elapsed, the sealer was removed, the solution decanted and the wells washed according to the washing procedure. Substrate solution (100 µl) was added to each well, the plate sealed and placed on a shaker for approximately 20 minutes. In standard wells, a blue pigment was observed with intensities proportional to increases in insulin concentrations. Stop solution (100 µl) was added to each well, and then shaken by hand to ensure complete mixing of the solution. At this stage, the blue pigment turned into a yellow colour. The intensity of the yellow colour was read at an absorbance value of 450 nm and 590 nm in a plate reader and the two readings subtracted from each other. The standard curve (Figure 20: an example of a standard curve) obtained was used to quantitatively determine the concentration of C-peptides in experimental serum samples.

Stellenbosch University https://scholar.sun.ac.za

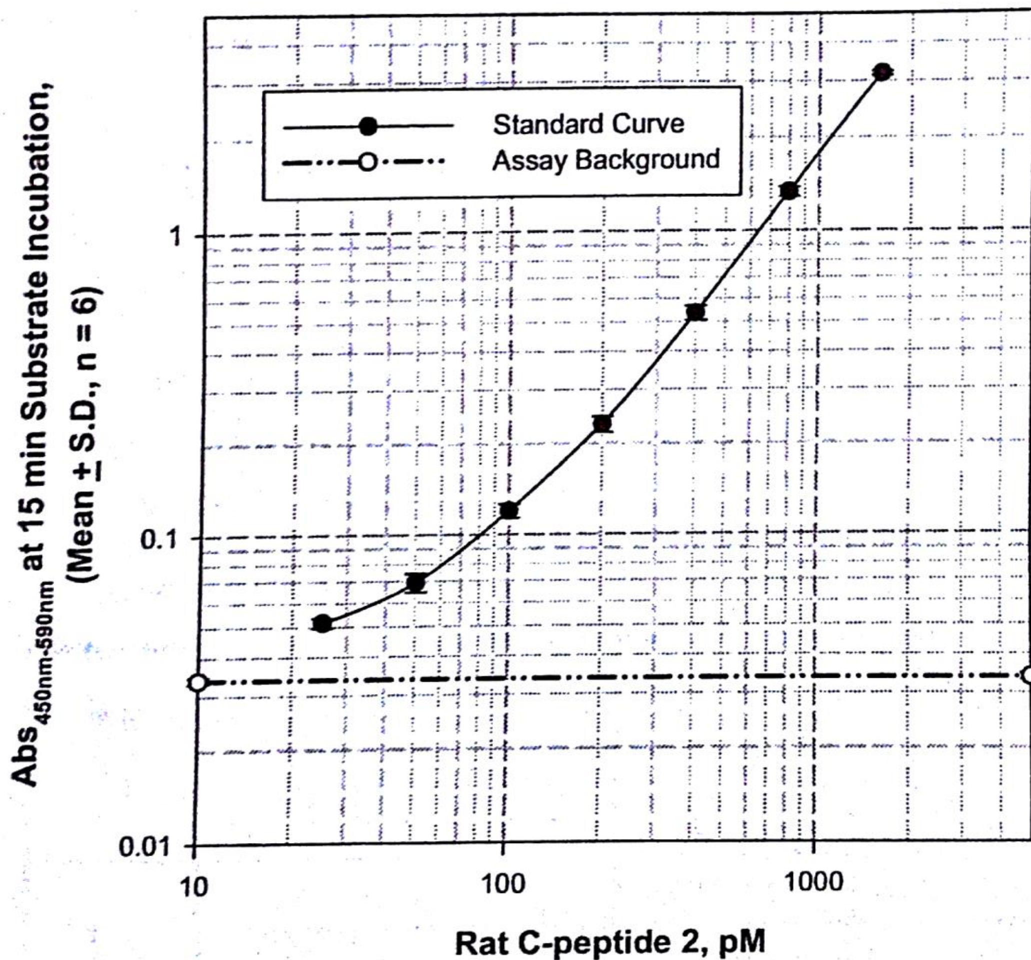



***Figure 20: A graph of a typical standard curve***

## 2.7. *Statistical Analysis*

Statistical analysis of data was performed using GraphPad Prism 5. All values are expressed as the mean ± standard error of the mean (SEM). For multiple comparisons, the ANOVA (two-way when appropriate) followed by the Bonferroni post-hoc test was applied. A p-value of less than 0.05 ($p<0.05$) was considered as statistically significant.

Stellenbosch University https://scholar.sun.ac.za

# CHAPTER 3

# Results

---

## 3.1. *Type 1 Diabetes*

The T1D model (STZ) was induced by using streptozotocin, a glucose moiety which is easily transported into pancreatic β-cells via GLUT 2 carrier proteins [*Wei et al., 2003; Motyl and McCabe, 2009*]. Streptozotocin exerts its toxic effects by damaging DNA, eventually, leading to β-cell dysfunction, hypoinsulinemia and hyperglycemia; all characteristics associated with diabetes [*Motyl and McCabe, 2009*]. Refer back to Figure 14 for an illustration of the experimental procedure.

### 3.1.1. *Biometric parameters*

#### 3.1.1.1. *Food and water/Buchu consumption*

On the first day of STZ administration; control and STZ animals were divided into their respective groups and treated with Buchu water at the start of the experiment (day 1) and three weeks after the initiation of the experiment (Buchu week 3). Each group contained 10 animals, 5 per cage. Animals that spontaneously normalized were excluded from the study. Food and water/Buchu water consumption were monitored for a period of 1 week and 20 days, respectively, 3 weeks after the start of the experiment. When comparing each cage, no significance was observed between any of the groups when inspecting food and water/Buchu water consumption (Figure 21 and Figure 22). However, a decrease in food consumption in STZ and control treated animals were

observed. This could be as a result of Buchu water having effects on the satiety centre thus reducing appetite. The bitter taste of Buchu water could have contributed to the reduced levels of Buchu water consumption seen in STZ and control animals.

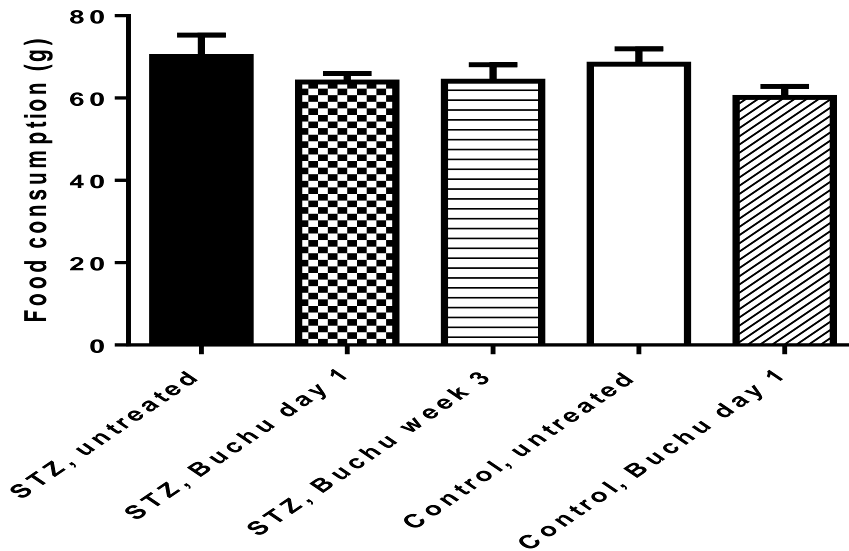



**Figure 21:** ***Food consumed by control and STZ animals, from week 3.*** Displayed is food consumption measured in grams (g) of streptozotocin (STZ) and control animals. The data is presented as a mean ± SEM. (n=10/group)

Stellenbosch University https://scholar.sun.ac.za

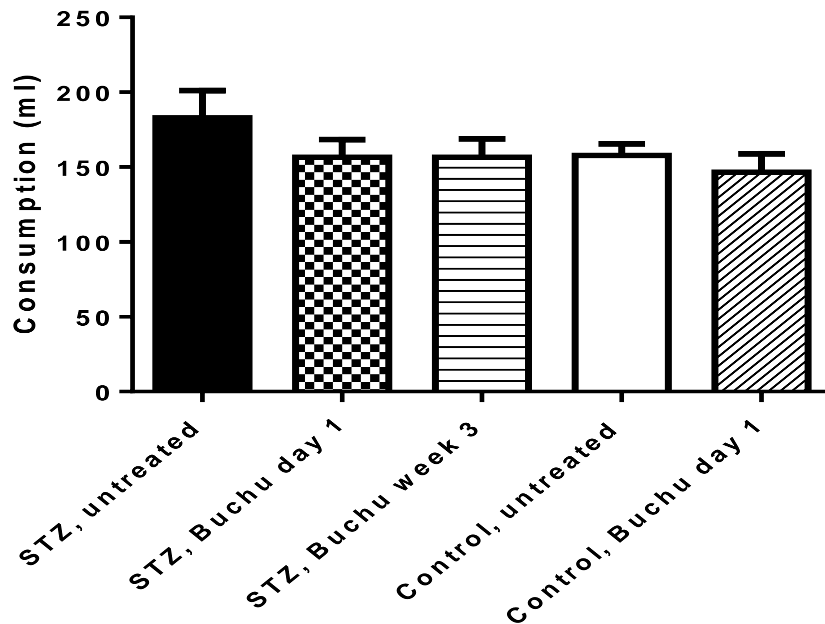



**Figure 22:** ***Water/Buchu water consumed by control and STZ animals, from week 3.*** Displayed is water/Buchu water consumption measured in millilitres (ml) of streptozotocin (STZ) and control animals. The data is presented as a mean ± SEM. (n=10/group)

#### 3.1.1.2. *Body weight and intra-peritoneal fat mass*

After 14 weeks of feeding, animals were anaesthetized and sacrificed. At this time point, their body weight and intra-peritoneal fat mass was determined. Measurements of each animal in their respective groups were recorded and plotted diagrammatically. When comparing each group, significantly lower body weight was observed in the STZ treated vs control animals (284.3 ± 9.9 vs 345.4 ± 7.6 g; $p<0.0001$) (Figure 23). This lower weight was exacerbated by Buchu water ingestion in the STZ animals (284.3 ± 9.9 vs 242.4 ± 10.9 g (day 1; $p<0.05$) and 240.3 ± 9.0g (week 3; $p<0.05$) respectively.

Similarly, control animals treated with Buchu water gained less weight than untreated animals (320.7 ± 6.2 vs 345.4 ± 7.6 g; p<0.05) (Figure 23)

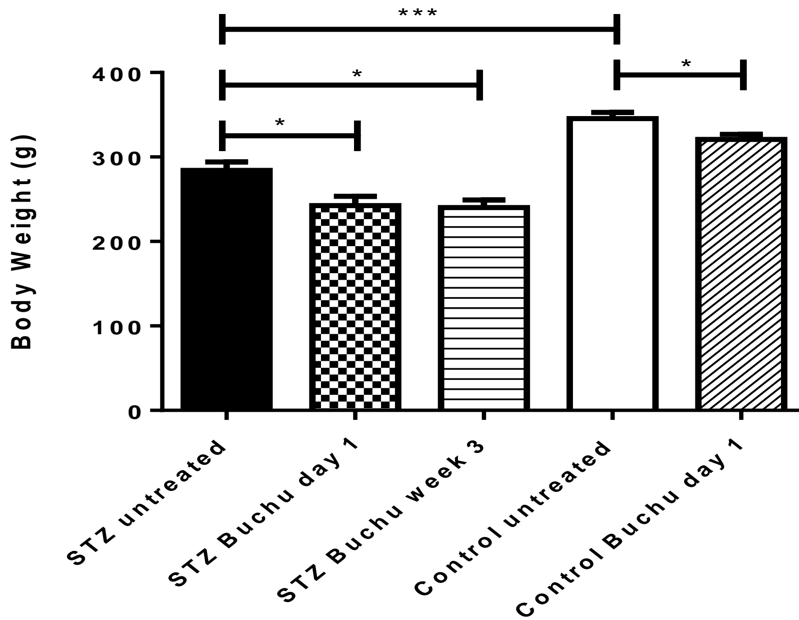



**Figure 23:** ***Body weight (BW) of control and STZ animals, with and without Buchu treatment, after 14 weeks.*** Displayed is body weight measured in grams (g) of streptozotocin (STZ) and control animals. The data is presented as a mean ± SEM. (n=10/group). *p<0.05, ***p<0.0001

A difference in body weight was observed. However, STZ animals treated with Buchu presented with significantly more intra-peritoneal fat than the untreated animals (14.79±2.5g (day 1), 13.41±1.6g (week 3) vs 9.66±0.7g respectively.

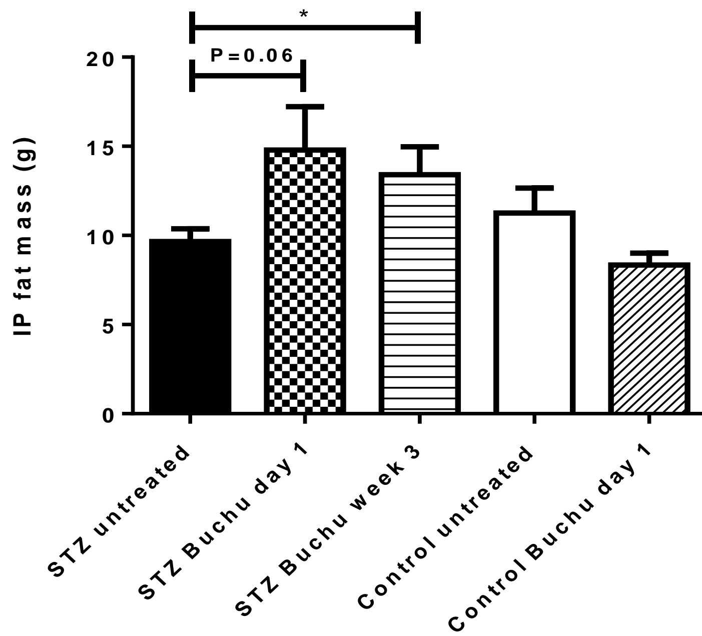



**Figure 24:** ***Intra-peritoneal (IP) fat of control and STZ animals, with and without Buchu treatment, after 14 weeks.*** Displayed is IP fat mass measured in grams (g) of streptozotocin (STZ) and control animals. The data is presented as a mean ± SEM. (n=8/group). *$p<0.05$

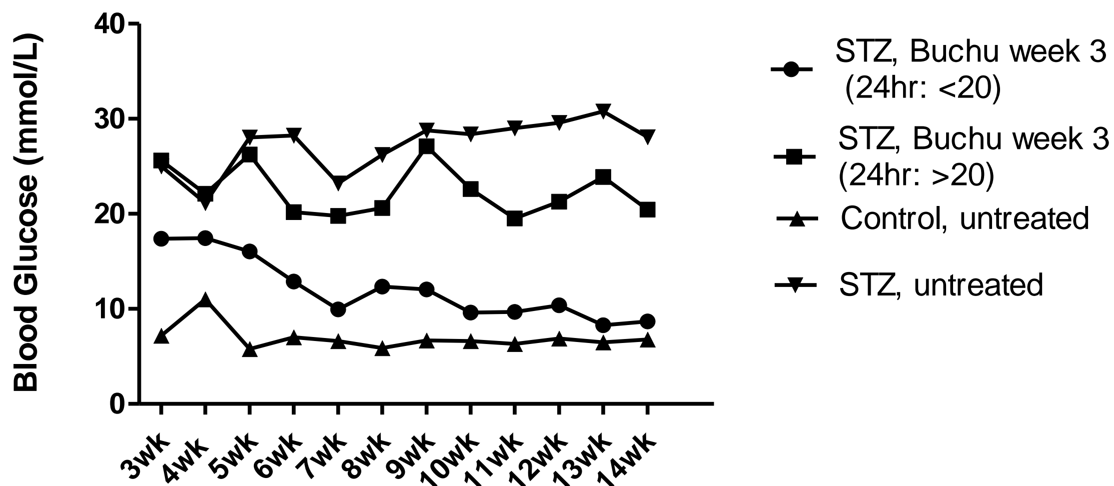
Stellenbosch University https://scholar.sun.ac.za

### 3.1.2. *Metabolic parameters*

#### 3.1.2.1. *Blood Glucose Levels*

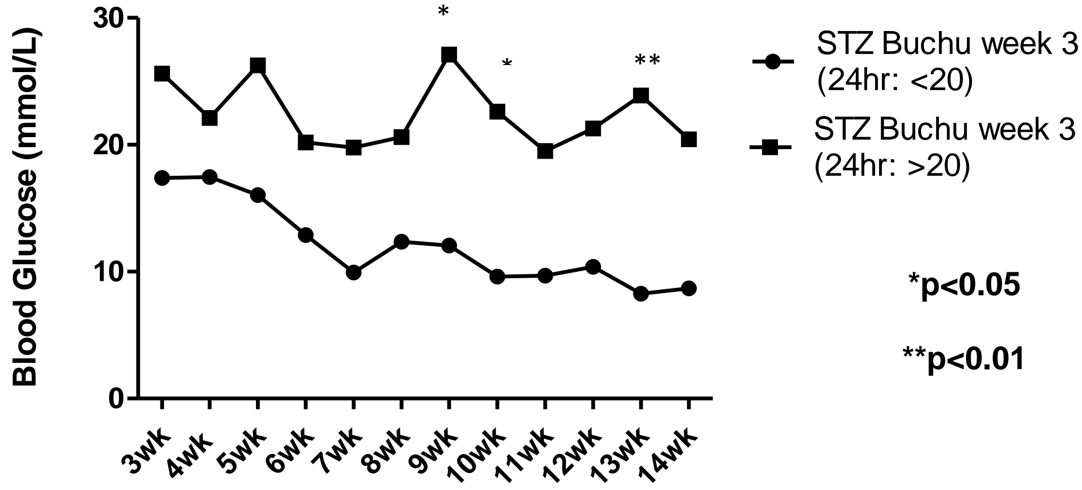
On day 1, animals were divided into 4 groups containing 5 animals per cage. Respective animals were treated with STZ from day 1 and Buchu from week 3. Non-fasting blood glucose levels of all animals were determined weekly for 12 weeks, starting at week 3. A drop of blood from a tail prick was collected. After 14 weeks, blood glucose levels of STZ animals treated with Buchu were significantly lower than untreated STZ animals. A significant decline was also seen in STZ animals with blood glucose levels more than 20 mmol/L at the start of experimentation (Figure 25 (B) and (C)).

