ATM EXPRESSSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS AS A BIOMARKER OF INSULIN RESISTANCE

By

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DECLARATION

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ABSTRACT

Introduction: The Ataxia Telangiectasia Mutated (ATM) gene codes for the 350 kDa ATM protein kinase. *ATM* gene mutations cause inactivity/deficiency of the ATM protein, resulting in the autosomal recessive disease Ataxia Telangiectasia (AT). AT patients are predisposed to developing insulin resistance or further progress to type 2 diabetes and are at high risk of developing ischaemic heart disease. Due to the prevalence of insulin resistance in AT patients, we investigated the relationship between ATM protein levels and the degree of insulin resistance and proposed it as a possible early diagnostic technique for insulin resistance.

Aim: To determine whether peripheral blood mononuclear cells (PBMCs) can be used to determine ATM levels in insulin resistant subjects and subsequently used as a biomarker of insulin resistance.

Objectives: (*i*) To standardise a protocol for the isolation of PBMCs from rat blood. (*ii*) To isolate rat PBMCs and determine the ATM levels using Western blotting. (*iii*) To determine differences between ATM levels in obese rats and compare them to controls. (*iv*) To analyse PBMCs from a female Black Xhosa population with different degrees of insulin resistance and to determine a relationship with ATM levels.

Methods: Male Wistar rats were fed an obesogenic diet (*od*) for 16 weeks to induce obesity and insulin resistance and compared to age-matched and young controls fed standard rat chow. Body weight and intraperitoneal (IP) fat mass were determined and oral glucose tolerance test (OGTT) was performed. PBMCs were isolated according to the standardised protocol and Western blotted for ATM and $P22^{phox}$. The Western blotting protocol was repeated with samples collected from patients.

Results from the animal model: 1. Effects of *obesity/insulin resistance* vs. age-matched controls: (*i*) larger IP fat mass; (*ii*) increased area under the curve of OGTT's; (*iii*) elevated basal glucose levels. (*iv*) The phospho-(P)/total-(T) ATM ratio was decreased.

2. *Effects of age:* (*i*) As expected, older animals weighed more while T-ATM was decreased, P-ATM increased and the P-/T-ATM ratio increased with age.

In PBMC's from patients, the following were observed: (*i*) Body mass index (BMI) was significantly higher in obese and pre-diabetic vs. control patients. (*ii*) The waist-to-hip ratio (WHR) of obese and pre-diabetic women was higher vs. controls. (*iii*) Trunk-to-limb fat mass (TF/LF) was increased in obese and pre-diabetics vs. controls but (*iv*) no differences in the lipid profiles were observed

except for increased triglyceride levels between young pre-diabetic patients vs. their controls. (*v*) Fasting blood glucose of obese and pre-diabetic patients was significantly increased vs. controls. (*vi*) Significantly higher P-ATM levels were seen for obese and pre-diabetic vs. control patients. T-ATM levels increased with the state of insulin resistance.

Effects of age: (*i*) BMI was significantly higher between young (Y) and middle aged (MA) control, obese and pre-diabetic groups vs. their respective controls while (*ii*) WHR of Y obese and Y pre-diabetic vs. Y controls also increased significantly. (*iii*) The TF/LF ratio was increased between Y and MA control and Y obese, Y pre-diabetic, MA obese and MA pre-diabetic women vs. their respective controls. Furthermore (*iv*) the blood glucose levels of Y pre-diabetics were increased vs. Y control. (*v*) The P-ATM levels was increased in Y pre-diabetic vs. Y control and therefore did not increase with age but the T-ATM levels significantly increased with age.

Conclusion: ATM levels can be measured in PBMCs and are affected by the insulin resistant state and age. Unfortunately, due to the variation in ATM levels under different degrees of insulin resistance, it would be difficult to use ATM as a biomarker of insulin resistance.

OPSOMMING

Inleiding: Die Ataxia Telangiectasia gemuteerde (ATM) geen kodeer vir die 350 kDa ATM proteïen kinase. Mutasies in die *ATM* geen lei tot uitdrukking van geen of onaktiewe proteïen wat die outosomaal resessiewe siekte, Ataxia Telangiectasia (AT), veroorsaak. AT pasiënte is voorbeskik om insulienweerstandigheid of tipe 2 diabetes te ontwikkel en het 'n hoë risiko vir iskemiese hartsiektes. Na aanleiding van die prevalensie van insulienweerstandigheid in AT pasiënte, het ons die verhouding tussen ATM proteïenvlakke en die graad van insulienweerstandigheid bestudeer en dit voorgestel as 'n moontlike vroeë diagnostiese tegniek vir insulienweerstandigheid.

Doel: Om te bepaal of perifere bloed mononukluêre selle (PBMCs) gebruik kan word om ATM vlakke in insulienweerstandige subjekte te meet en te bepaal of dit as biomerker vir insulienweerstandigheid gebruik kan word.

Objektiewes: Om (*i*) 'n protokol vir die isolasie van PBMCs uit rot bloed te standardiseer. (*ii*) Rot PBMCs te isoleer en die ATM vlakke met behulp van Westerse kladtegniede te bepaal. (*iii*) Verskille tussen ATM vlakke in vetsugtige rotte te vergelyk met kontrole rotte. (*iv*) PBMCs verkry van Swart vroulike Xhosa pasiënte met verskillende grade van insulienweerstandigheid te analiseer en die verhouding met ATM uitdrukking te bepaal.

Metodes: Manlike Wistarrotte is vir 16 weke met 'n obesogene dieet gevoer en vergelyk met ouderdomsgepaarde en jong kontrole diere op standaard rotkos. Liggaamsgewig en intraperitoneale (IP) vetgewig is bepaal en 'n orale glukosetoleransie toets (OGTT) uitgevoer. PBMCs is geïsoleer deur die gestandardiseerde metode en ATM en P22phox deur Westerse kladtegnieke bepaal. Die Westerse kladtegniek is op die pasiënte monsters herhaal.

Resultate verkry van die diermodel: 1. Effekte van *obesiteit/insulienweerstandigheid* vs. ouderdomsgepaarde kontroles: (*i*) groter IP vetmassa; (*ii*) verhoogde area onder die kurwe van die OGTT's (*iii*) verhoogde basale bloedglukosevlakke is gemeet. (*iv*) Die fosfo-(P)/totale (T) ATM verhouding was verlaag.

2. *Effekte van ouderdom:* (*i*) soos verwag, het die ouer diere meer geweeg terwyl T-ATM vlakke verlaag het, P-ATM verhoog en die P/T verhouding beduidend verhoog het met ouderdom.

In PBMC's van pasiënte is die volgende waargeneem: (*i*) die liggaamsmassaindeks (LMI) was beduidend hoër in die vetsugtige en pre-diabetiese pasiënte vs. kontroles. (*ii*) die middel-tot-heup (MTH) verhouding van vetsugtige en pre-diabetiese vroue was hoër as die kontroles. (*iii*) Die romp-tot-ledemaat (RV/LV) vetmassa was verhoog in die vetsugtige en pre-diabetiese vroue vs.

kontroles maar (*iv*) geen verskille in die lipiedprofiele is waargeneem nie, buiten verhoogde trigliseriedvlakke tussen jong pre-diabetiese pasiënte vs. hulle kontroles. (*v*) Vastende bloedglukose van vetsugtige en pre-diabetiese pasiënte was beduidend hoër as kontroles. (*vi*) Beduidend hoër P-ATM vlakke is waargeneem in die vetsugtige en pre-diabetiese vs. kontrole pasiënte. T-ATM vlakke het verhoog met die staat van insulienweerstandigheid.

Effekte van ouderdom: (*i*) LMI was beduidend hoër in die jong (J) en middeljarige (MJ) kontrole, vetsugtige en pre-diabetiese groepe vs. hulle onderskeie kontroles terwyl (*ii*) die MTH van die J vetsugtige en pre-diabetiese vs. J kontroles ook beduidend hoër was. (*iii*) Die RV/LV verhouding het toegeneem tussen J en MJ kontrole en J vetsugtig, J pre-diabeties, MJ vetsutgig en MJ pre-diabetiese vroue vs. hulle onderskeie kontroles. Verder (*iv*) was die bloedglukosevlakke van die J pre-diabetiese pasiënte verhoog teenoor J kontroles. (*v*) Die P-ATM vlakke was verhoog in die J pre-diabetiese vs. J kontroles en het dus nie met ouderdom verhoog nie maar die totale vlakke van ATM het beduidend toegeneem met ouderdom.

Gevolgtrekking: ATM vlakke kan in PBMCs gemeet word en die vlakke word beïnvloed deur die insulienweerstandige staat sowel as ouderdom. Ongelukkig, as gevolg van variasies in ATM vlakke in die verskillende grade van insulienweerstandigheid, sal dit problematies wees om ATM as ‘n biomarker van insulienweerstandigheid te gebruik.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µl	Microlitre
4E-BP1	Eukaryotic translation initiation factor 4E – binding protein 1
AMP	5' adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APS	Ammonium persulfate
ASCVD	Atherosclerotic cardiovascular disease
AT	Ataxia Telangiectasia
ATM 1981S-P	ATM serine-1981 phosphorylation
ATM	Ataxia Telangiectasia Mutated
AU	Arbitrary Unit
AUC	Area under the curve
BBB	Blood-brain barrier
BER	Base excision repair
BMI	Body Mass Index
BMI-SDS	BMI standard deviation score
BSA	Bovine serum albumin
CCS	Childhood cancer survivors
CGD	Chronic granulomatous disease
cm	Centimetre
CNS	Central nervous system

DBP	Diastolic blood pressure
dL	Decilitre
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DSBR	Double-strand break repair
DXA	Dual energy X-ray absorptiometry
EDTA	Ethylenediaminetetraacetic acid
eIF-4E	Eukaryotic translation initiation factor 4E
FFA	Free fatty acid
g	Gram
g	Relative centrifugal force
GLUT4	Glucose transporter type 4
GWAS	Genome-wide association study
H2AX	Histone 2A gene
HDL	High-density lipoproteins
HEK	Human embryonic kidney
HFD	High-fat diet
HOMA-IR	Homeostasis model assessment for insulin resistance
HR	High responding
HRP	Horseradish peroxidase
HRR	Homologous recombinational repair
HSA	Human Serum Albumin

HTR	HDL cholesterol/total cholesterol ratio
ICAM-1	Intracellular adhesion molecule-1
IFG	Impaired fasting glucose
IgG	Immunoglobulin G
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IP	Intraperitoneal
IRS-1	Insulin receptor substrate 1
J	Jong
kDa	Kilodalton
kg	Kilogram
kJ	Kilojoule
LDL	Low-density lipoproteins
LF	Limb fat mass
LH	Luteinizing hormone
LMI	Liggaamsmassaindeks
LR	Low responding
m	Metre
M	Molar
MA	Middle aged
mA	Milliamperes
MCP-1	Macrophage chemoattractant protein-1
MEF	Mice embryonic fibroblasts
mg	Milligram

min	Minute
MJ	Middeljarige
mL	Millilitre
mm	Millimetre
mM	Millimolar
mmHg	Millimetre per mercury
mmol/L	Millimoles per litre
mRNA	Messenger Ribonucleic acid
mROS	Mitochondrial reactive oxygen species
MTH	Middel-tot-heup
mTOR	Mammalian target of Rapamycin
MUFA	Monounsaturated fatty acids
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NCD	Non-communicable disease
NER	Nucleotide excision repair
NF-κB	Nuclear factor-kappa B
NHEJ	Non-homologous end joining
nm	Nanometre
NO	Nitric oxide
NOXs	NADPH oxidases
od	Obesogenic diet
OD	Optical density
OGTT	Oral glucose tolerance test

P	Phosphorylated or fosfo
PAGE	Polyacrylamide Gel Electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Positive control
PCOS	Polycystic ovary syndrome
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
rpm	Revolutions per minute
RR	Relative risk
RT-PCR	Real time polymerase chain reaction
RV/LV	Romp-tot-ledemaat vetmassa
s	Second
SADHS	South Africa Demographic and Health Survey
SAT	Saturated fatty acids
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SHBG	Sex hormone binding globulin
SIP	Stock Isotonic Percoll

SNP	Singular nucleotide polymorphism
SSB	Single-strand break
SSBR	Single-strand break repair
T	Total or totale
T1D	Type I Diabetes mellitus
T2D	Type II Diabetes mellitus
TAG	Triglycerides
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TF	Trunk fat mass
Thr	Threonine
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
V	Volt
v/v	volume/volume
vs.	versus
WHO	World Health Organisation
WHR	Waist to hip ratio
Y	Young
β	Beta
γ-H2AX	Gamma H2AX

CHAPTER 1: LITERATURE REVIEW

1. Ataxia Telangiectasia

Ataxia Telangiectasia (AT) also known as Louis-Bar syndrome is an autosomal recessive disease caused by mutations in the AT Mutated (*ATM)* gene and the deficiency of the ATM protein kinase (Foroughizadeh et al. 2012). The mutations in the *ATM* gene are hereditary and cause the expression of inactive or no ATM protein (Lavin and Shiloh, 1997; Yang *et al.*, 2011).

1.1 AT symptoms and characteristics

AT patients characteristically present with insulin resistance, an increased risk of developing type II diabetes mellitus (T2D) (Ristow, 2004; Lavin *et al.*, 2007), cerebellar degeneration, immunodeficiency and an increased predisposition for developing cancer (Llorca *et al.*, 2003), premature ageing (Shiloh and Lederman, 2017) and ischaemic heart disease (van Os *et al.*, 2016). Ataxia refers to the loss of coordinated body movements which occurs in AT patients due to cerebellar ataxia and subsequent neurodegeneration (Khalil, Tummala and Zhelev, 2012). Ataxic gait and truncal movements are seen when AT children start walking and the cerebellar degeneration processes affecting speech and movement causing immobilisation by the end of 10 years of age (Lavin and Shiloh, 1997). Telangiectasia is when dilated capillaries and veins become visible under the skin and on the nose, ears, conjunctiva of the eye and behind the knees (Khalil, Tummala and Zhelev, 2012) usually between the ages of 2 and 8 years (McFarlin, Strober and Waldmann, 1972; Boder E., 1985).

2. Overview of ATM gene and protein

2.1 ATM gene

ATM is a human tumour suppressor gene which codes for the 350 kDa protein, Ataxia Telangiectasia Mutated protein kinase (ATM) (Llorca *et al.*, 2003). The ATM protein kinase was discovered when the deficiency thereof caused the characteristics found in the AT disease. The ATM gene was mapped and localised to chromosome 11q22-23 (Gatti *et al.*, 1988). Mutations in a specific gene were found to cause the phenotypes seen in AT patients and was named the *ATM* gene (Savitsky *et al.*, 1995).

2.2 ATM protein kinase

ATM forms part of the phosphatidylinositol 3 (PI3) kinase protein family (Zhou *et al.*, 2011) and is a complex protein taking part in a number of physiological processes such as cell cycle regulation (Llorca *et al.*, 2003) metabolic regulation, oxidative stress, cell proliferation, transcriptional

modulation, protein degradation and DNA double-strand break repair (DSBR) (Khalil, Tummala and Zhelev, 2012). ATM is the main regulator in the response to Deoxyribonucleic acid (DNA) damage as well as regulating cell-cycle checkpoint activation, DNA repair and changes in metabolism due to DNA double-strand breaks (DSBs) (Paull, 2015). ATM exists as an inactive dimer but splits into its monomers when activated by DNA DSBs (Bakkenist and Kastan, 2003).

ATM is expressed as a serine/threonine (Ser/Thr) protein kinase in the nucleus (Chen and Lee, 1996; Scott *et al.*, 1998). During attacks to the genome, existing ATM is activated by phosphorylation of serine residues (Bakkenist and Kastan, 2003; Kozlov *et al.*, 2006) causing the protein to dissociate into its monomers for DNA repair (Bakkenist and Kastan, 2003). ATM is considered a housekeeping gene and is predominantly located in the nucleus (Gately *et al.*, 1998) where its basal levels exist as phosphorylated at Ser-1981 (Khalil, Tummala and Zhelev, 2012).

2.3 Localisation and functions of ATM

According to Khalil *et al.* (2012) a number of studies have investigated the localisation of ATM where it was found in compartments such as peroxisomes, endosomes, Golgi apparatus, plasma membrane, nucleus and the cytoplasm and cytoplasm vesicles. According to Yang *et al.* (2011), ATM's activation by DNA DSBs and subsequent cell cycle arrest is only due to the functioning of the ATM located in the nucleus and cannot explain the other characteristic disorders seen in AT patients. Most of the identified functions of ATM were deduced from its amino acid sequence, however more than 90% of its sequence's function has not yet been identified (Yang *et al.*, 2011).

Nuclear ATM

A study was done to determine the localisation of ATM where cells were sub-fractionated into cytoplasmic, microsomal and nucleic fractions and it was found to localise in the nucleus and microsomal fractions (Watters *et al.*, 1997). ATM located in the nucleus specifically responds to DSBs of DNA (Abraham, 2001). It furthermore functions as a cell-cycle checkpoints regulating protein when activated by the DNA DSBs where it phosphorylates downstream effectors (Llorca *et al.*, 2003). The phosphorylated downstream effectors and proteins stop the cell cycle, recruits DNA repair proteins (Khalil, Tummala and Zhelev, 2012) or, during severe DNA damage, apoptosis is initiated (Lee and McKinnon, 2000). Yang *et al*. (2011) suggested that, as ATM functions as a regulator of cell-cycle checkpoints and repairs damaged DNA, this would explain why AT patients are prone to developing cancer.

Cytoplasmic ATM

As previously stated by Yang *et al.* (2011) it was suggested that other characteristics seen in AT patients could be attributed to ATM localised elsewhere in the cell. In studies done in neuronal cells, ATM was found to be mainly localised in the cytoplasm (Oka and Takashima, 1998; Barlow *et al.*, 2000; Boehrs *et al.*, 2007). It was also reported that ATM is present in the cytoplasm of proliferating cells where it interacts with beta (β)-adaptin (a cytoplasmic vesicle trafficking protein) (Lim *et al.*, 1998). Furthermore, links between cytoplasmic ATM and a role in insulin signalling was found (Yang and Kastan, 2000; Viniegra *et al.*, 2005; Halaby *et al.*, 2008).

Mitochondrial ATM

According to Valentin-Vega *et al*. (2012), a fraction of ATM is localised in the mitochondria and is activated during mitochondrial dysfunction. A study investigating mitochondrial dysfunction in AT, analysed mice thymocytes and found an increase in the number of altered mitochondria under AT conditions (Valentin-Vega *et al.*, 2012). Furthermore, thymic cells from ATM null mice were found to contain higher mitochondrial mass and increases in mitochondrial reactive oxygen species (mROS). Valentin-Vega *et al.* (2012) deduced from their data that the increase in mitochondrial mass is possibly due to a decrease in mitophagy. Overall it was found that ATM deficiency caused mitochondria to be abnormal and dysfunctional; increased production of mROS and decreased mitophagy. Subsequently it was hypothesised that ATM may play a role in mitochondrial function and reactive oxygen species (ROS) production (Valentin-Vega *et al.*, 2012).

3. ATM in insulin signalling

The relationship between ATM and insulin signalling has not been fully established and is still largely been researched. Studies investigating the function of ATM in the cytoplasm have discovered its role as an insulin-responsive protein and hypothesised that a defect in this response could be responsible for the development of insulin resistance and T2D in AT patients (Yang and Kastan, 2000).

Yang and Kastan (2000) investigated the phosphorylation of eukaryotic translation initiation factor 4E (eIF-4E) - binding protein 1 (4E-BP1) causing the release of eIF-4E through insulin treatment for protein synthesis. The study reported that (*i*) ATM phosphorylates 4E-BP1 at Ser-111 *in vitro* and insulin treatment caused the phosphorylation of 4E-PB1 at Ser-111 *in vivo* ATM-dependently (Yang and Kastan, 2000) (*Figure 1.1*) . These findings were in agreement with a previous study, where insulin treatment was found to cause kinase activity that phosphorylated Ser-111 of 4E-BP1

(Heesom *et al.*, 1998). This kinase activity resembled that of ATM (Yang and Kastan, 2000) and was inhibited by wortmannin (PI3 kinase inhibitor) but not by rapamycin (an inhibitor of the mammalian target of rapamycin (mTOR)) (reviewed in (Yang and Kastan, 2000)). Insulin treatment was found to cause a two-fold increase in ATM kinase activity in HEK (human embryonic kidney) 293 cells irrespective of the substrate and a three-fold increase in 3T3 L1 mice cells, differentiated into adipocytes. It was suggested that (*ii*) 4E-BP1 phosphorylation occurs on Thr-36 and Thr-45 via the PI3K/protein kinase B (PKB)/mTOR pathway and on Ser-111 ATM-dependently (Yang and Kastan, 2000) (*Figure 1.1*).

For the relevance of this review, the PI3K/PKB/mTOR pathway will not be discussed in detail.

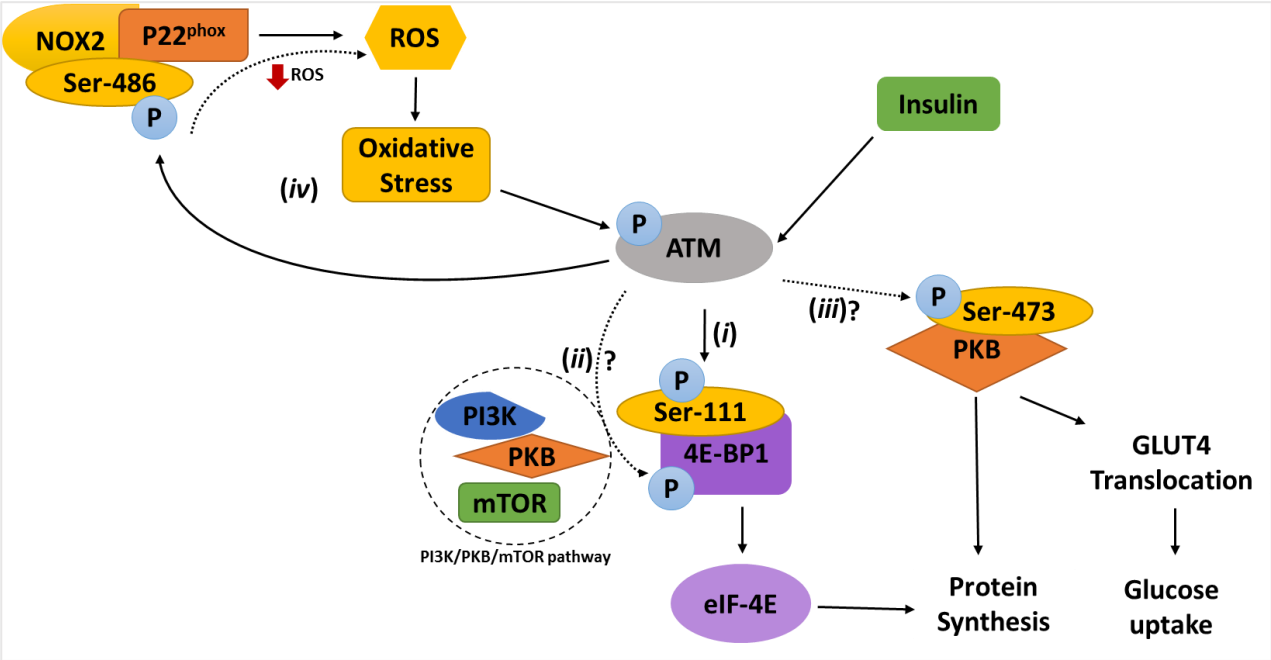

Figure 1.1: *Pathways with ATM as a role playing protein. Dotted arrows indicate multiple reactions in between. Dotted pathways with question marks have yet to be fully investigated. Ataxia Telangiectasia Mutated (ATM), Protein kinase B (PKB), AMP-activated protein kinase, Phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), Eukaryotic translation initiation factor 4E – binding protein 1 (4E-BP1), Eukaryotic translation initiation factor 4E (eIF-4E), NADPH oxidase 2 (NOX2), Reactive oxygen species (ROS), glucose transporter 4 (GLUT4), Serine (Ser), phosphorylated (P).*

A study by Viniegra *et al.* (2005) investigating the relationship between PKB and ATM, found that (*iii*) ATM induced phosphorylation of PKB Ser-473 during insulin treatment; however they were not able to determine whether ATM phosphorylated PKB directly (*Figure 1.1*). It was concluded that, although ATM does not seem to phosphorylate PKB directly, it acts as an upstream regulator of PKB Ser-473 phosphorylation (Viniegra *et al.*, 2005). Following insulin release, PKB is activated in the PI3K pathway and is responsible for protein translation (Lawrence and Abraham, 1997;

Sonenberg, Hershey and Mathews, 2000) as well as initiating glucose transporter type 4 (GLUT4) translocation to the membrane for glucose uptake (Pessin and Saltiel, 2000; Bryant, Govers and James, 2002).

A study by Halaby and colleagues (2008) investigated ATM expression and PKB phosphorylation in muscle tissue of rats fed with an insulin resistance-inducing high-fat diet. The results showed a decrease in ATM expression in the insulin resistant animals when compared to the controls. Halaby *et al.* (2008) suggested that low PKB activity is the main factor responsible for the development of ineffective glucose uptake and insulin resistance in the rats fed a high-fat diet, however the stage of the insulin signalling pathway at which deficiency starts, could not be determined. It was hypothesised that, as common characteristics of AT patients are insulin resistance that could develop into T2D and glucose intolerance, it could be plausible that decreased ATM expression levels may contribute to the development of insulin resistance in the high-fat diet rat model by causing the decrease in PKB activity (Halaby *et al.*, 2008). It was found that there was no decrease in the expression or activation of the insulin receptor substrate 1 (IRS-1) in AT cells when compared to normal cells and it was suggested that AT cells have a possible defect in intracellular insulin signalling (Halaby *et al.*, 2008). Furthermore, it was suggested that, as PKB is responsible for GLUT4 translocation for glucose uptake, the deficiency of ATM may down regulate the activation of PKB and subsequently the translocation of GLUT4, ultimately leading to glucose intolerance and insulin resistance symptoms seen in AT patients and in high-fat diet rat models (Halaby *et al.*, 2008).

4. ATM and adipocyte function

Takagi and colleagues (2015) hypothesised that adipocyte dysfunction is responsible for glucose intolerance and insulin resistance in AT individuals. In a study using knockout *ATM* mice, the mice were found to be insulin resistant and to have less subcutaneous adipose tissue when compared to wild-type mice (Takagi *et al.*, 2015). With further *in vitro* investigation in mice embryonic fibroblasts (MEF), it was found that adipocyte differentiation was impaired in $ATM^{-/-}$ cells due to a deficiency of important transcription factors for the induction of adipocyte differentiation (Takagi *et al.*, 2015). Takagi and colleagues (2015) further reported an ATM function not previously characterised, where ATM regulates important adipocyte transcription factors.

5. ATM and insulin resistance

Insulin resistance is commonly observed amongst AT patients. According to Yang *et al.* (2011), AT patients usually die within the first 30 years of age while T2D is usually diagnosed later in life, normally from 40 years of age, and it has been speculated that the percentage of AT patients found to have T2D may have been misrepresented (Robinson and Kessling, 1992). An oral glucose tolerance test (OGTT) study was performed by Yang et al. (2011), where AT individuals were found to show symptoms of glucose intolerance, hyperinsulinaemia and hyperglycaemia. An article in 1970 already stated that 10 out of 17 AT patients developed T2D (Schalch, McFarlin and Barlow, 1970). T2D develops when the β-cells of the pancreas, which secrete insulin, are unable to compensate for the increased glucose plasma levels (Wilcox, 2005). Further studies are needed for the investigation of other functions of ATM as a large number of ATM's signalling pathways, especially those pathways involved in insulin signalling and glucose metabolism, are not fully understood.

The first genome-wide association study (GWAS) on glycaemic response to metformin treatment in type 2 diabetics, identified a singular nucleotide polymorphism (SNP) at the locus containing the *ATM* gene (Zhou *et al.*, 2011). With further investigation, the inhibition of the ATM protein was found to down-regulate the phosphorylation and activation of the 5' adenosine monophosphate (AMP)-activated protein kinase (AMPK) in response to metformin, which is the first line drug for T2D. Zhou and colleagues (2011) concluded that variation in the *ATM* gene causes a variation in the glycaemic response to metformin treatment.

6. $P22^{phox}$

$P22^{phox}$ is a 22 kDa transmembrane protein that forms part of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases referred to as NOXs and together generate superoxide (Stasia, 2016). $P22^{phox}$ can form a complex with NOX1, NOX2, NOX3 and NOX4 and these complexes were found to be important sources of ROS and are associated with a number of diseases such as cardiovascular disease and cerebrovascular disease (Stasia, 2016). $P22^{phox}$ forms a complex with NOX2 and forms cytochrome b_{558} and is mainly expressed in phagocytes where its function is to eliminate microorganisms during fungal and bacterial infections (Stasia, 2016). The importance of NOXs in eliminating ingested pathogens, via phagocytosis, was proven by chronic granulomatous disease (CGD) (Stasia, 2016), which is caused by a defect of the oxidase complex of phagocytes where patients characteristically suffer from frequent, life threatening infections (van den Berg *et al.*, 2009). The absence of $P22^{phox}$ in X-linked CGD further motivated NOXs importance

(reviewed in (Stasia, 2016). Due to the discovery of NOXs as an important source of ROS, its role in cardiovascular diseases, namely hypertension, diabetes, kidney disease, heart failure, atherosclerosis and cerebrovascular disease is now broadly accepted (reviewed in (Stasia, 2016). While inactive, the NOX complexes are dormant and are activated when superoxide needs to be synthesised for phagocytosis (Stasia, 2016). Activation occurs by the assembly of all the subunits with cytochrome b_{558} which are all initiated by phosphorylation (reviewed in (Stasia, 2016)). The main function of $P22^{phox}$ is to assist with the maturation and stabilisation of the heterodimer that $P22^{phox}$ forms with the NOX enzymes (Stasia, 2016). Once assembled, electron transfer is initiated from NADPH to oxygen which results in oxidising species production used in phagocytosis of pathogens.

