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The Atherogenic Effects of Type 2 Diabetes Mellitus on the Abdominal Aorta of the Zucker Diabetic Sprague Dawley Rat

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of MSc (MED) in Anatomical Sciences.

Johannesburg 2019

DECLARATION

I declare that this dissertation is my own work. It is being submitted for the Degree of Master of Science (MED) in the school of Anatomical Sciences, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signature of Candidate: 

On this 30th day of August 2019

ABSTRACT

The current study has focused on Type 2 Diabetes Mellitus (T2DM) and its atherogenic effects on the abdominal aorta in the Zucker Diabetic Sprague Dawley (ZDSD) rats (experimental group) in comparison to normal Sprague Dawley (SD) rats (control group).

T2DM, one of the leading chronic diseases world-wide, greatly affects the population and the economy. Secondary morbidities, such as atherosclerosis, are common in advanced stages of T2DM, and increase the risk for complications such as embolism, aneurysms and stroke.

Atherosclerosis is implicated in the majority of deaths among diabetic patients and is recognised as a major component in the pathogenesis of T2DM. Inflammation has been found to play a key role in atherosclerotic plaque formation. It is also pointed out that collagens, especially type 8, are dominant in vascular calcification and tissue remodelling, as a result of atherosclerosis, and as yet have received little attention.

Previous investigations into T2DM have mainly focused on radiological techniques and very few studies have employed histological and immunohistochemical (IHC) techniques for investigating atherosclerosis.

This study employed Haematoxylin & Eosin (H&E) and Weigert's stains to illuminate and document arterial wall changes in diabetic animals and their controls, noting both the qualitative and quantitative changes in the cell layers. With the use of IHC, the presence of inflammation and arterial injury was also illuminated through labelling for Cluster of differentiation 40 (CD40) and Collagen type 8 alpha 2 (Col8A2) antibodies respectively.

An observational case-control approach was employed. When observed, the experimental group was found to have significant arterial wall changes, together with significant presence of both CD40 and Col8A2, while the contrary was true for their non-diabetic counterparts.

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NOMENCLATURE

Adipokine: cell signalling proteins (cytokines) secreted by adipose tissue.

Fasting blood glucose: a measure of the blood glucose level after 8 hours of not eating.

Hyperglyacaemia: an elevated blood glucose level, that is equal to or more than 7.0 mmol/l.

Normoglycaemia: a normal blood glucose level, that is between 3.9-6.9 mmol/l.

Oral glucose tolerance test (OGTT): a test employed to diagnose diabetes mellitus, whereby a 75g glucose solution is given to the subject for ingestion, after taking fasting blood glucose; then blood glucose levels are measured at 15, 30, 60 and 120 minutes intervals respectively.

Post prandial blood glucose: a measure of the blood glucose level at a specific time after eating a meal, e.g. a 2 hour post prandial blood glucose level is measured exactly 2 hours after eating a meal.

Random blood glucose: a measure of the blood glucose level regardless of the time the last meal was taken.

Tunica adventitia: the outermost layer in a blood vessel, composed primarily by connective tissue.

Tunica intima: the innermost layer of a blood vessel**,** consisting of endothelium, basal lamina and sub-endothelial layer.

Tunica media: the middle layer of a blood vessel, consisting of a circumferential arrangement of smooth muscle cells, separated by lamellae or sheets of elastin.