**(A)**

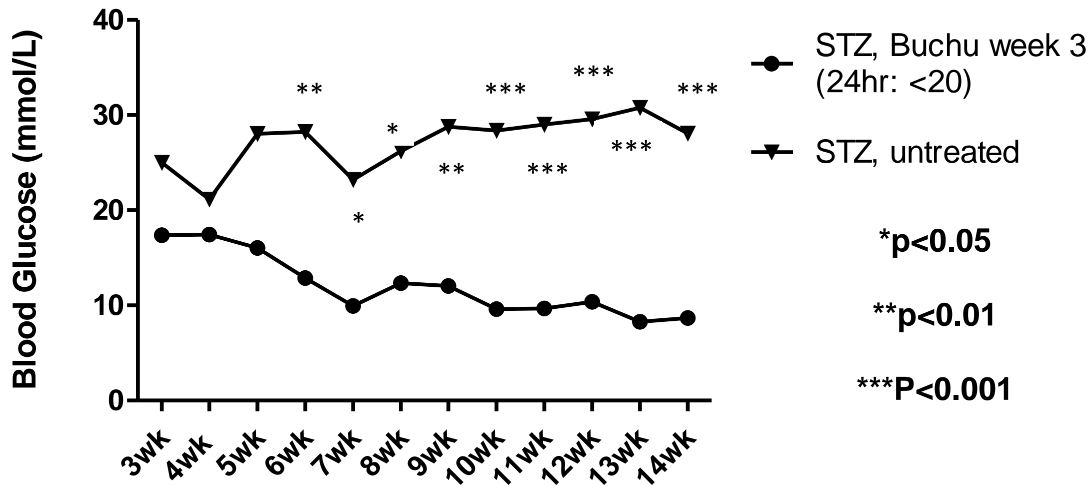


Stellenbosch University https://scholar.sun.ac.za

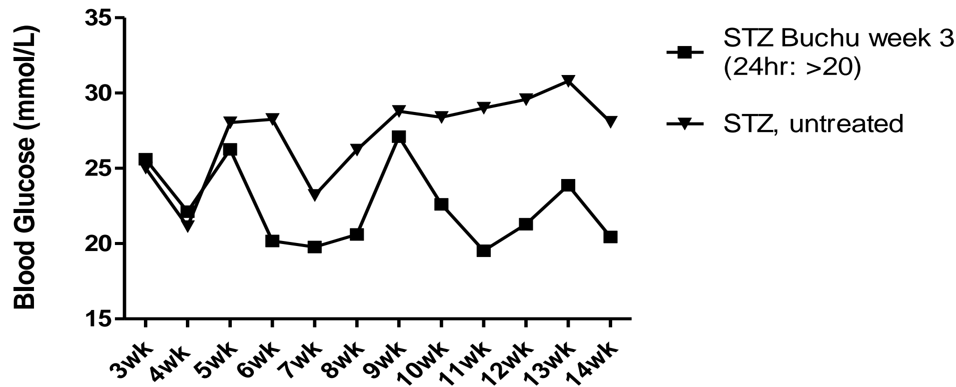
**(B)**




**(C)**



**(D)**




**(E)**

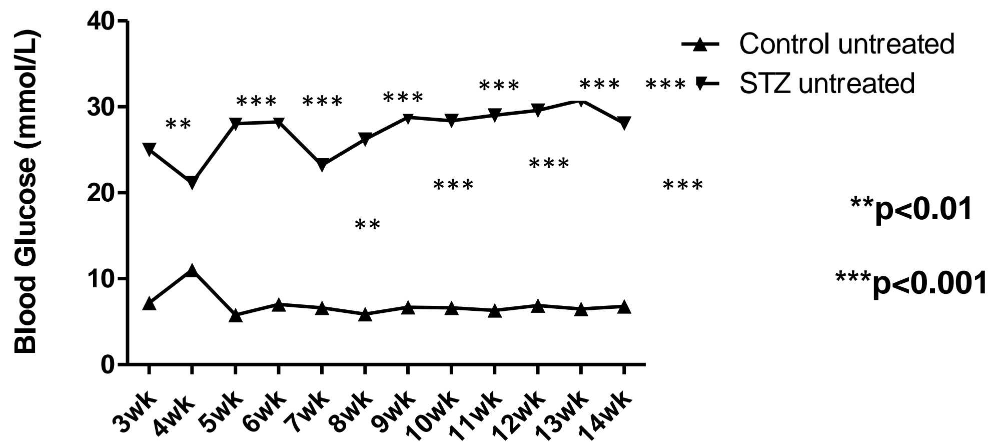


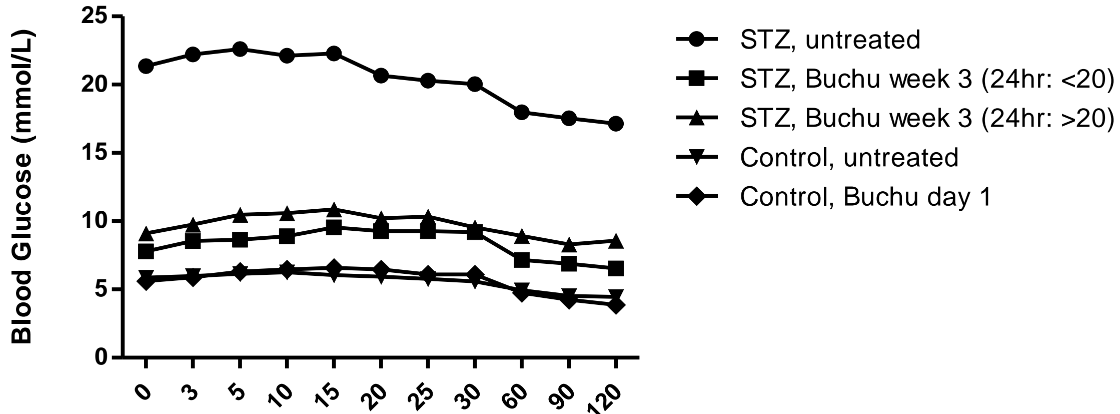

**Figure 25:** ***Weekly blood glucose levels of control and STZ animals, with and without Buchu treatment, for 12 weeks. (*A)** Representation of all groups, **(B)** Comparing treated streptozotocin animals with blood glucose levels at the onset of experimentation greater than (>20) and less than 20 mmol/L (<20), **(C)** Comparing treated streptozotocin animals (blood glucose levels < 20 mmol/L) with untreated streptozotocin animals, **(D)** Comparing untreated streptozotocin animals with treated streptozotocin animals (blood glucose levels > 20 mmol/L), and **(E)** Comparing untreated control and streptozotocin animals. Blood glucose levels were measured in a non-fasting state and expressed in mmol/L. The data is presented as a mean ± SEM. (n= 5/group).

Stellenbosch University https://scholar.sun.ac.za

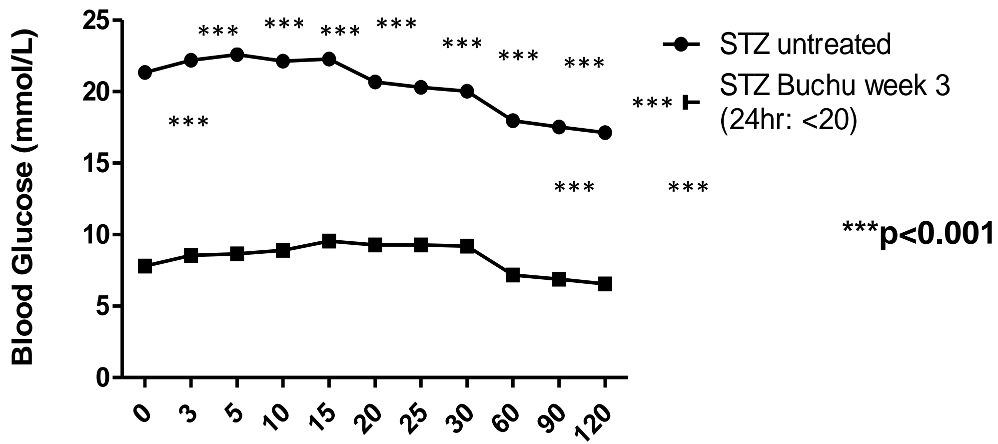
### 3.1.2.2. *Intraperitoneal glucose tolerance test (IPGTT)*

At 13 weeks, all animals following their respective treatment regimes, were fasted overnight. The next day, intraperitoneal glucose tolerance tests were performed on each animal for a period of 2 hours. When comparing treated and untreated controls, no significant difference was seen (Figure 26 (F)). However, a significant decrease (***$p<0.001$) in blood glucose levels were observed when comparing treated and untreated streptozotocin animals (Figure 26 (B) and (C)). At the 2 hour mark, averages of each group were calculated and plotted using a bar graph (Figure 26 (G)). From this graph it is clear that the consumption of Buchu water resulted in an improved ability to handle a glucose load. (STZ=17.1±0.9, STZ+Buchu=7.6±0.9, control=3.8±0.1 and control+Buchu=4.5±0.1mmol/L).

**(A)**

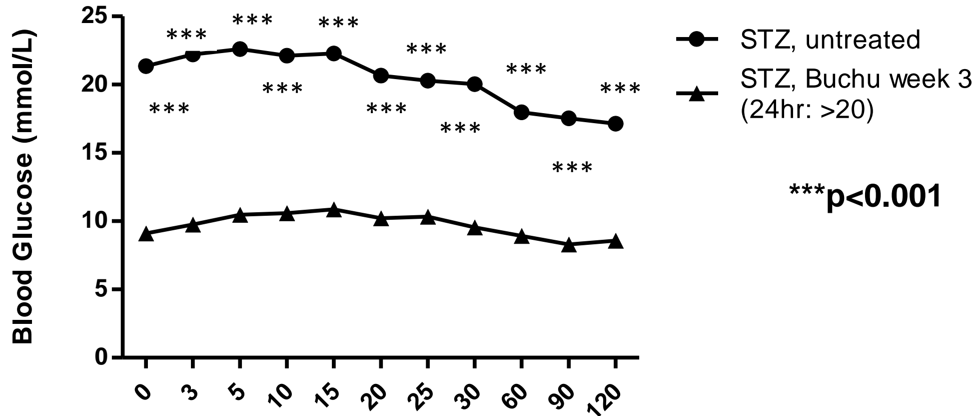



**(B)**

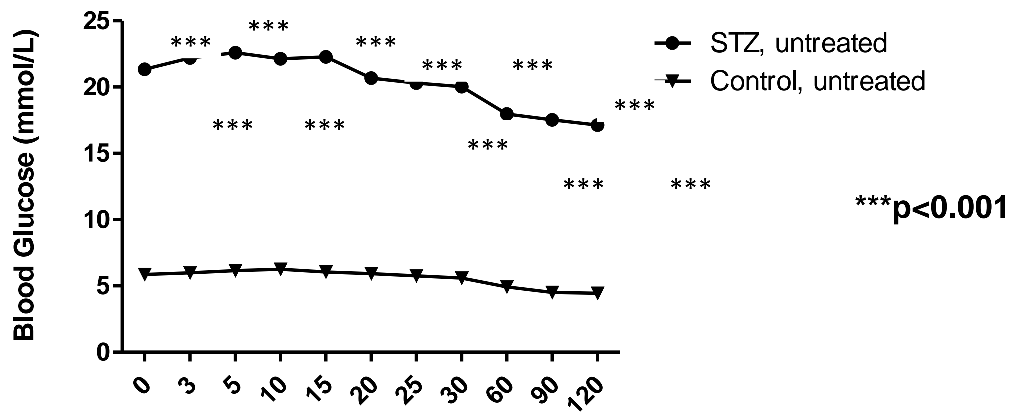


Stellenbosch University https://scholar.sun.ac.za

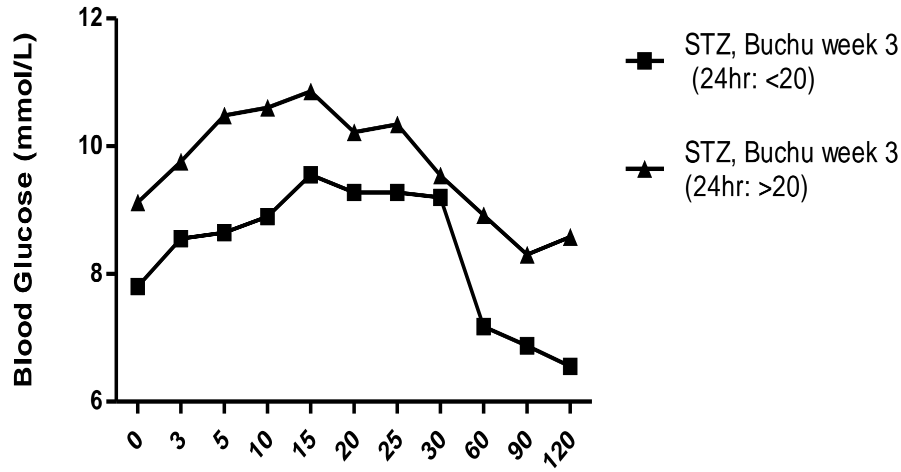
**(C)**



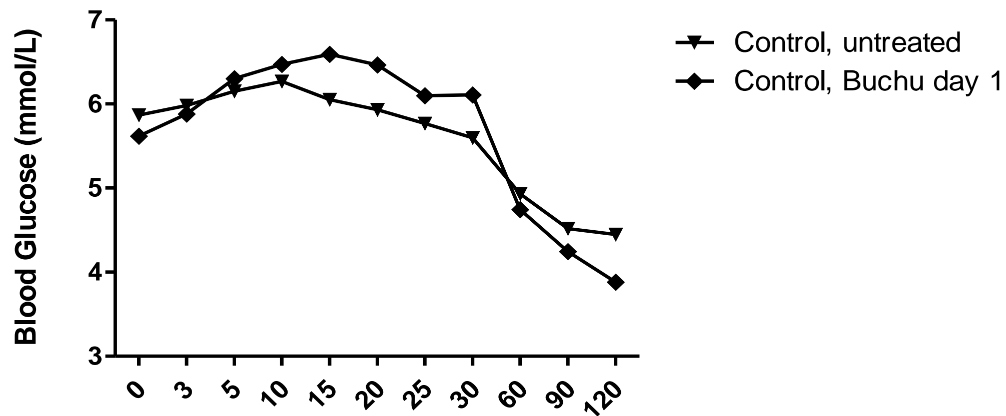

**(D)**



(E)




(F)



**(G)**

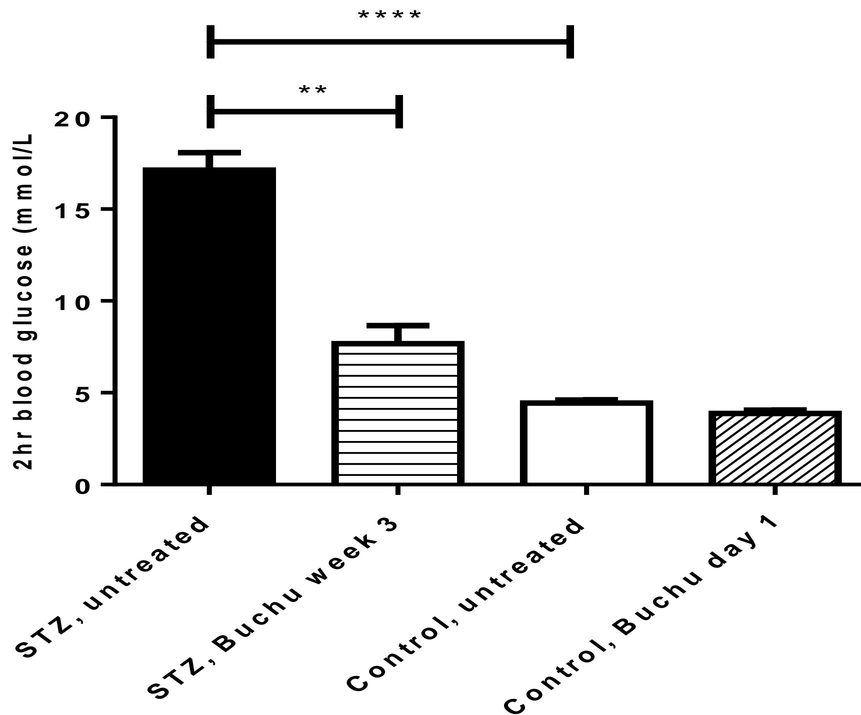



**Figure 26:** ***Intraperitoneal glucose tolerance test of control and STZ animals, with and without Buchu treatment, at 13 weeks.*** **(A)** Representation of all groups, **(B)** Comparing treated streptozotocin animals (blood glucose levels < 20 mmol/L) with untreated streptozotocin animals, **(C)** Comparing treated streptozotocin animals (blood glucose levels > 20 mmol/L) with untreated streptozotocin animals, **(D)** Comparing untreated streptozotocin and control animals, **(E)** Comparing treated streptozotocin animals with blood glucose levels greater than 20 mmol/L and less than 20 mmol/L, **(F)** Comparing treated and untreated control groups, and **(G)** IPGTT 2 hour values of control and streptozotocin groups. Blood glucose levels were measured in mmol/L. The data is presented as a mean ± SEM. (n= 4-10/group). **P<0.01, ****P<0.0001.