A study by Delbosc *et al.* (2005) investigated the importance of ROS production in the development of cardiovascular complications that are associated with insulin resistance, using fructose-fed rats. The insulin resistant fructose-fed rats showed significant superoxide anion production which they hypothesised to be due to NOX. With further investigation, immunoblotting of $P22^{phox}$ in left ventricular tissue, showed an overexpression in insulin resistant rats (Delbosc *et al.*, 2005). It was concluded that raised cardiac ROS production is linked to overexpression of $P22^{phox}$ and that this was dependent on NOX which is proven through elevated $P22^{phox}$ levels in high fructose - fed rats (Delbosc *et al.*, 2005).

A recent study found that (*iv*) once NOXs, specifically NOX2 is activated, ROS production takes place and superoxide is produced leading to oxidative stress, which activates ATM via phosphorylation. Phosphorylated ATM subsequently phosphorylates Ser-486 in NOX2 causing a conformational change and a decrease in ROS production (Beaumel *et al.*, 2017) (*Figure 1.1*).

7. Insulin resistance

Insulin resistance develops when the cells have a decreased sensitivity to the secretion of normal or high levels of insulin in the blood causing a decrease in the overall physiological responses (Wilcox, 2005). Insulin resistance is found in association with a number of other diseases, known as clinical syndromes, such as T2D, cardiovascular disease, hypertension, certain cancers and the metabolic syndrome (Reaven, 2004).

Pre-diabetic state: Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are considered pre-diabetic states (Bacha *et al.*, 2010). Pre-diabetes characteristically presents with

elevated fasting blood glucose or abnormal glucose tolerance or both and is associated with an increased predisposition for developing T2D in adults (American Diabetes Association, 2004b).

T2D: Diabetes mellitus is the insufficient metabolism of glucose subsequently initiating impaired glucose homeostasis of the blood (Ristow, 2004). T2D, specifically, is defined as a non-insulin-dependent diabetes (type II) and is the most common form of diabetes mellitus which commonly manifests during middle age and older ages or in the presence of factors such as a sedentary lifestyle, obesity and the metabolic syndrome (Ristow, 2004).

7.1 Insulin resistance and diseases

Insulin resistance is also highly prevalent in diseases such as obesity (Lee and Lee, 2014; Oh *et al.*, 2017), non-alcoholic fatty liver disease (NAFLD) (Paniagua *et al.*, 2014), which is considered the most common liver disorder in the world (Pala *et al.*, 2014) as well as polycystic ovary syndrome (PCOS) (Dunaif *et al.*, 1989), which is the most prevalent endocrinopathy amongst women of reproductive age (Pala *et al.*, 2014).

7.1.1 Obesity

Obesity is a state of excessive and/ or abnormal adiposity (Wang *et al.*, 2017) and is associated with other metabolic disorders such as T2D; cardiovascular disease and cancer (Hajer, van Haeften and Visseren, 2008). Risk factors such as adipose tissue dysfunction, mitochondrial dysfunction, gut microbiota dysbiosis and myofibre types have also been found to have an association with insulin resistance and obesity (Wang *et al.*, 2017). During obesity, adipose tissue has stored triglycerides to its fullest capacity and cannot store more lipids, causing lipolysis and an increase in free fatty acids (FFAs) in adipose tissue (Wang *et al.*, 2017). Obese patients are in a constant state of low-grade inflammation and more prone to contracting infections (Dicker *et al.*, 2013). Body Mass Index (BMI) is an index of weight in kilograms (kg) over height in metres squared (m^2) and is used for diagnosing adult obesity (Jung *et al.*, 2016). Individuals with a BMI exceeding 30 kg/m^2 are considered obese. The South Africa Demographic and Health Survey (SADHS) Key Indicator Report stated in 2016 and 2017, that 68% of women and 31% of men are overweight (BMI ≥25kg/m^2) or obese with 1 in 5 having a BMI ≥ 35 kg/m^2 classifying them as severely obese (National Department of Health (NDoH) *et al.*, 2017). Obese individuals characteristically present with waist circumferences exceeding 102 cm in men and 88 cm in women are considered abdominally obese (Hough, 2004).

Childhood overweight and obesity are increasing worldwide and is speculated to increase to 9% by 2020 (de Onis, Blössner and Borghi, 2010). Excessive weight amongst the young population may develop into obesity-related conditions at an earlier age (Moreno *et al.*, 2008; de Onis, Blössner and Borghi, 2010), namely T2D, cardiovascular disease (Baker, Olsen and Sørensen, 2008) and hypertension to name a few (Wabitsch, 2000; Reinehr and Wabitsch, 2011). With this in mind, obesity is considered a main health problem with a big effect on children, subsequently leading to disease in adulthood (Owen *et al.*, 2005).

a) Leptin and obesity

Leptin is a circulating peptide hormone produced by adipose cells and controls body weight by regulating food intake and metabolism (Friedman and Halaas, 1998). Leptin resistance is associated with increased plasma leptin levels in obesity and is more common than leptin deficiency, that develops into extreme obesity (reviewed in (Bacha *et al.*, 2010)). A number of studies have reported a directly proportional relationship between leptin and adipose tissue mass (Maffei *et al.*, 1995; Considine *et al.*, 1996; Zimmet *et al.*, 1996; Mahabir *et al.*, 2007). A Chinese population-based study found an independent association between leptin and all measures of adiposity (Zuo *et al.*, 2013). The results showed that insulin resistant overweight or obese participants had significantly higher leptin concentration levels, which was almost double, when compared to non-insulin resistant overweight or obese participants with the same level of adiposity in both sexes. Zuo *et al.* (2013) concluded that there is an association between leptin levels and insulin resistance independent of the level of obesity and hypothesised that the relationship seen between high levels of serum leptin in insulin resistance may explain the variation in metabolic risk amongst individuals with the same level of obesity. Zimmet *et al.* (1996) suggested that the variation in leptin concentration was possibly also influenced by other factors such as physical activity, nutrition, fat distribution, genotype, insulin or other hormones and not only the degree of obesity (Zimmet *et al.*, 1996). In a recent study the possibility of triglycerides causing leptin and insulin resistance in brain receptors were investigated where it was hypothesised to cause central insulin and leptin receptor resistance (Banks *et al.*, 2018). Leptin is able to cross the blood-brain barrier (BBB) and binds to its receptors in the brain where it stimulates weight loss, the production of heat and aids the central nervous system (CNS) for cognitive brain function (reviewed in (Banks *et al.*, 2018)). However, resistance of these receptors have been linked to increased eating, obesity and impaired cognition (Banks *et al.*, 2018). A previous study found triglycerides cause peripheral leptin resistance (Banks *et al.*, 2004). Banks

and colleagues (2018) found that triglycerides were able to cross the BBB and inhibit insulin and leptin from activating there canonical signalling pathways, subsequently affecting eating and cognitive brain function.

b) Dysfunctional adipose tissue

Adipose tissue becomes dysfunctional when it is unable to store all the excess FFAs, resulting in a decrease in production of adiponectin and an increased production of inflammatory cytokines (Coppack *et al.*, 1992; Skurk *et al.*, 2007). Adiponectin is an adipokine produced by adipose tissue and is responsible for the regulation of carbohydrate and lipid metabolism as well as regulating insulin sensitivity (Inoue and Tsugane, 2012). When adipose tissue is unable to store more lipids; lipolysis increases FFA levels in the adipose tissue (Wang *et al.*, 2017). FFAs are able to bind to macrophage toll-like receptor (TLR) 4 initiating inflammation (Shi *et al.*, 2006) as well as activation of macrophages (Wang *et al.*, 2017) present in adipose tissue. The activation of macrophages activates the nuclear factor-kappa B (NF-κB) pathway and subsequent synthesis and release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) (Suganami, Nishida and Ogawa, 2005; Suganami *et al.*, 2007). TNF-α activates adipocytes promoting lipolysis and the gene expression of pro-inflammatory cytokines such as interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1) and intracellular adhesion molecule-1 (ICAM-1) of which the latter two further promote the translocation of macrophages into the adipose tissue (Ruan *et al.*, 2002; Kanda *et al.*, 2006; Permana, Menge and Reaven, 2006; Amano *et al.*, 2014). In an obese state FFAs are constantly available allowing for the constant activation of macrophages and TLR-4 ultimately leading to an inflammatory state (Wang *et al.*, 2017). The inflammatory state promotes an imbalance in adiponectin secretion and proinflammatory markers secretion, subsequently leading to increased glucose production by the liver and a decrease in glucose uptake into adipose tissue and muscles via GLUT4 as well as the attenuation of the insulin signalling cascade (Yao *et al.*, 2016). The decrease in the downstream effects of insulin signalling promotes lipolysis of adipose tissue leading to deposition of lipids in non-adipose tissue, such as the liver and skeletal muscle, contributing to systemic insulin resistance (Rutkowski, Stern and Scherer, 2015). The surplus FFAs undergo FA oxidation ultimately producing substrates that are responsible for the development of insulin resistant conditions such as impaired glucose uptake and glucose oxidation (Randle *et al.*, 1963).

7.1.2 Cardiovascular diseases

Atherosclerosis is known to be a main risk factor for cardiovascular disease (Espach *et al.*, 2015). A number of metabolic factors have been found to be associated with an increased risk for atherosclerosis of which hyperglycaemia and insulin resistance are two abnormalities also directly associated with T2D (King and Wakasaki, 1999). T2D is recognised as a metabolic state which aggravates underlying mechanisms in the development of atherosclerosis and heart failure. Atherosclerotic cardiovascular disease (ASCVD) is the main cause of mortality and disability amongst diabetic patients (Low Wang *et al.*, 2016). Important indicators of ASCVD in T2D are coronary heart disease (advanced atherosclerosis), ischaemic stroke, peripheral artery disease and heart failure (Low Wang *et al.*, 2016). Diabetic individuals have been found to have a high prevalence of coronary and peripheral artery disease; however the lowering of glucose levels has yet to show an improvement in cardiovascular event rates (reviewed in (Low Wang *et al.*, 2016). T2D has a higher atherosclerotic plaque burden, higher percentage atheroma volume (the proportion of the total vessel wall volume occupied by atherosclerotic plaque) (Puri *et al.*, 2013) and smaller lumen diameters in the coronary arteries when compared to non-diabetic individuals (Nicholls *et al.*, 2008). Hyperglycaemia reduces endothelial function and decreases the availability of nitric oxide (NO) (Williams *et al.*, 1998). The high blood glucose concentration promotes inflammation of macrophages and enhances further inflammatory responses (Nishizawa and Bornfeldt, 2012). In an experiment comparing constant hyperglycaemia to induced episodes of hyperglycaemia followed by normoglycaemia over a period of 24 hours, it was found that the induced episodes of hyperglycaemia followed by normoglycaemia decreased endothelial function and increased oxidative stress (Ceriello *et al.*, 2008). Individuals with insulin resistance also experience increased rates of hypertension, dyslipidaemia and impaired glucose tolerance (Mikhail, 2009) which contribute to the development and progression of atherosclerosis (Low Wang *et al.*, 2016).

Endothelial function is weakened in both type I diabetes mellitus (T1D) and T2D (Johnstone *et al.*, 1993; Williams *et al.*, 1996). Studies have found that a short period of exposure to high concentrations of glucose is enough to decrease the bioavailability of nitric oxide (NO), as reviewed by Low Wang *et al.* (2016). Endothelial dysfunction aids the development of thrombosis, adhesion of leukocytes and platelets and inflammation (Potenza, Addabbo and Montagnani, 2009). During compensatory hyperinsulinaemic conditions there is an increased production of vasoconstrictors, angiotensin II and endothelin-1, which contribute to the development of

endothelial dysfunction and hypertension (Potenza *et al.*, 2005; Sarafidis and Bakris, 2007; Kobayashi *et al.*, 2008).

ATM is activated by oxidative stress and DNA damage which are both associated with atherosclerosis (Espach *et al.*, 2015). Studies have found that decreased ATM expression simulates clinical characteristics seen in the metabolic syndrome and some of these symptoms were ameliorated with anti-oxidants and ATM activators (reviewed by Espach *et al.*, 2015). The c-Jun N-terminal kinase (JNK) protein is responsible for the phosphorylation of Ser-307 of the insulin receptor substrate-1 (IRS-1), subsequently disrupting insulin signalling and aiding the development of insulin resistance (reviewed by Espach *et al*., 2015). Increased JNK activity was observed in the aorta, macrophages, adipose tissue, skeletal muscle and liver of ATM deficient mice (Schneider *et al.*, 2006). Furthermore, JNK1 deficient mice were protected from developing insulin resistance and obesity (Hirosumi *et al.*, 2002). Activator protein-1 (AP-1) is a transcription factor, which can be activated by JNK, and plays a role in lipoprotein lipase expression which has been identified as a contributing factor to the development of atherosclerosis (Mead and Ramji, 2002; Schneider *et al.*, 2006). As reviewed by Espach and colleagues (2015), the ATM/p53 pathway is one of the mechanisms that aids the development of insulin resistance and atherosclerosis. A study investigating a drug for atherosclerosis, where wild-type mice were fed a high fat diet, mice were found to have decreased atherosclerosis, however in mice deficient of p53 there were no effects, confirming that the benefits from ATM activity relied on the presence of p53 (Razani, Feng and Semenkovich, 2010). ATM phosphorylates p53 at Ser-15 in humans and Ser-18 in mice, where a mutation in mice caused an increase in inflammation cytokine expression and a decrease in antioxidant expression (reviewed by Espach *et al*., 2015). Furthermore, these mice developed glucose intolerance and insulin resistance at 6 months and the researchers hypothesised that the accumulation of oxidative damage disrupted glucose homeostasis (Armata *et al.*, 2010). As reviewed by Espach *et al.* (2015), due to the short average life span of AT patients, they do not live long enough to see the problematic effects of deficient functional ATM expression on the cardiovascular system. However, in a personal communication from Prof Y Shiloh (Israel) who has a large base of AT patients, his patients all suffer from ischaemic heart disease (unpublished data).

Defective ATM signalling has been found to cause symptoms similar to that of the metabolic syndrome, which is a contributing condition to cardiovascular disease (Espach *et al.*, 2015). Ten to forty percent of the heart's energy originates from glucose metabolism under ischaemic heart conditions, the heart mainly uses glucose for energy. Under normal conditions however, the

heart's main energy source is lipids (Gertz *et al.*, 1988; Taegtmeyer, 2000). Espach *et al.* (2015) proposes that abnormal glucose metabolism would visibly affect cardiovascular function, which would be more prominent during ischaemia, and as ATM has a role in glucose metabolism and insulin signalling pathways, the deficiency of ATM may aggravate cardiovascular dysfunction.

7.1.3 Metabolic syndrome

Metabolic syndrome or syndrome X consists of a group of disorders that occur simultaneously (Samson and Garber, 2014). A number of studies were done to define metabolic syndrome. The definitions differed but all agreed on the components of abdominal obesity or overall obesity, impaired glucose metabolism, hypertension and dyslipidaemia being the most common characteristics of metabolic syndrome (Samson and Garber, 2014). However, epidemiological studies found that not all patients with metabolic syndrome were insulin resistant (Mikhail, 2009). Insulin resistance is a characteristic that contributes to a number of other characteristics seen in metabolic syndrome such as, hyperglycaemia, dyslipidaemia and obesity (Mikhail, 2009).

In the early development of insulin resistance, β cells of the pancreas increase the secretion of insulin causing hyperinsulinaemia to maintain normal blood glucose levels (euglycaemia). However, when the degree of insulin resistance increases, the β cells are unable to secrete sufficient insulin according to the body's physiological demands, subsequently developing glucose intolerance and hyperglycaemia (Mikhail, 2009). Hyperinsulinaemia and hyperglycaemia may further contribute to the symptoms seen in metabolic syndrome as they can further aggravate insulin resistance. Insulin suppresses lipolysis under normal physiological conditions (Eckel, Grundy and Zimmet, 2005) however, insulin resistance promotes the lipolysis of adipose tissue and the release of FFAs into circulation (Mikhail, 2009) (as discussed in *Section 6.1.1b*). A common characteristic of metabolic syndrome dyslipidaemia is the modifications in lipoprotein composition which often occur during hypertriglyceridaemia, leading to an increase of low-density lipoproteins (LDL) and a decrease of high-density lipoproteins (HDL) in circulation (Eckel, Grundy and Zimmet, 2005). Obesity is considered as a contributing factor to the development of hypertension, dyslipidaemia and insulin resistance seen in metabolic syndrome, however, studies have found that obesity alone cannot be the cause for all the symptoms seen in metabolic syndrome and that other factors are contributing to this disease (Mikhail, 2009).

7.1.4 NAFLD

NAFLD is the most common liver disease internationally (Pala *et al.*, 2014) and is the general diagnostic term used for a spectrum of liver pathologies ranging from non-alcoholic fatty liver to non-alcoholic steatohepatitis, cirrhosis, fibrosis and hepatocellular carcinoma (Olaywi *et al.*, 2013). NAFLD is commonly present with other characteristics found in metabolic syndrome, namely obesity and insulin resistance (Paniagua *et al.*, 2014) and, vice versa, insulin resistance is present in these liver pathologies (Pala *et al.*, 2014). Although the underlying mechanism is not fully understood, it is suggested that insulin resistance induces triglyceride and FFA accumulation in the liver, stimulating oxidative stress and the release of pro-inflammatory cytokines causing injury of hepatocytes (Mazza *et al.*, 2012). The hallmark characteristic of NAFLD occurs when the rate of liver fatty acid uptake from circulation and *de novo* fatty acid synthesis are more than the rate of fatty acid oxidation and exportation (Fabbrini, Sullivan and Klein, 2010). According to Paniagua *et al.* (2014), it has become more evident that NAFLD patients have dysfunctional adipose tissue, especially a decrease in disposal and storing of fatty acids as well as an increase in peripheral adipose tissue lipolysis and irregular adipocytokine secretion, which they propose to have a direct impact on the development of liver insulin resistance and NAFLD.

A study by Paniagua *et al.* (2014) investigated transcriptional changes in adipose tissue of NAFLD patients as a function of their insulin resistant status, as well as the transcriptional acute response of adipose tissue genes to dietary and insulin treatments in the cohort of participants with the highest insulin resistance. The study found that, after multiple comparisons, only the messenger ribonucleic acid (mRNA) of leptin expression was significantly decreased in white adipose tissue of insulin resistant participants, when compared to that of the insulin sensitive participants. The mRNA expression correlated with leptin serum levels and was decreased irrespective of the higher body weight of insulin resistant participants. It was also found that, for energy intake, the ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SAT) were lower in insulin resistant than the insulin sensitive participants. The study was unable to conclude that the liver enzyme profiles could be used to segregate patients according to insulin sensitivity as there were no differences between insulin resistant and insulin sensitive participants. However, it was concluded that insulin sensitivity is directly linked to adiponectin levels and higher MUFA/SAT ratio intake and inversely to white blood cells in NAFLD individuals (white and red blood cells were found to be higher in insulin resistant participants).

7.1.5 PCOS

PCOS is an endocrinopathy characterised by menstrual disorders, anovulatory infertility and increased levels of male hormones (Pasquali, Gambineri and Pagotto, 2006). It was reported that 50 – 60% of PCOS patients suffer from insulin resistance (Dunaif *et al.*, 1989; Legro, Finegood and Dunaif, 1998) and that this is due to their impaired insulin secretion (Dunaif *et al.*, 1989). The impaired insulin secretion has an early onset risk of progressing to T2D which is earlier when compared to non-PCOS individuals (Dunaif *et al.*, 1989). According to Azziz (2002), insulin resistance aids the development of compensatory hyperinsulinaemia, which appears to be a main factor in hyperandrogenism of PCOS. Hyperinsulinaemia stimulates ovarian theca androgen secretion, acanthosis nigricans (excess growth and discolouration of skin basal cells) and dysfunctional liver and peripheral lipid metabolism (Azziz, 2002). Insulin sensitising medication has been found to improve clinical characteristics in PCOS patients, suggesting insulin resistance and hyperinsulinaemia as the underlying cause for many of PCOS' characteristics (Azziz, 2002).

A study by Park *et al.* (2001) compared the insulin resistance between controls, obese with T2D and PCOS women. No statistical significance was seen between the groups' age, waist to hip ratio (WHR), BMI, body fat, lipid profile and ideal body weight. However, basal serum insulin levels were found to be higher and statistically significant in the PCOS group along with higher luteinizing hormone (LH) and testosterone levels and lower sex hormone binding globulin (SHBG). The study concluded that PCOS women were insulin resistant independent of adiposity (Park *et al.*, 2001). In a study by Brower *et al.* (2013) the relationship between the severity of menstrual dysfunction and insulin resistance in PCOS patients were investigated, where the homeostasis model assessment for insulin resistance (HOMA-IR) was used to measure the degree of insulin resistance. PCOS individuals with menstrual cycles longer than 35 days had a mean HOMA-IR significantly higher than that of the controls and individuals with the highest HOMA-IR levels were those with a menstrual cycle longer than 3 months (Brower *et al.*, 2013).

7.1.6 Cancer

A study by Steinberger *et al.* (2012) found that childhood cancer survivors (CCS) are more insulin resistant and have higher levels of cardiovascular risk factors when compared to their healthy sibling before reaching adulthood. In this study childhood cancer survivors in remission for at least 5 years between the ages of 9 and 18 years were recruited along with age-matched controls that have never had cancer. The participants underwent extensive measuring of parameters to determine body fat composition, insulin resistance and insulin sensitivity. The study found that

CCS had a lower level of insulin sensitivity and suggested these lower levels may influence the early increase in cardiovascular risk factors and that the difference between the CCS and controls, risk factor levels will become more apparent with age (Steinberger *et al.*, 2012).

A review article reported on a meta-analysis of a number of studies performed in different countries focusing on the association between factors related with insulin resistance and cancer risk. The analysis showed the risk per 5 kg/m^2 increase in BMI with a calculated relative risk (RR) for cancers of the oesophagus (RR = 1.5), thyroid (RR = 1.3), colon (RR = 1.2), kidney (RR = 1.2) and liver (RR = 1.2) in men and cancers of the endometrium (RR = 1.6), gallbladder (RR = 1.6), oesophagus (RR = 1.5) and kidney (RR = 1.3) in women (Inoue and Tsugane, 2012). This suggests a relationship between an increase in BMI and the prevalence of developing cancer.

8. Cancer and AT

The *ATM* gene is also known as a tumour suppressor gene and is considered a breast cancer predisposition gene (Foroughizadeh *et al.*, 2012). AT patients have a number of clinical characteristics, one of which is the predisposition for developing cancer (Foroughizadeh *et al.*, 2012). ATM's main known functions are to repair DSBs in the DNA and to control signalling pathways of cell-cycle checkpoints (Foroughizadeh *et al.*, 2012). The hallmarks of cancer include sustaining proliferation, avoiding growth suppressors and immune destruction, allowing unlimited cell proliferation (replicative immortality), resisting cell death, tumour-promoting inflammation, deregulating cellular energetics, instability and mutations of the genome, activation of invasion and metastasis and inducing angiogenesis (Hanahan and Weinberg, 2011). As ATM repairs breaks in DNA and regulates signalling pathways of cell-cycle checkpoints, it could be suggested that the deficiency of ATM as observed in AT patients, would allow multiple genome mutations, as well as uncontrolled cell proliferation with abnormal cell-cycle checkpoint signalling increasing the probability of developing cancer. According to Foroughizadeh and colleagues (2012), females with a heterozygous AT gene have a fivefold increased risk of developing breast cancer when compared to the general population. Due to ATM's function of desensitising the cell against genotoxic attacks (damaging to genetic material), AT patients are radiosensitive to ionising radiation and AT cancer patients receive a decreased dose of radiation in cancer treatment, as the normal doses used for non-AT cancer patients could be lethal (Khalil, Tummala and Zhelev, 2012). According to Khalil *et al.* (2012) a number of cancers have an established link to ATM with cancer been the most frequent cause of death in AT patients. As one of the characteristics of AT individuals is a feeble immune system, the most common forms of cancers they develop are of the immune system,

namely lymphomas and leukaemias with non-Hodgkin's lymphoma and acute lymphocytic leukaemia being the most common (Khalil, Tummala and Zhelev, 2012). Cancers of the immune system are common in young AT patients, however, older AT patients have a higher predisposition of developing malignancies in other organ systems such as (*i*) breast, (*ii*) stomach, (*iii*) brain (known as a medulloblastoma) and (iv) the skin (basal cell carcinoma which is a type of skin cancer of the basal cells) (Meyn, 1999). According to Khalil *et al.* (2012), leukaemias were found to be of T cell origin and lymphomas of B cell origin. B and T cells are lymphocytes and form part of white blood cells circulating in the blood throughout the body.

9. Peripheral blood mononuclear cells

9.1 Genetic material

Blood constantly circulates around the body connecting all biological systems at a physiological level and is defined as a fluid connective tissue consisting of cells suspended in a fluid matrix (Liew *et al.*, 2005). Blood contains white blood cells namely, neutrophils, monocytes, lymphocytes (B and T cells), basophils and eosinophils, which form part of the first line of defence in the immune system (Liew *et al.*, 2005). Peripheral blood mononuclear cells (PBMCs) are white blood cells consisting of monocytes and lymphocytes (Reynés *et al.*, 2016) which contain a single round nucleus with DNA found to express approximately 80% of the human genome when compared to other tissues (Liew *et al.*, 2005). Because they express a large percentage of the genome, PBMCs can be used for genetic analysis in comparison to more invasive sampling of other tissues (Reynés *et al.*, 2016). This circulating blood cells' gene expression varies amongst persons from which a gene expression profile can be constructed. Changes in these gene expression profiles were found to be characteristic of a number of diseases and disorders (Tsuang *et al.*, 2005).

9.2 Environmental stimuli of gene expression

According to Reynés *et al.* (2016) PBMCs gene expression can reflect the metabolic effects of different diets, such as high-fat diets, with reference to the digestion of lipids and cholesterol specifically. These researchers also found that obesity and T2D affect PBMCs' gene expression of certain cytokines (Reynés *et al.*, 2016). This may also be affected by outside stimuli such as exercises and smoking (Liew *et al.*, 2005). Thus the gene expression profiles of PBMCs can be used as an indication of its micro- and macro-environment (Liew *et al.*, 2005).

9.3 Gene expression profiles

Gene expression profiles are compiled following the isolation of PBMCs from whole blood by centrifugation with a density gradient medium. DNA and RNA are extracted from the PBMCs using commercially available reagents and a gene expression profile is constructed using microarray analysis and real time reverse transcription polymerase chain reaction (RT-PCR) (Jung *et al.*, 2016). The gene expression profiles obtained from PBMCs are considered indicative of the physiological conditions of organs due to blood's constant contact with certain areas in the body (Reynés et al. 2016). According to Liew *et al.* (2005) a number of studies have found that alterations in these gene expression profiles may be used as a diagnosing indicator for a number of diseases such as hypertension, arthritis, lupus and cancer as well as variations in gene expression when exposed to external stimuli such as smoking, exercises or other forms of stress. Interestingly, a study conducted by Tsuang *et al.* (2005) demonstrated that psychiatric disorders also alter the gene expression profiles in PBMCs. A number of researchers suggest the use of PBMCs' gene expression for the prognosis and diagnosis of certain diseases as the sampling of this tissue is less invasive and can be collected more frequently and in larger quantities than liver or lung biopsies (Liew *et al.*, 2005; Reynés *et al.*, 2016).

9.4 Gene expression for diagnostics

According to Reynés *et al.* (2016) the analysis of gene expression in PBMCs has become an important technique for investigations into the interactions between nutrition and genetics (nutrigenomics). As previously mentioned, the metabolic effects of diets high in lipids and cholesterol (hyperlipidic) were found to alter PBMCs' gene expression, specifically from altered cholesterol metabolism, lipid metabolism and the inflammatory responses (Reynés *et al.*, 2016). Reynés and colleagues (2016) mentioned studies that found PBMCs are susceptible to specific nutrients such as polyunsaturated fatty acids (PUFA) and polyphenols as well as changes in feeding conditions. A study was conducted where obese Spanish boys between the ages of 7 and 15 years, were fed a moderate energy restriction diet, after which baseline anthropometric measurements were compared to measurements following intervention (Rendo-Urteaga *et al.*, 2015). The participants' response to the intervention was measured according to their changes in BMI standard deviation score (BMI-SDS) and divided into low responding (LR) and high responding (HR) groups. Rendo-Urteaga *et al.* (2015) found that the HR group had lower levels of inflammatory and immune response-related genes compared to LR. It was hypothesised that the LR group may have a more developed pro-inflammatory phenotype (Rendo-Urteaga *et al.*, 2015). Following the

intervention the LR group was found to have an overexpression of genes involved in cell proliferation, motility, survival, differentiation, haemostasis, immune response and inflammation. Obese individuals have been found to be in a constant pro-inflammatory state (Xu *et al.*, 2003) and researchers have found that PBMCs' gene expression of inflammatory cytokines are affected in obese and diabetic conditions (Tsiotra *et al.*, 2007, 2008).