µm: Micrometre

s: Standard deviation

Definitions for the above mentioned terms were taken from:

1. Eckel, J (2018).
2. Mayo clinic (2019).
3. Ross & Pawlina (2006).
4. World Health Organisation (2018).

List of abbreviations

CD40: Cluster of differentiation 40

Col8A2: Collagen type 8 alpha 2

g: grams

H2O2: Hydrogen peroxide

H&E: Haematoxylin & Eosin

IHC: immunohistochemistry

mg/dl: milligrams per decilitre

ml: millilitres

ml/min: millilitres per minute

mm: millimetre

mmol/l: millimole per litre

OGTT: oral glucose tolerance test

SD: Sprague dawley rats

T2DM: Type 2 diabetes mellitus

TC: Total cholesterol

TGs: Triglycerides

ZDSD: Zucker diabetic Sprague dawley rats

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CHAPTER 1: INTRODUCTION

Diabetes mellitus is one of the known leading chronic diseases world-wide and greatly affects the population and the economy at large (Mooventhan, 2017). This chronic disorder is a major cause of mortality, morbidity, and health care expenditure and addressing it represents one of the greatest global health challenges of our time (Jaacks *et al.*, 2016). The incidence of diabetes is increasing drastically (Torres-Espinola *et al.*, 2015) and the World Health Organisation (WHO, 2018) estimates that by 2025, there will be more than 300 million patients with diabetes (5.4% of the world population) (Mohammadi *et al*., 2017). Diabetes mellitus is the ninth major cause of mortality and more than 80% of people diagnosed with the metabolic disorder have type 2 diabetes mellitus (T2DM) (Zheng *et al*., 2018). The vast majority of people affected by T2DM live in low and middle-income countries (Jaacks *et al*., 2016), and this is attributed to the low socio-economic status in these regions.

Diabetes mellitus is a metabolic disorder characterised mainly by hyperglycaemia (Hamed, 2016). Diabetes mellitus occurs as a result of insulin deficiency (type 1 diabetes mellitus) or insulin resistance (type 2 diabetes mellitus) (Artinano & Castro, 2009). In type 1 diabetes mellitus (T1DM) the cause is often genetic, whereby the beta cells in the pancreas do not produce sufficient insulin to facilitate the absorption of glucose into the cells; thereby resulting in hyperglycaemia (Hami *et al.*, 2011). With T2DM, the cause is often lifestyle induced and is related to obesity and an unhealthy lifestyle (Jiao *et al.*, 2014). T2DM begins with insulin resistance, in which the body's cells cannot respond to insulin; therefore resulting in hyperglycaemia (Jiao *et al*., 2014). In the absence of treatment, poor management and progression of the disease; glucose remains in the blood circulation and cannot be utilised by the cells. It is then that the pathology of diabetes mellitus manifests and complications are inevitable.

The current study has focused on T2DM and the resulting pathology that leads to comorbidities and complications, including, atherosclerosis, aneurysms and ultimately death; and how these can be understood further and possibly, improvement in management strategies can be sought.

1.1 Literature review

1.1.1 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is a global pandemic and an incompletely understood metabolic condition, chiefly characterised by hyperglycaemia (Chaudhury *et al.*, 2017) and insulin resistance in peripheral tissues; which ultimately results in progressive dysfunction of beta pancreatic cells (Areosa *et al*., 2017). It is a lifestyle disease and comes about as a result of high fat diets, lack of exercise or sedentary lifestyles (Loeken, 2005) and increased alcohol intake; which then result in obesity, insulin resistance and persistent hyperglycaemia (Rios *et al*., 2015). In T2DM, the body is somewhat able to produce insulin; however, several processes induce abnormalities in a manner that either hinders hormone production or cells are unable to mediate the effects of insulin (Nowotny *et al*., 2015). Secondary morbidities include hypertension and atherosclerosis, and are common in advanced stages of T2DM (Chaudhury *et al.*, 2017). These increase the risk of further complications such as embolism, aneurysms and stroke (Mohamadi *et al*., 2007).