Stellenbosch University https://scholar.sun.ac.za

### 3.1.2.3. *Serum Insulin levels*

Following 14 weeks on diet, no significant difference in fasting serum insulin levels were observed when treated and untreated STZ animals were compared, as well as between treated and untreated control animals (Figure 27).

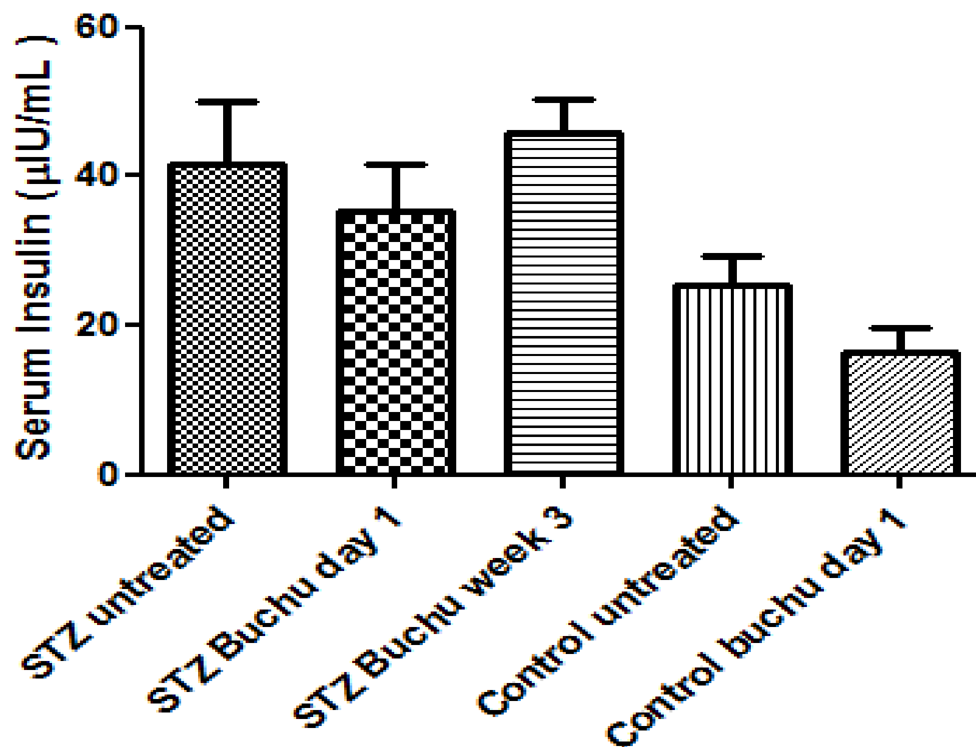



**Figure 27:** ***Fasting serum insulin levels in control and STZ animals, with and without Buchu treatment, after 14 weeks.*** Displayed, are fasting serum insulin levels of treated and untreated streptozotocin (STZ) and control animals. The data is presented as a mean ± SEM. (n=5-7/group).

Stellenbosch University https://scholar.sun.ac.za

### 3.2. *Type 2 Diabetes*

The T2D model (DIO) was generated by feeding animals with a high caloric diet containing normal rat chow supplemented with sucrose and condensed milk for a period of 16 weeks. This diet correlates with changes seen in humans, changing from a rural to a more westernised diet. The diet induced obesity and insulin resistance. Below is a table (Table 9) indicating the macronutrient composition of diets consumed by control and DIO animals.

***Table 9: Diet composition of controls versus DIO animals [Du Toit et al., 2008; Pickavance et al., 1999]***

| | Control | DIO |
|---|---|---|
| **Carbohydrates** | 60% | 65% |
| **Protein** | 30% | 19% |
| **Fat** | 10% | 16% |
| **KJ/day** | ± 380KJ/day | ± 575KJ/day |

Stellenbosch University https://scholar.sun.ac.za

### 3.2.1. *Biometric parameters*

#### 3.2.1.1. *Food and water/Buchu consumption*

On day 1, control and DIO animals were divided up into their respective groups, treated with Buchu at various time points. Each group contained 8 animals, 4 per cage. Food consumption was monitored for a period of 1 week while water/Buchu consumption was monitored daily for 10 days, both readings were recorded from week 8. There were no difference in the amount of food consumed (Figure 28). Furthermore, no significance was observed between any of the groups when inspecting water/Buchu water consumption (Figure 29).

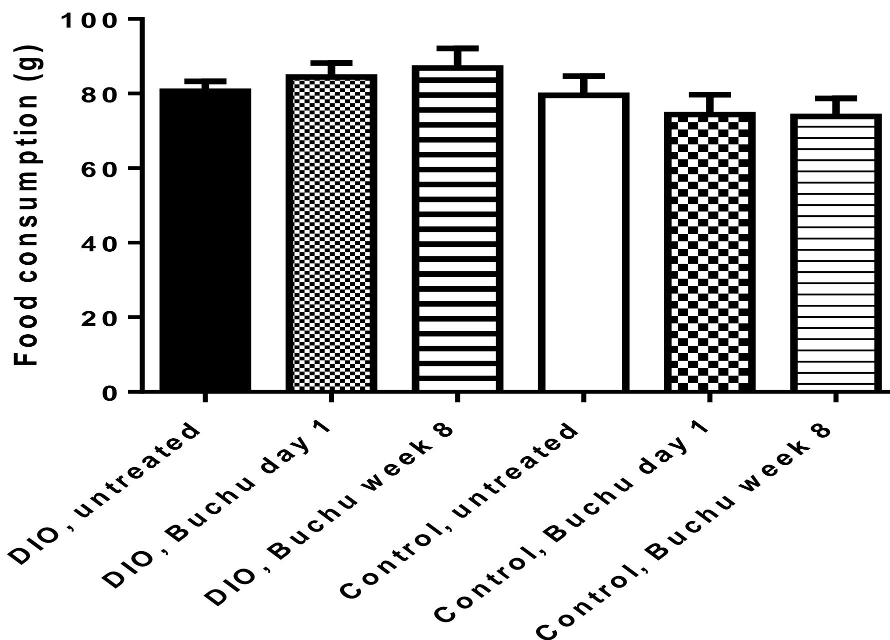



**Figure 28:** ***Food consumed by control and DIO animals, from week 8.*** Displayed is food consumption measured in grams (g) of diet induced obese (DIO) and control animals. The data is presented as a mean ± SEM. (n=8/group).

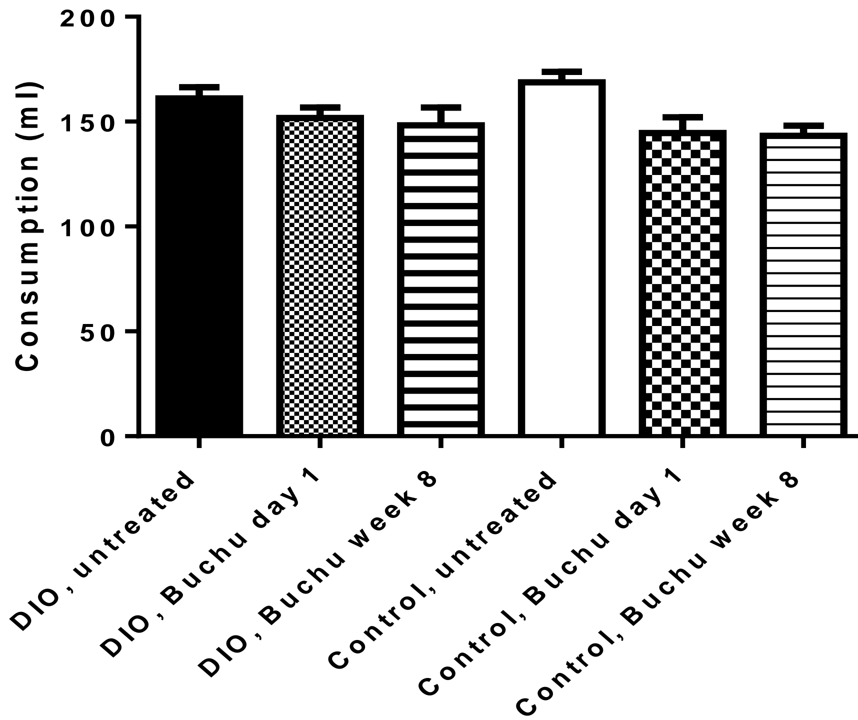



**Figure 29:** ***Water/Buchu water consumed by control and DIO animals, from week 8.*** Displayed is water/Buchu consumption measured in millilitres (ml) of diet induced obese (DIO) and control animals. The data is presented as a mean ± SEM. (n=8/group)

Stellenbosch University https://scholar.sun.ac.za

### 3.2.1.2. *Body weight and intra-peritoneal fat mass*

After 16 weeks of feeding, animals were anaesthetized and sacrificed. Their body weight and intra-peritoneal fat mass were weighed. The DIO animals gained significantly more weight than their control counterparts (507.4±12.3 vs 403.6±20.1g, $p<0.0001$, n=5). When comparing each group, a significant difference in body weight was also observed between untreated and treated DIO animals (507.4±12.3 vs 460.4±8.4 (day 1) and 455±9.7g (wk8) $p<0.05$, n=5/group. Metformin treatment also resulted in significant less weight gain vs the untreated DIO animals (415.8±14.9 vs 507.4±12.3g, $p<0.0001$, n=5/group) (Figure 30).

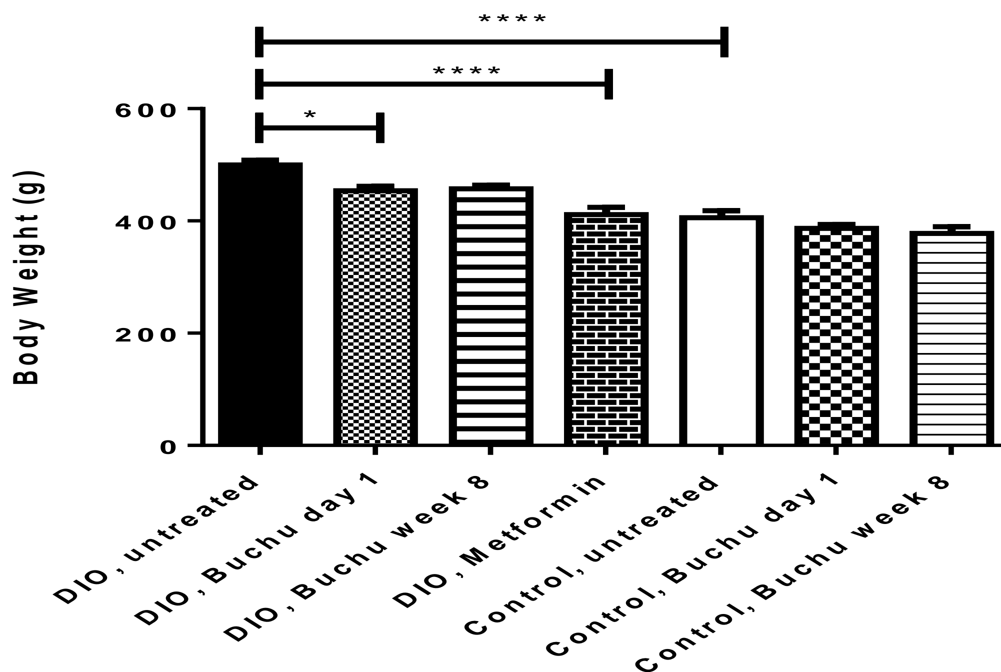



**Figure 30:** ***Body weight (BW) of control and DIO animals, with and without Buchu treatment, after 16 weeks.*** Displayed is body weight measured in grams (g) of diet induced obese (DIO) and control animals. The data is presented as a mean ± SEM. (n=5/group). *$p<0.05$, ****$p<0.0001$.

Stellenbosch University https://scholar.sun.ac.za

The DIO animals presented with significantly more intra-peritoneal fat mass than all the control groups (Figure 31). DIO=24.24±3.8g vs control=10.6±0.9g, control with Buchu from day 1 = 10.4±0.5g and control with Buchu from wk 8 = 9.3±0.5g, $p<0.001$, n=5/group. In addition, metformin resulted in significantly less intra-peritoneal fat mass gain with animals presenting with 12.1±1.3g vs DIO=24.24±3.8g, $p<0.05$ and n=5/group.

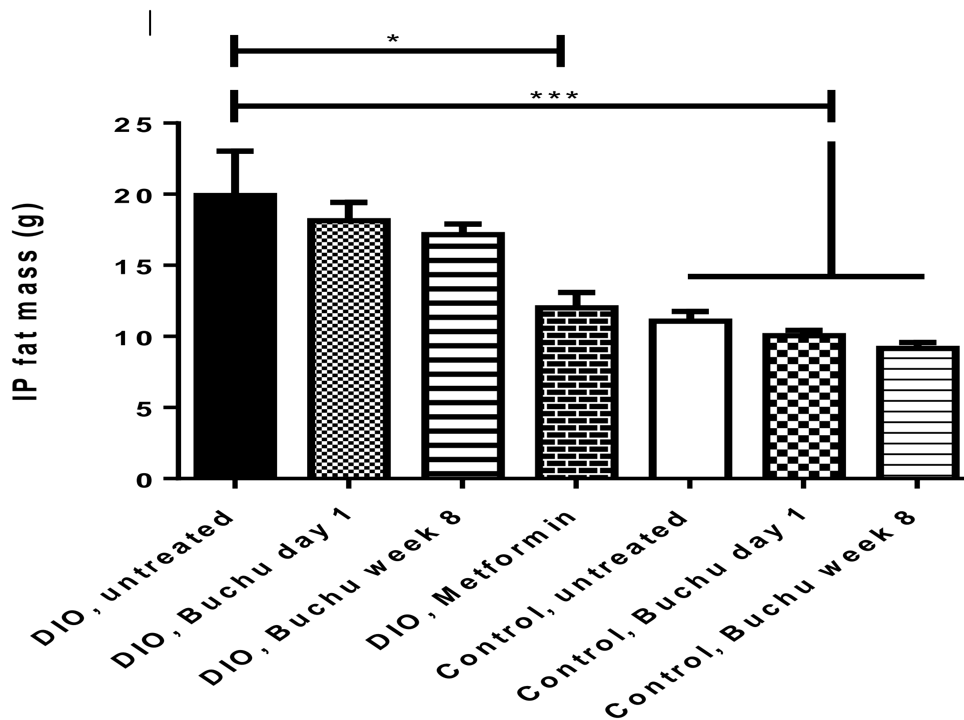



**Figure 31:** ***Intra-peritoneal (IP) fat of control and DIO animals, with and without Buchu treatment, after 16 weeks.*** Displayed is IP fat mass measured in grams (g) of diet induced obese (DIO) and control animals. The data is presented as a mean ± SEM. (n=6-8/group). *$p<0.05$, ***$p<0.001$.