10. Ageing

10.1 Ageing and DNA damage and repair

DNA is constantly susceptible to injury by various agents. Damaged DNA can accumulate and aid the generation of errors in DNA replication and mutations, which is thought to contribute to ageing, cancer development and neurodegeneration (Jeppesen, Bohr and Stevnsner, 2011). Depending on the type of DNA damage one of four repair pathways are activated namely, base excision repair (BER), nucleotide excision repair (NER), mismatch repair or DSBR (Garm *et al.*, 2013). DSBs are considered the most dangerous type of DNA lesion as it can cause genome rearrangements, however this type of lesion is not as common as single-strand breaks (SSBs) (Garm *et al.*, 2013). DSBR is molecularly switched on by phosphorylation causing DSB formation followed by gamma phosphorylation (Ser-139) of the histone 2A (H2AX), now γ-H2AX (Garm *et al.*, 2013). In the first hour following DSB formation γ-H2AX surrounds the break and is involved in DSB signalling response (Muslimovic *et al.*, 2008). Several other DSB damage response proteins are activated downstream and subsequently affects DNA repair, cell cycle checkpoints, telomere maintenance and transcription (Garm *et al.*, 2013). Following DSB, DSBR occurs via two mechanisms namely, non-homologous end joining (NHEJ), which works throughout the cell cycle, and homologous recombinational repair (HRR), which repairs DSBs at phases S and G_2 of the cell cycle (Garm *et al.*, 2013).

Studies regarding single-strand break repair (SSBR) in PBMCs from donors of different ages, showed little or no effect of age, however other studies have suggested age-associated increases of SSBs in human PBMCs while others have reported no age effect (reviewed in (Garm *et al.*, 2013)). A number of studies in human PBMCs and age-related NHEJ function found a decrease in binding of a specific complex and Garm *et al.* (2013) proposed these studies suggest changes in DNA repair with age. As PBMCs and blood cells are frequently replaced it was suggested that it may not be as susceptible to ageing as organ cells (Garm *et al.*, 2013), however, old age has been associated with age-associated decreases in immune repertoire and function (Gill, Rutherfurd and Cross, 2001). Garm *et al.* (2013) reviewed a number of studies looking at DNA repair and its

association with age and reported that some studies found there is a decrease in the ability to repair DNA damage with the progression in age. The results of Garm and colleagues (2013) suggested that SSB repair ability can be maintained up to the age of 77 in human PBMCs. Additionally, no statistical significance was seen for age-related changes in endogenous SSBs, however, the results suggested a tendency of DSBR and γ-H2AX response to decline with age but none of the associations were statistically significant.

10.2 Premature ageing in AT

Multiple similarities have been seen between elderly patients and AT patients with regards to clinical, pathological and the CNS effects, and AT has been proposed as a premature ageing model (Boder E., 1985). AT patients also have multiple defects of the immune system and this has been suggested to be indicative of premature ageing of the immune system in AT individuals (Exley *et al.*, 2011; Carney *et al.*, 2012). AT was proposed as a model for the ageing of the immune system (Exley *et al.*, 2011). Younger AT patients were found to have health problems normally related to people of middle to elderly age. A study on 53 AT patients, between the ages of 5.9 – 26.1 years, found that 43% had increased serum transaminase, 39% had fatty liver and 33% developed more severe liver diseases (Weiss *et al.*, 2016) while another study found an increased incidence of dyslipidaemia and diabetes (Nissenkorn *et al.*, 2016). Similarities were seen between brain function of aged individuals and AT individuals experiencing neurodegeneration (Barzilai, Schumacher and Shiloh, 2017). With the characteristic of premature ageing in AT, Shiloh and Lederman (2017) suggested that premature cell senescence may be responsible. Shiloh and Lederman (2017) defined the hallmark of cellular senescence as the irreversible arrest of cell proliferation. Cell senescence can be stimulated by a number of stresses and has been suggested as an important factor for development, wound healing, tissue repair and ageing (reviewed in (Shiloh and Lederman, 2017)). AT patients suffer from age-related abnormalities such as telangiectasia of the eye, length-dependent neuropathy causing foot deformation, cerebellar atrophy, intracerebral telangiectasia and cutaneous granulomas (Shiloh and Lederman, 2017).

As previously discussed, ATM has multiple functions in pathways related to the cell cycle, cell-cycle check point regulation and cell proliferation, metabolism, DNA repair, transcription and protein degradation. The literature suggests the susceptibility of DNA repair to ageing and subsequently a possible decrease in bioavailability and/or expression of the ATM protein.

11. Motivation for this study

Non-communicable diseases (NCDs) are increasing worldwide with 70% of all deaths been due to NCD in 2015 of which 4% was deaths due to T2D worldwide (World Health Organization, 2017). In NCD Country Profiles 2014 compiled by the World Health Organisation (WHO), it was reported for South Africa that approximately 43% of deaths were due to NCD of which the four main contributors were cancer (7%), T2D (6%), cardiovascular diseases (18%) and chronic respiratory diseases (3%) (WHO, 2014) which are linked to metabolic disorders such as insulin resistance, glucose intolerance, hyperinsulinaemia, obesity and dyslipidaemia.

A recent study investigated the potential of ATM serine-1981 phosphorylation (ATM 1981S-P) as a biomarker for radiation, radiation toxicity and DNA damage (Bakkenist, Beumer and Schmitz, 2015). These investigators stated that there is a need for a biomarker for radiation exposure following the release of radioactive material, a predictive biomarker for radiation toxicity following treatment for tumours (oncology) and pharmacodynamics biomarkers for chemotherapy related DNA damage and the inhibition of signalling kinases for DNA damage. A biomarker is a characteristic that can be measured and used as an indicator of normal physiological processes, pathological processes or a pharmacological response to a treatment (Bakkenist, Beumer and Schmitz, 2015). Bakkenist and colleagues (2015) found that ATM 1981S-P levels increased in PBMCs following cancer radiation treatment. A previous study found that ATM 1981S-P is induced in the genome of PBMCs following drug-induced DNA damage (Bakkenist *et al.*, 2015).

This review focused on literature discussing ATM and its role in insulin signalling and glucose metabolism due to the genetic link found between the glycaemic response and metformin treatment in type II diabetics (Zhou *et al.*, 2011) along with the development of insulin resistance, T2D and cancer in AT individuals. Research in PBMCs and the compiling of gene expression profiles and its use in the diagnosis of diseases and as biomarkers of pathology were also discussed. The effects of ageing on genetic material were linked to the premature ageing characteristic seen in AT patients. From literature it is evident that a diagnostic technique is needed for the early diagnosis of insulin resistance as it is a common characteristic found in a number of other prevalent diseases. PBMCs' ability to express the human genome makes it an easily available source of protein expression. As AT commonly presents with insulin resistance and an increased prevalence for developing T2D along with the results found by Zhou *et al.* (2011), linking the *ATM* gene and ATM protein to the glycaemic response, it could be a plausible target for determining a patient's degree of insulin resistance.

Thus this study proposes ATM protein expression from PBMCs as a diagnostic technique which could be used as an indicator of the measure of insulin resistance or the propensity to develop T2D in patients. PBMCs are an eligible source of protein expression as the study by Bakkenist *et al.* (2015) proved ATM was present in PBMCs. As insulin resistance is common in a number of NCD, sufferers of these diseases would also benefit from this diagnostic technique.

12. Aim and Objectives

Aim: To determine whether a) PBMCs can be used to determine ATM levels in insulin resistant subjects and b) whether these levels can be used as a biomarker for peripheral insulin resistance.

Animal study:

Objective 1: Standardise a protocol to isolate PBMCs from rat blood.

Objective 2: Isolate PBMCs from rat blood and determine the ATM expression levels using Western blotting and a total ATM specific antibody.

Objective 3: Use the PBMCs isolated from insulin resistant obese rats and those of age-matched control rats to determine whether there is any difference in ATM expression.

Human study:

Objective 1: Analyse PBMCs from a biobank that was collected from a female Black Xhosa population spanning normal, obese and pre-diabetic (metabolic syndrome) subjects and correlate the expression of ATM with the measure of insulin resistance.

Objective 2: Broaden the ethnicity of human subjects and collect and analyse samples from Mixed Race subjects.

CHAPTER 2: MATERIALS AND METHODS

The investigation was divided into animal-based experiments and human-based experiments. The animal experiments were to (*i*) set up and optimise a protocol to isolate PBMCs, (*ii*) determine whether the ATM protein expression levels can be measured in these PBMCs; (*iii*) determine whether obesity changes the expression of ATM in PBMCs and (*iv*) to determine whether any changes of ATM expression in PBMCs reflect changes observed in the heart. Samples were analysed by means of Western blotting and statistical analysis was done to determine significance.

1. Setting up of PBMCs isolation protocol in an animal model

During the setting up of the protocol an existing protocol for the isolation of human PBMCs were used as a guide (protocol from Division of Endocrinology, Department of Medicine, Faculty of Medicine and Health Sciences at Stellenbosch University). This protocol made use of a density gradient medium Histopaque®-1077 at a density of 1.077 gram (g)/millilitre (mL). According to the product information it could be used for the separation of small volumes of blood to collect lymphocytes and mononuclear cells.

1.1 Whole animal blood collection

Blood samples were collected at random from male Wistar rats sacrificed for other laboratory experiments. Samples of at least 5 mL of whole blood were collected with a Pasteur pipette from the chest cavity following the removal of the heart. Blood was collected in a 10 mL K2 Ethylenediaminetetraacetic acid (EDTA) blood collection tube (SG Vac, The Scientific Group). The tube was inverted a number of times to ensure that the blood mixed with the anticoagulant to prevent clotting.

1.2 Percoll PLUS® as density gradient medium

The separation of whole blood samples were attempted with Percoll PLUS® (density 1.130 g/mL) (GE Healthcare Life Sciences) which was available in the laboratory. Following separation, the medium was found to be too dense to allow separation of the blood. A Stock Isotonic Percoll (SIP) was prepared at a density of 1.07 g/mL, NaCl at a concentration of 1.5 Molar (M) and a density of 1.058 g/mL was used to dilute the Percoll PLUS at a dilution ratio of 9 parts Percoll to 1 part 1.5M NaCl (GE Healthcare Life Sciences, 2014). Multiple separations were done with the SIP solution, however clarity of separations varied, possibly due to the centrifugation speed, especially the centrifuge speed of deceleration which disturbed the separated layers, causing them to mix (*Figure 2.1*). Another possible reason for unclear separations may have been due to the prepared

SIP not been the correct density. Separations were attempted using the slow start/stop option on the centrifuge (Eppendorf Centrifuge 5403) which produced more defined layers.

1.3 Comparison between Percoll PLUS® and Histopaque®-1077

Due to the variations in separation results, a comparison was made between Percoll PLUS® and Histopaque®-1077 (Sigma-Aldrich). The centrifugation times and speeds were used from the existing Human PBMCs Isolation protocol. For the separation of the blood, 4 mL of gradient medium was pipetted into a sterile 15 mL conical tube and 4 mL of whole rat blood was slowly pipetted on top of the gradient medium to prevent the two layers from mixing. The blood was centrifuged at 1850 rpm for 30 min at room temperature using the slow start/stop function. The separation done with Histopaque®-1077 was clearer than that done with the SIP preparation (*Figure 2.1*).

1.4 Centrifugation optimisation

Following the separation of the blood the buffy coat (white blood cells situated at the interface between the blood plasma and the Histopaque®) was then transferred with a Pasteur pipette to a new sterile 15 mL tube with 5 mL sterile Phosphate Buffered Saline (PBS) (137 millimolar (mM) NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 dissolved in 800 mL distilled water (dH_2O), pH to 7.4 and final volume of 1 litre (L) with dH_2O) and centrifuged at 1450 rpm for 10 min at the room temperature with the slow start/stop. The pellet formed following separation with Histopaque was bigger and contained less platelets than the separation done with the SIP. Thus Histopaque was used as the density gradient medium for the animal experiments along with the centrifuge settings, explained in the following paragraph.

1.5 Centrifugation specifications

For clear isolation of PBMCs, the specific settings used were 1850 rpm for 30 min at room temperature to separate blood into its layers. The buffy coat was washed with 5 mL PBS at 1450 rpm for 10 min at room temperature to collect the pellet.

1.6 Storage and preparation of samples for lysates

Following collection of the buffy coat in the form of a pellet, the PBS was discarded. Rat PBMCs sample pellets were suspended and stored in 1 mL freezing medium (100 microlitres (µl) Dimethyl sulfoxide (DMSO), 200 µl Bovine Serum Albumin (BSA) (50 milligram (mg) dissolved in 1 mL dH_2O) and 700 µl Dulbecco's Modified Eagle's Medium (DMEM)) in a 1.5 mL Eppendorf tube at - 20 degrees Celsius (°C) overnight and then - 80°C until analysis. However, as samples were analysed

with the Western blotting technique, later samples were stored in 100 µl fresh lysis buffer (20 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, 1 mM beta (β)-glycerophosphate, 2.5 mM tetra-Na-Pirophosphate, 1 mM Na_3VO_4 (freshly prepared weekly, 0.018 g/10 mL), 50 microgram (µg)/mL PMSF, 10 µg/mL Leupeptin, 10 µg/mL Aprotinin and 1% Triton X-100 in a solution of total 5 mL dH_2O) in a 1.5 mL Eppendorf tube at - 80°C. This volume was later reduced to 50 µl to increase the protein concentration of each sample.

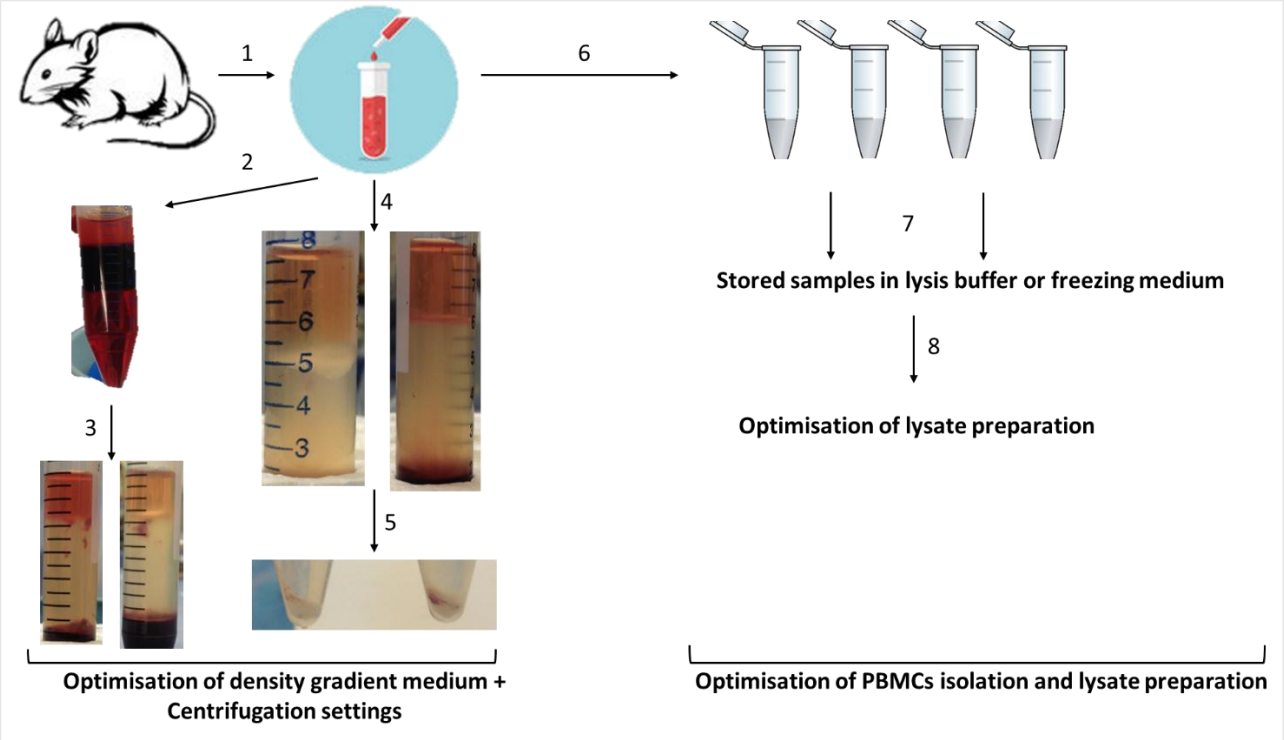

Figure 2. 1: *Setting up of rat PBMCs isolation protocol. 1) Collected whole blood from rats into EDTA tubes. 2) Separated blood with Percoll PLUS® (1.130 g/mL). 3) Separated blood with SIP (approximately 1.07 g/mL) which produced differences in separation with the same SIP. 4) Compared separation with SIP (right) to Histopaque®-1077 (left). 5) Washed buffy coat with PBS to compare clarity of isolation. 6) Isolated multiple samples with Histopaque® to optimise isolation technique. 7) Stored samples in freezing medium (- 20°C overnight then - 80°C) or lysis buffer (- 80°C). 8) Optimised isolation of protein from freezing medium and lysis buffer stored samples for lysate preparation.*

1.7 Lysing cells for protein isolation

A new set of samples were collected and separated using Histopaque-1077. Samples were stored in freezing medium and later the storage medium was changed to lysis buffer. For analysis of samples stored in the freezing medium, samples were thawed and centrifuged at 2000 relative centrifugal force (g) for 7 min at 4°C (Sigma 1-14K Microcentrifuge). The supernatant was aspirated and discarded without disturbing the pellet. Cells were washed with 1 mL PBS and centrifuged at 2000 x g for 7 min at 4°C. Supernatant was aspirated and 100 µl fresh lysis buffer

was added to each sample with ¼ - ½ scoop (depending on pellet size) of Zirconium oxide beads (0.15 millimetre (mm)) (NEXT Advance). The samples stored in lysis buffer had a scoop of beads added while still frozen and sealed with Parafilm® M Laboratory Film (Lasec). Samples were homogenised with a Bullet Blender® (NEXT Advance) for 1 min at speed 8 with 5 min rest on ice. This was repeated twice with a final rest period of 20 min. Samples were centrifuged at 15000 rpm for 20 min at 4°C and the supernatant was transferred to a new 1.5 mL sterile Eppendorf tube. The supernatants were stored at - 80°C.

1.8 Bradford assay

The Bradford assay (Bradford, 1976) is a protein determination assay and was used for the measuring of each samples' protein concentration. BSA was used as the standard solution (1 in 4 dilution of BSA stock solution in dH_2O and further diluted to construct a standard curve from 5 to 60 µg protein (*Table 2.1*). This was assayed in duplicate along with a blank of dH_2O. The assay was set up using plastic tubes.

Table 2. 1: *The dilutions of the BSA stock solution for the different protein concentrations (in µg).*

BSA standard	BSA stock (ul)	dH_2O (ul)
Blank	0	100
5 (in duplicate)	5	95
10	10	90
20	20	80
40	40	60
60	60	40

The samples underwent a serial dilution where 10 µl of each sample was diluted with 90 µl dH_2O to dilute the concentration of the detergents used in the lysis buffer as they may otherwise interfere with the assay. 10 µl of the diluted sample was further diluted with 90 µl dH_2O. Samples were also assayed in duplicate. The assay was set-up as shown in *Figure 2.2*.

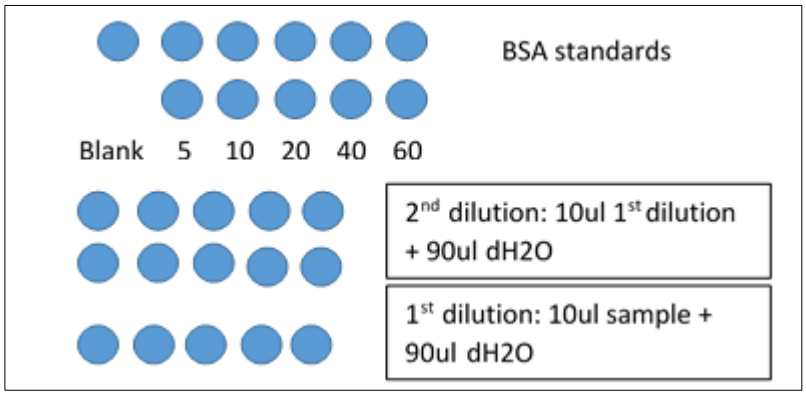

Figure 2. 2: *Set up of BSA standards and dilution of samples for the Bradford assay.*

A 1:4 dilution with dH_2O of a Bradford stock solution (0.06 mM Coomassie Brilliant Blue G-250, 95% ethanol and 50% volume/volume (v/v) phosphoric acid) was filtered through a double layer of Whatman® Qualitative 1 filter paper (Whatman Limited) and 900 µl was pipetted into each BSA standard tube and each sample. Colour was allowed to develop for 20 min (start timer when adding Bradford solution to first tube) after which the spectrophotometer (Spectronic® 20 Genesys™) absorbance reading was set to 0 with the blank and then the optical density (OD) of each BSA standard and sample were determined at 595 nanometre (nm) wavelength.

1.9 Protein determination calculations

Microsoft Excel was used to calculate and construct a standard curve graph from the BSA standards' absorbance readings. The equation of this line was used to calculate the µg of protein in each sample, taking the dilution of the samples into account. The samples were adjusted with lysis buffer to all contain the same amount of protein per volume unit. Following analysis of the samples, each sample was found to have very low protein concentrations and thus the lysis buffer volume was decreased to 50 µl for future sample collection to concentrate the samples.

1.10 Preparation of lysate samples for Sodium Dodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE)

The lysates were mixed with a 2x concentrated Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-Mercaptoethanol, 0.004% Bromophenol blue, 0.125 M Tris-HCl) (Laemmli, 1970) (prepared by mixing 850 µl sample buffer stock solution with 150 µl 2-Mercaptoethanol) in a 2:1 ratio. Samples were boiled for 4 min and then centrifuged for 6 seconds (s) to collect sample in the bottom of the Eppendorf tube. Aliquots of samples were made as samples are thawed and then boiled before been used for blotting. If the remaining volume of sample is frozen and then thawed for another experiment some of the proteins would denature when boiled again. Samples were then stored at - 80°C.

1.11 Western blotting

Western blotting is a separation technique used to separate a sample's proteins according to size. In SDS-PAGE, the samples are denatured and the gel contains SDS. The protocol makes use of current that flows through the gel and separates the proteins according to their size and their electrical charge. A set of samples were collected for the optimisation of the Western blotting of lysates obtained from PBMCs. These samples were stored directly in 50 µl lysis buffer at - 80°C.

1.11.1 Positive control

A positive control known to contain the proteins of interest was used in each experiment. The positive control was prepared from a freeze clamped heart harvested from a sacrificed control rat. A small piece of tissue, approximately 100 mg, was transferred to a lysate tube containing 700 µl lysis buffer (on ice). Tissue was homogenised with a SilentCrusher M homogeniser (Heidolph Instruments) at 20 rpm for 4 s with a rest period on ice before repeating. The lysate of the positive control's protein concentration was calculated to contain 50 µg of protein per 15 µl volume per well.

1.11.2 Casting of gels

For the separation of the proteins in the samples, two 15-well, 7.5%, 0.75 mm thick electrophoresis mini gels with a 4% stacking gel (*Table 2.2*) were cast using a standard Bio-Rad mini Protean III system. The stain-free solution was added to ensure visibility of the proteins in the Bio-Rad ChemiDoc ™ system. The Tetramethylethylenediamine (TEMED) (Sigma Life Science) was added just before the solution was pipetted into the casts as it is responsible for gel polymerisation in combination with ammonium persulfate (APS). The running gel was pipetted into the cast and a layer of butanol was layered on top to enable a straight meniscus. The gel was allowed to set (at least 30 min) and the butanol was rinsed out with dH_2O once the gel had set. TEMED was added to stacking gel mix and then pipetted into the cast after which the 15-well comb was inserted and allowed to set (at least 15 min). The comb was removed once gels have set and wells were rinsed with dH_2O.

Table 2. 2: *The composition of the 7.5% running gel and the 4% stacking gel used for Western blotting.*

Reagent	Stock	7.5 % gel	4 % gel
dH_2O		5.525 – 50 ul	3.05 ml
Stain-free solution		50 ul	-
Tris-HCl (pH8.8)	1.5 M	2.5 ml	-
Tris-HCl (pH6.8)	0.5 M	-	1.25 ml
SDS	10 %	100 ul	50 ul
Acrylamide	40 %	1.875 ml	500 ul
APS	10 %	50 ul	50 ul
TEMED	99 %	20 ul	10 ul

1.11.3 Protein separation

Samples were allowed to thaw and then boiled for 4 min. Sample was centrifuged to collect in the bottom of the tube and to remove any particulate matter. The system for running the gel was

filled with running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Using a Hamilton syringe (Hamilton Company) 15 µl of each lysate along with the positive control was loaded into the wells. A volume of 5 – 7 µl of a 31 – 460 kilodalton (kDa) molecular weight marker (HiMark™ Pre-Stained Protein Standard) (Thermo Fisher Scientific) was loaded into the first lane as an indicator for the size of the proteins. The system was run for 10 min at 100 volts (V) and 200 milliamperes (mA) and then for 50 min at 200 V and 200 mA until the Coomassie blue front reached the bottom of the gel. The running buffer was discarded and the gels were removed from the casts and kept in a transfer buffer specially modified for ATM. The transfer buffer composition: 25 mM Tris, 192 mM glycine and 20% (v/v) MeOH, for the transfer buffer modified for ATM contained half the amount of MeOH. The gel was exposed using the ChemiDoc™ XRS+ system (Bio-Rad) and the image saved. The proteins separated in the lanes are visible with the ChemiDoc™ due to the Stain-free solution (Aldrich Chemistry) used in the gel.

1.11.4 Protein transfer

For the protein transfer, the gel was allowed to swell in transfer buffer for approximately 15 – 30 min to allow optimal transfer of large proteins. A transfer sandwich was made by stacking a sponge, 2 pieces of blotting paper, the gel with the molecular ladder on the right, a polyvinylidene difluoride (PVDF) Immobilon®-P Transfer Membrane (Merck Millipore Ltd), 2 pieces of blotting paper (chromatography paper, grade: FN 100, 195 g/m^2, Lasec) and another sponge, after which the transfer cassette was closed and inserted into the transfer system. The transfer occurs from the cathode (-) to the anode (+) thus the current should pass through the gel containing the proteins to transfer them to the membrane. Both transfer sandwiches were inserted into the system and an ice pack was inserted to keep the system cool. The system was filled with transfer buffer. The system ran for 1 hour 30 min at 200 V and 200 mA and the ice pack was changed after 45 min to ensure that the system remained cold. Once the transfer was completed, the buffer was collected to be reused and the membrane was collected and kept in Tris-buffered saline (TBS)-Tween solution (10x dilution of TBS stock (200 mM Tris (pH7.6), 1.37 M NaCl) with 0.1% Tween 20). The membrane was visualised with the ChemiDoc™ system and an image was saved. To fix the proteins to the membrane, it was dipped in methanol for 30 s and allowed to air dry.

1.11.5 Visualisation of proteins

The membrane was blocked with 5% milk (5 mL fat free milk diluted in 95 mL TBS-Tween) for 1 hour on a lab rotator at room temperature. The membrane was then washed with TBS-Tween thrice for 5 min on a lab rotator. The ATM protein was probed for with total (T)-ATM and

phosphorylated (P)-ATM antibodies (Cell Signaling Technology). Primary antibodies were diluted in a Primary Antibody Signal Boost (Merck Millipore) in a ratio of 1:1000. Membranes were exposed to the antibody overnight on a lab rotator at 4°C. The next day, the membranes were removed from antibody and washed with TBS-Tween as in the previous wash. The membranes were treated with anti-rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) as secondary antibody in a ratio of 1:4000 in Secondary Antibody Signal Boost (Merck Millipore). Membranes were treated for 1 hour on a lab rotator at room temperature. Membranes were washed with TBS-Tween and treated with 1.5 mL of the Clarity™ Western ECL Substrates (Bio-Rad) reagents for 5 min before exposure in the ChemiDoc™ system. Following exposure, the protein bands became visible and an image was saved.

1.11.6 Normalisation

Normalisation was performed by comparing the total protein in each lane that was transferred to the PVDF membrane to the band intensity of that lane on the membrane treated with the specific antibody using the Image Lab™ Software (Bio-Rad). The software uses the first lane as the standard and compares all the other lanes to lane 1 and generates a normalisation factor (*Figure 2.3*). The software generated an analysis table containing the normalisation factor and normalisation volume (intensity). The normalisation factor is the corrective value calculated by the software in comparison to lane 1. The normalisation volume (intensity) is then calculated by multiplying the original volume (intensity) of each lane by the normalisation factor and generating the value that is indicative of the amount of the specific protein in each band. The use of the ChemiDoc™ normalisation system corrects for possible unequal loading of samples and negates the use of a loading control such as β-tubulin.

1.11.7 Statistical analysis

The data from the Image Lab was exported to Microsoft Excel where the average of all control samples was calculated. The fold change relative to the control was calculated by dividing each normalisation volume (intensity) by the average of the control. This data was exported to GraphPad Prism 5 software where statistical analysis was performed by either a one-way analysis of variance (ANOVA) or a Student's t-test

All steps used during the optimisation of the Western blotting technique were used during all other blotting of samples. Modifications were made to the protocol where necessary.