In South Africa, the prevalence of T2DM is estimated at about 9%; a total of over 2 million people above the age of 30 years (Bertram *et al.*, 2013). According to the WHO diagnostic criteria, T2DM is confirmed by a fasting blood glucose reading that is equal to or greater than 7.0 mmol/l (Agyemang, *et al*., 2016). Parameters such as total cholesterol and triglyceride levels are also measured, as hyperlipidaemia is implicated in the initial stages of the pathogenesis seen in this metabolic disorder (Mooradian, 2009). The relationship between diabetes mellitus and the increased triglycerides (triglycerideamia) remains unclear (Amani *et al*., 2016), although the insulin resistance seen in T2DM is thought to result in lipid changes known as diabetic dyslipidaemia. In diabetic dyslipidaemia, increased plasma triglycerides and small dense low-density lipoprotein cholesterol levels are observed, leading to the pathogenic processes (Mooradian, 2009); however, other researchers suggest that relatively normal cholesterol levels are seen in patients with T2DM. The normal cholesterol and triglyceride levels seen in some diabetic patients hide the atherogenic profile of these lipids and it is important to rectify the misconception that triglyceride concentration is a poor indicator of cardiovascular disease (Schofield *et al*., 2016).

T2DM has been found to be a significant and independent risk factor for coronary artery disease, brain injury and peripheral arterial disease (Aronson & Rayfield, 2002) and hence it is imperative to study and understand the disease further. Treatment options are available and

when taken accordingly, improve the quality of life, to some extent. Despite having treatment options for the disease, complications of T2DM are still resulting in increased morbidity and mortality rates; showing the need for further understanding and improvement in the management of the disease.

Type 2 diabetes mellitus treatment

Glucose control remains the cornerstone in the management of patients with T2DM. Studies have already determined that reducing hyperglycemia decreases the onset and progression of microvascular complications, however, the impact of glucose control on cardiovascular complications remains uncertain (Inzucchi *et al*., 2015). Examples of T2DM treatment include Sodium–Glucose Cotransporter 2 inhibitors, Thiazolidinediones, Dipeptidyl Peptidase 4 (DPP4) inhibitors, Biguanides, Sulfonylureas, Meglitinides, Dopamine-2 agonists, etc (Inzucchi *et al*., 2015). Some of these medications are briefly explained below to illustrate their mechanisms of action; confirming that most, if not all, are targeted at lowering blood glucose levels solely and neglect other properties such as inflammation and collagen which are now implicated to play a role in the disease progression.

Sodium–Glucose Cotransporter 2 (SGLT 2) Inhibitors

Canagliflozin is an example of T2DM treatment protocols (Scheen, 2015) and inhibits sodium-glucose cotransporter 2 (SGLT2) in the proximal nephron (Watts *et al*., 2016). It blocks glucose reabsorption by the kidney and therefore increasing glycosuria (Watts *et al*., 2016).

Thiazolidinediones

Thiazolidinediones are commonly used in the treatment T2DM and Rosiglitazone is an example (Dawed *et al.*, 2016). Thiazolidinediones activate the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR) gamma, and therefore increase insulin sensitivity at the insulin receptors (Dawed *et al.*, 2016). This results in a decrease in blood glucose levels.

Dipeptidyl Peptidase 4 (DPP-4) Inhibitors

DPP-4 inhibitors are widely used to treat T2DM and Sitagliptin is an example (Deacon, 2018). DPP-4 inhibitors work by inhibiting DPP-4 activity and increasing postprandial active incretin (GLP-1, GIP) concentrations; therefore increasing insulin production and decreasing glucagon secretion (Deacon, 2018). This results in reduced blood glucose levels.

Biguanides

Biguanides are a class of drugs that are insulin-sensitizing and are used in the treatment of T2DM (Herrera-Martinez *et al*., 2018). Metformin is an example and is targeted at decreasing hepatic glucose production.

Sulfonylureas

For many years, the mainstay of T2DM treatment was Sulfonylureas (Pfeiffer, 2016) but it was later discovered that this class of drugs is associated with a 4,5 fold increase in the risk of severe hypoglycaemia (Yu *et al*., 2017). Gliclazide is an example and works by closing ATP-sensitive potassium (KATP) channels on beta-cell plasma membranes; thereby increasing insulin secretion, which results in glucose absorption (Pfeiffer, 2016).