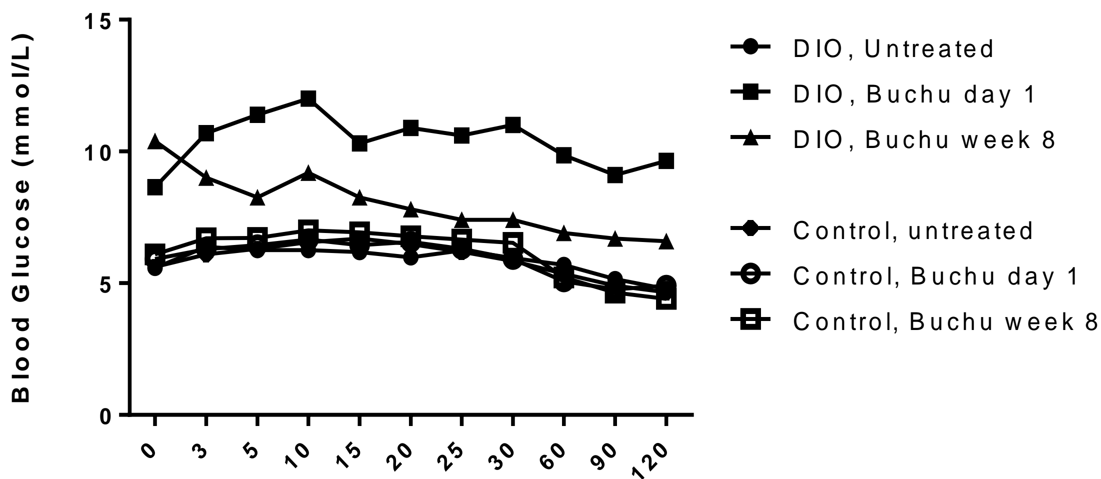
Stellenbosch University https://scholar.sun.ac.za

### 3.2.2. *Metabolic parameters*

#### 3.2.2.1. *Intraperitoneal glucose tolerance test (IPGTT)*

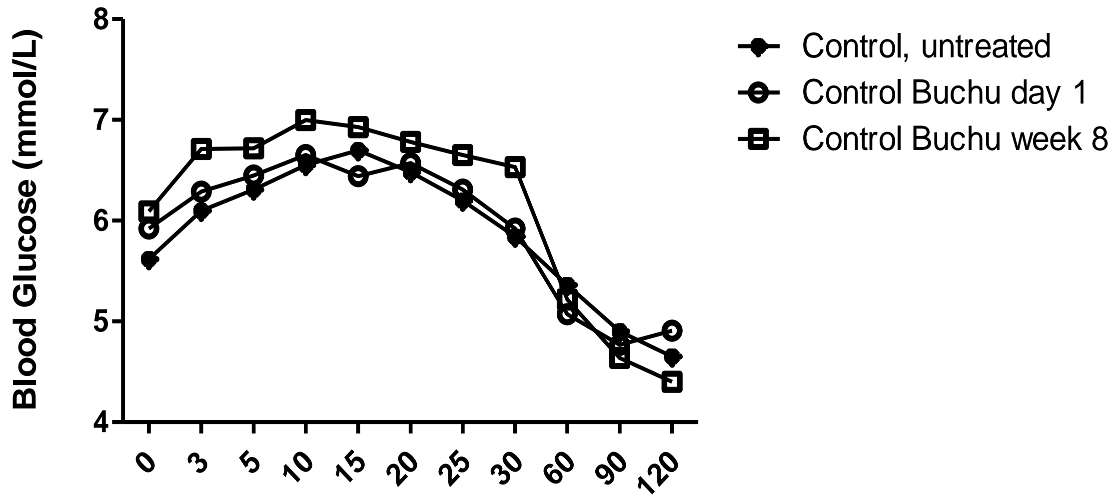
At 15 weeks, all animals following their respective treatment regimens were fasted overnight. The next day, intraperitoneal glucose tolerance tests were performed on each animal for a period of 2 hours. When comparing treated and untreated controls, no significant differences were seen (Figure 32 (B)). However, a significant difference in blood glucose levels were observed when comparing treated and untreated DIO animals (Figure 32 (D) and (E)).

**(A)**

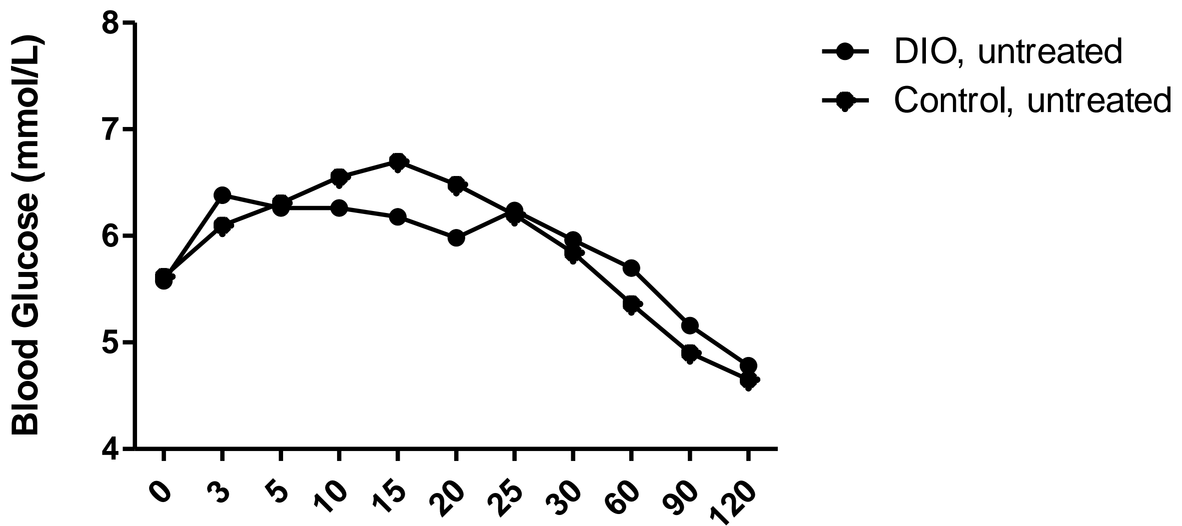


Stellenbosch University https://scholar.sun.ac.za

(B)

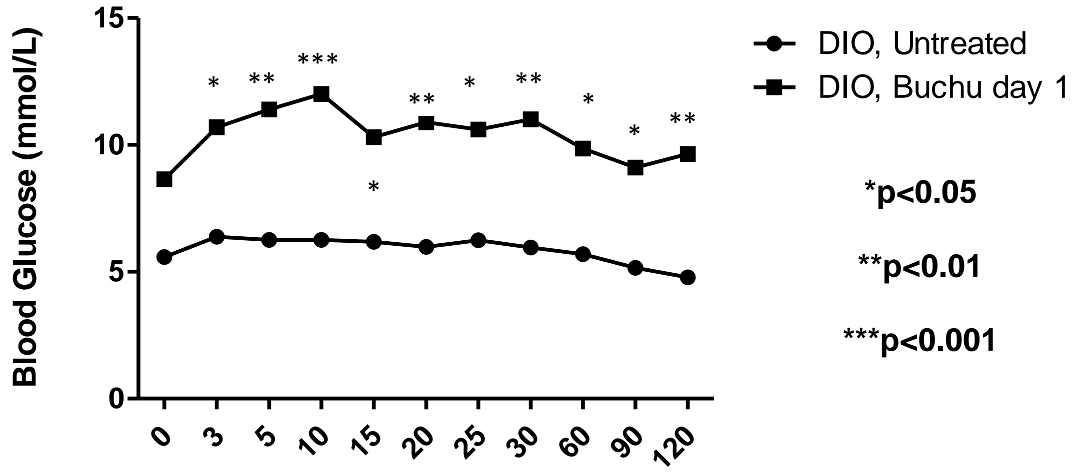



(C)

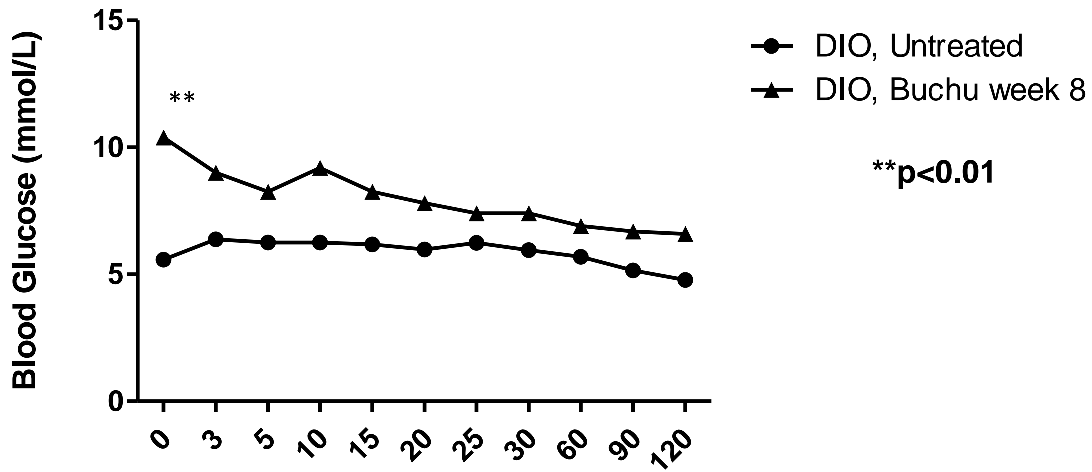


Stellenbosch University https://scholar.sun.ac.za

(D)




(E)



**(F)**

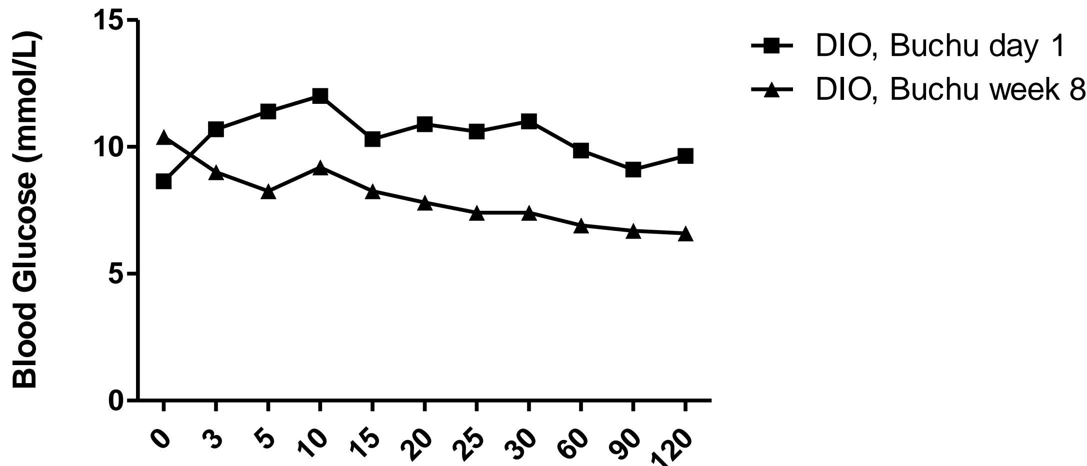



**Figure 32:** ***Intraperitoneal glucose tolerance test of control and DIO animals, with and without Buchu treatment, at 15 weeks. (*A)** Representation of all groups, **(B)** Comparing all control groups, **(C)** Comparing untreated DIO and control groups, **(D)** Comparing untreated DIO animals with treated DIO animals (buchu water from day 1), **(E)** Comparing untreated DIO animals with treated DIO animals (buchu water from week 8), **(F) c**omparing DIO animals treated with Buchu water from day 1 and week 8. Blood glucose levels were measured in mmol/L. The data is presented as a mean ± SEM. (n: 2-10/group)

### 3.2.2.2. *2-Deoxy-D-3[H] glucose (2DG) uptake by soleus muscle*

At 16 weeks, 4 animals per group were anaesthetized, the soleus muscles were rapidly isolated and sliced into five thin longitudinal strips. Each strip was placed into a specific flat-bottomed incubation tube containing incubation medium and various other reagents (Table 6). The purpose of this procedure was to determine muscle sensitivity to insulin.

Stellenbosch University  https://scholar.sun.ac.za

Neither basal glucose uptake levels nor insulin sensitivity differed in these muscles between the DIO and control animals or with ingestion of Buchu water by the animals. (Figure 33)

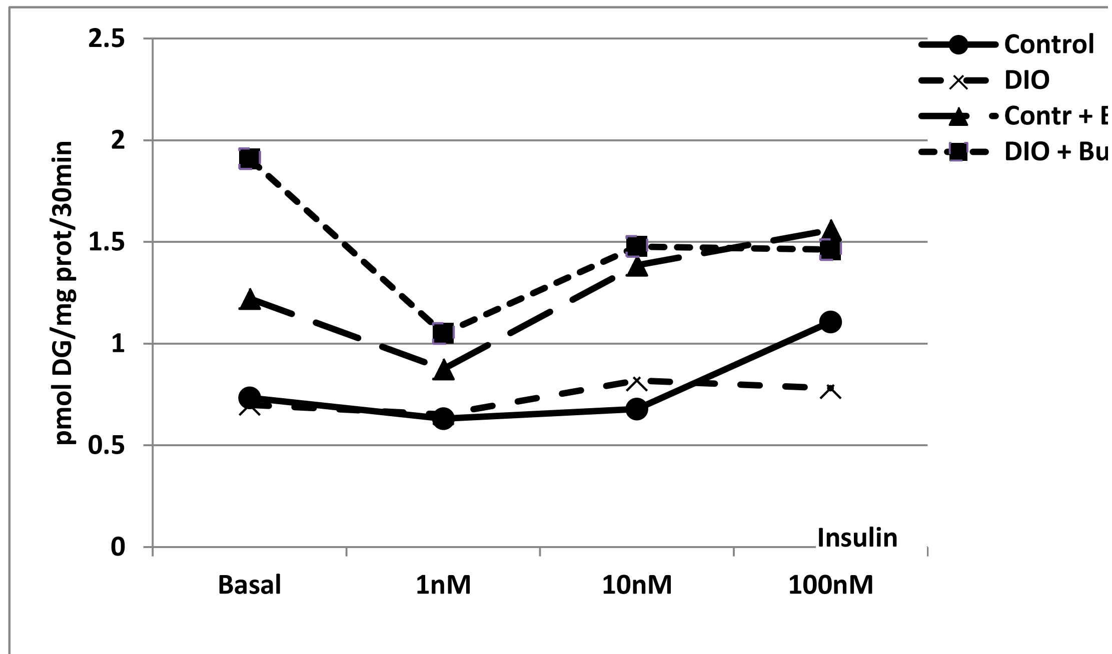


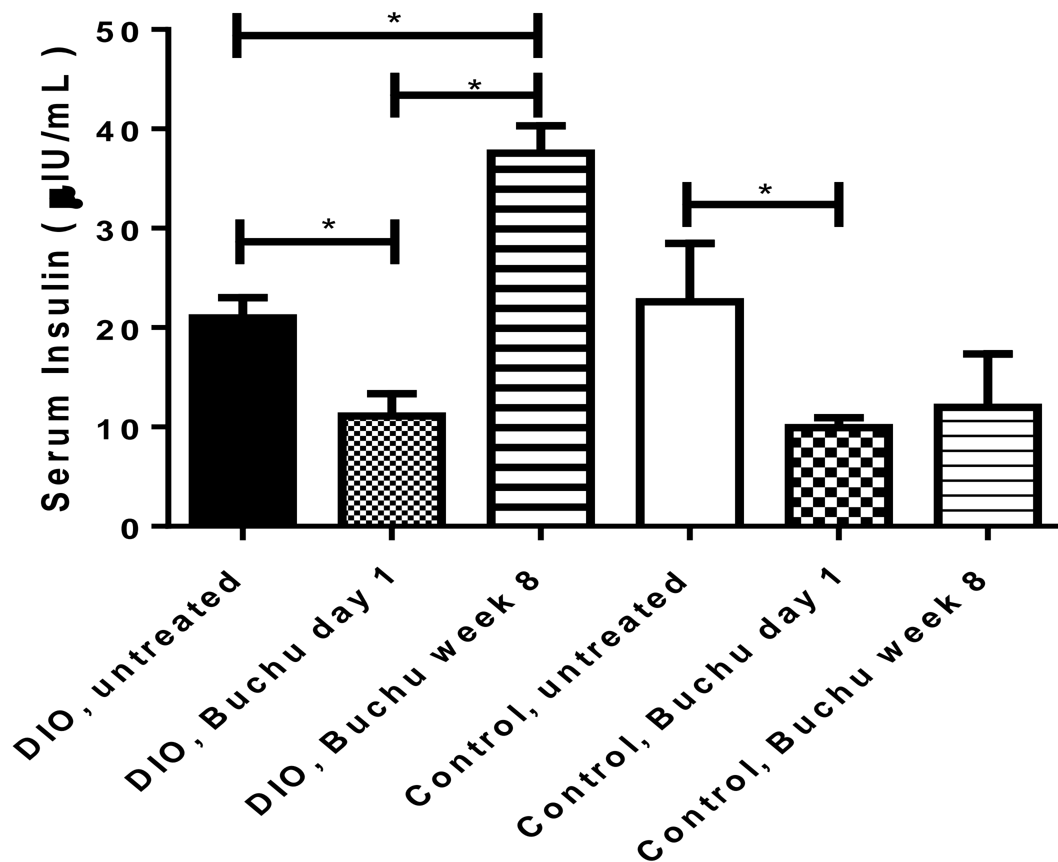

**Figure 33*: Glucose uptake by the soleus muscle of control and DIO animals, with and without Buchu treatment, after 16 weeks.*** Insulin sensitivity of the soleus muscle was determined by measuring the ability of the muscle to accumulate radio-labelled deoxy-glucose over a set time period. The data is presented as a mean ± SEM. (n= 4/group)

### 3.2.2.3. *Serum insulin levels*

Following 16 weeks on diet, serum insulin levels were not significantly different between the control and DIO animals without Buchu treatment (22.58±5.8 vs 20.99±2). However, starting Buchu water treatment at the onset of the change in diet, resulted in lower insulin levels (DIO: 20.99±2 vs 11.12±2; Control: 22.58±5.8 vs 9.95±0.9).

Starting the Buchu water treatment on week 8 after onset of the obesity-inducing diet, could not lower insulin levels in the DIO animals. To the contrary, insulin levels were raised significantly (22.58±5.8 vs 37.5±2.7) (Figure 34A). Figure 34B depicts a 2-way ANOVA analysis of the effect of Buchu water on insulin levels in both control and DIO animals when used from day 1, therefore for the full duration of 16 weeks of the respective diets.