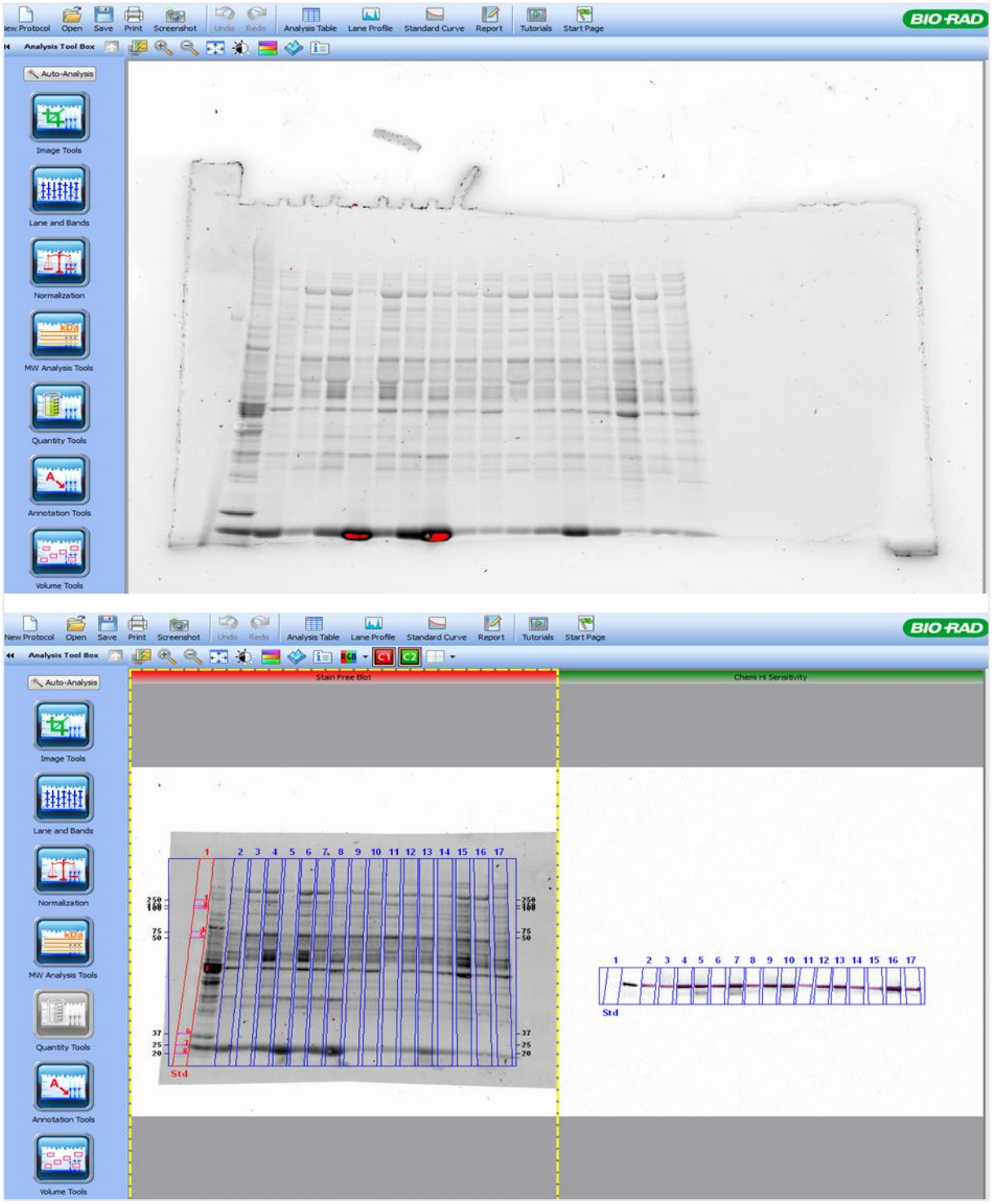

Figure 2. 3: *Normalisation. Top image depicts the gel showing the separated proteins. Transferred proteins are detected on the Stain Free Blot (bottom left) where lanes are detected for total lane protein. Chemiluminescent bands are detected from the antibody probed membrane and normalised (bottom right).*

2. Animal study

2.1 Diet treatment of animals

Male Wistar rats were fed either a standard chow (control) or an obesogenic diet (*od*) (*Table 2.3*), to induce insulin resistance, for 16 weeks. The diet is sometimes referred to as a high-fat diet (HFD) as it induces obesity in the rats. The diet was started when animals were 6 weeks old. The original cohort contained 20 rats of which two died and two provided insufficient volumes of blood to do further experiments. The final experimental animals were 7 Controls and 9 *od* animals (*Figure 2.4*).

Ethical approval was obtained from the Research Ethics Committee: Animal Care and Use of Stellenbosch University (Number: SU-ACUD16-00080). Handling of animals were conducted according to the accepted standards as stipulated in the South African National Standard for The Care and Use of Animals for Scientific Purposes, South African Bureau of Standards (SANS 10386:2008).

Table 2. 3: *Composition of standard chow and od diets.*

	Fat (g/100g)	Cholesterol (mg/100g)	% Proteins	% Carbohydrates	Sugar (g/100g)	Kilojoules (kJ/100g)
Control	4.8	3	17.1	34.6	6.6	1272
od	11.5	13	8.3	42	24.4	1364

2.2 OGTT

The OGTT is used to determine an animal or patient's level of insulin sensitivity. At 15 weeks, animals were fasted overnight and OGTTs were done. To ensure ethical handling, rats were injected with 0.1 ml Eutha-naze (53 mg/kg Sodium pentobarbitone) (Bayer Animal Health) for anaesthesia. A drop of blood was collected from a tail prick and basal glucose levels were measured with a hand held glucometer. Rats were gavaged with 1 g/kg sucrose each and blood glucose levels were monitored for 2 hours. Blood glucose levels were measured with a drop of blood from the same tail prick at 3, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min for each rat. Rats were left to recover for 1 week before sacrificing at week 16 of the diet and 22 weeks of age.

2.3 Sacrificing of animals and blood collection

During the diet period, rats were weighed before been anaesthetised for sacrifice. Animals were anaesthetised with 160 mg/kg Sodium pentobarbitone (Eutha-naze). Rats were checked to be unresponsive by pinching the foot (pedal reflex) or touching the eyes. The non-fasting blood glucose levels were measured with a glucometer by means of a tail prick. The heart was removed

and snap frozen in liquid nitrogen. Blood was collected from the chest cavity and PBMCs were isolated and stored in 50 µl lysis buffer in marked Eppendorf tubes and stored at - 80°C until protein determination. Intraperitoneal (IP) fat was removed and divided into abdominal and epididymal fat depots and weighed.

2.4 Bradford assay and Western blotting

Protein determination was done using the Bradford assay protocol as described previously. A standard curve was constructed using BSA standards and the absorbance readings were taken to determine the µg of protein in each sample. The samples were used to make lysates containing 30 µg of protein per 15 µl. All 16 samples were run simultaneously on a 26 well Bio-Rad Criterion™ TGX Stain-Free™ Precast Gel with a 4-20% gradient. The lysate prepared from the rat heart tissue was used as a positive control. The protocol used during Western blotting optimisation was used and T-ATM and P-ATM protein expression was investigated. As samples were run on one membrane, the membrane was first treated with T-ATM antibody and then stripped.

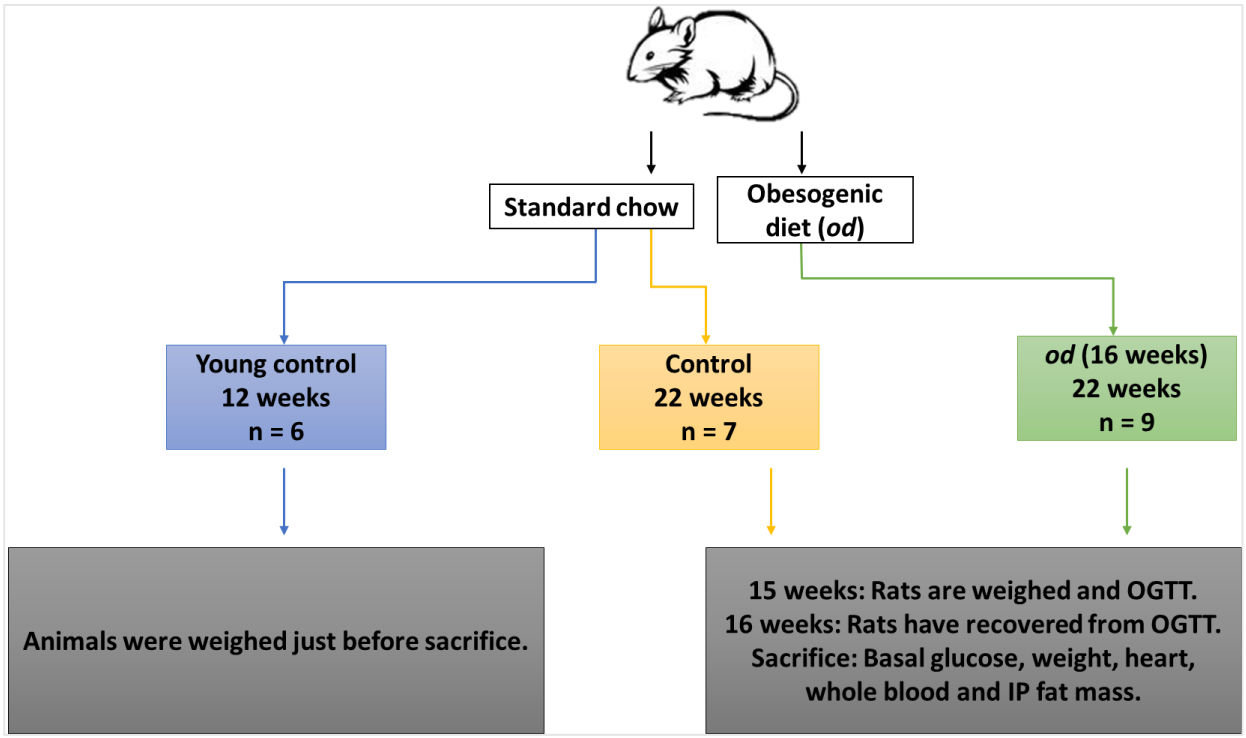

Figure 2. 4: *Overview of group divisions in animal experiments.*

2.5 Stripping of membrane and blocking

The membrane was stripped by exposing it to two 5 min sessions of washing with dH_2O followed by 7 min of washing with 0.2 M NaOH and followed by another two 5 min sessions of washing with dH_2O, all on a lab rotator at room temperature. The membrane was blocked with 5% milk for 2

hours on a lab rotator at room temperature and then washed with TBS-Tween before been treated with P-ATM antibody.

2.6 Comparison between 22-week and 12-week animals

To investigate whether age (weeks) had a role in the protein expression levels of the ATM kinase protein, an additional 6 control Wistar rats (standard chow diet) were sacrificed at 12 weeks (young controls) (*Figure 2.4*). All 22 samples were run on a precast gradient gel along with the positive control. The membrane was probed with T-ATM antibody, stripped and then probed with P-ATM antibody. As the P-ATM bands were not visible following stripping the samples were run on a new gel and the membrane was specifically probed with P-ATM antibody.

3. Human study

3.1 Patient recruitment

Patients were recruited by researchers in the Division of Endocrinology, Department of Medicine, Faculty of Medicine and Health Sciences at Stellenbosch University. The patients were recruited at Tygerberg Hospital, Cape Town and were a Western Cape female black Xhosa population aged ≤ 45 years (Ethics # N15/07/066 (PI: Dr M van de Vyver)).

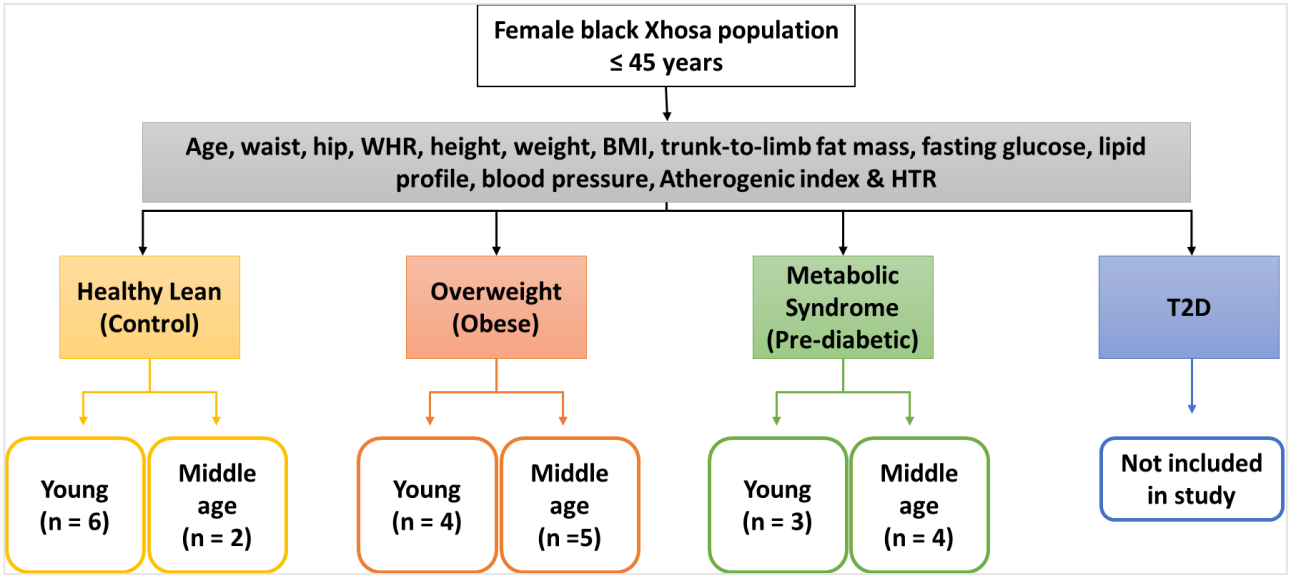

Figure 2. 5: *Overview of group divisions for the human experiments.*

3.2 Biometric measurements and calculations

Patients' age (years), weight (kg), height (m) BMI (kg/m^2), hip and waist measurements (cm), WHR, trunk:limb fat mass (TF/LF) (Dual energy X-ray absorptiometry (DXA), fasting blood glucose in millimoles per litre (mmol/L), total cholesterol (mmol/L), LDL (mmol/L), HDL (mmol/L), triglycerides (TAG) (mmol/L), systolic blood pressure (SBP) in millimetre of mercury (mmHg) and

diastolic blood pressure (DBP) (mmHg) were measured of each patient. The Atherogenic index was calculated for each patient according to the following formula: (total cholesterol – HDL)/HDL. HTR was calculated according to the following formula: HDL cholesterol/total cholesterol ratio and presented as a percentage.

A risk score was calculated according to visceral adiposity, metabolic risk factors and inflammatory markers. Visceral adiposity: WHR ≥ 0.85, TF/LF > 1 was given a risk score of 0.5 and 1 respectively. Metabolic risk factors: TAG ≥ 1.7 mmol/L, HDL < 1.29 mmol/L, systolic blood pressure ≥ 130 mmHg and diastolic blood pressure ≥ 85 mmHg, fasting blood glucose ≥ 5.6 mmol/L each was given a risk score of 1, 1, 1 (0.5 for systolic and diastolic) and 1 respectively. C-reactive protein was measured as an inflammatory marker and where ≥15 mg/decilitre (dL) was given a risk score of 1.

Patients were divided into groups for healthy lean with a risk score ≤ 1 (no visceral adiposity, one or no metabolic risk factors and normal BMI), overweight/obese with a risk score between 0-3.5 (less than two metabolic risk factors and no visceral adiposity or normal BMI), metabolic syndrome with a risk score between 3-7 (more than two metabolic risk factors, visceral adiposity and previous diagnosis of type 2 diabetes (T2D)) and type 2 diabetics (previous diagnosis in medical history). For this study, healthy lean will be the controls, overweight/obese will be obese and metabolic syndrome will be pre-diabetic (*Figure 2.5*). The T2D samples did not form part of this study.

3.3 Blood collection and PBMCs isolation

Blood was collected as in the animal studies and separated by centrifugation. The PBMCs were collected and stored in 1 mL freezing medium (200 µl Human Serum Albumin (HSA), 100 µl DMSO, 700 µl DMEM) at - 80°C. Samples were stored at - 20°C overnight and then stored at - 80°C. Human samples were collected from a biobank (made available by Dr Marí van de Vyver).

3.4 Western Blotting

3.4.1 Sample preparation

In preparation for western blotting, samples were thawed and centrifuged at 2000 x g for 7 min and then an additional 4 min at 4°C to ensure all suspended cells were pelleted. The freezing medium was aspirated and the pellet was suspended in 100 µl ice cold PBS for washing. Samples were centrifuged 7 min at 4°C at 2000 x g. The supernatant was aspirated without disturbing the pellet and the pellet was suspended in 50 µl ice cold fresh lysis buffer (samples were kept on ice at all times). Zirconium beads were added according to the size of the pellets. Each Eppendorf tube

was sealed with Parafilm and put into the Bullet Blender®. Samples were homogenised as in the animal study before the supernatant (approximately 50 µl) were transferred to new tubes.

3.4.2 Bradford assay

The Bradford assay was modified due to the small pellet size and volume of each sample. For the 1st dilution 5 µl sample was diluted in 45 µl dH_2O instead of 10 µl in 90 µl dH_2O. In the 2nd dilution 10 µl of the 1st dilution was diluted in 90 µl dH_2O. The rest of the protocol was maintained as in the animal study. Bradford solution was prepared as previously explained and filtered. 900 µl was added to the BSA standards and dilutions of samples and colour was allowed to develop for 20 min before absorbance readings were taken at 595 nm. Protein concentrations were calculated in Microsoft Excel. All the samples' protein concentrations were only sufficient to make lysates containing 15 µg of protein if 21 µl of the sample was loaded on the gel.

3.4.3 Lysate protein concentration optimisation

Due to the low protein concentrations, new PBMCs were isolated from rat whole blood. Lysates were prepared to contain 15 µg of protein. A standard 10 well 7.5% mini gel was cast and 21 µl of lysate was loaded on the gel as well as 25 µl (maximum volume of well) of the same sample along with 21 µl and 25 µl of the 50 µg positive control. This was to determine whether the difference in volume loaded would affect the band intensity. The gel was run and bands were normalised.

3.4.4 Human protein separation

Lysates containing 15 µg of protein were prepared from the human samples and 21 µl of each lysate was loaded for the groups: control (n= 8), obese (n= 9) and pre-diabetic (n= 7). Within each group data was split according to age with young being <30 years and middle aged (MA) been 30-45 years. A lysate prepared from available mouse heart tissue was used as a positive control to load on gel. Gel electrophoresis, protein separation and transfer to PVDF membranes were done according to the standard protocol as used in the animal study. T- and P-ATM was probed for and normalised according to the total lane protein.

4. Western blotting of $P22^{phox}$

$P22^{phox}$ is a protein directly associated with NOXs and is used an indicator of the level of ROS production. As previously discussed under obese conditions, patients have low-grade inflammation. Inflammation enhances the production of ROS (Zhou *et al.*, 2006; Hollyfield *et al.*, 2008) and the inhibition of $P22^{phox}$ was shown to decrease ROS generation, the activation of NOXs and inflammatory cytokine production (Qiu *et al.*, 2015). Thus $P22^{phox}$ was probed for to

investigate inflammation and oxidative stress and whether there are any relationships with the degree of insulin resistance as well as the lipid profile.

$P22^{phox}$ primary antibody was prepared in a 1:500 dilution in TBS-Tween with 1% milk. As $P22^{phox}$ is 22 kDa, the bottom half of all animal and human study's Western blotting membranes were probed for $P22^{phox}$.

5. Statistical analysis

Normalisation of protein bands were done according to the total protein membrane and was done using Bio-Rad Image Lab™ software and data was exported to Microsoft Excel. Fold change relative to control was calculated in Microsoft Excel. Data was transferred to GraphPad Prism 5 software where one-way ANOVA or two-way ANOVA (where applicable), Bonferroni's Multiple Comparison Test and Student t Tests (where applicable) were performed. The data was used to construct column graphs with error bars and data was expressed as Mean ± Standard Error of the Mean (SEM). Analysis generating a p-value ≤ 0.05 was considered statistically significant.

CHAPTER 3: RESULTS

This chapter reports the results of the animal and human PBMCs analysis.

1. Animal study

Rats were obtained at 6 weeks of age and fed an *od* diet to induce obesity and insulin resistance and compared to a normal standard chow-fed age-matched control for 16 weeks. Data collected was used to investigate the relationship between ATM levels and the degree of insulin resistance.

1.1 Biometric data

Body weight, IP fat mass, OGTT and basal blood glucose levels for controls, *od* and young control animals were measured. All data is expressed as Mean ± SEM.

1.1.1 Body weight

A significant difference in body weight (g) was seen between the young controls versus (vs.) the age-matched controls (302.00 ± 8.33; vs. 411.30 ± 19.08 g; n= 5-7) with a $p<0.0001$. No significant difference was seen between the body weight of the age-matched controls vs. *od* (411.30 ± 19.08 vs. 456.90 ± 12.23 g; n= 7-9) rats following treatment with the different diets (*Figure 3.1*).

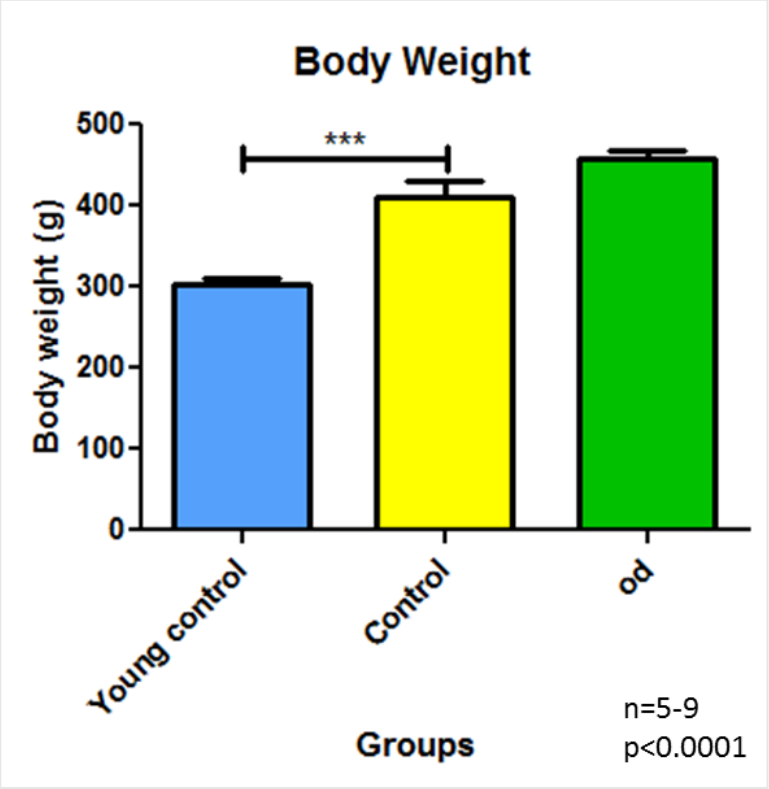

Figure 3. 1: *Body weight (g) of experimental groups at sacrifice.*

1.1.2 IP fat mass

IP fat mass was measured after sacrificing after 16 weeks of diet. A significant difference was seen between the IP fat mass (g) of *od* vs. control (25.62 ± 1.50 vs. 14.17 ± 1.43 g; n= 7-9), p<0.0001 (*Figure 3.2*).

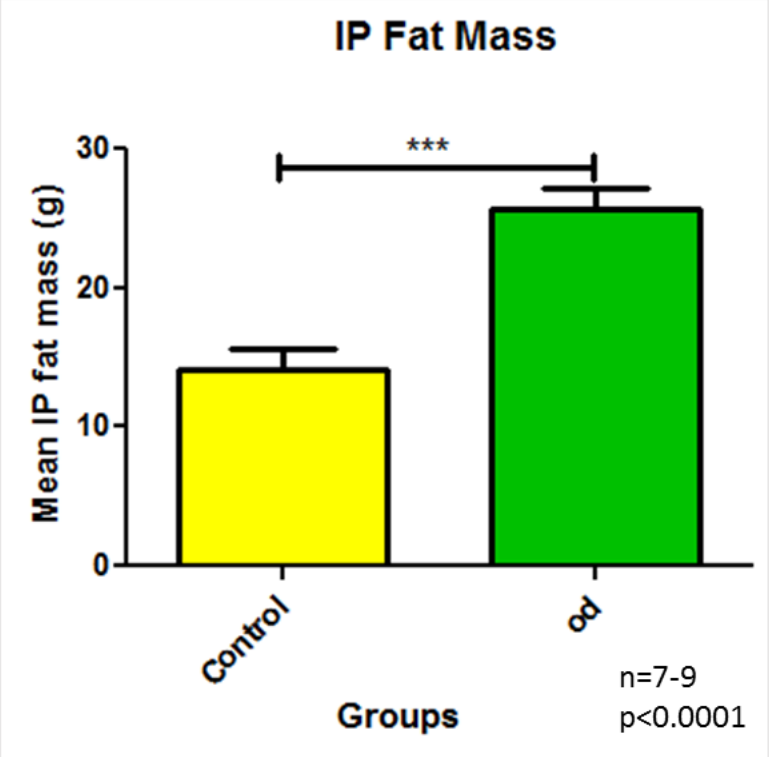

Figure 3. 2: *Mean IP fat mass (g) of od vs. age-matched controls after 16 weeks of diet treatment.*

1.1.3 OGTT

OGTTs were recorded in week 16 following overnight fasting. A two-way ANOVA was performed that indicated an overall significant difference between the blood glucose levels (mmol/L) of the controls and *od* following treatment of 16 weeks with the respective diets with $p<0.0001$, n= 12 per group (*Figure 3.3*). A significant difference was seen between the area under the curve (AUC) of the *od* when compared to the age-matched controls (793.3 vs. 731.2), $p<0.05$, n= 12 per group. A two-way ANOVA with repeated measures was performed to determine significance in the OGTT ($p<0.0001$) and was expressed as Mean ± SEM (*Table 3.1*). No statistical significance was seen when comparing the mean ± SEM of the *od* to that of the controls for each time point, n= 12 per time point per group.

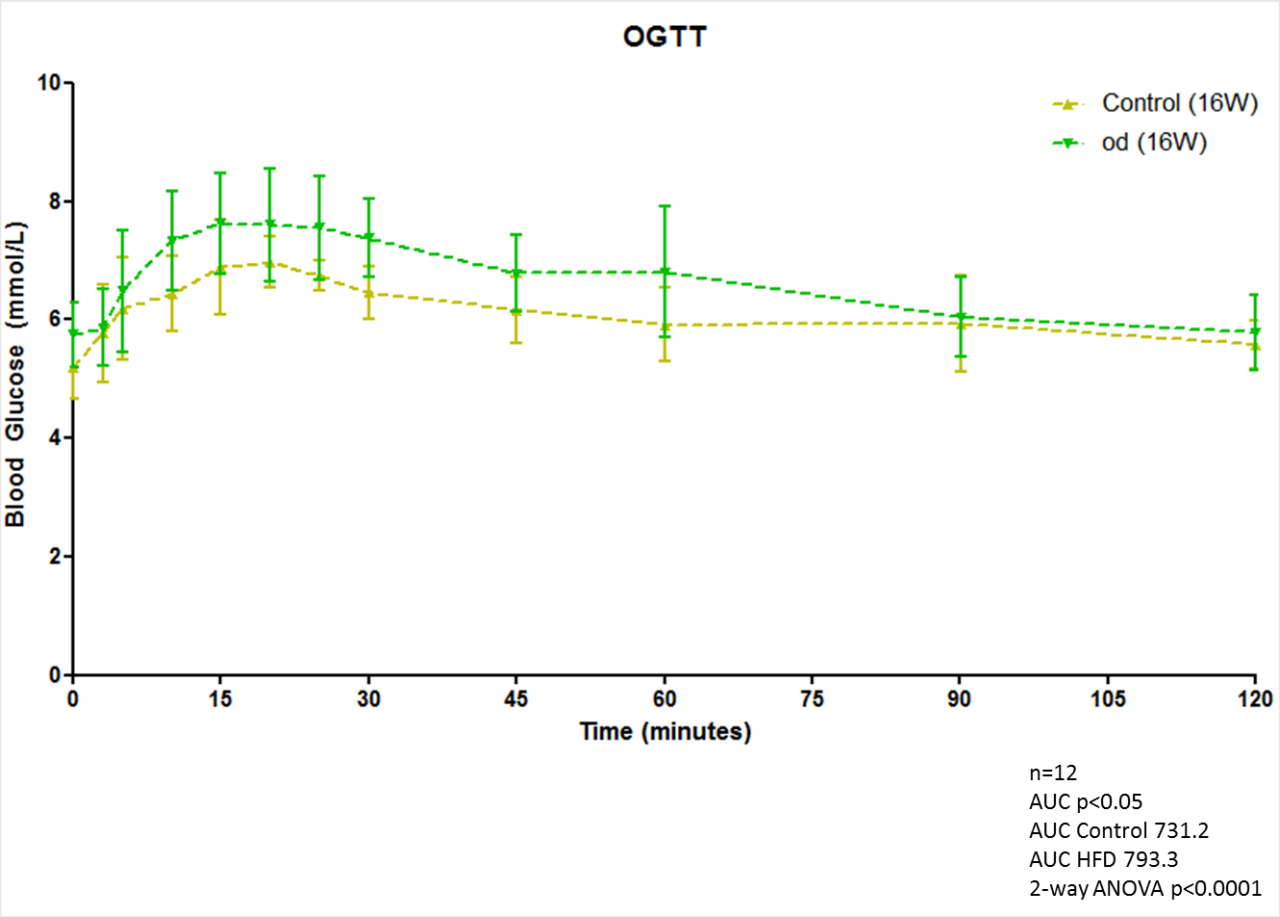

Figure 3. 3: *OGTT (mmol/L blood glucose) of controls vs. od measured at time periods 0 min, 3 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 45 min, 60 min, 90 min and 120 min measured in week 15 after administration of 1 g/kg sucrose.*

Table 3. 1: *OGTT (mmol/L blood glucose) values of controls and od animals.*

Time (min)	Mean ± SEM	
	Control	***od***
0	5.21 ± 0.16	5.75 ± 0.16
3	5.78 ± 0.24	5.87 ± 0.19
5	6.19 ± 0.25	6.48 ± 0.30
10	6.43 ± 0.18	7.34 ± 0.24
15	6.89 ± 0.23	7.63 ± 0.24
20	6.98 ± 0.12	7.60 ± 0.27
25	6.74 ± 0.07	7.56 ± 0.25
30	6.46 ± 0.13	7.38 ± 0.19
45	6.18 ± 0.16	6.78 ± 0.19
60	5.92 ± 0.18	6.81 ± 0.32
90	5.93 ± 0.23	6.04 ± 0.19
120	5.58 ± 0.11	5.78 ± 0.18

Data expressed as Mean ± SEM

1.1.4 Basal blood glucose levels

Following 16 weeks of treatment with the separate diets the basal fasting blood glucose levels were measured. A significant difference was seen between the basal blood glucose levels (mmol/L) of the *od* vs. controls (5.75 ± 0.16 vs. 5.21 ± 0.16 mmol/L); $p<0.05$, both with n= 12 (*Figure 3.4*).

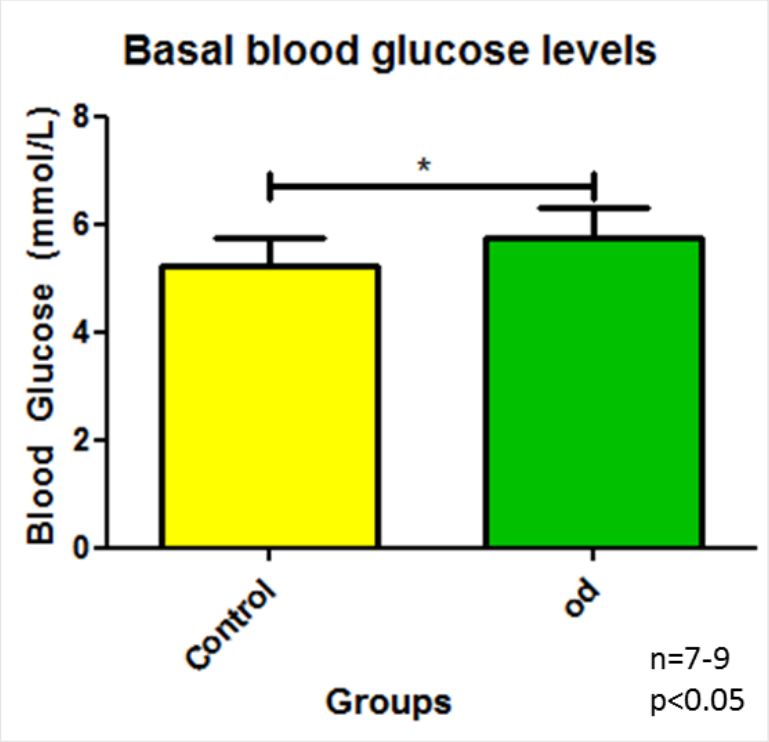

Figure 3. 4: *Baseline blood glucose levels (mmol/L) of fasted od vs. age-matched controls (at 0 min of OGTT) (in week 15).*

1.2 Western blotting data

1.2.1 ATM

a) T-ATM levels

No significant difference was seen between the levels of T-ATM when comparing the *od* (n= 9) to the age-matched controls (n= 7) (*Figure 3.5*).

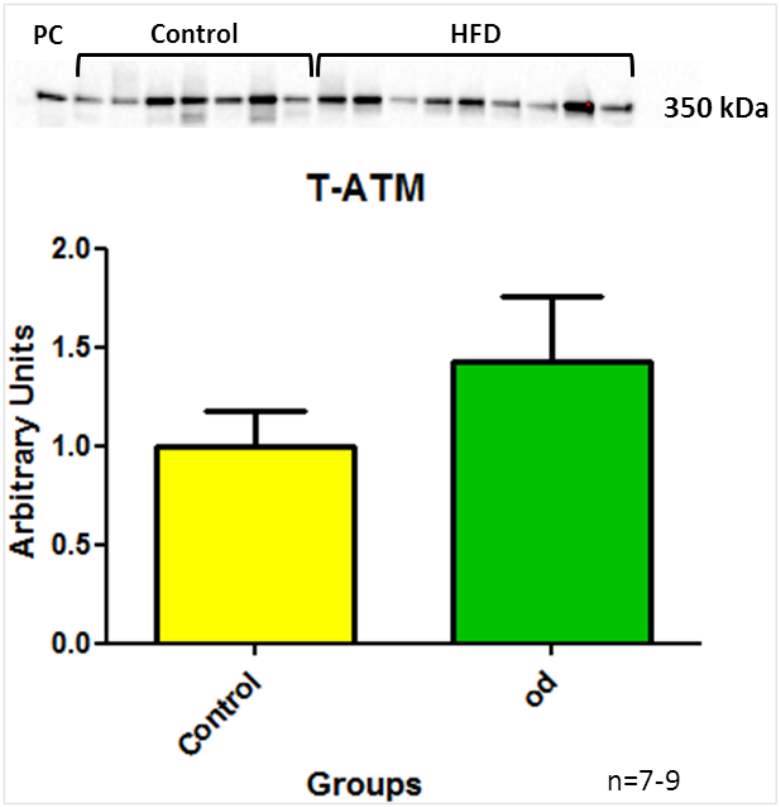

Figure 3. 5: *T-ATM levels (Arbitrary Units) of od vs. age-matched controls. PC Positive control.*

b) P-ATM levels

No significant difference was seen between the levels of P-ATM when comparing the *od* (n= 9) to the age-matched controls (n= 7) (*Figure 3.6*).

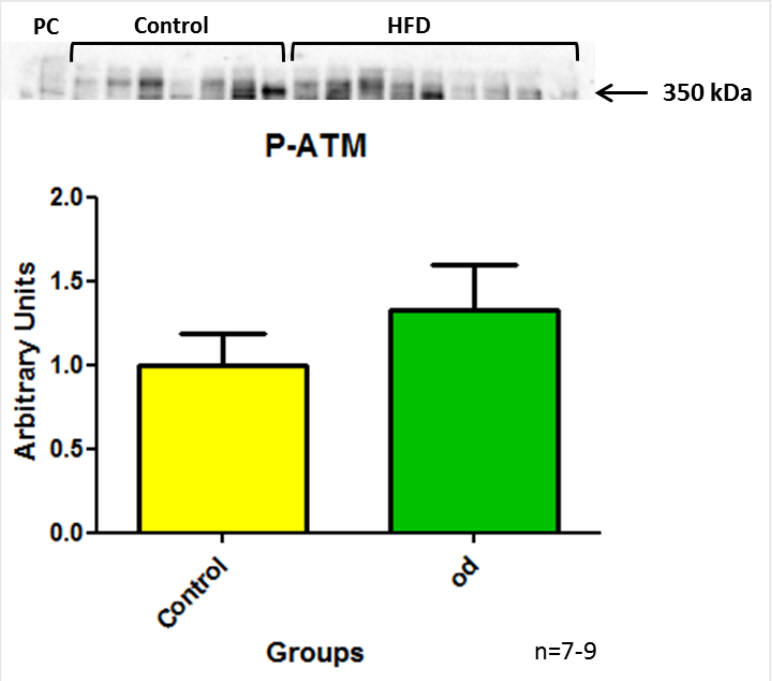

Figure 3. 6: *P-ATM levels (Arbitrary Units) of od vs. age-matched controls. PC Positive control.*

c) P-ATM/T-ATM ratio

No significant difference was seen between the P-ATM/T-ATM ratios of the *od* (n= 9) vs. the age-matched controls (n= 7) (*Figure 3.7*).

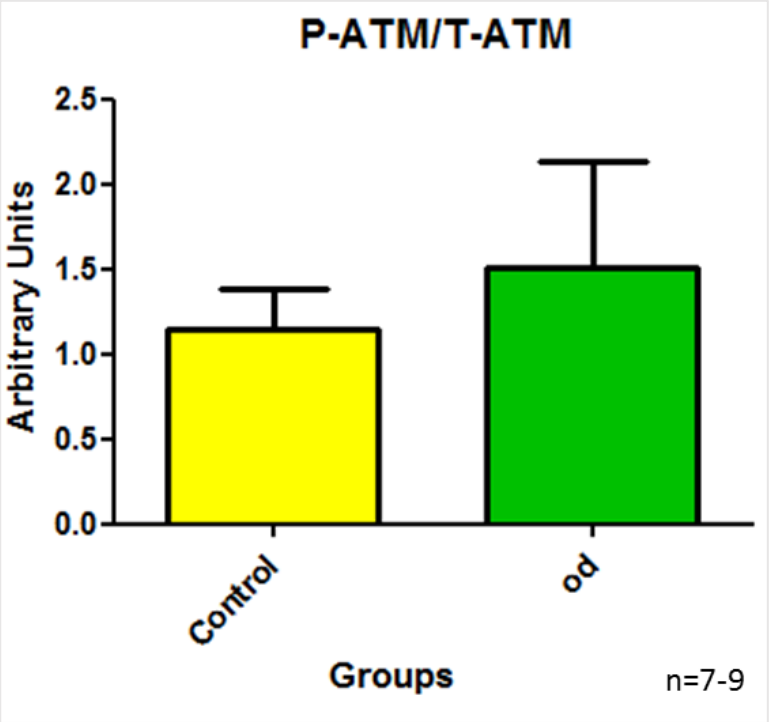

Figure 3. 7: *P-ATM/T-ATM ratio (Arbitrary Units) for od vs. age-matched controls.*

1.2.2 Age comparison

An age comparison was performed between the total and phosphorylated ATM protein levels of age-matched controls fed the control diet for 22 weeks of age and young controls fed the same diet for 12 weeks.

a) T-ATM levels

Significant difference was seen between the T-ATM protein levels of the young controls (n= 6) compared to age-matched controls (n= 7) expressed as Arbitrary Units (AU) (2.677 ± 0.6661 vs. 1.000 ± 0.1925 AU; p= 0.025) (*Figure 3.8*). No significant difference was seen between the T-ATM protein levels of the *od* (n= 9) and age-matched controls.

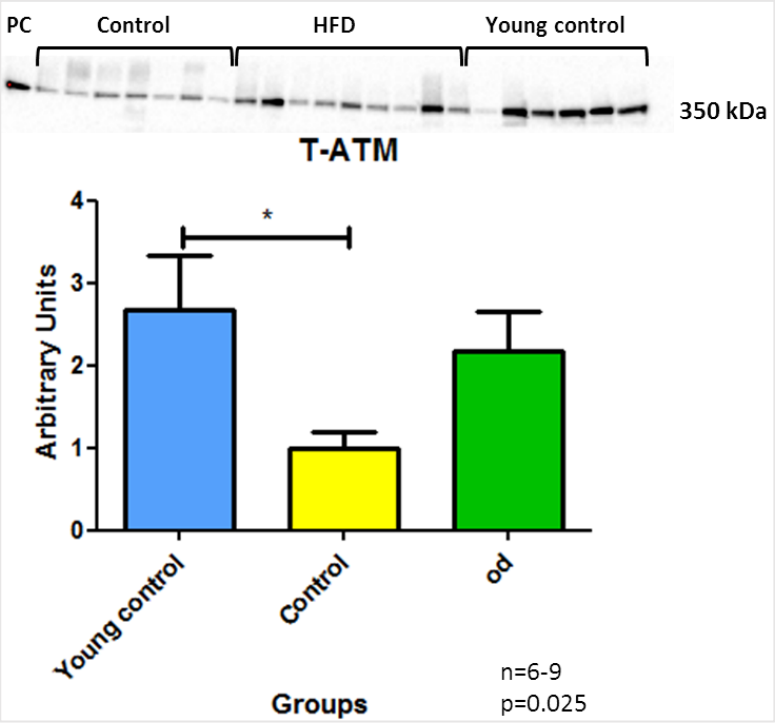

Figure 3. 8: *T-ATM levels (Arbitrary Units) of age-matched controls (22 weeks) vs. young controls (12 weeks). PC Positive control.*

b) P-ATM levels

A significant difference was seen between the P-ATM levels of the young controls (n= 6) when compared to the age-matched controls (22 weeks) (n= 7) (0.4708 ± 0.1106 vs. 1.000 ± 0.1484 AU; n= 6-7; p= 0.018) (*Figure 3.9*).

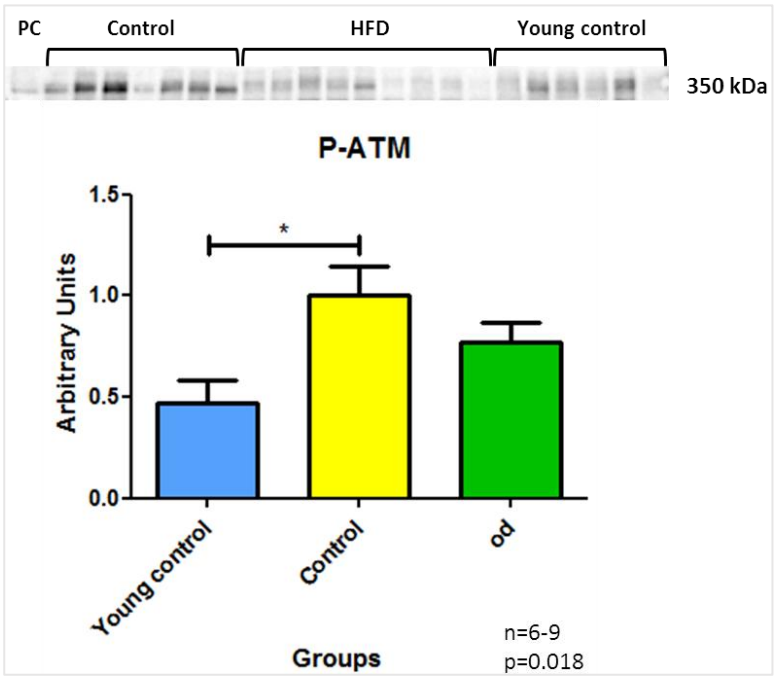

Figure 3. 9: *P-ATM levels (Arbitrary Units) of age-matched controls (22 weeks) vs. young controls (12 weeks). PC Positive control.*

c) p-ATM/T-ATM ratio

Significant differences were seen between P-ATM/T-ATM ratio (Arbitrary Units) of *od* vs. age-matched controls (0.51 ± 0.13; n= 9 vs. 1.25 ± 0.25 AU; n= 7-9) with p= 0.0127 and young controls vs. age-matched controls (0.43 ± 0.28; n= 6 vs. 1.25 ± 0.25 AU; n= 6-7) with p= 0.0476 (*Figure 3.10*).

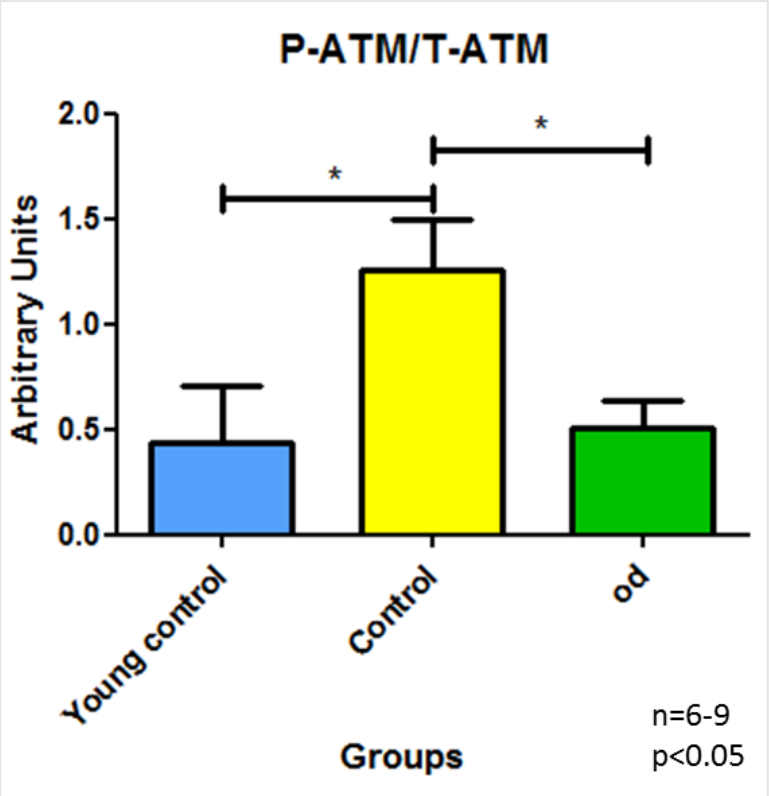

Figure 3. 10: *P-ATM/T-ATM ratio (Arbitrary Units) of groups.*

2. Human study

Female black Xhosa patients were recruited, data was collected and participants were divided into young and middle aged groups. Data was used to investigate the relationship between ATM levels and the degree of insulin resistance.

2.1 Biometric measurements

Biometric data was analysed within each experimental group namely, control (n= 8), obese (n= 9) and pre-diabetic (n= 7) as well as according to age groups of young (Y) (<30 years) and middle aged (MA) (30 – 45 years) within each group.

2.1.1 BMI

a) BMI vs. degree of insulin resistance.

Significant differences were seen between the BMI (kg/m^2) of the obese vs. control (39.04 ± 1.46 vs. 21.35 ± 0.85; n= 8-9) and pre-diabetic vs. control (41.29 ± 1.30 vs. 21.35 ± 0.85; n= 7-8), p<0.0001 (*Figure 3.11*).

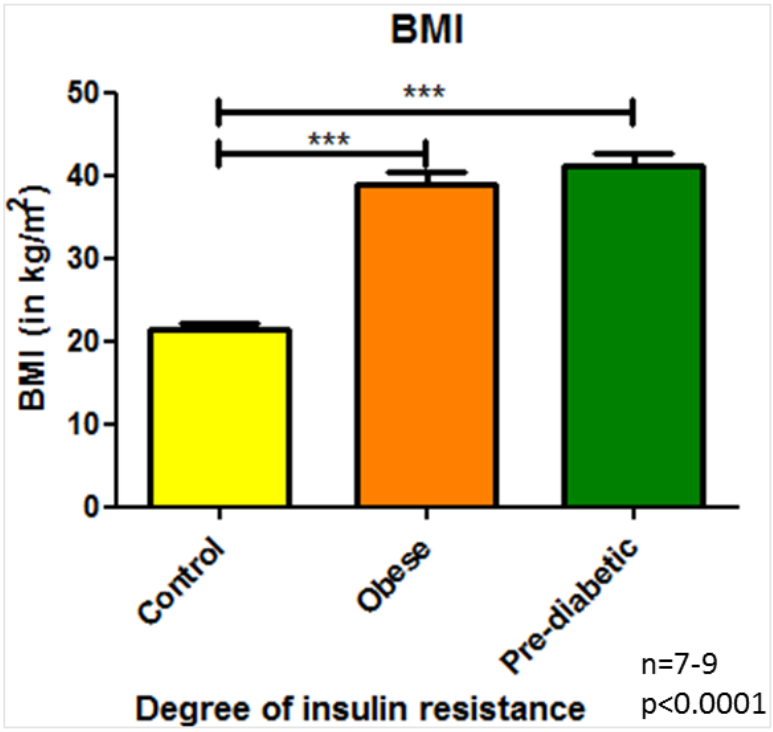

Figure 3. 11: *BMI (in kg/m^2) vs. the degree of insulin resistance.*

b) BMI vs. age groups vs. degree of insulin resistance.

Significant differences were seen in the BMI (kg/m^2) of the Y obese vs. the MA obese (35.25 ± 0.82 vs. 42.07 ± 1.44; n= 4-5), p= 0.0064, as well as the Y pre-diabetic vs. the MA pre-diabetic (44.45 ± 0.39; vs. 38.92 ± 1.354; n= 3-4), p=0.0196. Significant difference was seen between Y obese vs. Y control (35.25 ± 0.82 vs. 21.47 ± 1.12; n= 4-6), as well as Y pre-diabetic vs. Y control (44.45 ± 0.39 vs. 21.47 ± 1.12; n= 3-6), both with a p<0.0001. Significant differences were seen between MA obese vs. MA control (42.07 ± 1.44 vs. 20.99 ± 1.09; n= 2-5), p= 0.0004, as well as the MA pre-diabetic vs. the MA control (38.92 ± 1.35 vs. 20.99 ± 1.09; n= 2-4), p= 0.0011 (*Figure 3.12*). According to a two-way ANOVA age had an overall interaction with the degree of insulin resistance with a p=0.0005 as well as the degree of insulin resistance with a p<0.0001, n= 2-6.

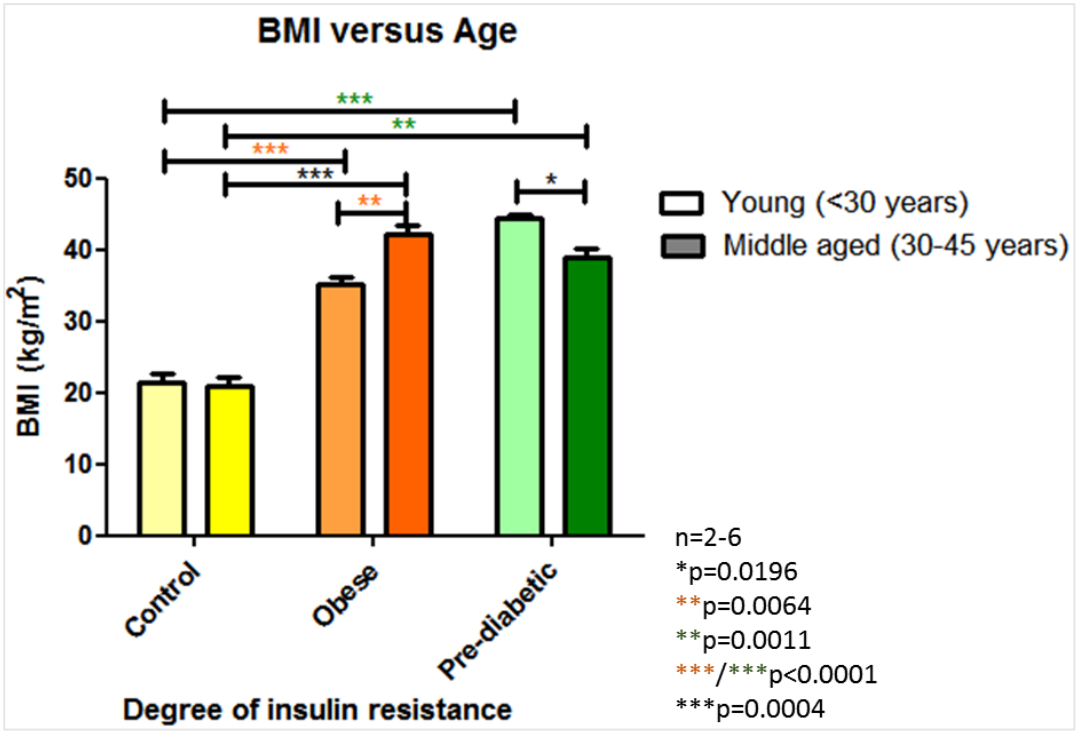

Figure 3. 12: *BMI (kg/m^2) vs. age vs. the degree of insulin resistance.*

2.2.2 WHR

a) WHR vs. degree of insulin resistance.

Significant differences were seen between the WHR (cm) of the obese vs. the control (0.85 ± 0.011 vs. 0.75 ± 0.016 cm; n= 8-9) as well as the pre-diabetic vs. the control (0.90 ± 0.016 vs. 0.75 ± 0.016 cm; n= 7-8), p<0.0001 (*Figure 3.13*).

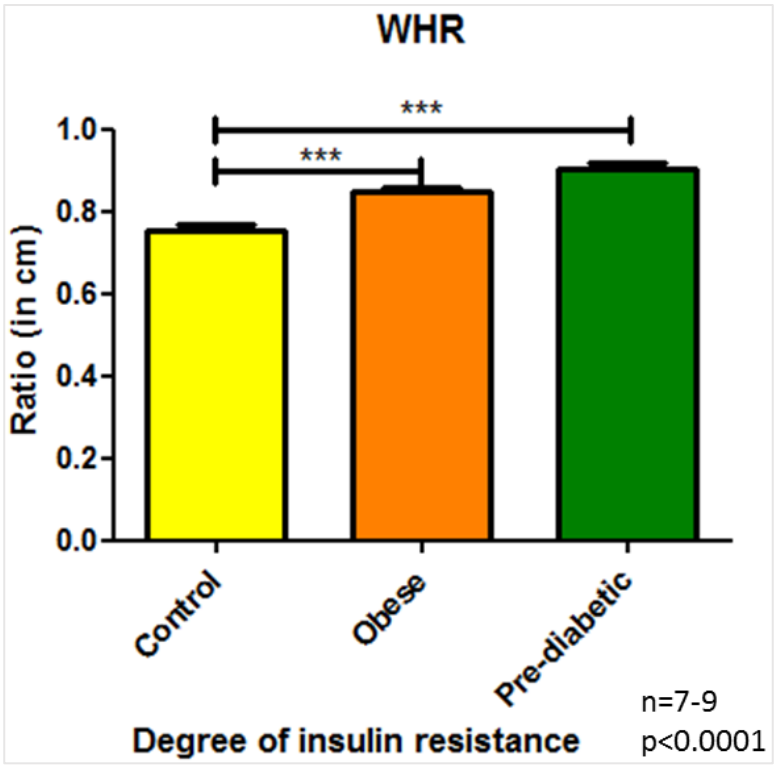

Figure 3. 13: *WHR (cm) vs. the degree of insulin resistance.*

b) WHR vs. age groups vs. degree of insulin resistance.

Significant differences were seen in the WHR (cm) of the Y obese vs. Y control (0.84 ± 0.01 vs. 0.75 ± 0.012 cm; n= 4-6), p= 0.0007, as well as the Y pre-diabetic vs. Y control (0.90 ± 0.010 vs. 0.75 ± 0.012 cm; n= 3-6), p<0.0001 (*Figure 3.14*). According to a two-way ANOVA, the degree of insulin resistance had an overall significant effect with a p<0.0001, n= 2-6.

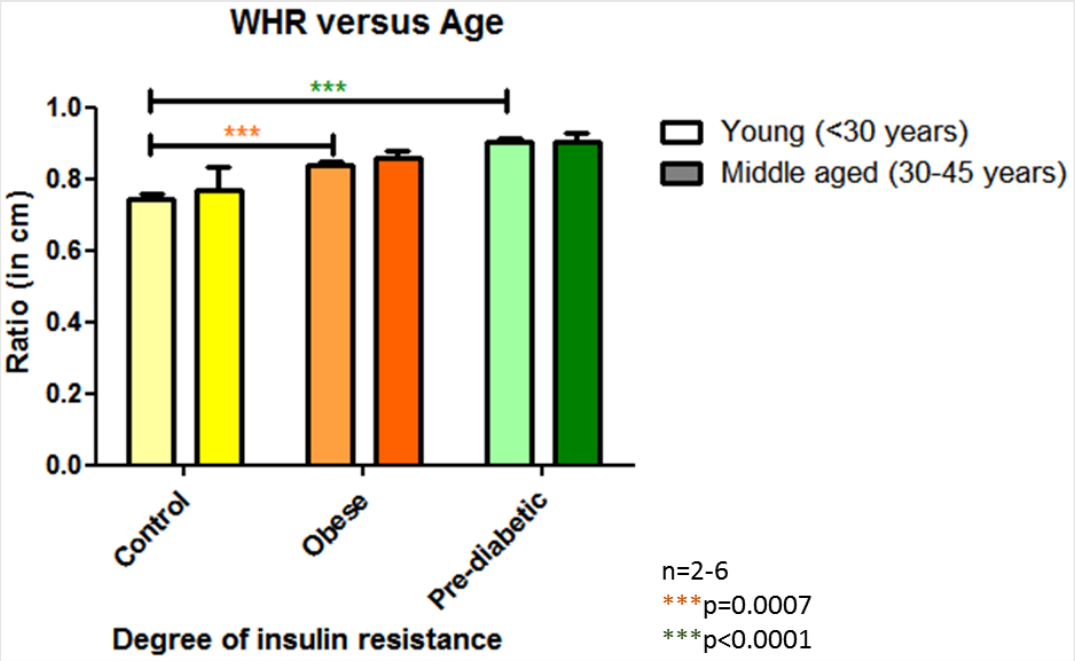

Figure 3. 14: *WHR (cm) vs. age vs. the degree of insulin resistance.*

2.2.3 TF/LF

a) TF/LF ratio vs. degree of insulin resistance.

Significant differences were seen for TF/LF ratio (measured by DXA) between obese vs. control (1.07 ± 0.087 vs. 0.72 ± 0.056; n= 8-9), p= 0.0046, and pre-diabetic vs. control (1.15 ± 0.09 vs. 0.72 ± 0.056; n= 6-8), p= 0.0009 (*Figure 3.15*).