**A)**



Stellenbosch University https://scholar.sun.ac.za

**B)**

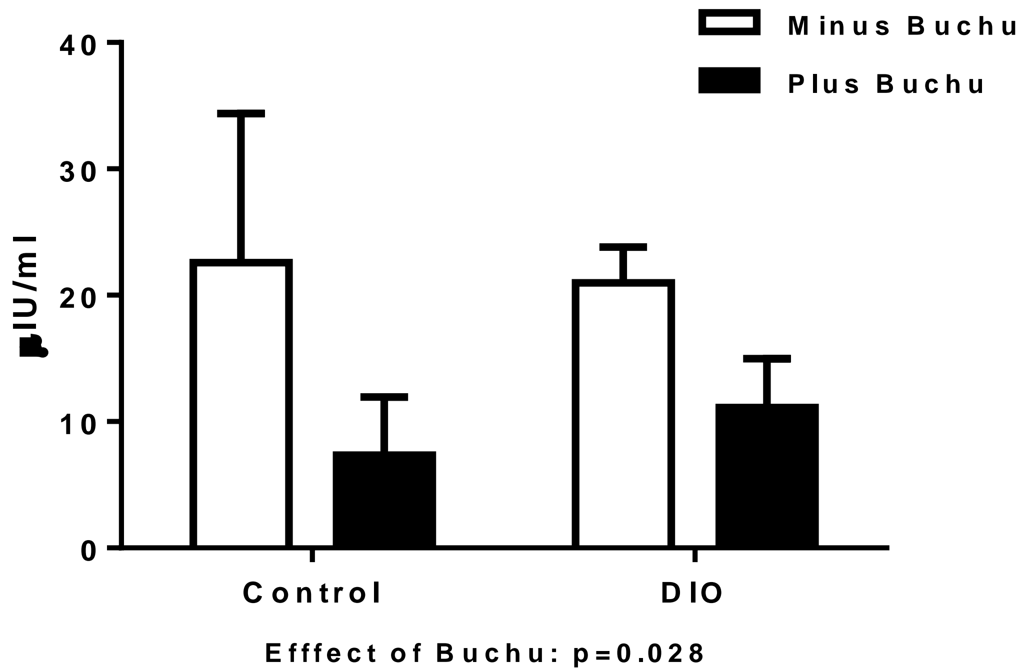



**Figure 34:** ***Fasting insulin levels in control and DIO animals after 16 weeks.*** Fasting blood was drawn under anaesthesia directly from the carotid artery and insulin levels determined using a RIA assay as described in Materials and Methods. The data is presented as a mean ± SEM. (n=4/group) *p<0.05

Stellenbosch University https://scholar.sun.ac.za

### 3.2.2.4. *C-peptide levels*

C-peptide levels of blood collected from the animals treated with Buchu water from day 1

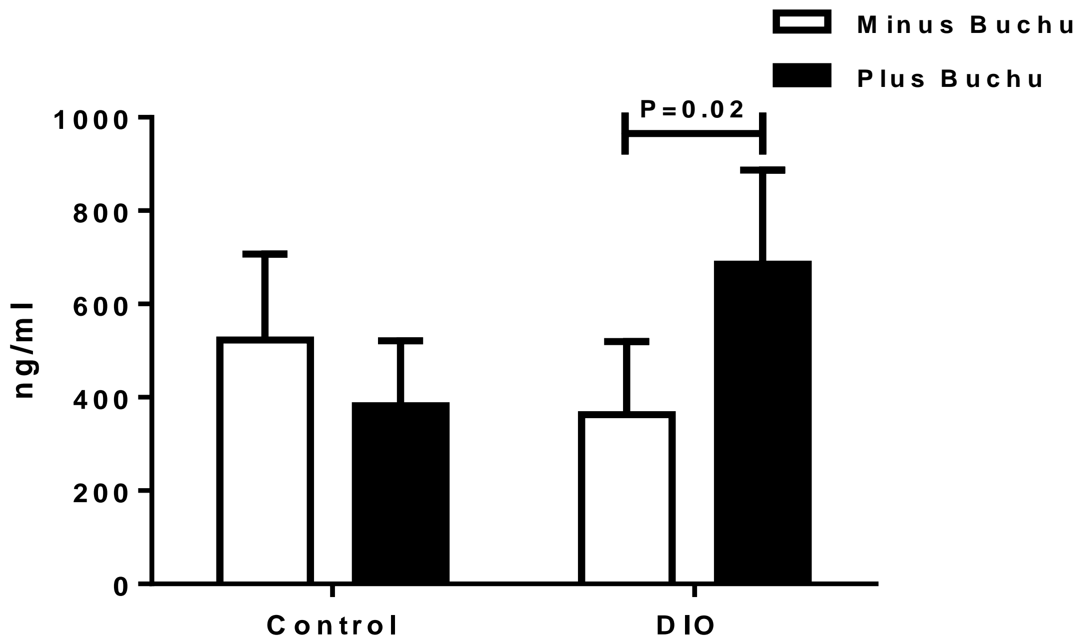



**Figure 35: C-peptide levels in control and DIO animals after 16 weeks.** A significant difference was found in C-peptide levels of treated vs untreated DIO animals. The data is presented as a mean ± SEM. (n=4-6/group).

Stellenbosch University https://scholar.sun.ac.za

#### 3.2.2.5 *Pancreatic Transcription Factors*

Following 16 weeks on their respective diets, animals were anaesthetized and pancreatic tissue isolated and stored at -80°C. These tissues were used to determine the effect of Buchu treatment on the expression of Pdx-1 and MafA in DIO and control animals. Ponceau staining was used as a loading control.

##### 3.2.2.5.1. *Pdx-1*

Pdx-1 expression was downregulated in DIO pancreata compared to controls. A significance increase in Pdx-1 expression was observed when comparing treated and untreated DIO animals, while the total effect of Buchu treatment as indicated by a 2-way ANOVA, on Pdx-1 expression was significant (Figure 36).

Stellenbosch University https://scholar.sun.ac.za

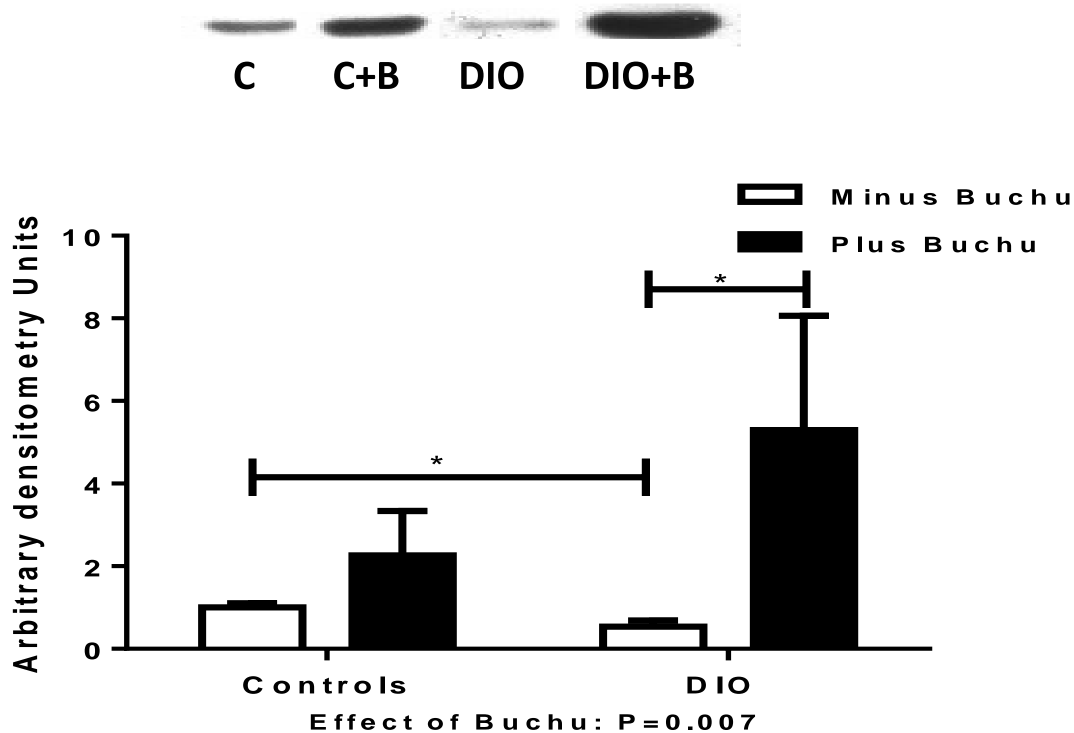



**Figure 36: *Total Pdx-1 expression in control and DIO groups after 16 weeks*.** Shown is an example of a western blot representing Pdx-1 expression as well as a graph depicting protein expression in arbitrary units. The data is presented as mean ± SEM. The data is presented as a mean ± SEM. (n = 4/group) *p<0.05

**3.2.2.5.2. *MafA***

MafA expression was downregulated in DIO pancreata compared to controls. A significant increase in MafA expression was observed when comparing treated and untreated DIO animals. A 2-way ANOVA indicated a significant effect of Buchu water treatment on the expression of MafA (Figure 37).

Stellenbosch University https://scholar.sun.ac.za

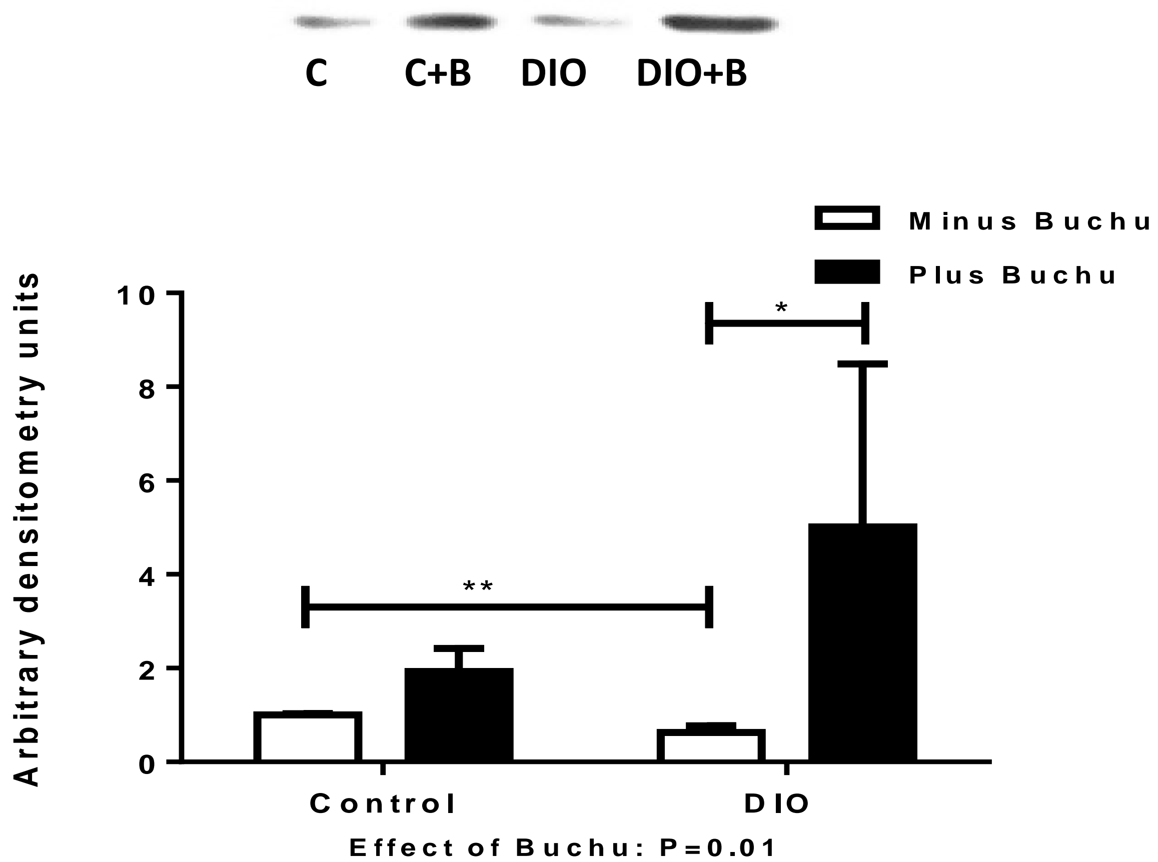



**Figure 37:** ***Total MafA expression in control and DIO groups after 16 weeks.*** Shown is an example of a western blot representing MafA expression as well as a graph depicting protein expression in arbitrary units. The data is presented as mean ± SEM. The data is presented as a mean ± SEM. (n = 4/group) *p<0.05, **p<0.01

Stellenbosch University https://scholar.sun.ac.za

# *CHAPTER 4*

# *Discussion*

---

It has been estimated that 347 million people worldwide have diabetes mellitus, with approximately 80% of diabetic deaths occurring in low and middle income countries [*Mathers and Loncar, 2006; WHO, 2014*]. Diabetes mellitus is a chronic disease associated with disastrous complications, wreaking havoc amongst individuals of all ages. According to the World Health Organisation (WHO), diabetes will be the 7$^{th}$ leading cause of death by the year 2030. As a result, much research has been conducted on the pathophysiology of diabetes mellitus as well as the establishment of safer, more convenient treatment options.

The people of South Africa are faced with many challenges on a daily basis, especially where healthcare is concerned. In developing countries, treating chronic diseases are either too expensive or inaccessible. South Africa is a country rich in floral biodiversity, shifting attention toward the medicinal use of indigenous plants [*Light et al., 2005*]. There are many complex issues surrounding the use of indigenous plants in medicine. One of these issues stem from the fact that the use of medicinal plants were based on hearsay [*Light et al., 2005*].

The indigenous plant of interest for this study belongs to the genus *Agathosma* and is collectively known as Buchu. It is claimed that Buchu exert various beneficial effects

Stellenbosch University https://scholar.sun.ac.za

which still need to be scientifically validated. A study reviewed by Moolla and Viljoen successfully validated that Buchu exerts anti-microbial, anti-inflammatory, anti-oxidant and diuretic effects [*Moolla and Viljoen, 2008*]. Whether buchu has any anti-diabetic properties, remains a question which demands scientific investigation.

The aim of our study was therefore to investigate the anecdotal claims of anti-diabetic effects experienced by individuals using Buchu water. Buchu water is the by-product of the extraction of Buchu oil. The latter is mainly isolated from the leaves of *Agathosma crenulata* and *Agathosma betulina*, both endemic to the mountains of the Western Cape in South Africa. The oil is used as a flavour enhancer in the perfume and food industries. The aqueous solution is currently marketed as a herbal remedy to treat various ailments e.g., urinary infections, rheumatism, arthritis and obesity. People ingesting the water started claiming insulin sensitizing potential necessitating scientific validation of these claims.

Moolla and Viljoen (2008) comprehensively reviewed the phytochemical composition of Buchu oil.  In Buchu oil, there is only one family of substances that may be harmful – the pulegones.  However, during fractionation, the pulegones are extracted in the oil fraction with less than 0.1% present in the water fraction. In addition to pulegone Buchu oil consists of limonene, menthone and diosphenol. Currently, little is known about the composition of Buchu water as well as the active substance present.

Stellenbosch University https://scholar.sun.ac.za

**Main findings:**

One of the main findings of this study is that ingestion of Buchu water results in weight loss despite unchanged food consumption. This occurred in both the models used as well as control animals. In the obese animals, this weight loss was due to the loss of intra-peritoneal fat.

A second important finding is that the ingestion of Buchu water in all instances, whether given as treatment or as prophylactic, resulted in normalization of glucose levels in a type-1 diabetic model with residual beta-cell mass.

An insulin-sensitizing effect has not been clearly established in the muscle of DIO animals but this may be because of a large variation in values obtained as well as the use of a slow-twitch muscle.

A definite effect on pancreatic insulin secretion has been demonstrated by raised C-peptide levels in DIO treated animals.

## 4. Diabetic models

### 4.1. Streptozotocin-induced type 1 diabetic model

Type 1 Diabetes is an organ specific autoimmune disease caused by the immunological destruction of insulin producing pancreatic β-cells [*Narendran et al., 2003; Atkinson and Eisenbarth, 2001*]. The destruction of β-cells minimizes β-cell mass, thus, exposing the body to hyperglycemia. High blood glucose levels are associated with a vast array of detrimental effects such as glucotoxicity and ketoacidosis.

Stellenbosch University https://scholar.sun.ac.za

Streptozotocin is a glucosamine compound, derived from a soil microbe called *Streptomyces achromogenes,* which is easily transported into pancreatic β-cells via glucose transporter 2, where it exerts its toxic effects by damaging DNA [*Wei et al., 2003; Motyl and McCabe, 2009; Arora et al., 2009*]. This leads to β-cell dysfunction, hypoinsulinemia and hyperglycemia; all characteristics associated with diabetes [*Motyl and McCabe, 2009*]. The severity of type 1 diabetes depends on the species of the animal, age and amount of STZ administrated intravenously or intraperitoneally [*Arora et al., 2009*]. In this study, we successfully developed a type 1 diabetic model by injecting rats with a single dose of STZ (40 mg/kg), intra-peritoneally. In these animals, approximately 50 % of the total pancreatic β-cell mass was ablated [*George et al., 2011]* leading to blood glucose levels within the range of >10<20 mmol/L. Diabetic animals were further divided into a group with blood sugar values less than 20mmol/L and those with values exceeding 20mmol/L. This was clearly seen when weekly evaluating blood glucose levels of STZ animals after 11 weeks treatment (Figure 25). Additionally, fasting blood glucose levels of untreated STZ animals at week 13 were significantly higher compared to treated STZ animals (Figure 25 and Figure 26).

The streptozotocin-induced type 1 diabetic model was further used to determine the effect of Buchu water on certain biometric and metabolic parameters.