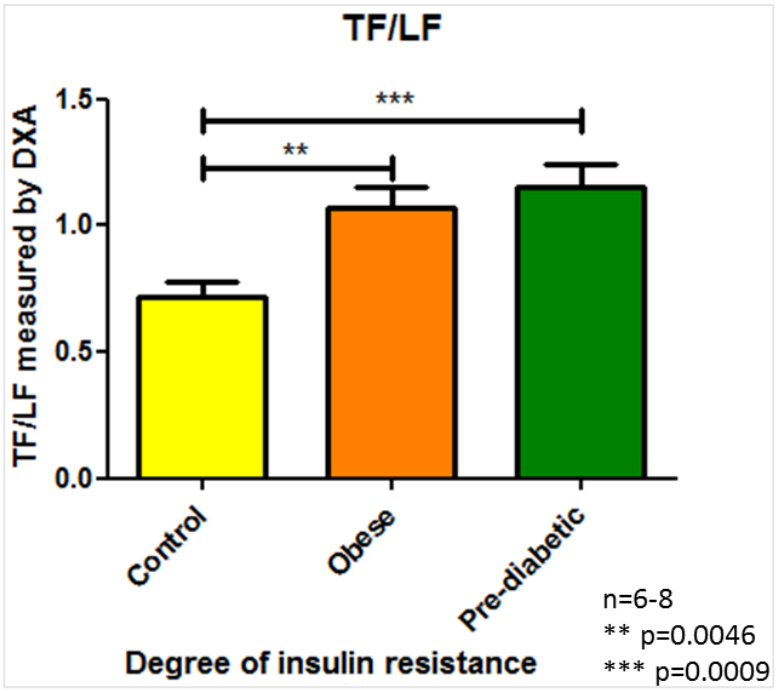

Figure 3. 15: *TF/LF ratio (measured by DXA) vs. the degree of insulin resistance.*

b) TF/LF ratio vs. age vs. degree of insulin resistance.

Significant difference was seen for the TF/LF ratio of the MA control vs. Y control (0.51 ± 0.07 vs. 0.79 ± 0.041; n= 2-6), p= 0.015; Y obese vs. Y control (1.19 ± 0.21 vs. 0.79 ± 0.041; n= 3-6), p= 0.032; Y pre-diabetic vs. Y control (1.28 ± 0.04 vs. 0.79 ± 0.041; n= 2-6), p= 0.0007; MA obese vs. MA control (0.99 ± 0.065 vs. 0.51 ± 0.07; n= 2-5), p= 0.0086, and MA pre-diabetic vs. MA control (1.09 ± 0.12 vs. 0.51 ± 0.07; n= 2-4), p= 0.0356 (*Figure 3.16*). According to a two-way ANOVA, age had an overall significant effect with p= 0.0249 and degree of insulin resistance had an overall significant effect of p= 0.0004, n= 2-6.

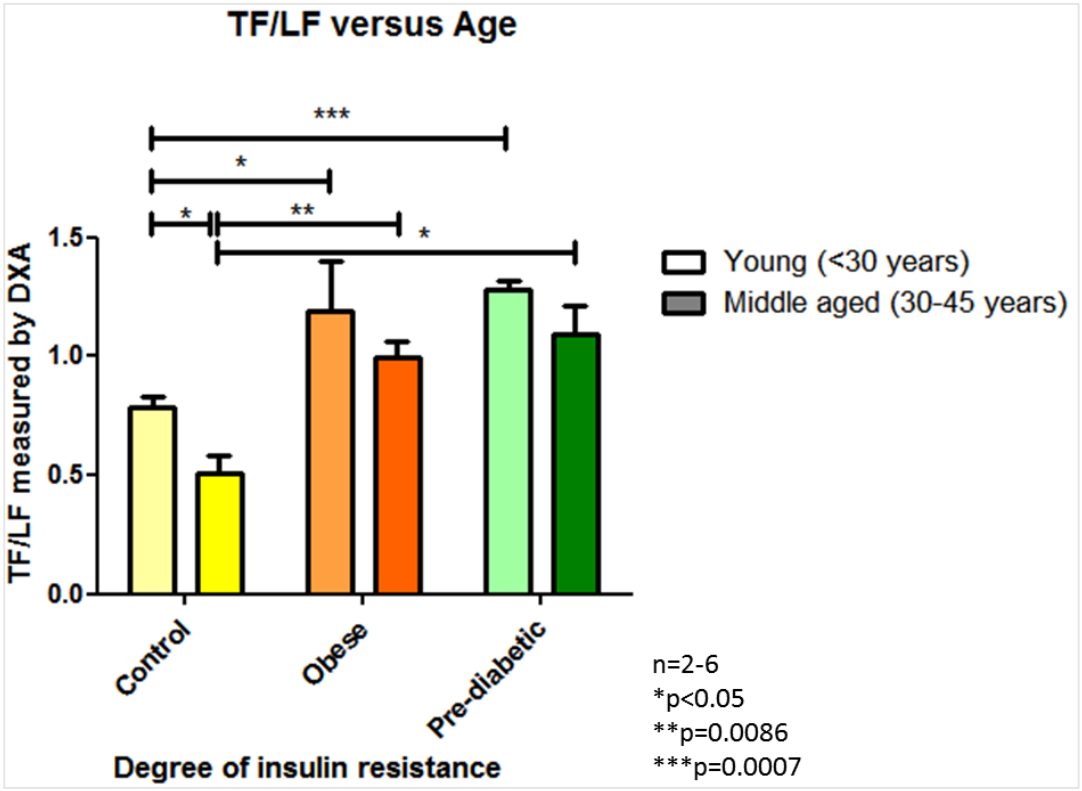

Figure 3. 16: *TF/LF ratio (measured by DXA) vs. age vs. the degree of insulin resistance.*

2.2.4 Lipid profile

The total cholesterol, HDL, LDL and triglyceride levels were measured for each patient. Data was analysed according to the degree of insulin resistance as well as age groups within each degree of insulin resistance.

a) Total cholesterol

i) Total cholesterol vs. degree of insulin resistance.

No significant difference was seen in the total cholesterol levels (mmol/L) of the obese (n= 9) and pre-diabetic (n= 7) groups when compared to the controls (n= 8) (*Figure 3.17*).

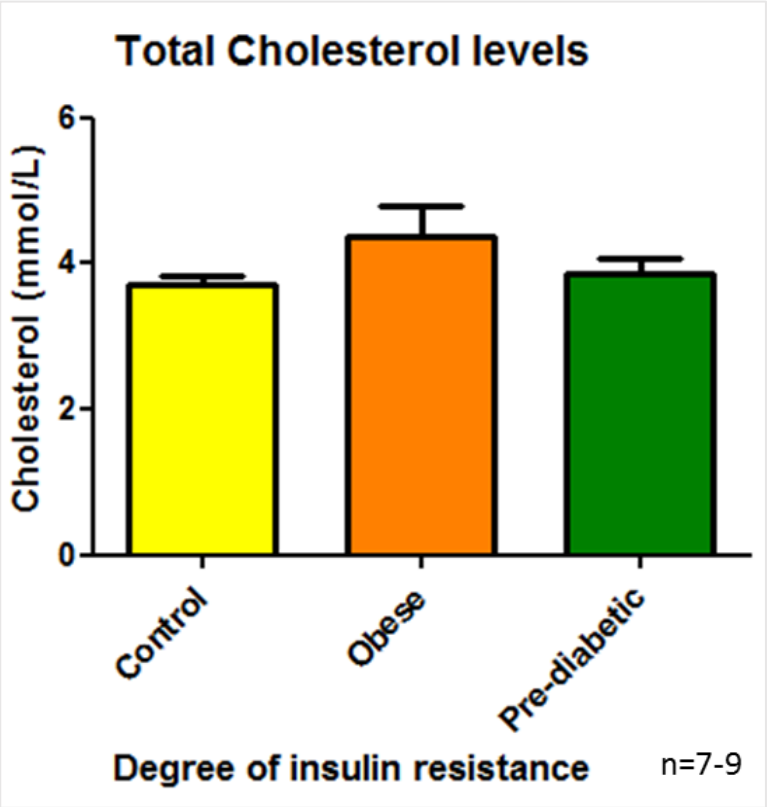

Figure 3. 17: *Total cholesterol levels (mmol/L) vs. the degree of insulin resistance.*

ii) Total cholesterol vs. age vs. degree of insulin resistance.

No significant differences were seen in the total cholesterol levels (mmol/L) when comparing age within each group or when comparing to the age controls, n= 6 for Y control, n= 2 for MA control, n= 4 for Y obese, n= 5 for MA obese, n= 3 for Y pre-diabetic and n= 4 for MA pre-diabetic (*Figure 3.18*).

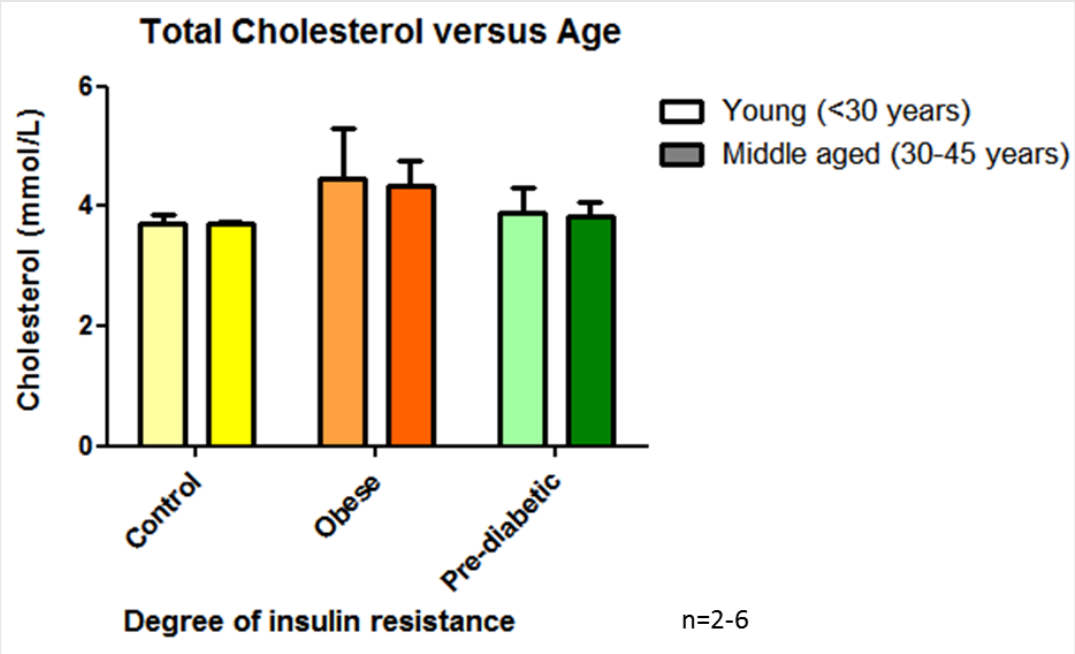

Figure 3. 18: *Total cholesterol levels (mmol/L) vs. age vs. the degree of insulin resistance.*

b) LDL

i) LDL cholesterol levels vs. degree of insulin resistance.

No significant differences were seen between the LDL cholesterol levels (mmol/L) obese (n= 9) and pre-diabetic (n= 7) groups when compared to the controls (n= 8) (*Figure 3.19*).

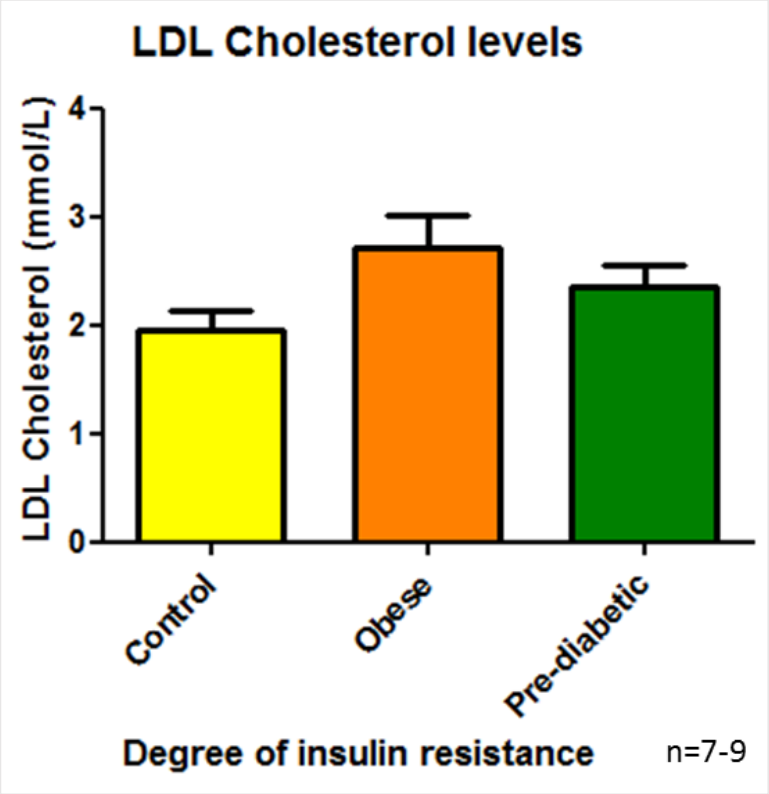

Figure 3. 19: *LDL cholesterol levels (mmol/L) vs. the degree of insulin resistance.*

ii) LDL cholesterol levels vs. age vs. degree of insulin resistance.

No significant difference was seen in the LDL cholesterol levels (mmol/L) when comparing age within each group or when comparing to age controls, n= 6 for Y control, n= 2 for MA control, n= 4 for Y obese, n= 5 for MA obese, n= 3 for Y pre-diabetic and n= 4 for MA pre-diabetic (*Figure 3.20*).

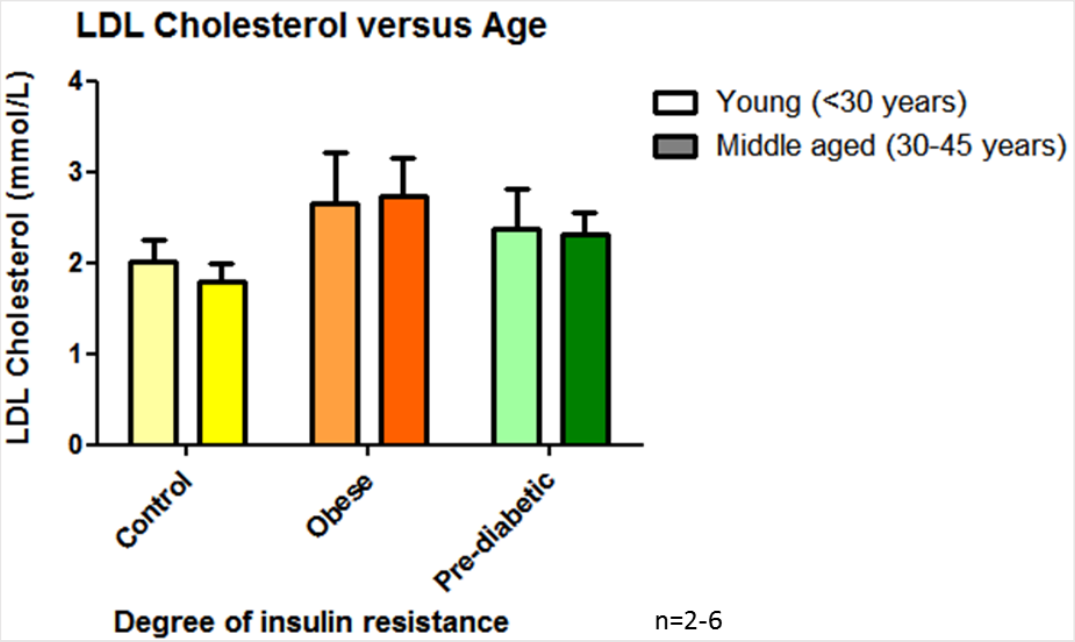

Figure 3. 20: *LDL cholesterol levels (mmol/L) vs. age vs. the degree of insulin resistance.*

c) HDL

i) HDL cholesterol levels vs. degree of insulin resistance.

No significant differences were seen in the HDL cholesterol levels (mmol/L) of the obese (n= 9) or pre-diabetic (n= 7) groups when compared to the controls (n= 8) (*Figure 3.21*).

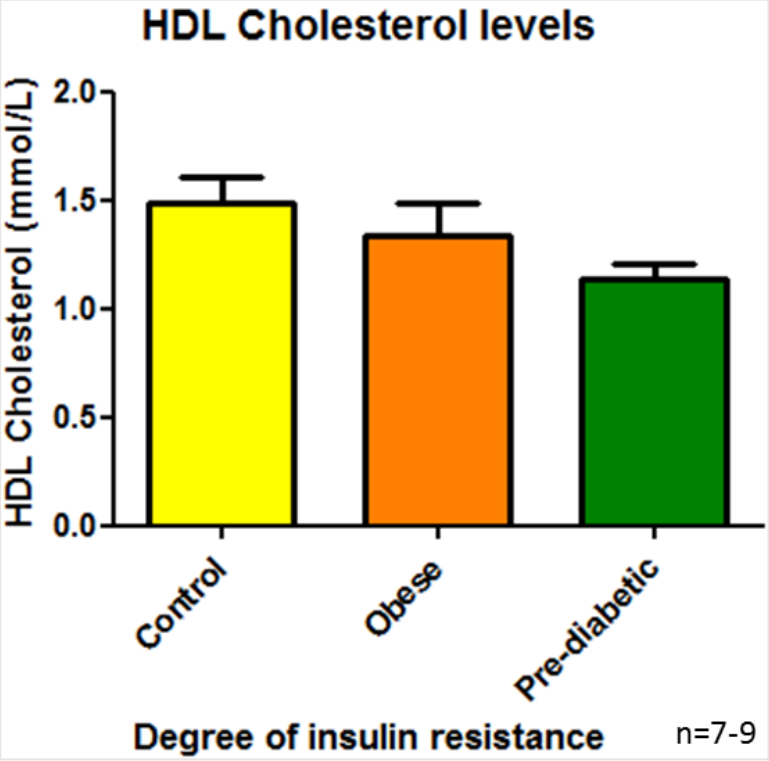

Figure 3. 21: *HDL cholesterol levels (mmol/L) vs. the degree of insulin resistance.*

ii) HDL cholesterol levels vs. age vs. degree of insulin resistance.

No significant difference was seen in the HDL cholesterol levels (mmol/L) when comparing age within each group or when comparing to age controls, n= 6 for Y control, n= 2 for MA control, n= 4 for Y obese, n= 5 for MA obese, n= 3 for Y pre-diabetic and n= 4 for MA pre-diabetic (*Figure 3.22*).

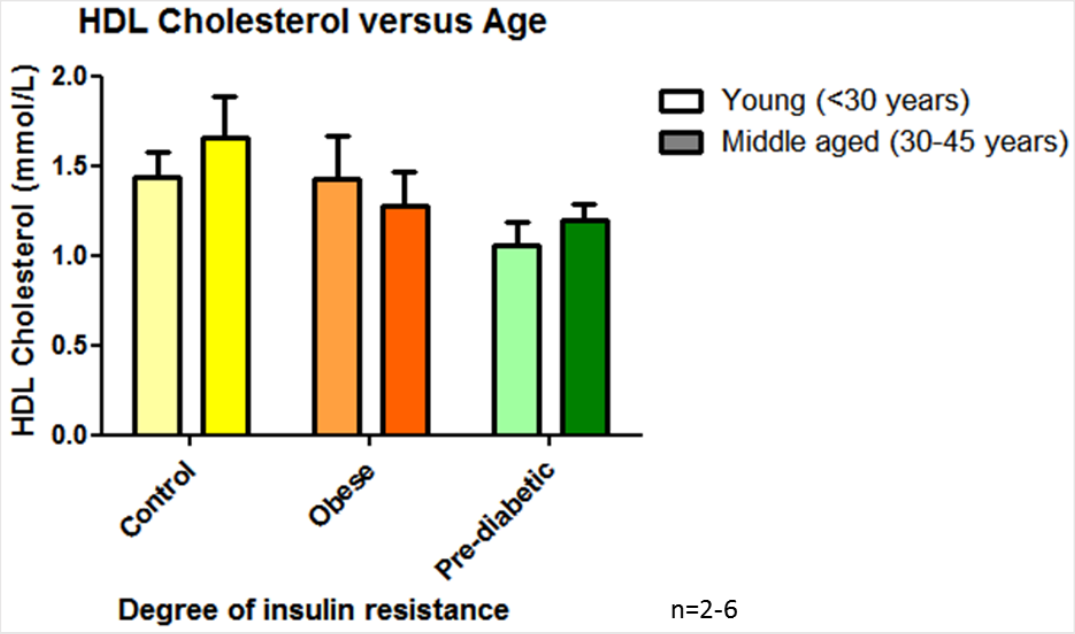

Figure 3. 22: *HDL cholesterol vs. age vs. the degree of insulin resistance.*

d) Triglycerides

i) Triglyceride levels vs. degree of insulin resistance.

No significant differences were seen in the triglyceride levels of the obese (n= 9) and pre-diabetic (n= 7) groups when compared to the controls (n= 8) (*Figure 3.23*).

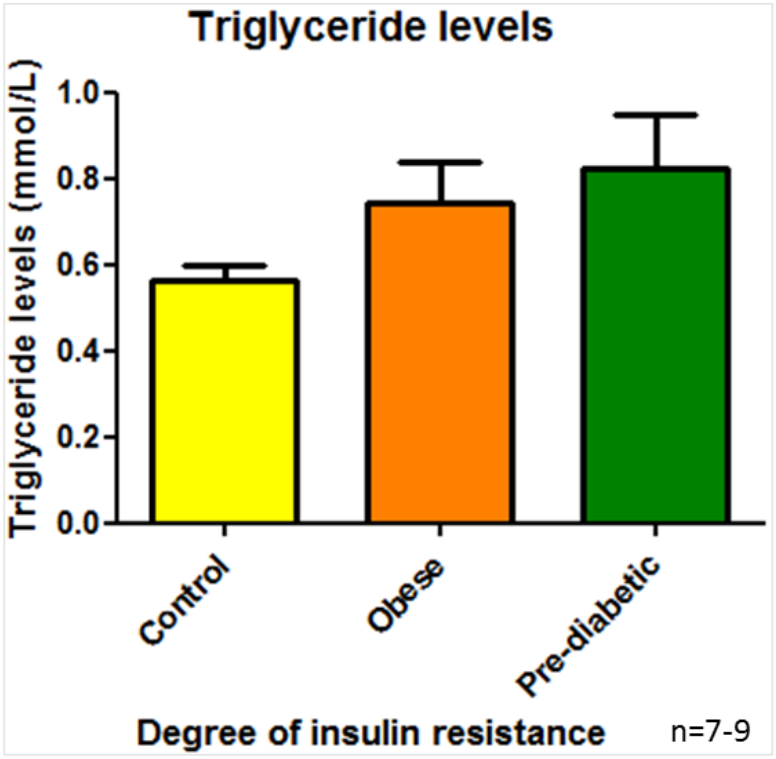

Figure 3. 23: *Triglyceride levels (mmol/L) vs. the degree of insulin resistance.*

ii) Triglyceride levels vs. age vs. degree of insulin resistance.

Significant difference was seen for triglyceride levels (mmol/L) between the Y pre-diabetic and Y control (1.00 ± 0.26 vs. 0.56 ± 0.044 mmol/L; n= 3-6), p= 0.0479 (*Figure 3.24*).

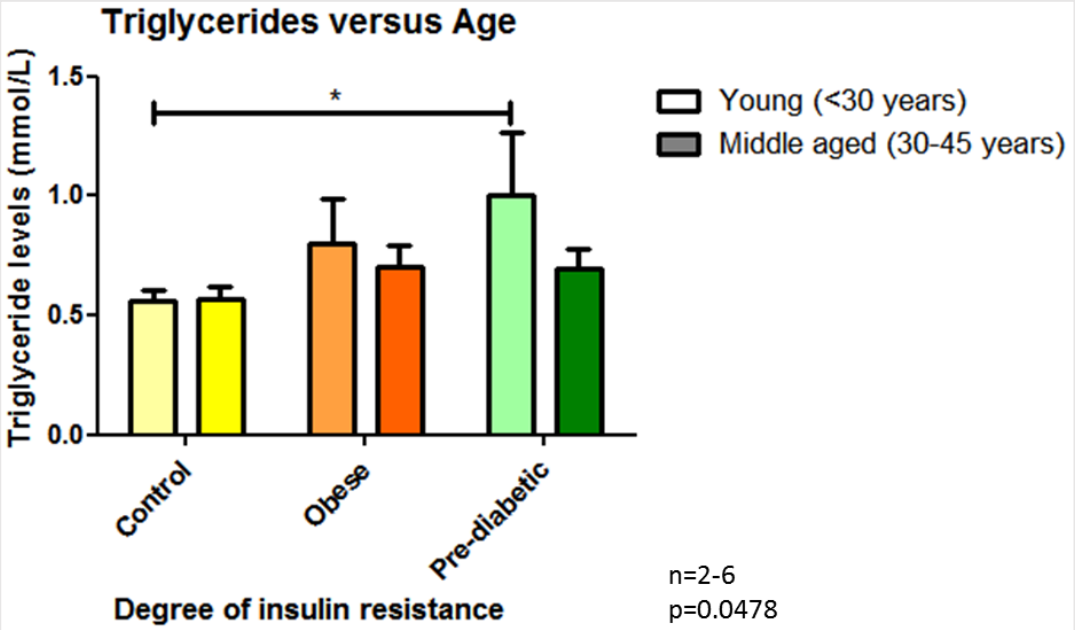

Figure 3. 24: *Triglyceride levels (mmol/L) vs. age vs. the degree of insulin resistance.*

2.2.5 Fasted blood glucose

a) Fasted blood glucose levels vs. degree of insulin resistance.

Statistical differences were seen for fasting blood glucose (mmol/L) of obese vs. control (5.10 ± 0.21 vs. 4.41 ± 0.14 mmol/L; n= 8-9), p= 0.0170, as well as pre-diabetic vs. control (5.54 ± 0.23 vs. 4.41 ± 0.14 mmol/L; n= 7-8), p= 0.0008 (*Figure 3.25*).

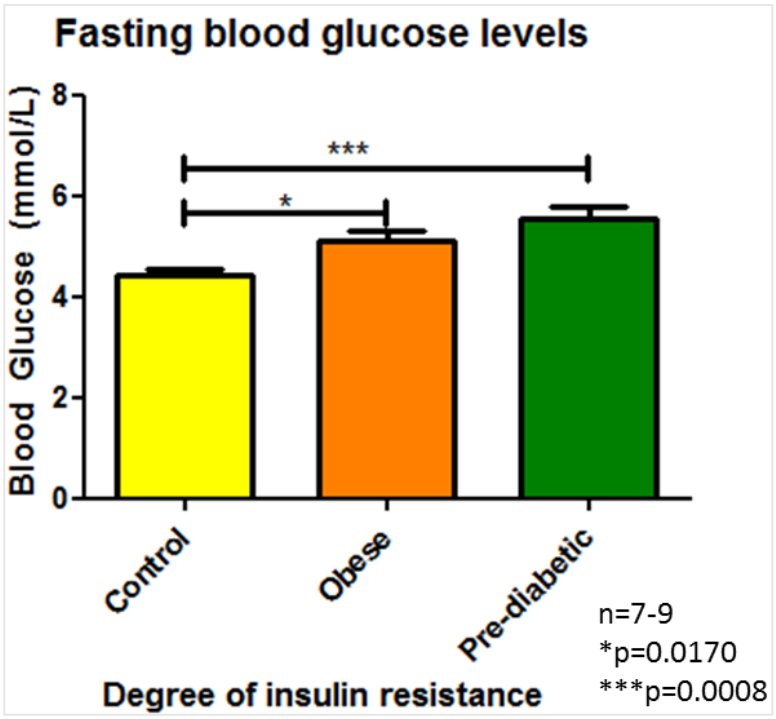

Figure 3. 25: *Fasting blood glucose levels (mmol/L) vs. the degree of insulin resistance.*

b) Fasted blood glucose vs. age vs. degree of insulin resistance.

Significant difference was seen between the Y pre-diabetic vs. Y control (5.90 ± 0.15 vs. 4.48 ± 0.13 mmol/L; n= 3-6), p= 0.0003 (*Figure 3.26*). According to a two-way ANOVA, the degree of insulin resistance had an overall effect with p= 0.003, n= 2-6.

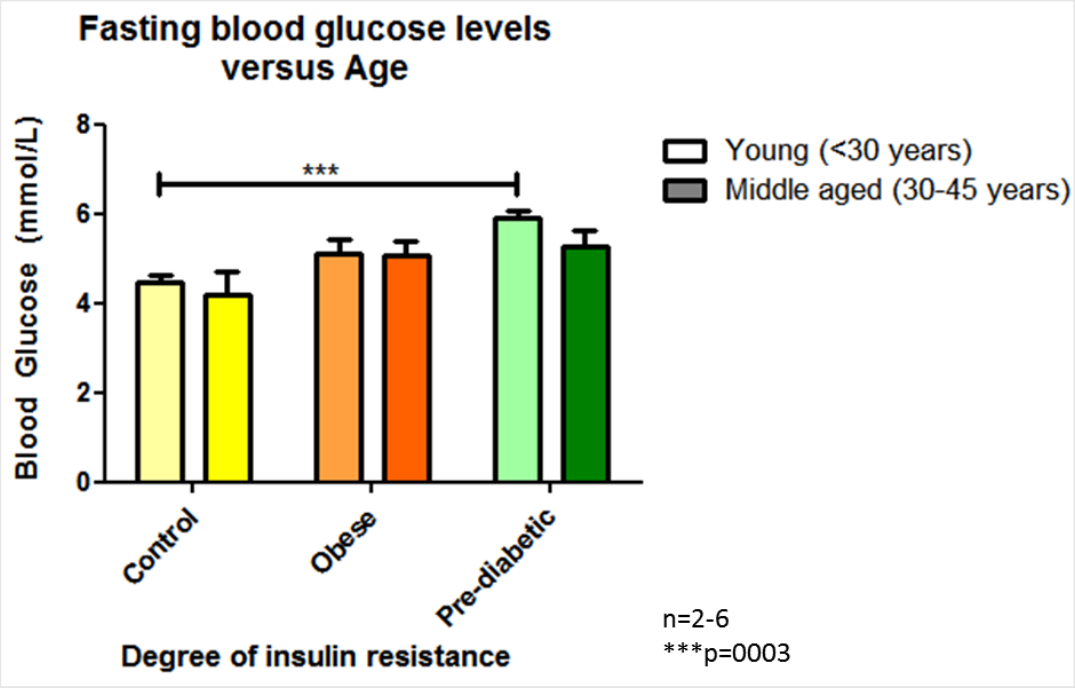

Figure 3. 26: *Fasting blood glucose levels (mmol/L) vs. age vs. the degree of insulin resistance.*

2.2.6 Blood pressure, Atherogenic Index and HTR

A significant difference was seen between the pre-diabetic vs. control with reference to SBP (mmHg) (136.1 ± 8.57 vs. 108.4 ± 4.47 mmHg; n= 7-8; *p<0.05), DBP (mmHg) (88.71 ± 2.48 vs. 68.38 ± 2.53 mmHg; n= 8-9; ***p<0.0001) and HTR (30.14 ± 2.84 vs. 40.60 ± 3.78; n= 7-8; *p<0.05). A significant difference was seen between obese vs. control for Atherogenic Index (2.416 ± 0.26 vs. 1.62 ± 0.26; n= 8-9; *p<0.05). No significant differences were seen between obese vs. control for SBP, DBP or HTR as well as for Atherogenic Index for pre-diabetic vs. control (*Table 3.2*).