#### 4.1.1. The effect of Buchu water on biometric parameters

In the current study, water/Buchu water and food consumption were monitored to determine whether all treated animals consumed equal amounts of Buchu water and

Stellenbosch University https://scholar.sun.ac.za

whether this consumption might influence their food intake. Daily measurements showed no significant differences within or between groups (Figure 21 and Figure 22).

After 14 weeks of feeding, all animals were anaesthetized with sodium pentobarbital and sacrificed. At this point, body weight and intra-peritoneal fat mass were measured. The data retrieved was plotted and clear differences in body weight between STZ treated and untreated animals were evident (Figure 23). As would be expected, the STZ animals gained less weight than their control counterparts but in both STZ animals, as well as in the control animals, ingestion of Buchu water resulted in significant lower weight gain over the treatment period [*Singh et al., 2006*]. The lower weight gain seen in STZ animals are not good and could result in ketoacidosis. However, when evaluating intra-peritoneal fat, the STZ animals ingesting Buchu water, accumulated more fat than untreated animals (Figure 24). In the setting of type 1 diabetes, intra-peritoneal fat accumulation but loss of body weight with equal food consumption, might point towards either muscle wasting or a higher thermogenic state. It is possible that Buchu water might act as a thermogenic supplement by increasing the metabolic rate of cells leading to weight loss [*Jitomir et al., 2008; Preuss et al.,2002*]. This is an aspect of the results that needs further investigation.

#### 4.1.2. The effect of Buchu water on metabolic parameters

Non-fasting blood glucose levels of all animals were measured and recorded weekly for 13 weeks. From the data gathered, Buchu treatment resulted in a decrease in blood glucose levels over time with the most significant decrease observed in STZ animals

Stellenbosch University https://scholar.sun.ac.za

that had a lower blood glucose level (< 20 mmol/L) at the onset of experimentation (Figure 25).

At week 13, animals were fasted overnight then anaesthetized in order to perform intra-peritoneal glucose tolerance tests. All STZ animals that were treated with Buchu water, presented with normalized IPGTT curves (Figure 26). This was underscored by a 2hr value not significantly different from control animals (Figure 26G). It can therefore be concluded that, in this STZ model of type 1 diabetes, treatment with Buchu water normalized blood glucose values and served as an effective treatment in the absence of any other treatment.

Despite this normalization of the blood glucose values, we could not detect any significant differences in the insulin secretion of these animals (Figure 27). It is therefore possible that the Buchu water treatment may have lowered either the uptake of glucose from the intestinal mucosa by the SGLT-1 transporter [*Steel and Hediger, 1998; Srichamroen, 2007*] or it may have enhanced glucose excretion by inhibition of the SGLT-2 glucose transporters in the kidney [Review: *Rosenwasser et al., 2013*]. It has been shown that inhibitors of SGLT-2 improve glucose control to an extent comparable to other hypoglycemic agents while simultaneously reducing body weight. [Review: *Rosenwasser et al., 2013*]. These inhibitors, like canagliflozin or dapagliflozin, are of the most recent substances approved for treatment of diabetes. In addition, we have noticed that the insulin levels of STZ untreated animals were very high, this could have occurred as a result of inadequate insulin breakdown.

Stellenbosch University https://scholar.sun.ac.za

### 4.2. Diet-induced obese type 2 diabetic model

Type 2 Diabetes is a multi-factorial disorder that accounts for 90-95% of all diabetic cases worldwide, making it the most common type of diabetes known [*Stumvoll et al., 2005*]. T2D occurs as a result of insufficient insulin production by pancreatic β-cells or reduced insulin action at target tissues. A strong correlation exists between T2D and obesity, while obese individuals undergo metabolic changes which encompass dyslipidemia, hypertension, insulin resistance and proinflammation [*Chen and Morris, 2007; Stumbo et al., 2005]*.

In our second model we set out to test treatment with Buchu using a type 2 diabetic model in which male Wistar rats were fed a high caloric diet for 16 weeks, while control animals were fed normal rat chow for the duration of the experiment. The high caloric diet consisted of normal rat chow supplemented with condensed milk and sucrose. In this respect, DIO animals consumed more energy than control animals per day. Several studies have validated that an energy imbalance, where energy intake exceeds energy expenditure, is linked to obesity and insulin resistance [*Chen and Morris, 2007; Pulgarón, 2013*]. Although this diet was sufficient in establishing hyperglycemia, it did not result in T2D as the fasting blood glucose values were still below 8 mmol/L.

#### 4.2.1. The effect of Buchu water on biometric parameters

Food consumption was recorded over a period of 7 days with no differences in food consumption (Figure 28). In addition, water/ Buchu water consumption was monitored

Stellenbosch University https://scholar.sun.ac.za

for the duration of 20 days with no significant differences present within or between groups (Figure 29).

Body weight gain in untreated DIO animals was significantly higher than that of the untreated controls (Figure 30) despite similar food intake. This can be ascribed to the higher caloric density of the food that they ingested (Table 9). Additionally, when evaluating intra-peritoneal fat, a significant difference was seen when comparing the untreated DIO and control groups (Figure 31). It would therefore seem that the DIO animals became centrally obese.

Similar to what was observed in the STZ animals, the ingestion of Buchu water from day 1 of the change in diet by the DIO animals, resulted in significantly less weight gain, similar to the positive control, metformin. Metformin acts by lowering glucose production by the liver thereby curbing appetite, resulting in weight loss [*Hundal et al., 2000; Lee and Morley, 2012*]. However, treatment with Buchu water from week 8 of the diet, could not induce a significant reduction in weight gain. This effect was also not seen in the control animals treated with Buchu water. However, there is a clear trend for lower IP fat gain in both the DIO and the control animals ingesting Buchu water. This effect of lower weight gain induced by Buchu water despite equal food consumption, is not clearly understood but warrants an investigation of the fat depots of these animals and the accompanying adipokines that may affect this, e.g. leptin levels.

Stellenbosch University https://scholar.sun.ac.za

### 4.2.2. Anti-diabetic and insulin sensitizing effects of Buchu water

#### 4.2.2.1. The effect of Buchu water on insulin secretion and glucose homeostasis

When pancreatic β-cells are exposed to hyperglycemia they adapt by increasing metabolism and ATP production [*Layden et al., 2010*]. This adaptation causes a surge in the ATP/ADP ratio, resulting in the closure of the ATP-sensitive potassium channels leading to an influx of calcium. This results in increased insulin release via exocytosis [*Layden et al., 2010; Li et al., 2007*]. Insulin secreted by β-cells is readily available due to prior synthesis and storage in immature secretory vesicles containing endoprotease and carboxypeptidase E [*Robinson, 2013*]. Once β-cells are exposed to appropriate stimuli, proinsulin is cleaved releasing insulin and C-peptides into circulation [*Halban and Irminger, 2003*]. Insulin is a potent anabolic hormone, responsible for disposal of excess glucose in response to hyperglycemia [*Satiel and Pessin, 2002*]. Insulin lowers blood glucose levels by stimulating glucose uptake by peripheral tissue and lowering all processes involved in glucose production.

In the current study, IPGTTs done on various groups showed a significantly higher fasting blood glucose level in DIO treated animals compared to untreated DIOs , with no significant differences between untreated control and DIO animals (Figure 32). When monitoring glucose uptake in the soleus muscle under stimulated and basal conditions, no significant difference between groups was present (Figure 33). Interestingly, a significant increase in serum insulin levels of Buchu treated DIO animals at week 8 was observed when comparing this group to its untreated counterpart (Figure 34). However,

Stellenbosch University https://scholar.sun.ac.za

blood glucose levels were higher in DIO animals, even after treatment and although not significant the DIO muscle appears to be insulin resistant (Figure 33). This may account for increased insulin values.

#### 4.2.3. Buchu water as an insulin sensitizing agent/ promoter

There are many factors capable of reducing insulin sensitivity at peripheral tissues such as insulin resistance and defects in insulin signalling. A strong relationship exists between insulin resistance and obesity which are associated with a variety of co-morbidities such as type 2 diabetes [*Stumbo et al., 2005; Sahani et al., 2010; Chen and Morris, 2007*]. In obese individuals energy intake exceeds energy expenditure, leading to an accumulation of free fatty acids. In these individuals, adipose tissue can no longer effectively clear circulating free fatty acids due to these tissues reaching their optimum storage capacity [*Goossens, 2008*]. Lipids would then accumulate in non-adipose tissue but with time they too will lose the ability to oxidize fat effectively [*Goossens, 2008*]. The accumulation of lipids in non-adipose tissue has detrimental effects on insulin sensitivity eventually causing insulin resistance within these tissues. For possible mechanisms in which free fatty acids causes insulin resistance refer back to section 1.6.2.1 of the literature review. In response to insulin resistance, β-cells would secrete more insulin to overcome resistance. This compensatory mechanism usually results in hyperinsulinemia, which is accompanied by glucose intolerance, hyperlipidemia and hyperglycemia. The manifestation of insulin resistance at insulin's target tissue usually occurs due to an impairment in insulin action. However, analyzing insulin sensitivity of soleus muscle did not show clear signs of peripheral insulin resistance in our DIO

Stellenbosch University https://scholar.sun.ac.za

model, nor did ingestion of Buchu water affect this, although there was a strong trend to enhanced insulin stimulated glucose uptake, the variation in data was quite large. A possible effect of the Buchu water on insulin sensitivity is underscored by the significant lowering of insulin levels with treatment for the full 16 weeks. An effect on insulin secretion induced by Buchu water treatment was clearly demonstrated by the fact that c-peptide levels were significantly raised in DIO treated animals. This effect was further investigated by analyzing pancreatic tissue for transcription factors known to be associated with enhanced insulin production.

### 4.2.4. Buchu water promotes β-cell neogenic activity

Adult β-cell mass are dynamic components which readily adapt to changes in insulin demand or glucose concentrations. To maintain an optimal glucose level, an exquisite balance exists between insulin biosynthesis, stimulation and secretion. β-cell mass is influenced by an interplay between β-cell growth and loss. For this study, we will only be focusing on β-cell growth, specifically, neogenesis.

β-cell neogenesis is a mechanism that triggers the generation of new β-cells from precursor cells [*Paris, 2004*]. These precursor cells originate from ductal cells (budding from duct epithelium), differentiated pancreatic cells through a process called transdifferentiation and intra islet precursors [*Paris, 2004*]. Under normal conditions, β-cell neogenesis is at its optimum working capacity prenatally and for a short period of time after birth [*Masiello, 2006*]. As individuals age, neogenesis stabilizes while apoptotic activity increases, leading to a reduction in β-cell mass [*Masiello, 2006*].

Stellenbosch University https://scholar.sun.ac.za

There are various transcription factors promoting β-cell neogenesis by targeting β-cell differentiation, proliferation, and/or pancreatic development [*Rocques et al., 2007*].

In our study we determined the effect of Buchu treatment on the expression of transcriptional factors involved with β-cell neogenesis, namely, Pdx-1 and MafA. The levels of both of these transcription factors were significantly lower in pancreata from the DIO animals. This could therefore explain why the DIO model did not present with enhanced insulin secretion as would be expected. The overall effect of Buchu treatment was significant while increasing Pdx-1 and MafA expression (Figure 36 and Figure 37). This observation warrants further investigation as it is realized that stimulation of neogenesis in the adult pancreas may lead to pancreatic hyperplasia, β-cell maturation/survival and eventual pancreatic dysfunction.

### 4.3. Adverse effects of Buchu water

Buchu is an indigenous plant (herb) species from the Cape regions of South Africa which has been used commercially in tea, perfume, herbal tonics and lotions [*Moolla et al., 2007*]. In recent years, the herb's medicinal uses were explored and research has accredited these plants with the possession of diuretic, anti-inflammatory and anti-septic properties [*Moolla and Viljoen, 2008*]. It should be noted that even though Buchu is a naturally occurring plant it does not necessarily classify it as safe. However, Buchu is approved by the American FDA for human consumption. Many studies have reported adverse effects of herbal remedies [*Johnson W, 2007; Peters D, 2009*].

Stellenbosch University https://scholar.sun.ac.za

# *CHAPTER 5*

# *Conclusion*

---

## 5.1 Conclusion

This study, with regard to the type 1 diabetic model, has confirmed the anti-diabetic effect of Buchu water by significantly lowering blood glucose levels of fasted and non-fasted blood and normalizing IPGTT curves. This was however not so evident in the DIO model utilized. Despite this, animals lost weight which was mainly intra-peritoneal fat in these animals.

## 5.2 Limitations of this study

The current study was done on male Wister rats only. Effects on female animals may be different. In respect to the DIO model, the effect of Buchu water on insulin resistance via adipose tissue secretes (adipokines and cytokines) was not investigated. Such an analysis could have shed more light on the metabolic effects observed. The effects on pancreatic tissue of the type 1 diabetic animals were also not studied. In view of the results obtained in the DIO animals, this is deemed important. Determination of the active compound present in Buchu water would help in explaining the results obtained.

Stellenbosch University https://scholar.sun.ac.za

### 5.3 Future perspectives

- The liver tissue of animals after ingestion of Buchu water should be studied to ascertain metabolic changes and possible toxicity.
- The kidneys should be studied especially with respect to the expression of the SGLT-2 glucose transporter. This should be accompanied by a study of the urine itself to determine kidney function.
- The studies on the soleus muscle which is a slow twitch muscle, should be extended to e.g. the gastrocnemius muscle. The latter may show more pronounced changes.
- The effect of Buchu water on weight loss should be studied in more detail.
- The effect of Buchu water on β-cell neogenesis in pancreata of STZ animals should be studied.

Stellenbosch University https://scholar.sun.ac.za

# CHAPTER 6

# References

## i. Journal articles

**A**ballay LR, Eynard AR, Diaz Mdel P, Navarro A, Munoz SE. Overweight and obesity: a review of their relationship to metabolic syndrome, cardiovascular disease, and cancer in South America. *Nutrition Review* 2013; 71(3): 168-179.

**A**ballay LR, Osella AR, Celi A, del Pilar Díaz M. Overweight and obesity: Prevalence and their association with some social characteristics in a random sample population-based study in Córdoba city, Argentina. *Obesity Research & Clinical Practice* 2009;3(2):75-83.

**A**froze, S., Meng, F., Jensen, K., McDaniel, K., Rahal, K., Onori, P., Gaudio, E., Alpini, G., Glaser,S. The physiological roles of secretin and its receptor. *Annals of Translational Medicine* 2013;1(3):29

**A**ndres R, Cader G, Zierler KL. The quantitatively minor role of carbohydrate in oxidative metabolism by skeletal muscle in intact man in the basal state; measurements of oxygen and glucose uptake and carbon dioxide and lactate production in the forearm. *The Journal of Clinical Investigation* 1956;35(6):671-682.

**A**rdestani A, Sauter NS, Paroni F. Neutralizing Interleukin-1 beta (IL-1 beta) Induces beta-Cell Survival by Maintaining PDX1 Protein Nuclear Localization. *Journal of Biological chemistry 2011*;286(19):17144-17155.

**A**rora S, Ojha SK, Vohora D. Characterisation of Streptozotocin Induced Diabetes Mellitus in Swiss Albino Mice. *Global Journal of Pharmacology* 2009;3(2):81-84.

**A**snaghi L, Bruno P, Priulla M, Nicolin A. mTOR: a protein kinase switching between life and death. *Pharmacological Research* 2004;50(6):545-549.

**A**tkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *The Lancet* 2001;358(9277):221-229.

**B**ailey CJ. Insulin resistance and antidiabetic drugs. *Biochemical Pharmacology* 1999;58(10):1511-1520.

Stellenbosch University https://scholar.sun.ac.za

**B**ayir H, Kagan VE. Bench-to-bedside review: Mitochondrial injury, oxidative stress and apoptosis--there is nothing more practical than a good theory. *Critical Care* 2008;12(1):206.

**B**raccini L, Ciraolo E, Martini M, Pirali T, Germena G, Rolfo K. PI3K keeps the balance between metabolism and cancer. *Advances in Biological Regulation* 2012;52(3):389-405.

**B**radford MM. Rapid sensitive method for quantion of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976;71:248-254

**B**ucci D, Rodriguez-Gil JE, Vallorani C, Spinaci M, Galeati G, Tamanini C. GLUTs and mammalian sperm metabolism. *Journal of Andrology* 2011;32(4):348-355.

**B**uchanan TA. Pancreatic beta-cell loss and preservation in type 2 diabetes. *Clinical Therapeutics* 2003;25:B32-B46.

**C**hang L, Adams RD, Saltiel AR. The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. *Proceedings of the National Academy of Sciences* 2002;99(20):12835-12840.

**C**hen H. Cellular inflammatory responses: Novel insights for obesity and insulin resistance. *Pharmacological Research* 2006;53(6):469-477.

**C**hen H, Morris MJ. Maternal smoking—A contributor to the obesity epidemic? *Obesity Research & Clinical Practice* 2007;1(3):155-163.

**C**hen H, Martin B, Cai H. Pancreas++: Automated Quantification of Pancreatic Islet Cells in Microscopy Images. *Frontiers in Physiology* 2012;3:482.

**C**hiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* 2001;410(6831):944-948.

**C**ho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw III EB, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 2001; 292:1728-1731

**C**how LML, Baker SJ. PTEN function in normal and neoplastic growth. *Cancer Letters* 2006;241(2):184-196.

**C**ipok M, Aga-Mizrachi S, Bak A, Feurstein T, Steinhart R, Brodie C. Protein kinase Cα regulates insulin receptor signaling in skeletal muscle. *Biochemical and Biophysical Research Communications* 2006;345(2):817-824.

Stellenbosch University https://scholar.sun.ac.za

**C**laiborn KC, Sachdeva MM, Cannon CE, Groff DN, Singer JD, Stoffers DA. Pcif1 modulates Pdx1 protein stability and pancreatic beta cell function and survival in mice. *The Journal of Clinical Investigation* 2010;120(10):3713-3721.

**C**olin Mathers DL. Projections of Global Mortality and Burden of Disease from 2002 to 2030. *PLOS Medicine* 2006;3(11):e442.

**C**ollins BS, Kelly CT, Fogarty WM, Doyle EM. The high maltose-producing alpha-amylase of the thermophilic actinomycete, Thermomonospora curvata. *Applied Microbiology and Biotechnology* 1993;39(1):31-35.

**C**ondé C, Gloire G, Piette J. Enzymatic and non-enzymatic activities of SHIP-1 in signal transduction and cancer. *Biochemical Pharmacology* 2011;82(10):1320-1334.

**C**ornu M, Albert V, Hall MN. mTOR in aging, metabolism, and cancer. *Current Opinion in Genetics & Development* 2013;23(1):53-62.

**D**anaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ. 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(9785):31-40.

**D**as D, Khan PP, Maitra S. Participation of PI3-kinase/Akt signalling in insulin stimulation of p34cdc2 activation in zebrafish oocyte: Phosphodiesterase 3 as a potential downstream target. *Molecular and Cellular Endocrinology* 2013;374(1–2):46-55.

**D**oble BW, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. Journal of Cell Science 2003;116(7):1175-86.

**D**odd M. Obesity and time-inconsistent preferences. *Obesity Research & Clinical Practice* 2008;2(2):83-89.

**D**ominici FP, Argentino DP, Muñoz MC, Miquet JG, Sotelo AI, Turyn D. Influence of the crosstalk between growth hormone and insulin signalling on the modulation of insulin sensitivity. *Growth Hormone & IGF Research* 2005;15(5):324-336.

**D**ong XC, Copps KD, Guo S, Li Y, Kollipara R, DePinho RA. Inactivation of hepatic Foxo1 by insulin signaling is required for adaptive nutrient homeostasis and endocrine growth regulation. *Cell Metabolism* 2008;8(1):65-76.

**D**oyle ME, Egan JM. Mechanisms of Action of GLP-1 in the Pancreas. *Pharmacology & Therapeutics* 2007;113(3):546–593.

Stellenbosch University https://scholar.sun.ac.za

**D**u Toit EF, Smith W, Muller C, Strijdom H, Stouthammer B, Woodiwiss AJ, Norton GR and Lochner A. Myocardial susceptibility to ischemic-reperfusion injury in a prediabetic model of dietary-induced obesity. *American Journal of Physiology - Heart and Circulatory Physiology* 2008;294(5): H2336-H2343.

**E**l-Bacha T, Da Poian A, Luz M. Dynamic adaptation of nutrient utilization in humans. *Nature Education* 2010;3(9):8.

**E**lmquist JK. Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. *International Journal of Obesity* 2001;25(5):S78-82.

**E**mbi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *European Journal of Biochemistry* 1980;107(2):519 -527.

**E**nriquez YR, Giri M, Rottiers R, Christophe A. Fatty acid composition of erythrocyte phospholipids is related to insulin levels, secretion and resistance in obese type 2 diabetics on Metformin. *Clinica Chimica Acta* 2004;346(2):145-152.

**E**spinosa A, Garcia A, Hartel S, Hidalgo C, Jaimovich E. NADPH oxidase and hydrogen peroxide mediate insulin-induced calcium increase in skeletal muscle cells. *The Journal of Biological Chemistry* 2009;284(4):2568-2575.

**F**abian TK, Hermann P, Beck A, Fejerdy P, Fabian G. Salivary defense proteins: their network and role in innate and acquired oral immunity. *International Journal of Molecular Sciences* 2012;13(4):4295-4320.

**F**antuzzi G. Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical Immunology* 2005;115(5):911-919.

**F**elig P, Wahren J. Fuel homeostasis in exercise. *The New England Journal of Medicine* 1975;293(21):1078-1084.

**F**ieker A, Philpott J, Armand M. Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clinical and Experimental Gastroenterology* 2011;4:55-73.

**F**illmore N, Lopaschuk GD. Targeting mitochondrial oxidative metabolism as an approach to treat heart failure. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2013 4;1833(4):857-865.

**F**inn PF, Dice JF. Proteolytic and lipolytic responses to starvation. *Nutrition* 2006;22(7–8):830-844.

Stellenbosch University https://scholar.sun.ac.za

**F**reireich, EJ, et al. Quantitative comparison of toxicity of anticancer agents in mouse, rat, dog, monkey and man. *Cancer chemotherapy reports* 1996;50(4):219-244.

**F**rick NT, Bystriansky JS, Ip YK, Chew SF, Ballantyne JS. Lipid, ketone body and oxidative metabolism in the African lungfish, Protopterus dolloi following 60 days of fasting and aestivation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 2008;151(1):93-101.

**F**riedman J. The Function of Leptin in Nutrition, Weight, and Physiology. *Nutrition Reviews* 2002;60(10):S1-S14.

**F**röjdö S, Vidal H, Pirola L. Alterations of insulin signaling in type 2 diabetes: A review of the current evidence from humans. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 2009;1792(2):83-92.

**F**ryer LGD, Hajduch E, Rencurel F, Salt IP, Hundal HS, Hardie GD, and Carling D. Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase. *Diabetes* 2000;49:1978-1985.

**F**ujimoto K, Ford EL, Tran H, Wice BM, Crosby SD, Dorn GW,2nd. Loss of Nix in Pdx1-deficient mice prevents apoptotic and necrotic beta cell death and diabetes. *Journal of Clinical Investigation* 2010;120(11):4031-4039.

**F**ujimoto K, Polonsky KS. Pdx1 and other factors that regulate pancreatic beta-cell survival. *Diabetes, Obesity and Metabolism* 2009;11(4):30-37.

**G**astaldelli A. Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Diabetes Research and Clinical Practice* 2011;93,1(0):S60-S65.

**G**eorge C, Lochner A, Huisamen B. The efficacy of Prosopis glandulosa as antidiabetic treatment in rat models of diabetes and insulin resistance. *Journal of Ethnopharmacology* 2011;137:298-304

**G**ericke A, Munson M, Ross AH. Regulation of the PTEN phosphatase. *Gene* 2006;374(0):1-9.

**G**lobal status report on noncommunicable diseases 2010. World Health Organization, 2011.

**G**olay A, Ybarra J. Link between obesity and type 2 diabetes. *Best Practice & Research Clinical Endocrinology & Metabolism* 2005;19(4):649-663.

**G**oossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiology & Behavior* 2008;94(2):206-218.

Stellenbosch University https://scholar.sun.ac.za

**G**uillausseau P-, Meas T, Virally M, Laloi-Michelin M, Médeau V, Kevorkian J-. Abnormalities in insulin secretion in type 2 diabetes mellitus. *Diabetes Metabolism* 2008 2;34,2(0):S43-S48.

**G**uo S, Dunn SL, White MF. The reciprocal stability of FOXO1 and IRS2 creates a regulatory circuit that controls insulin signaling. *Journal of Molecular Endocrinology* 2006;20(12):3389-3399.

**G**upta A, Dey CS. PTEN and SHIP2 regulates PI3K/Akt pathway through focal adhesion kinase. *Molecular and Cellular Endocrinology* 2009;309(1–2):55-62.

**H**agopian K, Ramsey JJ, Weindruch R. Krebs cycle enzymes from livers of old mice are differentially regulated by caloric restriction. *Experimental Gerontology* 2004;39(8):1145-1154.

**H**alban PA, **I**rminger JC. Mutant proinsulin that cannot be converted is secreted efficiently from primary rat beta-cells via the regulated pathway. *Molecular Biology of the Cell* 2003;14(3):1195-1203.

**H**ardie, DG. AMP-activated protein kinase: the guardian of cardiac energy status. *The Journal of Clinical Investigation* 2004;114(4): 465-468.

**H**ardie DG, Carling D. The AMP-activated protein kinase: fuel gauge of the mammalian cell? *European Journal of Biochemistry* 1997;246:259-273.

**H**artley T, Brumell J, Volchuk A. Emerging roles for the ubiquitin-proteasome system and autophagy in pancreatic beta-cells. *American Journal of Physiology - Endocrinology and Metabolism* 2009;296(1):E1-10.

**H**ayashi T, Hirshman MF, Kurth EJ, Winder WW, and Goodyear LJ. Evidence for 5'AMP-Activated Protein Kinase Mediation of the Effect of Muscle Contraction on Glucose Transport. *Diabetes* 1998;47:1369-1373.

**H**e J, de la Monte S, Wands JR. The p85beta regulatory subunit of PI3K serves as a substrate for PTEN protein phosphatase activity during insulin mediated signaling. *Biochemical and Biophysical Research Communications* 2010;397(3):513-519.

**H**olloway GP, Bonen A, Spriet LL. Regulation of skeletal muscle mitochondrial fatty acid metabolism in lean and obese individuals. *The American Journal of Clinical Nutrition* 2009;89(1):455S-62S.

**H**otamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993 Jan 1;259(5091):87-91.

Stellenbosch University https://scholar.sun.ac.za

**H**uang P, Altshuller YM, Hou JC, Pessin JE, Frohman MA. Insulin-stimulated Plasma Membrane Fusion of Glut4 Glucose Transporter-containing Vesicles Is Regulated by Phospholipase D1Dâƒž. *Molecular Biology of the Cell* 2005;16(6):2614-2623.

**H**undal RS, Krssak M, Dufour S. Mechanism by Which Metformin Reduces Glucose Production in Type 2 Diabetes. *Diabetes* 2000;49(12):2063-2069.

**H**unter CS, Maestro MA, Raum JC, Guo M, Thompson FH,3rd, Ferrer J, et al. Hnf1alpha (MODY3) regulates beta-cell-enriched MafA transcription factor expression. *Journal of Molecular Endocrinology* 2011;25(2):339-347.

**H**unter SJ, Garvey WT. Insulin action and insulin resistance: diseases involving defects in insulin receptors, signal transduction, and the glucose transport effector system. *American Journal of Medicine* 1998;105(4):331-345.

**I**slam S. One hundred fourty years after the discovery of islets by Paul Langerhans, *Islets*, a new journal dedicated to these mini-organs, is born. *Landes Bioscience* 2009;1(1).

**J**aswal JS, Keung W, Wang W, Ussher JR, Lopaschuk GD. Targeting fatty acid and carbohydrate oxidation — A novel therapeutic intervention in the ischemic and failing heart. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2011;1813(7):1333-1350.

**J**iang Z, Song J, Qi F, Xiao A, An X, Liu NA, et al. Exdpf is a key regulator of exocrine pancreas development controlled by retinoic acid and ptf1a in zebrafish. *PLOS Biology* 2008;6(11):e293.

**J**itomir J, Nassar E, Culbertson J. The acute effects of the thermogenic supplement Meltdown on energy expenditure, fat oxidation, and hemodynamic responses in young, healthy males. *Journal of the International Society of Sports Nutrition* 2008;5(23):1186/1550-2783-5-23.

**J**ohnson W. Final report on the safety assessment of Capsicum annuum extract, Capsicum annuum fruit extract, Capsicum annuum resin, Capsicum annuum fruit powder, Capsicum frutescens fruit, Capsicum frutescens fruit extract, Capsicum frutescens resin, and Capsaicin. International Journal of Toxicology 2007; 26:3-106.

**J**orgensen MC, Ahnfelt-Ronne J, Hald J, Madsen OD, Serup P, Hecksher-Sorensen J. An illustrated review of early pancreas development in the mouse. *Endocrine Reviews* 2007;28(6):685-705.

**K**ahn BB, Flier JS. Obesity and insulin resistance. *Journal of Clinical Investigation* 2000;106(4):473-481.

Stellenbosch University https://scholar.sun.ac.za

**K**alesnikoff J, Lam V, Krystal G. SHIP represses mast cell activation and reveals that IgE alone triggers signaling pathways which enhance normal mast cell survival. *Molecular Immunology* 2002;38(16–18):1201-1206.

**K**annan MB, Solovieva V, Blank V. The small MAF transcription factors MAFF, MAFG and MAFK: Current knowledge and perspectives. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 2012;1823(10):1841-1846.

**K**ataoka K, Han SI, Shioda S, Hirai M, Nishizawa M, Handa H. MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene. *Journal of Biological Chemistry* 2002;277(51):49903-49910.

**K**elley DE, Mokan M, Simoneau JA, Mandarino LJ. Interaction between glucose and free fatty acid metabolism in human skeletal muscle. *Journal of Clinical Investigation* 1993;92(1):91-98.

**K**ennah M, Yau TY, Nodwell M, Krystal G, Andersen RJ, Ong CJ, et al. Activation of SHIP via a small molecule agonist kills multiple myeloma cells. *Experimental Hematology* 2009;37(11):1274-1283.

**K**essler G, Friedman J. Metabolism of Fatty Acids and Glucose. *American Heart Association* 1998;98:1350a-1353.

**K**ieffer TJ, Habener JF. The adipoinsular axis: effects of leptin on pancreatic beta-cells. *American Journal of Physiology - Endocrinology and Metabolism* 2000;278:E1-E14.

**K**im JP, Battaile KP. Burning fat: the structural basis of fatty acid β-oxidation. *Current Opinion in Structural Biology* 2002;12(6):721-728.

**K**im SK, Novak RF. The role of intracellular signaling in insulin-mediated regulation of drug metabolizing enzyme gene and protein expression. Pharmacology & Therapeutics 2007;113(1):88-120.

**K**oonen DPY, Glatz JFC, Bonen A, Luiken JJFP. Long-chain fatty acid uptake and FAT/CD36 translocation in heart and skeletal muscle. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 2005;1736:163–180.

**K**otronen A, Juurinen L, Tiikkainen M, Vehkavaara S, Yki–Järvinen H. Increased Liver Fat, Impaired Insulin Clearance, and Hepatic and Adipose Tissue Insulin Resistance in Type 2 Diabetes. *Gastroenterology* 2008;135(1):122-130.

**K**oubaa M, Cocuron J, Thomasset B, Alonso AP. Highlighting the tricarboxylic acid cycle: Liquid and gas chromatography–mass spectrometry analyses of 13C-labeled organic acids. *Analytical Biochemistry* 2013;436(2):151-159.

Stellenbosch University https://scholar.sun.ac.za

**K**ovacs P, Stumvoll M. Fatty acids and insulin resistance in muscle and liver. *Best Practice & Research Clinical Endocrinology & Metabolism* 2005;19(4):625-635.

**K**udo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *Journal of Biological Chemistry* 1995;270:17513-17520.

**L**ambert P, Bingley PJ. What is Type 1 Diabetes? *Medicine* 2002;30(1):1-5.

**L**ayden B, Durai V, Lowe W. G-Protein-Coupled Receptors, Pancreatic Islets, and Diabetes. *Nature Education* 2010;3(9):13.

**L**azar DF, Saltiel AR. Lipid phosphatases as drug discovery targets for type 2 diabetes. *Nature Reviews Drug Discovery* 2006;5(4):333-342.

**L**ea W, Abbas AS, Sprecher H, Vockley J, Schulz H. Long-chain acyl-CoA dehydrogenase is a key enzyme in the mitochondrial beta-oxidation of unsaturated fatty acids. *Biochimica et Biophysica Acta* 2000;1485(2-3):121-128.

**L**eahy JL. Pathogenesis of type 2 diabetes mellitus. *Archives of Medical Research* 2005;36(3):197-209.

**L**ee, A., & Morley, J. E. Metformin Decreases Food Consumption and Induces Weight Loss in Subjects with Obesity with Type II Non-Insulin-Dependent Diabetes. *Obesity research* 2012;6(1):47-53.

**L**eoni L, Roman BB. MR Imaging of Pancreatic Islets: Tracking Isolation, Transplantation and Function. *Current Pharmaceutical Design* 2010;16:1582-1594.

**L**i C, Tsai S, Chou P. Relative role of insulin resistance and β-cell dysfunction in the progression to type 2 diabetes—The Kinmen Study. *Diabetes Research and Clinical Practice* 2003;59(3):225-232.

**L**i Y, Wang P, Xu J, Gorelick F, Yamazaki H, Andrews N, et al. Regulation of insulin secretion and GLUT4 trafficking by the calcium sensor synaptotagmin VII. *Biochemical and Biophysical Research Communications* 2007;362(3):658-664.

**L**ight ME, Sparg SG, Stafford GI, van Staden J. Riding the wave: South Africa's contribution to ethnopharmacological research over the last 25 years. *Journal of Ethnopharmacology* 2005;100(1-2):127-130.

**L**iu J, Kimura A, Baumann CA, Saltiel AR. APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. *Molecular and Cellular Biology* 2002;22(11):3599-3609.

Stellenbosch University https://scholar.sun.ac.za

**L**oberg RD, Vesely E, Brosius FC 3rd. Enhanced glycogen synthase kinase-3beta activity mediates hypoxia-induced apoptosis of vascular smooth muscle cells and is prevented by glucose transport and metabolism. *Journal of Biological Chemistry* 2002; 277(44):41667-73.

**L**ockwood DH, Amatruda JM. Cellular alterations responsible for insulin resistance in obesity and type II diabetes mellitus. *The American Journal of Medicine* 1983;75(2):23-31.