Table 3. 2: *SBP, DSP, HTR and Atherogenic Index according to the degree of insulin resistance of human study participants.*

	Mean ± SEM		
	Control (n= 8)	**Obese (n= 9)**	**Pre-diabetic (n= 7)**
SBP (mmHg)	108.4 ± 4.47	121.3 ± 4.32	136.1 ± 8.57 *
DBP (mmHg)	68.38 ± 2.53	76.56 ± 3.20	88.71 ± 2.48 ***
HTR	40.60 ± 3.78	31.02 ± 2.92	30.14 ± 2.84 *
Atherogenic Index	1.62 ± 0.26	2.416 ± 0.26*	2.50 ± 0.33

** (p<0.05), *** (p<0.0001). Data expressed as mean ± SEM. Systolic blood pressure (SBP), diastolic blood pressure (DBP), HDL cholesterol/total cholesterol ratio (HTR).*

Statistical significance was seen for SBP of MA pre-diabetic vs. MA control (149.0 ± 8.54 vs. 107.0 ± 3.00 mmHg; n= 2-4; * p<0.05). According to a two-way ANOVA age (p= 0.0405) and the degree of insulin resistance (p= 0.0145) had an overall effect on SBP. Statistical significance was seen for DBP between Y obese vs. MA obese (68.00 ± 2.94 vs. 83.40 ± 2.31 mmHg; n= 4-5; ** p<0.01), Y pre-diabetic vs. Y control (84.33 ± 4.84 vs. 70.17 ± 2.96 mmHg; n= 3-6; *p<0.05), MA obese vs. MA control (83.40 ± 2.31 vs. 63.00 ± 3.00 mmHg; n= 2-5; ** p<0.01) and MA pre-diabetic vs. MA control (92.00 ± 1.16 vs. 63.00 ± 3.00 mmHg; n= 2-4; ***p=0.0003). According to a two-way ANOVA the degree of insulin resistance had an overall effect, p<0.0001, on DBP. No significant differences were seen in HTR or Atherogenic Index (*Table 3.3*).

Table 3. 3: *SBP, DSP, HTR and Atherogenic Index according to the degree of insulin resistance and age of human study participants.*

	Mean ± SEM					
	Control		**Obese**		**Pre-diabetic**	
	Y (n= 6)	**MA (n= 2)**	**Y (n= 4)**	**MA (n= 5)**	**Y (n= 3)**	**MA (n= 4)**
SBP (mmHg)	108.8 ± 6.05	107.0 ± 3.00	114.3 ± 3.07	127.0 ± 6.59	119.0 ± 10.54	149.0 ± 8.54 *
DBP (mmHg)	70.17 ± 2.96	63.00 ± 3.00	68.00 ± 2.94	83.40 ± 2.31**,***	84.33 ± 4.84 *	92.00 ± 1.16***
HTR	39.25 ± 4.78	44.67 ± 5.87	32.39 ± 2.82	29.93 ± 5.03	28.18 ± 6.02	31.61 ± 2.89
Atherogenic Index	1.736 ± 0.33	1.278 ± 0.30	2.161 ± 0.28	2.620 ± 0.42	2.847 ± 0.71	2.241 ± 0.28

(p<0.05), ** (p<0.01), * (p<0.001). Data expressed as mean ± SEM. Systolic blood pressure (SBP), diastolic blood pressure (DBP), HDL cholesterol/total cholesterol ratio (HTR), young (Y), middle aged (MA).*

2.2 Western blotting data

2.2.1 15 µg animal PBMCs optimisation

The human PBMCs samples were found to contain low concentrations of protein. Human PBMCs lysates could only be prepared with 15 µg of protein. A rat PBMCs lysate was prepared at the same concentration and tested to see if the protein bands would be visible. Sample was visible at 15 µg concentration and was compared to a positive control at 50 µg.

2.2.2 ATM

a) T-ATM levels

No significant differences were seen in the T-ATM levels of the human PBMCs samples from the obese (n = 9) and pre-diabetic (n= 7) groups when compared to the control (n= 8) (*Figure 3.27*).

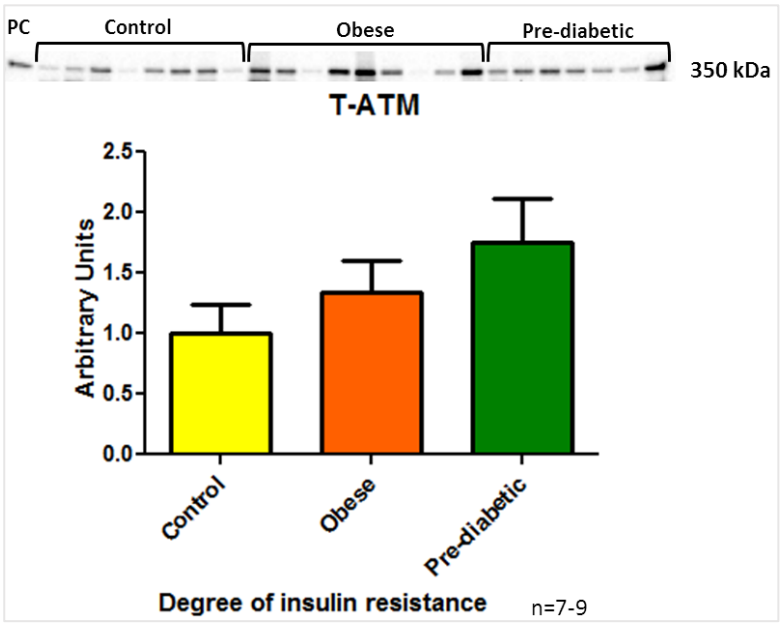

Figure 3. 27: *T-ATM levels (Arbitrary Units) vs. the degree of insulin resistance. PC Positive control.*

b) P-ATM levels

Significant differences were seen in the P-ATM levels of the human PBMCs samples from the obese vs. control (2.10 ± 0.41 vs. 1.00 ± 0.15 AU; n= 8-9), p= 0.0310, as well as the pre-diabetic vs. control (2.52 ± 0.62 vs. 1.00 ± 0.15 AU; n= 7-8), p= 0.0261 (*Figure 3.28*).

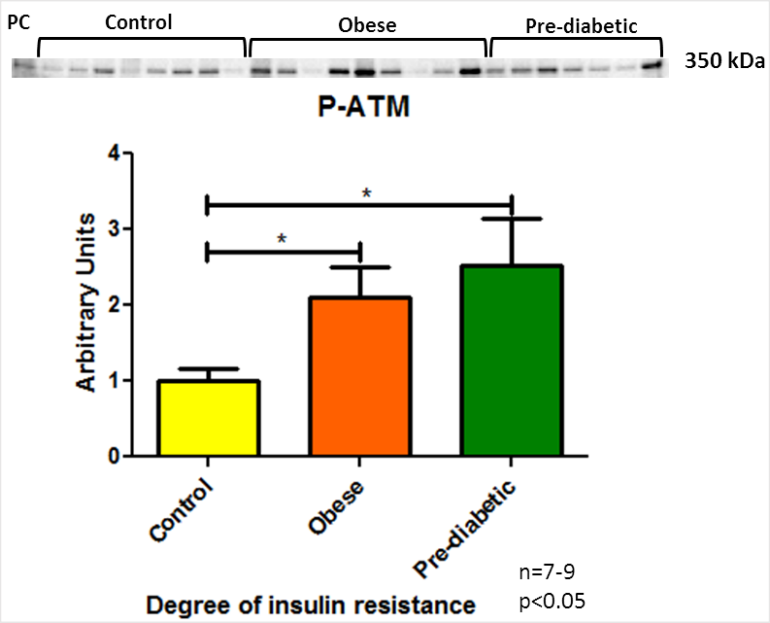

Figure 3. 28: *P-ATM levels (Arbitrary Units) vs. the degree of insulin resistance. PC Positive control.*

c) P-ATM/T-ATM ratio

No significant difference was seen between the P-ATM/T-ATM ratios of the human PBMCs samples when comparing obese (n= 9) or pre-diabetic (n= 7) groups to the control (n= 8) (*Figure 3.29*).

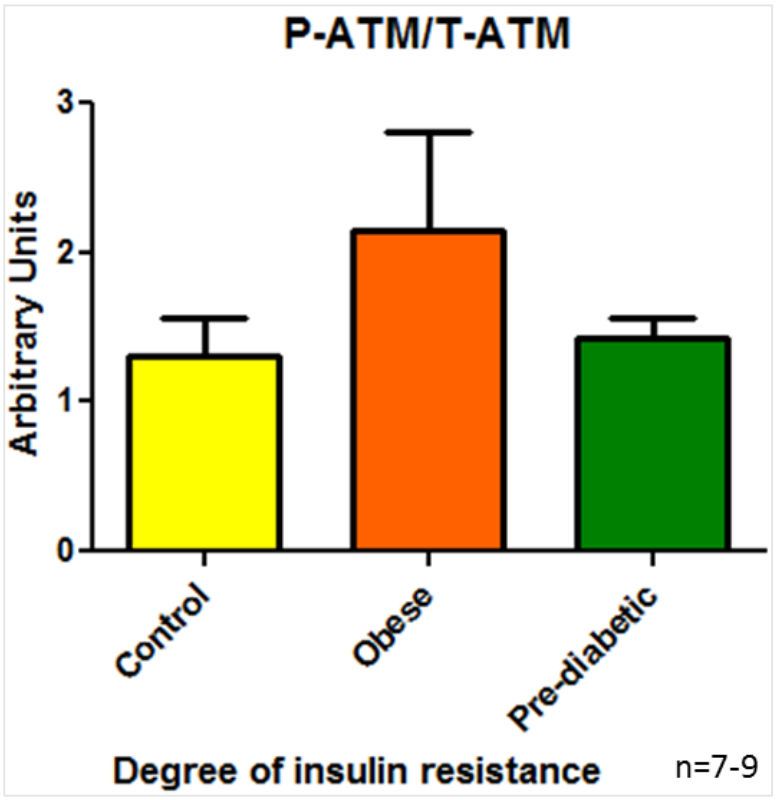

Figure 3. 29: *P-ATM/T-ATM ratio (Arbitrary Units) vs. the degree of insulin resistance.*

2.2.3 Comparison between Y and MA participants

a) T-ATM levels

No significant differences were seen in T-ATM levels when comparing age within degree of insulin resistance (*Figure 3.30*), with n= 6 for Y control, n= 2 for MA control, n= 4 for Y obese, n= 5 for MA obese, n= 3 for Y pre-diabetic and n= 4 for MA pre-diabetic.

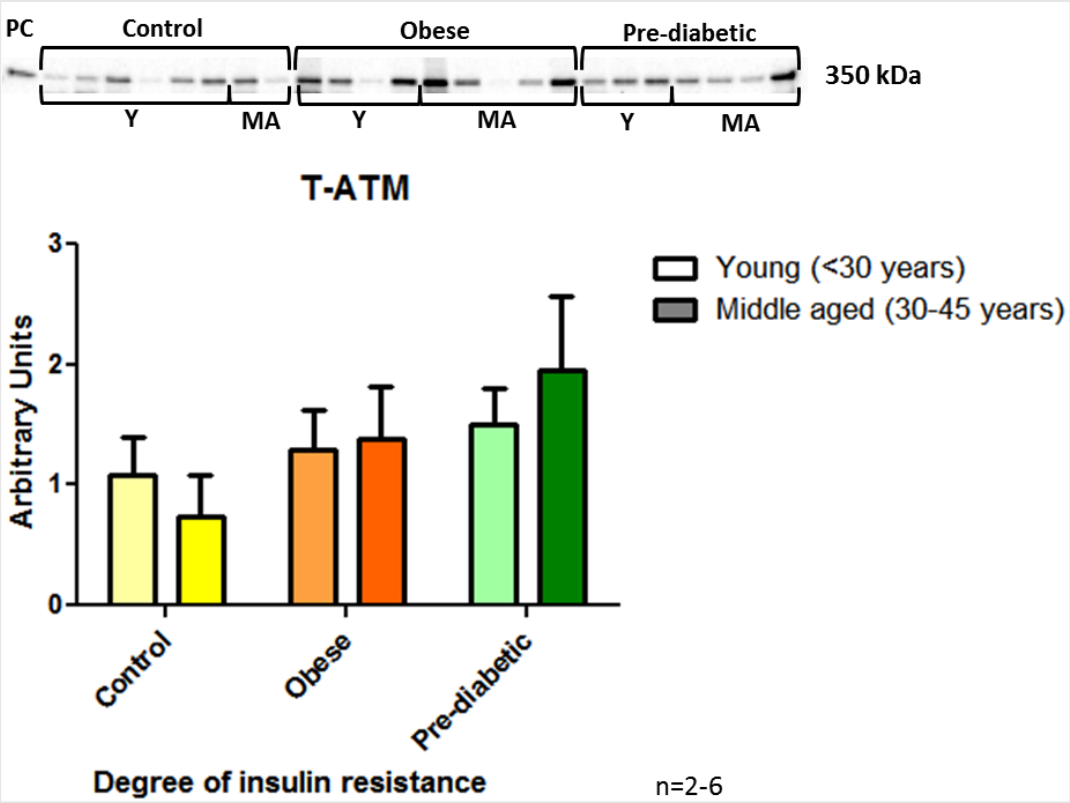

Figure 3. 30: *T-ATM levels (Arbitrary Units) vs. age vs. the degree of insulin resistance. PC Positive control, Y Young, MA Middle aged.*

b) P-ATM levels

Significant difference was seen in P-ATM levels between the Y pre-diabetic vs. the Y control (2.09 ± 0.55 vs. 1.03 ± 0.17 AU; n= 3-6), p= 0.0458 (*Figure 3.31*).

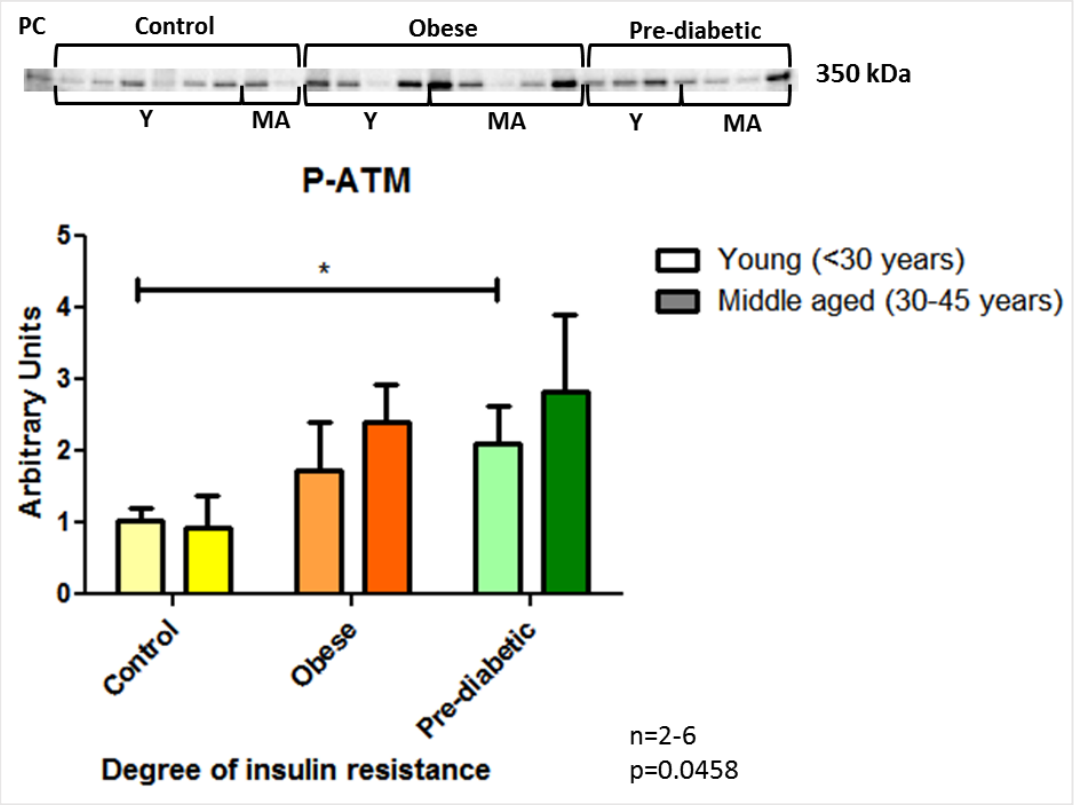

Figure 3. 31: *P-ATM levels (Arbitrary Units) vs. age vs. the degree of insulin resistance. PC Positive control, Y Young, MA Middle aged.*

c) P-ATM/T-ATM ratio

No significant difference was seen in the P-ATM/T-ATM ratio of the human PBMCs when comparing age within each degree of insulin resistance (*Figure 3.32*).

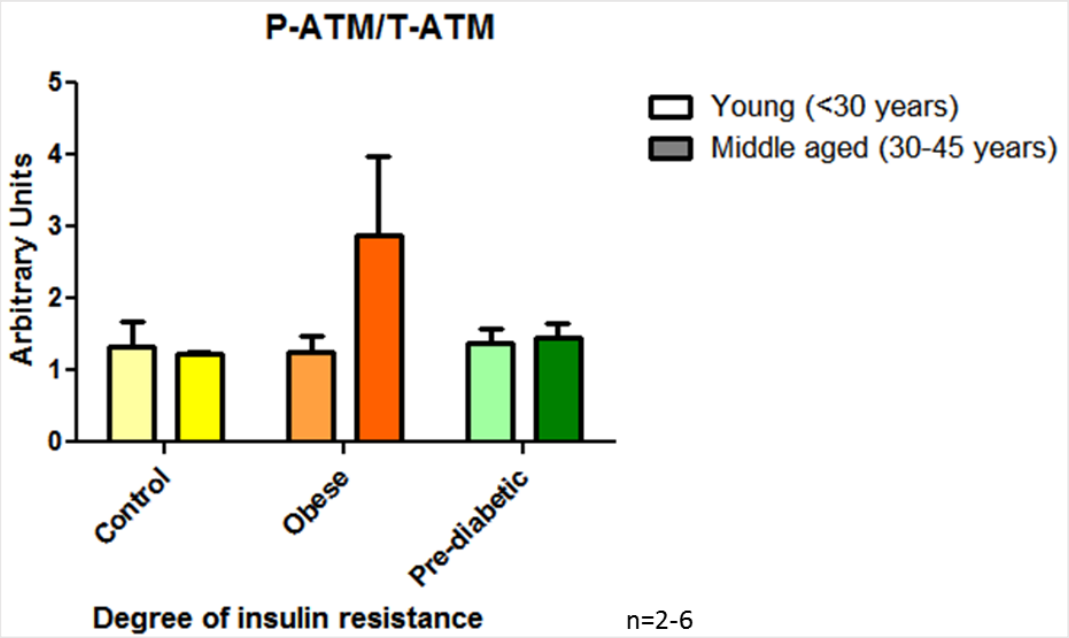

Figure 3. 32: *P-ATM/T-ATM ratio (Arbitrary Units) vs. age vs. the degree of insulin resistance.*

3. Western blotting for $P22^{phox}$

3.1 Control vs. *od* of 22 week old animal PBMCs

No significant difference was seen between the $P22^{phox}$ levels of the *od* (n= 9) when compared to the age-matched controls (n= 7) (*Figure 3.33*).

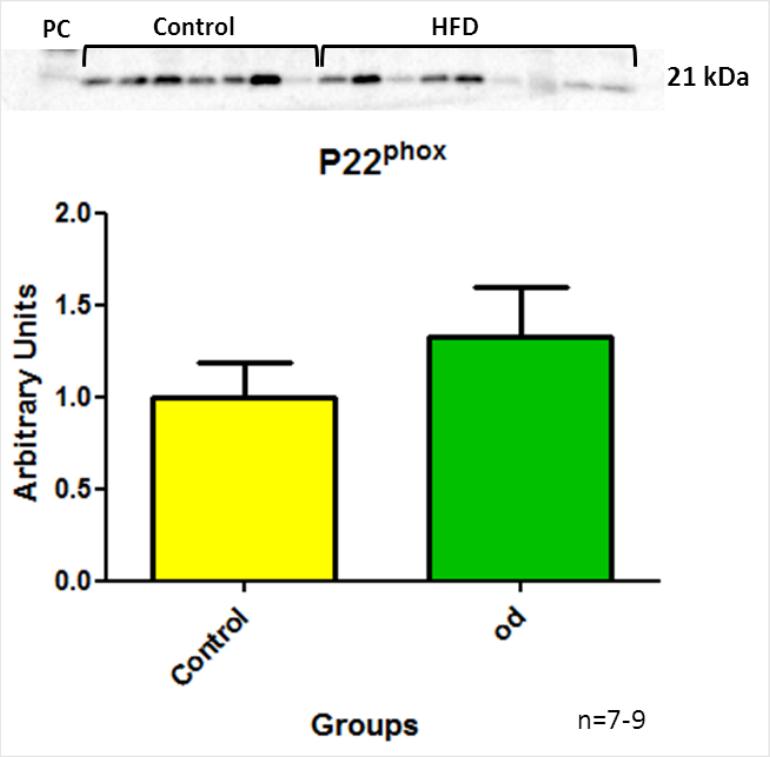

Figure 3. 33: *$P22^{phox}$ levels (Arbitrary Units) of od vs. age-matched controls. PC Positive control.*

3.2 Age comparison between animal PBMCs

No significant differences were seen between the $P22^{phox}$ levels of the young control (n= 6) vs. control (n= 7) or the control vs. *od* (n= 9) (*Figure 3.34*).

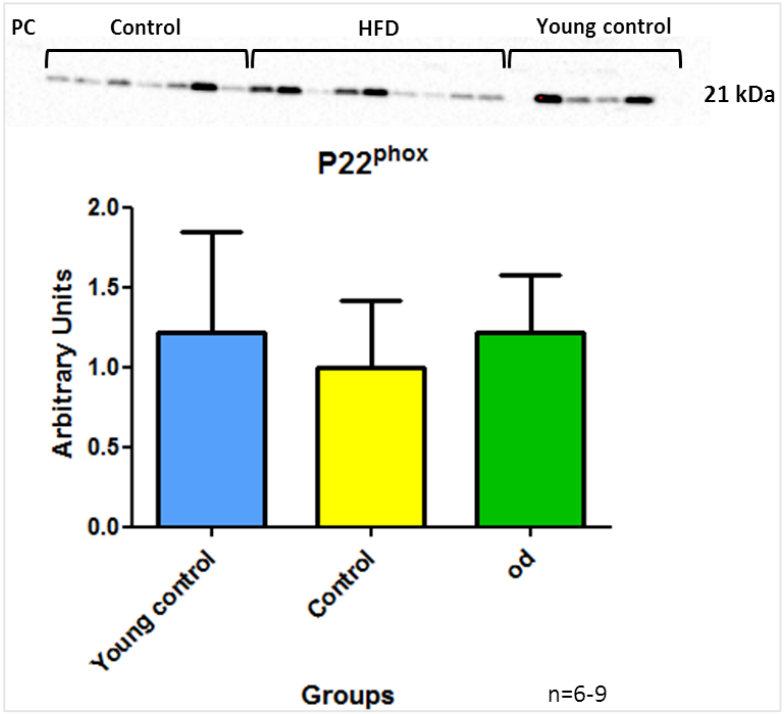

Figure 3. 34: *$P22^{phox}$ levels (Arbitrary Units) of groups. PC Positive control.*

3.3 Human PBMCs

No significant differences were seen in the $P22^{phox}$ levels of the control (n= 8) vs. obese (n= 9) or the control vs. pre-diabetic (n= 7) (*Figure 3.35*).

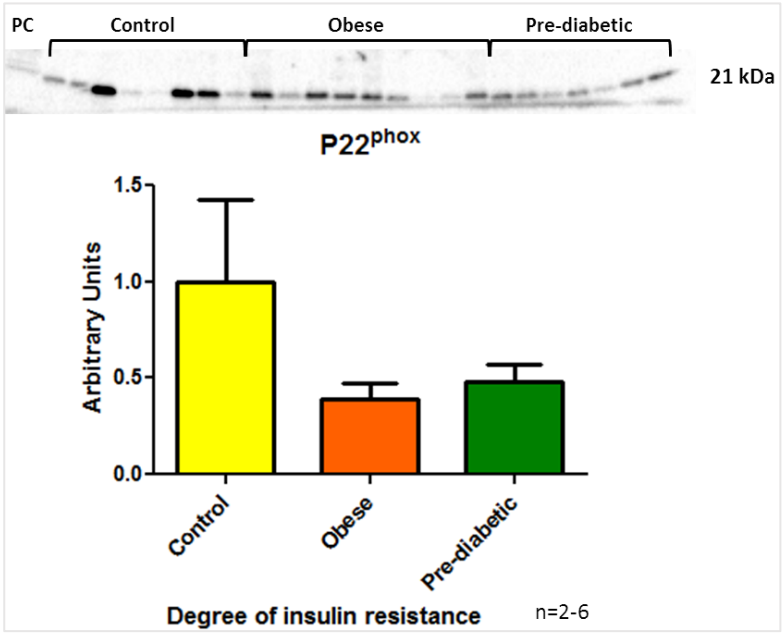

Figure 3. 35: *$P22^{phox}$ levels (Arbitrary Units) vs the degree of insulin resistance. PC Positive control.*

3.4 Degree of insulin resistance between human PBMCs vs. age

No significant differences were seen in the $P22^{phox}$ levels of the Y obese or MA obese or Y pre-diabetic or MA pre-diabetic when compared to the Y control or MA control or between Y and MA with in degree of insulin resistance (*Figure 3.36*).

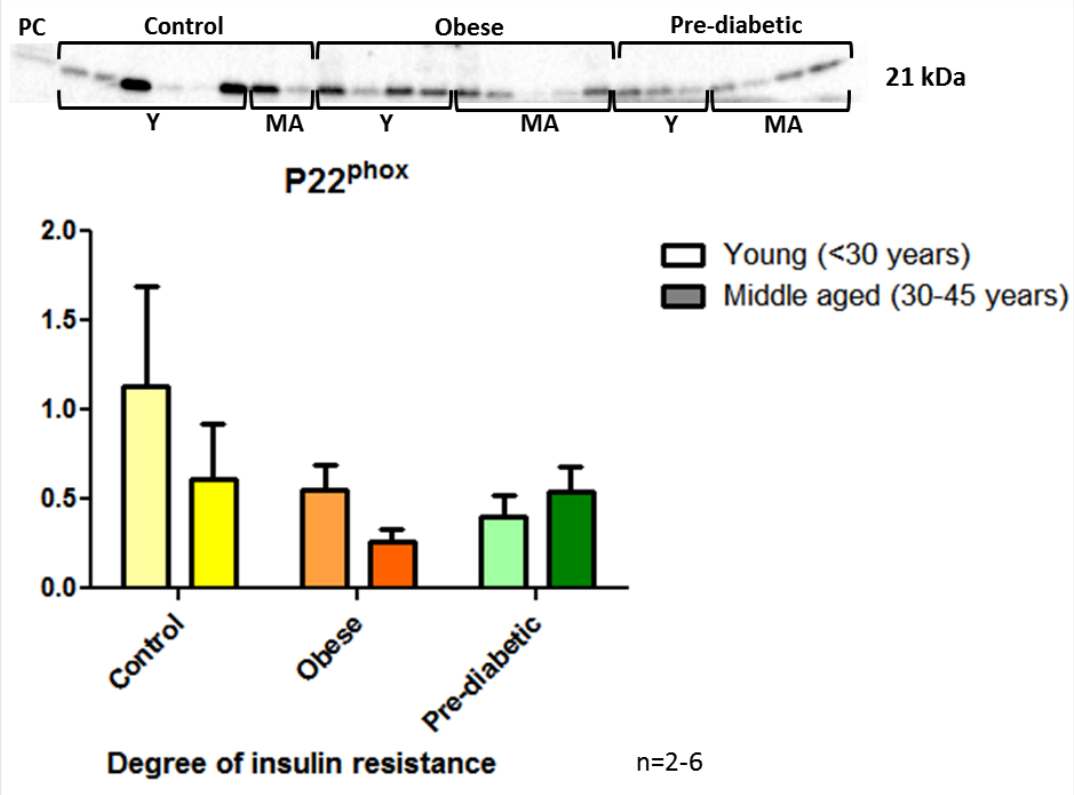

Figure 3. 36: *$P22^{phox}$ levels (Arbitrary Units) vs. the degree of insulin resistance. PC Positive control, Y Young, MA Middle aged.*

CHAPTER 4: DISCUSSION

This chapter discusses the findings of an animal model measuring ATM levels in PBMCs as well as the findings when repeating these methods using human PBMCs previously harvested and collected from a biobank. Furthermore, the chapter discusses the possibility of ATM levels as a biomarker for insulin resistance.