**L**ogsdon CD, Ji B. The role of protein synthesis and digestive enzymes in acinar cell injury. *Nature Reviews Gastroenterology & Hepatology* 2013;10(6):362-370.

**L**owry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 1951;193:265-275

**L**uiken JJ., et al. Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. *Diabetes* 2003;52:1627–1634.

**M**archetti P, Dotta F, Lauro D, Purrello F. An overview of pancreatic beta-cell defects in human type 2 diabetes: Implications for treatment. *Regulatory Peptides* 2008;146(1–3):4-11.

**M**asiello P. Animal models of type 2 diabetes with reduced pancreatic β-cell mass. *The International Journal of Biochemistry & Cell Biology* 2006;38(5–6):873-893.

**M**atsuo K, Palmer JB. Anatomy and physiology of feeding and swallowing: normal and abnormal. *Physical Medicine & Rehabilitation Clinics of North America* 2008;19(4):691-707.

**M**cCarthy AM, Elmendorf JS. GLUT4's itinerary in health & disease. *Indian Journal of Medical Research* 2007;125(3):373-388.

**M**oitra VK, Meiler SE. The diabetic surgical patient. *Current Opinion in Anaesthesiology* 2006;19(3):339-345.

**M**onnier L, Colette C, Owens DR. Type 2 diabetes: A well-characterised but suboptimally controlled disease. Can we bridge the divide? *Diabetes & Metabolism* 2008;34(3):207-216.

**M**oolla A, Van Vuuren SF, Van Zyl RL, Viljoen AM. Biological activity and toxicity profile of 17 Agathosma (Rutaceae) species. *South African Journal of Botany* 2007;73(4):588-592.

Stellenbosch University https://scholar.sun.ac.za

**M**oolla A, Viljoen AM. 'Buchu' – Agathosma betulina and Agathosma crenulata (Rutaceae): A review. *Journal of Ethnopharmacology* 2008;119(3):413-419.

**M**orisco C, Lembo G, Trimarco B. Insulin Resistance and Cardiovascular Risk: New Insights From Molecular and Cellular Biology. *Trends in Cardiovascular Medicine* 2006;16(6):183-188.

**M**otyl K, McCabe LR. Streptozotocin, type I diabetes severity and bone. *Biological Procedures Online* 2009;11:296-315.

**N**arendran P, Mannering SI, Harrison LC. Proinsulin—a pathogenic autoantigen in type 1 diabetes. *Autoimmunity Reviews* 2003;2(4):204-210.

**N**guyen DM and El-Serag HB. The epidemiology of obesity. *Gastroenterology Clinics of North America* 2010;39(1):1-7

**N**ielsen JN, Jorgensen SB, Frosig C, Viollet B, Andreelli F, Vaulont S, Kiens B, Richter EA and Wojtaszewski JFP. A possible role for AMP-activated protein kinase in exercise-induced glucose utilization: insights from humans and transgenic animals. *Biochemical Society Transactions* 2003;31(1):186-190

**N**ishimura W, Bonner-Weir S, Sharma A. Expression of MafA in pancreatic progenitors is detrimental for pancreatic development. *Developmental Biology* 2009;333(1):108-120.

**N**ovak I. Purinergic receptors in the endocrine and exocrine pancreas. *Purinergic Signal* 2008;4(3):237-253.

**O**lefsky JM, Garvey WT, Henry RR, Brillon D, Matthael S, Freidenberg GR. Cellular mechanisms of insulin resistance in non-insulin-dependent (type II) diabetes. *The American Journal of Medicine* 1988;85(1):86-105.

**O**valle F, Azziz R. Insulin resistance, polycystic ovary syndrome, and type 2 diabetes mellitus. *Fertility and Sterility* 2002;77(6):1095-1105.

**P**aris M, Tourrel-Cuzin C, Plachot C, Ktorza A. Review: pancreatic beta-cell neogenesis revisited. *Experimental Diabesity Research* 2004;5(2):111-121.

**P**arsons R. Human cancer, PTEN and the PI-3 kinase pathway. *Semin Cell Developmental Biology* 2004;15(2):171-176.

**P**elicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006;25(34):4633-4646.

**P**eters D. CAM: doing more good than harm. *Focus on Alternative and Complementary Therapies* 2009;14:176–8.

Stellenbosch University https://scholar.sun.ac.za

**P**etersen KF, Shulman GI. Etiology of Insulin Resistance. *The American Journal of Medicine* 2006;119(1):S10-S16.

**P**ickavance LC, Tadayyon M, Widdowson PS, Buckingham RE, & Wilding JP. Therapeutic Index for Rosiglitazone in Dietary Obese Rats: Separation of Efficacy and Haemodilution. *British Journal of Pharmacology* 1999;128(7):1570-1576.

**P**onticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D: Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO Journal* 1998; 17:1688-1699.

**P**owers MA, Pappas TN. Physiologic approaches to the control of obesity. *Annals of Surgery* 1989;209(3):255-260.

**P**rasad C, Rupar T, Prasad AN. Pyruvate dehydrogenase deficiency and epilepsy. *Brain and Development* 2011;33(10):856-865.

**P**reuss, H.G.DiFernando, D.Bagchi, M.Bagchi, D. Citrus aurantium as a thermogenic, weight-reduction replacement for ephedra: An overview. *Journal of Medicine*. 2002;33:247–264.

**P**rotti A, Singer M. Bench-to-bedside review: potential strategies to protect or reverse mitochondrial dysfunction in sepsis-induced organ failure. *Critical Care* 2006;10(5):228.

**P**ulgarón ER. Childhood Obesity: A Review of Increased Risk for Physical and Psychological Comorbidities. *Clinical Therapeutics* 2013;35(1):A18-A32.

**R**abe K, Lehrke M, Parhofer KG, Broedl UC. Adipokines and Insulin Resistance. *Journal of Molecular Medicine* 2008;14(11-12):741-751.

**R**andle PJ, Garland PB, Hales CN & Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1963;1:785–789.

**R**en J, Jin P, Wang E, Liu E, Harlan DM, Li X, et al. Pancreatic islet cell therapy for type I diabetes: understanding the effects of glucose stimulation on islets in order to produce better islets for transplantation. *Journal of Translational Medicine* 2007;5:1-5876-5-1.

**R**obinson R. A pair of crescent-shaped proteins shape vesicles at the golgi. *PLOS Biology* 2013;11(4):e1001543.

**R**ocques N, Abou Zeid N, Sii-Felice K, Lecoin L, Felder-Schmittbuhl M, Eychène A, et al. GSK-3-Mediated Phosphorylation Enhances Maf-Transforming Activity. *Molecular Cell* 2007;28(4):584-597.

Stellenbosch University https://scholar.sun.ac.za

**R**odgers JT, Haas W, Gygi SP, Puigserver P. Cdc2-like kinase 2 is an insulin-regulated suppressor of hepatic gluconeogenesis. *Cell Metabolism* 2010;11(1):23-34.

**R**osenwasser RF, Sultan S, Sutton D, Choksi R, Epstein BJ. SGLT-1 inhibitors and their potential in the treatment of diabetes. *Journal of Diabetes, Metabolic Syndrome and Obesity* 2013;6:453-67

**R**ossignol DA, Frye RE. Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis. *Molecular Psychiatry* 2012;17(3):290-314.

**R**ussell RR, Bergeron R, Shulman GI, Young LH. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *American Journal of Physiology* 1999;277:H643–H649.

**R**ustenbeck I. Desensitization of insulin secretion. *Biochemical Pharmacology* 2002;63(11):1921-1935.

**S**ahani R, Chakrabarty S, Bharati P. Temporal trends in overweight and obesity among Nicobarese adults in Nicobar Islands, India, 1960s–1999. *Obesity Research & Clinical Practice* 2010;4(2):e119-e125.

**S**akamoto K, McCarthy A, Smith D, Green KA, Hardie GD, Ashworth A, and Alessi DR. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO Journal* 2005;24:1810–1820.

**S**altiel AR, Pessin JE. Insulin signaling pathways in time and space. *Trends Cell Biology* 2002;12(2):65-71.

**S**ciascia Q, Pacheco D, McCoard SA. Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR)C1-dependent and independent cell signaling. *Journal of Dairy Science* 2013;96(4):2327-2338.

**S**egner H, Dölle A, Böhm R. Ketone Body Metabolism in the Carp Cyprinus carpio: Biochemical and 1H NMR Spectroscopical Analysis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 1997;116(2):257-262.

**S**esti G. Pathophysiology of insulin resistance. *Best Practice & Research Clinical Endocrinology & Metabolism* 2006;20(4):665-679.

**S**hah K, Desilva S, Abbruscato T. The Role of Glucose Transporters in Brain Disease: Diabetes and Alzheimer’s Disease. *International Journal of Molecular Sciences* 2012;13(10):12629-12655.

Stellenbosch University https://scholar.sun.ac.za

**S**hah P, Ardestani A, Dharmadhikari G, Laue S, Schumann DM, Kerr-Conte J, Pattou F, Klein T, Maedler K. The DPP-4 inhibitor linagliptin restores β-cell function and survival in human isolated islets through GLP-1 stabilization. *The Journal of Clinical Endocrinology & Metabolism* 2013;98(7):E1163-72.

**S**hulman GI. Cellular mechanisms of insulin resistance. *Journal of Clinical Investigation* 2000;106:171–176.

**S**impson L, Parsons R. PTEN: Life as a Tumor Suppressor. *Experimental Cell Research* 2001;264(1):29-41.

**S**impson RJ, Hammacher A, Smith DK, Matthews JM, Ward LD. Interleukin-6: Structure-function relationships. *Protein Science* 1997;6(5):929-955.

**S**ly LM, Rauh MJ, Kalesnikoff J, Büchse T, Krystal G. SHIP, SHIP2, and PTEN activities are regulated in vivo by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide. *Experimental Hematology* 2003;31(12):1170-1181.

**S**mith AJ, Daut J, Schwappach B. Membrane proteins as 14-3-3 clients in functional regulation and intracellular transport. *Physiology (Bethesda)* 2011;26(3):181-191.

**S**ingh J, Chonkar A, Bracken N, Adeghate E, Latt Z, Hussain M. Effect of streptozotocin-induced type 1 diabetes mellitus on contraction, calcium transient, cation contents in the isolated rat heart. *Annals of the New York Academy of Sciences* 2006;1084: 178–190.

**S**oria B, Tuduri E, Gonzalez A, Hmadcha A, Martin F, Nadal A. Pancreatic islet cells: a model for calcium-dependent peptide release. *HFSP Journal* 2010;4(2):52-60.

**S**richamroen A. Intestinal transport of monosaccharides. *Naresuan University Journal* 2007;15(2):127-135.

**S**tapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE. "Mammalian AMP-activated protein kinase subfamily". *Journal of Biological Chemistry1996*:271(2):611–4.

**S**teel A, Hediger MA. The Molecular Physiology of Sodium- and Proton-Coupled Solute Transporters. *News in Physiological Science* 1998;13:123-131.

**S**teiner DJ, Kim A, Miller K, Hara M. Pancreatic islet plasticity: Interspecies comparison of islet architecture and composition. *Islets* 2010;2(3):135-145.

**S**tumbo P, Hemingway D, Haynes WG. Dietary and Medical Therapy of Obesity. *Surgical Clinics of North America* 2005;85(4):703-723.

Stellenbosch University https://scholar.sun.ac.za

**S**tumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet* 2005;365(9467):1333-1346.

**S**tyskal J, Van Remmen H, Richardson A, Salmon AB. Oxidative stress and diabetes: What can we learn about insulin resistance from antioxidant mutant mouse models? *Free Radical Biology and Medicine* 2012;52(1):46-58.

**S**ugden MC, Caton PW, Holness MJ. PPAR control: it's SIRTainly as easy as PGC. *Journal of Endocrinology* 2010;204(2):93-104.

**S**werdlow RH. Does mitochondrial DNA play a role in Parkinson's disease? A review of cybrid and other supportive evidence. *Antioxidants & Redox Signaling* 2012;16(9):950-964.

**T**anti J, Jager J. Cellular mechanisms of insulin resistance: role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Current Opinion in Pharmacology* 2009;9(6):753-762.

**T**hong FS, Dugani CB, Klip A. Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology (Bethesda)* 2005;20:271-284.

**T**hrower SL, Bingley PJ. What is type 1 diabetes? *Medicine* 2010;38(11):592-596.

**T**imothy Garvey W, Birnbaum MJ. 1 Cellular insulin action and insulin resistance. *Baillière's Clinical Endocrinology and Metabolism* 1993;7(4):785-873.

**T**olman KG, Chandramouli J. Hepatotoxicity of the thiazolidinediones. *Clinical Liver Disease* 2003;7(2):369-79, vi.

**T**orpy J, Lynm C, Golub R. Pancreatitis. *The Journal of the American Medical Association* 2012;307(14):1542.

**T**urban S, Hajduch E. Protein kinase C isoforms: Mediators of reactive lipid metabolites in the development of insulin resistance. *FEBS Letters* 2011;585(2):269-274.

**T**urnbull DM, Bartlett K, Watmough NJ, Shepherd IM, Sherratt HS. Defects of fatty acid oxidation in skeletal muscle. *Journal of Inherited Metabolic Disease* 1987;10(1):105-112.

**T**urner RC, Matthews DR, Clark A, O'Rahilly S, Rudenski AS, Levy J. 3 Pathogenesis of NIDDM—a disease of deficient insulin secretion. *Baillière's Clinical Endocrinology and Metabolism* 1988;2(2):327-342.

**v**an Haeften TW. Early disturbances in insulin secretion in the development of type 2 diabetes mellitus. *Molecular Cell Endocrinology* 2002;197(1–2):197-204.

Stellenbosch University https://scholar.sun.ac.za

**V**irally M, Blicklé J-, Girard J, Halimi S, Simon D, Guillausseau P-. Type 2 diabetes mellitus: epidemiology, pathophysiology, unmet needs and therapeutical perspectives. *Diabetes & Metabolism* 2007;33(4):231-244.

**W**aite KA, Eng C. Protean PTEN: Form and Function. *The American Journal of Human Genetics* 2002;70(4):829-844.

**W**ang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR. Protein Kinase B/Akt Participates in GLUT4 Translocation by Insulin in L6 Myoblasts. *Molecular and Cellular Biology* 1999;19(6):4008-4018.

**W**ang X, Jiang X. PTEN: a default gate-keeping tumor suppressor with a versatile tail. *Cell Research* 2008;18(8):807-816.

**W**ei M, Ong L, Smith MT, Ross FB, Schmid K, Hoey AJ, et al. The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. *Heart, Lung and Circulation* 2003;12(1):44-50.

**W**orld Health Organization. Global Health Estimates: Deaths by Cause, Age, Sex and Country, 2000-2012. Geneva, WHO, 2014.

**W**ilcox G. Insulin and Insulin Resistance. *The Clinical Biochemist Reviews* 2005;26(2):19-39.

**W**inder WW. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *Journal of Applied Physiology* 2001;91:1017-1028.

**W**ojtaszewski JFP, MacDonald C, Nielsen JN, Hellsten Y, Hardie GD, Kemp BE, Kiens B, and Richter EA. Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *The American Journal of Physiology - Endocrinology and Metabolism* 2003;(284): E813–E822.

**W**oodgett JR, Cohen P. Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochimica et Biophysica Acta*1984;788(3):339-47.

**W**right EM. Glucose galactose malabsorption. *American Journal of Physiology* 1998;275(5):G879-G882.

**Y**amauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nature Medicine* 2007;13(3):332-339.

Stellenbosch University https://scholar.sun.ac.za

**Y**ang BT, Dayeh TA, Volkov PA, Kirkpatrick CL, Malmgren S, Jing X. Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes. *Molecular Endocrinology* 2012;26(7):1203-1212.

**Y**ang Y, Hua QX, Liu J, Shimizu EH, Choquette MH, Mackin RB. Solution structure of proinsulin: connecting domain flexibility and prohormone processing. *Journal of Biological Chemistry* 2010;285(11):7847-7851.

**Z**hao L, Guo M, Matsuoka TA, Hagman DK, Parazzoli SD, Poitout V. The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. *Journal of Biological Chemistry* 2005;280(12):11887-11894.

**Z**ick Y. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. *Trends Cell Biology* 2001;11(11):437-441.

**Z**iegler AG, Nepom GT. Prediction and Pathogenesis in Type 1 Diabetes. *Immunity* 2010;32(4):468-478.

**ii. Book**

**B**arker JM. Chapter 5 The Pathogenesis of Type 1 Diabetes. Handbook of Systemic Autoimmune Diseases: 45-59.

**B**erg JM, Tymoczko JL, Stryer L. Biochemistry. Glycolysis Is an Energy-Conversion Pathway in Many Organisms. 5th edition. New York: 2002

**O**pie LH. The heart physiology from cell to circulation. Philadelphia: 3rd edition:Lippincott-Raven: 1998

**R**aven PH, Johnson GB. Chapter 51 Fueling Body Activities: Digestion. 6th edition. Biology: 2001

**S**chulz H. Chapter 5 Oxidation of fatty acids in eukaryotes. New Comprehensive Biochemistry:127-150, 2008.

**Z**ubay G. Chapter 22 - Evolution of the Main Energy-Producing Pathway for Aerobic Metabolism: The Tricarboxylic Acid Cycle. Origins of Life on the Earth and in the Cosmos. 2nd edition. San Diego: Academic Press: 433-450, 2000.

**iii. Websites**

1. http://www.who.int/mediacentre/factsheets/fs311/en/print.html.