1. A summary of findings in the animal study

The first aim of the study was to isolate PBMCs from rat blood and test whether the ATM protein was expressed in such levels that it can be measured by Western blotting. As shown in *Chapter 2: Materials and Methods*, a method was successfully developed and T-ATM was measured by Western blotting.

As the main aim of the study was to test whether the ATM levels in PBMCs could reflect metabolic changes induced by obesity and insulin resistance, we utilised a rat model to test this before using PBMCs collected from patients. Previous animal studies done in this laboratory along with the current study have shown that this obesogenic diet (*Table 2.3*) caused increased body weight, IP fat mass and basal blood glucose levels and induces obesity and insulin resistance as determined by the OGTT (Reynés *et al.*, 2016). An increase in IP fat mass, OGTT and basal blood glucose biometric measurements were seen when comparing the *od* data to that of the age-matched controls. A significant increase in body weight was seen between that of the young controls compared to controls.

An overall increase was seen in the T- and P-ATM levels as well as the P-ATM/T-ATM ratio of the *od* animals when compared to the age-matched controls. This differs from related studies looking at adipocyte differentiation and glucose homeostasis in wild-type vs. knockout ATM mice showing decreased ATM expression in knockout mice (Takagi *et al.*, 2015). Studies done in our laboratory also showed a decrease in ATM levels with a HFD. Studies in humans, specifically with AT, have shown results that suggested an age-associated decrease in DNA repair that could possibly be attributed to the decreased activity of ATM with age (as discussed in *Chapter 1 Section 9.2,* (Garm *et al.*, 2013; Reynés *et al.*, 2016)), and which may explain the decreased activity of ATM in the age-matched controls compared to the young controls. Furthermore, the *od* model that we used may have caused oxidative stress or DNA damage that may have resulted in the higher T- and P-ATM levels.

1.1 Body weight and IP fat mass

It is widely known that consuming diets that are high in fats, cholesterol, refined sugar and carbohydrates induce a state of over-nutrition (obesogenic) or malnourishment (unbalanced diet) and causes an imbalance in the homeostasis of the metabolism (Lee and Lee, 2014). The increase in body weight is aggravated by the increase in energy consumption, with increased food intake, but low activity levels (sedentary lifestyle) which may explain the significant difference seen between the body weights of the age-matched controls when compared to the young controls (*Figure 3.1*). As discussed in *Chapter 1 Section 6.1.1*, lipids are stored in adipose tissue and in an obese state the adipose tissue reaches maximum capacity, inducing lipolysis and subsequently an increase in circulating FFAs (Wang *et al.*, 2017). Obese individuals have an increased fat deposition around the abdominal area, which was visible in the rats fed an *od* diet.

There was no significant difference seen between the body weight of the *od* and age-matched control animals however, there was significance when comparing the IP fat mass (*Figure 3.2*), which supports the findings that obese individuals have an increased waist circumference. The significant difference seen between the body weight of the young controls and controls is possibly due to age, as older rats may be larger in size.

1.2 OGTT and basal blood glucose levels

The OGTT is performed to determine the animal's ability to clear glucose from circulation, and is measured to determine glucose tolerance. Following overnight fasting of the animals and gavaging with 1 g/kg sucrose, animals fed *od* showed a slower rate of clearance of blood glucose (mmol/L) from circulation compared to the controls (*Figure 3.3*). This indicated glucose intolerance in the *od* animals and animals were considered to be insulin resistant, but not diabetic.

Glucose is one of the main sources of energy for the body that is manufactured during glycolysis, the metabolism of carbohydrates. Basal blood glucose levels are measured to determine whether there is normal glucose homeostasis and are measured following overnight fasting. According to the American Diabetes Association (2004a) normal fasting blood glucose is <5.6 mmol/L, impaired fasting glucose is between 5.6 – 6.9 mmol/L and diabetes is a fasting blood glucose of >7.0 mmol/L. Levels were measured at time point 0 min and *od* animals were shown to have a significantly higher level of circulating blood glucose when compared to age-matched controls (*Figure 3.4*). Increased basal blood glucose levels in animals fed the *od* when compared to the age-matched controls, suggest that the *od*-fed animals had a decreased glucose tolerance and insulin resistance. Hence, *od* animals' cells were not as sensitive to circulating glucose and subsequent

insulin activity and therefore could not maintain glucose homeostasis when compared to the controls. This is also indicative of high basal circulating blood glucose which is similar to conditions seen in insulin resistant patients.

1.3 Proteins in insulin signalling and obese conditions

1.3.1 ATM

As previously discussed, AT patients characteristically present with insulin resistance and have a higher prevalence of developing T2D. This suggested ATM plays a role in the insulin signalling pathway and glucose metabolism (Bar *et al.*, 1978). The discovery of PKB activation in an ATM-dependent manner due to insulin treatment or ionising radiation that initiates glucose uptake, was seen as proof of ATM's role in metabolic pathways (reviewed in (Khalil, Tummala and Zhelev, 2012)). Under obese conditions, and proven by the OGTT, the rats were shown to be insulin resistant.

This study's data is in agreement with literature, as Khalil *et al.* (2012) reviewed articles that reported insulin treatment or ionizing radiation activates ATM and subsequently the AMPK pathway or PBK pathway depending on the energy state of the cell and the level of ROS. Although not significant, the *od* animals showed, what appeared to be, a higher level of T- and P-ATM when compared to the controls (*Figure 3.5 – Figure 3.7*). From the review of Khalil et al. (2012), it is suggested that the possible increased levels of insulin could act as insulin treatment and subsequently increase ATM levels. However, under insulin resistant conditions, the cell has a decreased responsiveness to the stimulus of insulin and thus ATM is expected to be downregulated. The P-/T-ATM ratio is used to show how much of the total protein is active. No significance amongst the *od* vs. control ratios could suggest that although the *od* animals seemed to have an increased level of ATM, there were no significant differences between the groups.

AT patients characteristically present with premature ageing (reviewed in (Shiloh and Lederman, 2017)). Younger control animals were included to determine whether age would affect ATM levels. Studies investigating the parallel between rat age and human age found that rats reach reproductive maturity at 6 weeks and become socially mature at 5 – 6 months and that 1 month of rat age is equal to 2.5 – 3 human years (Andreollo *et al.*, 2012; Sengupta, 2013). When comparisons were made with young controls, age had an effect on ATM activity, with significantly higher T-ATM levels of young controls compared to controls and significantly higher P-ATM levels of age-matched controls compared to young controls (*Figure 3.8 and Figure 3.9*). The P-ATM differed from a previous analysis of the same age-matched and *od* samples (*Figure 3.6*) and may

be due to a difference of a month between running the samples for the T-ATM and then for the P-ATM. Control animals had a significantly higher P-/T-ATM ratio compared to young controls, suggesting higher basal activity in the older animals, possibly due to more DSB in the older animals (*Figure 3.10*). However, the *od* animals had a significantly lower ratio (*Figure 3.10*) that may be due to the insulin resistance state lowering some of the intermediates responsible for ATM activity as reported by Halaby *et al.* (2008).

1.3.2 P22^{phox}

The results obtained while probing for P22^{phox} suggests no ROS or inflammation in the *od* animals. Although not significant, *od* P22^{phox} levels appeared to be more than that of the age-matched controls (*Figure 3.33*). This could suggest that P22^{phox} levels were elevated under insulin resistant conditions but not enough to be statistically significant. Significant differences might have occurred over a longer period of time once animals developed diabetes and with larger n-values. As obese conditions induce inflammation, P22^{phox} is expected to be upregulated as inflammation causes the production of ROS which occurs via the NOXs.

In conclusion, this animal study found that ATM levels can be measured in PBMCs and that the ATM levels are (*i*) susceptible to changes in insulin sensitivity, namely the normal state of insulin signalling seen in the controls and young controls and the insulin resistant state seen in the *od* animals as well as (*ii*) susceptible to the effects of age. Both these conditions caused fluctuations in the P- and T-ATM levels as well as ATM activity indicated by the ratio.

2. A summary of findings in the human study

Patients' biometric data showed significant relationships between the degree of insulin resistance and the measurements taken. With the division into age groups, more significance was seen between the degree of insulin resistance and age within a measurement. Patients in the obese and pre-diabetic groups fitted into parameters commonly seen in obese with insulin resistance individuals, namely an increased BMI exceeding 30 kg/m^2, increased waist circumference exceeding the 88 cm obesity parameter for females (Hough, 2004) subsequently increasing the WHR, increased TF/LF and fasting blood glucose. Of the whole lipid profile, significance was only seen in the triglyceride levels when comparing the Y pre-diabetic group to the Y control.

No significance was seen in the T-ATM levels although the data seemed to have an increasing trend with an increase in the degree of insulin resistance. P-ATM levels showed significant

differences with an increase in the degree of insulin resistance as well as with the analysis with age groups.

2.1 Obesity-related findings

In South Africa NCDs are estimated to be responsible for 51% of deaths (WHO, 2018). The NCDs Country Profiles 2018 for South Africa reported that adult obesity at ages 18 and older were at 15% for males and 39% for females and adolescent obesity between the ages of 10 – 19 years were at 9% for males and 13% for females in 2016. It is widely known that an unbalanced diet high in carbohydrates, sugar and fat would induce obesity and subsequently induce the development of other comorbidities and that a sedentary lifestyle would aggravate this disease. In 2016, the prevalence of adult (18 years and older) physical inactivity was 26% amongst males and 48% amongst females (WHO, 2018). The WHO (2018) reported that in 2014, 24% of both adult males and females had raised blood glucose levels. Epidemiological studies have shown a relationship between obesity and T2D, which was further investigated where healthy lean individuals, without any previous record of obesity or diabetes, were fed an over-nutritious diet and were found to develop insulin resistance (Sims and Danforth, 1987).

BMI

BMI was significantly higher in the obese and pre-diabetic groups when compared to the controls, thus suggesting an increase in body weight with an increase in insulin resistance. As previously mentioned (*Chapter 1 Section 7.1.1b*), in an obese state there is an increase in circulating FFAs, which aids the development of insulin resistance. With a constant state of high blood glucose, the state of insulin resistance further develops until the patient is pre-diabetic. This may explain the increase in BMI with an increase in insulin resistance. Significant increases in BMI with the increase in insulin resistance was seen when differentiating between young and middle aged participants. Significance was also seen between the MA obese and pre-diabetics when compared to their respective controls. This may be explained by the probable decrease in physical activity that comes with age, which is also apparent when comparing the MA groups to the Y groups (*Figure 3.11 and Figure 3.12*).

WHR

The WHR is used to determine body fat distribution and a high ratio is normally indicative of abdominal adiposity, however, WHR can be influenced by other factors in the body and thus cannot be used in isolation (Sweeting, 2007). Adults with a high waist circumference have extra

morbidity along with diabetes and risk factors of cardiovascular disease (Lean, Han and Seidell, 1998). This study's findings are in agreement with literature. Participants' WHR was seen to be significantly higher in the obese and pre-diabetic compared to control as well as in the Y obese and pre-diabetic groups compared to Y controls with an increase in the degree of insulin resistance (*Figure 3.13 and Figure 3.14*) suggesting a high level of visceral adiposity in the Y obese and Y pre-diabetic groups. Four concepts are mentioned below that may explain the reason for the increased WHR with an increased degree of insulin resistance.

BMI and WHR in insulin resistance

A review article discussing the relationship between obesity and insulin resistance discussed recent concepts suggesting the possible reasons for the development of insulin resistance due to visceral adiposity (Hardy, Czecha and Corveraa, 2012). Four possible mechanisms through which visceral adiposity is linked to insulin resistance was discussed. Concept *i*) Visceral adipose tissue induces diabetes by secreting cytokines that induce the progression to insulin resistance in tissues like the liver and muscle; *ii*) visceral adipose accumulation is an indicator of the accumulation of ectopic lipids and lipotoxicity, causing insulin resistance of the liver and muscle; *iii*) excess accumulation of lipids in visceral adipose tissue induces diabetogenic properties such as the accumulation of macrophages in visceral adipose tissue which decreases insulin sensitivity and *iv*) peripheral tissue lipotoxicity and cytokine production in visceral adiposity contribute to insulin resistance (reviewed in (Hardy, Czecha and Corveraa, 2012)).

TF/LF

BMI and waist circumference are overall measures of obesity and central adiposity respectively (reviewed in (Wilson *et al.*, 2013)). However, these measures do not provide information on the exact total fat mass. DXA is a medical imaging technique that can provide scan data on the whole body as well as fat mass, lean mass, bone mineral density and bone mineral content (Wilson *et al.*, 2013). A study was done to investigate trunk to leg volume ratio from DXA whole body scans and its relationship to diabetes and metabolic abnormalities. This study found that the prevalence of diabetes, elevated triglycerides, low HDL, increased blood pressure and metabolic syndrome with its associated mortality were linked to significantly increased trunk to leg volume ratio quartile (Wilson *et al.*, 2013). Furthermore, amongst participants at a low risk with a normal BMI showed an increase of afore mentioned diseases with an increase in trunk to leg volume ratio. Although the current study used TF/LF instead of trunk to leg volume ratio the findings are in agreement

with literature, where an increase in TF/LF was seen with an increase in the degree of insulin resistance (*Figure 3.15*). If the TF/LF ratio is decreased, it indicates lower limb fat mass and more abdominal (trunk) fat mass. These findings showed a decrease in abdominal fat mass with age and an increase in abdominal fat mass with insulin resistance (*Figure 3.16*). Age and the degree of insulin resistance had an overall effect on TF/LF.

Fasting blood glucose

Fasting is considered as no calorie intake for at least 8 hours (American Diabetes Association, 2004a). As previously mentioned, normal fasting glucose is <5.6 mmol/L, impaired fasting glucose is between 5.6 – 6.9 mmol/L and is also referred to as pre-diabetes and diabetes is >7.0 mmol/L (American Diabetes Association, 2004a). For this study, fasting blood glucose was measured after overnight fasting or at least 4 hours prior to blood glucose measurement via a finger prick. As expected, fasting blood glucose levels increased significantly with an increase in the degree of insulin resistance suggesting elevated levels of circulating glucose and suggesting insulin resistance as glucose is not effectively cleared from circulation (*Figure 3.25*). Y pre-diabetic participants fasting blood glucose levels were significantly higher than their control (*Figure 3.26*). In addition, a two-way ANOVA showed the positive relationship between increased blood glucose levels and insulin resistance.

2.2 Lipid profile and obesity

A lipid profile is created following a period of overnight fasting of at least 12 – 14 hours followed by the collection of blood samples and the determination of total cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels (Nigam, 2011). According to Nieves *et al.* (2003) the lipoprotein profile is influenced by obesity and insulin sensitivity and in conditions of increased intra-abdominal fat and insulin resistance, patients have a more atherogenic lipid profile. An atherogenic profile has increased total cholesterol, LDL cholesterol and triglycerides and decreased HDL cholesterol (Nieves *et al.*, 2003). A study by Nieves and colleagues (2003), looked at the atherogenic lipoprotein profile of obese and insulin resistant individuals and linked it to their intra-abdominal fat. Density gradient ultracentrifugation was performed to determine the lipoprotein content of most intra-abdominally obese and insulin resistant group and compared with the least intra-abdominally lean and insulin sensitive group and significant differences were seen across most of the fractions across the density gradient (Nieves *et al.*, 2003). The study found that more abdominal obesity and decreased insulin sensitivity was associated with more cholesterol in the very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and

dense LDL fraction and less cholesterol in the HDL fractions. Furthermore, these findings were more strongly influenced by intra-abdominal fat than insulin sensitivity. Nieves and colleagues (2003) found that insulin sensitivity had no influence on cholesterol concentrations.

In the current study, no significant relationship was seen between the total cholesterol, LDL cholesterol, HDL cholesterol or triglycerides levels of obese or pre-diabetic patients and their controls irrespective of the age parameter. Significance was only seen between the Y pre-diabetic and Y control groups' triglyceride levels. From the data, the pre-diabetic group was shown to have the highest BMI, WHR and TF/LF compared to controls amongst the whole group once age was included. As previously discussed, obese individuals are unable to store surplus lipids which then circulate in the blood stream and may explain the elevated levels of triglycerides seen in the Y pre-diabetics. HDL cholesterol levels tended to decrease with the degree of insulin resistance and this was also seen when the age parameter was included. Furthermore, there was a strong trend for an increase in triglyceride levels with the degree of insulin resistance. Unfortunately, the available n-values of samples were very small, especially when divided into different age parameters. The study will benefit from expanding these measurements in future. As discussed above, no significance was seen in the lipoprotein levels possibly due to the non-effect of insulin sensitivity on cholesterol levels as seen by Nieves *et al.* (2003). They suggest that, although insulin resistance is associated with a lipid profile seen in obese conditions, it seems as though this association is due to insulin resistance's close relation to intra-abdominal fat.

2.3 Blood pressure, Atherogenic Index and HTR

According to Hardy *et al.* (2012), insulin resistance is considered a precursor for T2D and has been found to be associated with hypertension and dyslipidaemia. SBP and DBP were higher in the pre-diabetic group when compared to the controls suggesting a hypertensive state (*Table 3.2*). With the separation into ages, SBP was elevated in MA pre-diabetics and this is possibly due to the hyperinsulinaemic state, which was statistically supported with a two-way ANOVA indicating age and the degree of insulin resistance had a significant overall effect on SBP (*Table 3.3*). MA obese and Y and MA pre-diabetic groups showed higher DBP compared to controls, indicating the effect of insulin resistance on blood pressure and, as was shown with a two-way ANOVA, the degree of insulin resistance had an overall significant effect on DBP (*Table 3.3*). Furthermore, pre-diabetics also had a lower HTR compared to controls (*Table 3.2*), suggesting that pre-diabetics had lower levels of HDL and higher levels of total cholesterol compared to controls. With age, no significant difference were seen in the HTR or Atherogenic index. HTR is dependent on HDL cholesterol and

total cholesterol which did not show significance when analysed in the group and when analysed with age (*Figure 3.17, 3.18 and Figure 3.21, 3.22*). These results may also be due to the small sample sizes per age group that was available in the biobank. The Atherogenic index is calculated based on total and HDL cholesterol and as previously discussed there were no significant differences seen in these parameters.

2.4 Proteins in insulin signalling and obese conditions

2.4.1 ATM

The role of ATM in insulin signalling has been discussed in *Section 1.3.1*. Although not significant T-ATM seemed to increase with an increase in the degree of insulin resistance (*Figure 3.27*). There was a significant increase in P-ATM with an increase in the degree of insulin resistance (*Figure 3.28*). ATM can be activated by either oxidative stress or by metabolic signalling pathways or alternatively by DNA damage, resulting in elevated phosphorylation. In the insulin resistant state, especially in pre-diabetes, higher than normal insulin levels are expected which could have resulted in this increase in P-ATM. As no differences in $P22^{phox}$ was observed, there was no overproduction of ROS in these patients. Unfortunately, the insulin levels of the patients were not available, therefore it can only be speculated that this may have resulted in the higher levels of P-ATM in the pre-diabetic vs. controls.

For the Y and MA analysis the same data sets were used as for the degree of insulin resistance groups but split according to the age cut offs. Although not significant, the T-ATM levels seemed to increase with the degree of insulin resistance in both the Y and MA groups (*Figure 3.30*). The same was seen in the P-ATM data, however, significance was only seen between the Y pre-diabetic and Y control groups, this may be due to the low n – value for the Y pre-diabetic (n= 3) compared to the high n – value of the Y controls (n= 6) (*Figure 3.31*). The apparent implications of age on ATM expression and its use as a biomarker will be further discussed below.

2.4.2 $P22^{phox}$

As previously discussed (*Chapter 1 Section 6. $P22^{phox}$*), $P22^{phox}$ is used as an indicator of the level of ROS production as well as inflammation. $P22^{phox}$ was probed for to investigate whether cholesterol levels could be causing inflammation. No significant differences were seen in total, HDL or LDL cholesterol levels however, there was significance in the triglyceride results. Furthermore, no significance was seen in the $P22^{phox}$ data irrespective of the degree of insulin resistance or age.

3. ATM levels as a biomarker for insulin resistance

The GWAS study by Zhou and colleagues (2011) reported variation in glycaemic response to metformin treatment. They concluded that ATM acts upstream of AMPK where it plays a role in the response to metformin and when there are variations in the ATM gene there are variations in the glycaemic response and the activation of AMPK by metformin. Patients on metformin were unfortunately excluded from the current study.

Both triglyceride levels and blood glucose levels increased with the state of insulin resistance, coinciding with an increase in P-ATM but not in ATM expression levels, indicating that neither one influences the activity state of ATM. Age, on the other hand, seemed to affect the levels of ATM.

4. Overall summary

The *od* diet induced obesity and insulin resistance which was evident with the OGTT showing elevated basal blood glucose levels when compared to the controls (*Figure 3.4*) as well as a slower rate of glucose clearance from circulation which was not significant at any specific time point however, *od* had an overall effect on glucose clearance, suggesting decreased sensitivity to insulin (*Figure 3.3*). The *od* diet was intended to simulate a Westernised or malnourished/unbalanced diet and thus, induce the obese state which is similar to how humans develop metabolic related diseases. As expected, although not significant, the rats' body weight seemed to be more than that of the controls, however, the expected significant difference was seen when comparing the IP fat mass suggesting abdominal adiposity as is often seen amongst obese individuals (*Figure 3.1, Figure 3.2*).

The Western blotting results of the rat PBMCs, showed what appeared to be increased levels of T- and P-ATM, T/P ratio and $P22^{phox}$. However, none of these findings were statistically significant. It is possible that with longer exposure to the *od* diet animals would have developed significant differences in the levels of these proteins. However, as it was shown by the OGTT data that these animals were insulin resistant, it could be suggested that insulin resistant conditions upregulated ATM as well as $P22^{phox}$ activity. Although a decrease in ATM levels were expected, the high circulating glucose levels may have induced an increase in circulating insulin and may have simulated insulin treatment which would have activated ATM intracellularly as discussed by Khalil *et al.* (2012).

As previously mentioned and widely known, the current composition of what is known as a Westernised diet paired with a sedentary lifestyle has increased the prevalence of obesity

worldwide with a more apparent increase amongst children and adolescents (*Chapter 1 Section 7.1.1 Obesity*), (*Chapter 4 Section 2.1*). A large portion of participants formed part of the obese group (data not shown) of which a few were selected for analysis. It could be speculated that these participants may later develop further insulin resistance and be reclassified at pre-diabetic or T2D.

Speculative functioning of ATM under insulin resistant conditions

Insulin resistant animals and humans showed increased levels of T- and P-ATM. As previously discussed, Viniegra and colleagues (2005) found that (*i*) ATM indirectly phosphorylates PKB at Ser-473 following insulin treatment. It is possible that, although cells are supposed to be less sensitive to insulin, a possibly hyperinsulinaemic environment could act as insulin treatment causing an upregulation of ATM (*Figure 4.1*). According to Nishizawa and Bornfeldt (2012), (*ii*) insulin resistance conditions also cause inflammation due to high levels of circulating glucose which could subsequently cause the upregulation of the NOXs as well as $P22^{phox}$ which could explain the increase in $P22^{phox}$, however, these findings were not significant. The opposite was seen in the human study. This may be explained by a possibly longer exposure to insulin resistant conditions than the animals, causing the (*ii*) inflammatory condition and (*iii*) increased ROS production and oxidative stress which would increase the phosphorylation of ATM. This may explain the significant increases of P-ATM (*Figure 3.28*). Furthermore, the (*iv*) upregulated P-ATM would then work to decrease ROS production which may explain the non-significant decrease in $P22^{phox}$ with the increase in insulin resistance. Additionally, although no data was collected for PKB and 4E-BP1, from the literature (Khalil, Tummala and Zhelev, 2012), it could be hypothesised that (*v*) the upregulation of ATM would subsequently upregulate GLUT4 translocation as well as protein synthesis (*Figure 4.1*).

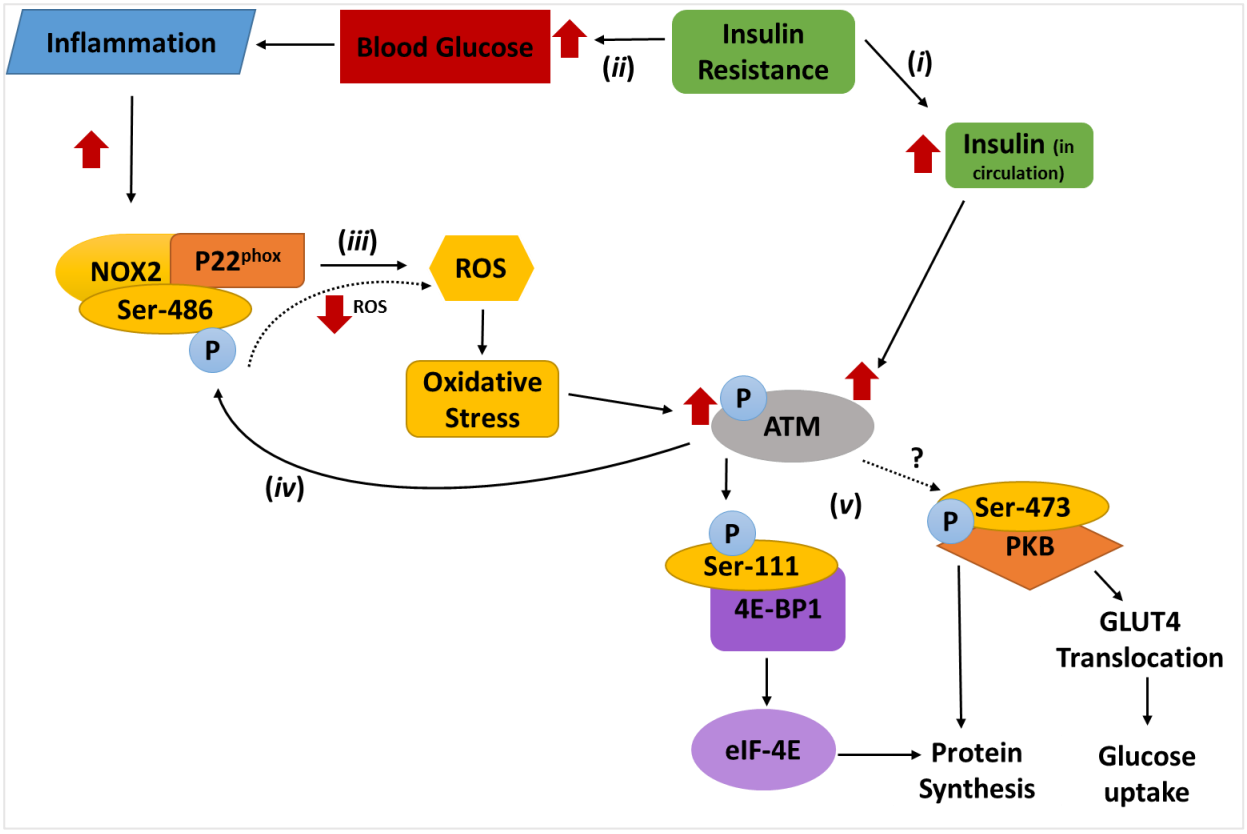

Figure 4. 1: *The functioning of ATM under insulin resistant conditions. Ataxia Telangiectasia Mutated (ATM), Protein kinase B (PKB), glucose transporter 4 (GLUT4), Serine (Ser), Eukaryotic translation initiation factor 4E – binding protein 1 (4E-BP1), Eukaryotic translation initiation factor 4E (eIF-4E), NADPH oxidase 2 (NOX2), Reactive oxygen species (ROS), phosphorylated (P).*

5. Conclusion

PBMCs are susceptible to changes in their micro- and macro-environments (Liew *et al.*, 2005) as was shown by the changes in ATM and $P22^{phox}$ levels in an obese or insulin resistant environment. An unbalanced diet induced the development of obesity and insulin resistance in both animals and humans with both species showing an increase in basal/fasting blood glucose levels, abdominal fat distribution (IP fat mass, WHR, TF/LF) and overall body weight. The inconclusive lipid profile results suggest further investigation into the relationship between obesity, insulin resistance and cholesterol. The findings did indicate an increase in a hypertensive state with the degree of insulin resistance as well as age. This study established a working protocol for the isolation of PBMCs from rat whole blood and ATM levels were successfully measured in PBMCs. This protocol was successfully repeated with human samples.

Due to the variation in ATM levels with the degree of insulin resistance and the compounding effect observed with age, it would be difficult to use ATM levels as a biomarker for insulin resistance. As previously discussed this variation may be due to gene variation affecting the glycaemic response.

CHAPTER 5: LIMITATIONS AND FUTURE RESEARCH

- Rats fed the *od* developed obesity and insulin resistance, however there were no animal groups that simulated pre-diabetes and T2D.
- The human study did not include a T2D group and the number of participants were very small.
- This study only included participants of the black Xhosa female population.
- The samples collected in the human study were shared amongst more than one project. Thus each sample used in this project contained very low protein concentrations and possibly may have affected the data outcomes.
- The animal study made use of male rats and the human study made use of female participants.
- Future animal studies: This study should be repeated with an animal study including a larger variation in the degree of insulin resistance and possibly a larger sample size per group.
- Future human studies: The human study should be repeated with a larger sample size of at least 100 participants. The study should include both male and female groups for each degree of insulin resistance and a T2D group should be included. Blood samples should be collected solely for this project in order to obtain the highest possible protein concentration. The study should also be expanded to include other ethnic groups (e.g. Caucasian, Mixed Race).

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