Meglitinides

Meglitinides have a similar action to Sulfonylureas but bind to different receptors on the pancreatic KATP (Xu & Rajaratnam, 2017). Repaglinide and Nateglinide are examples, and work by closing KATP channels on beta-cell plasma membranes; thereby increasing insulin secretion, which results in glucose absorption (Xu & Rajaratnam, 2017).

Dopamine-2 agonists

Bromocriptine is another treatment protocol example. It modulates hypothalamic regulation of metabolism and increases insulin sensitivity; therefore resulting in reduced blood glucose levels (Lamos *et al.*, 2016).

Schofield *et al.* (2016) have alluded to the idea that glycaemic control has essentially failed to significantly improve cardiovascular outcomes. Given the fact that such a vast variety of treatment options exist; the control of T2DM and diabetes mellitus as a whole should ideally be a success, but statistics prove to date that the gap for treatment and management of the disease requires further research. This study is a mere attempt at acquiring further understanding and possible mitigation of this global burden.

1.1.2 Atherosclerosis

Chief among the secondary comorbidities is atherosclerosis; a condition characterised by calcification and narrowing of arteries, also known as stenosis (Aronson & Rayfield, 2002. Atherosclerosis accounts for virtually 80% of all deaths among diabetic patients (Aronson & Rayfield, 2002). In its initial stages, atherosclerosis manifests as lesions which are

asymmetric, focal thickenings of the tunica intima and consists of cells, lipids, connective tissue elements such as collagen and debris (Hansson, 2005).These are commonly known as plaques or atheromata. Atherosclerosis is a major vascular complication of diabetes, constituting the main cause of morbidity and mortality in this common metabolic disorder (Bastaa *et al*., 2004).

For many years, the theory of atherosclerosis was based on high lipid content, where hypercholesteroleamia was implicated in about 40% of cardiovascular diseases (Russel, 2009), but recent studies suggest otherwise. Similarly, the notion of high glucose levels (hyperglycaemia) were thought to induce a large number of alterations at the cellular level of vascular tissue that potentially accelerate the atherogenic process, but recent research suggests that other molecular processes are also at play. For instance, in a study done by Hansson (2005), it is suggested that immune and inflammatory cells that are blood-borne were found to play an important role in the formation of the plaque, and the remaining constituents were smooth muscle and endothelial cells.

One of the important pathways involved in the pathogenesis of atherosclerosis in diabetes is thought to be the increase in non-enzymatic glycation of proteins and lipids, with the irreversible formation and deposition of advanced glycation end products (Bastaa *et al*., 2004). Glycation, in contrast to glycosylation, which is enzyme mediated, means the binding of a sugar molecule to a protein or lipid molecule without the regulation of an enzyme (Chan-Sik *et al.*, 2016). A second form of damage promoting atherosclerosis is that advanced glycation end products accumulation can cause circulating blood cells to adhere to the vessel wall, thus, further compromising the affected blood vessels (Bastaa *et al*., 2004).

Arteries in atherosclerosis will exhibit, among other features, a reduced luminal diameter (stenosis) due to a combination of changes including deposition of plaques within the tunica intima and abnormal smooth muscle cell activity (Adiguzel *et al*., 2009). Other features include a reduced elastic capacity due to marked deterioration; possible rapture of the internal elastic lamina, and eventual calcification (Adiguzel *et al*., 2009), leading to increased risk of ischemia and aneurisms (Adiguzel *et al*., 2009). This combination of factors can lead to deprivation of much needed blood, of particular significance when involving the abdominal aorta; one of the largest arteries that convey blood from the heart to the rest of the body (Ross & Pawlina, 2006). It is therefore imperative that the nature of the causal relationship between T2DM, a rapidly rising disease, and atherosclerosis is very well understood.

1.1.3 Inflammation in atherogenesis

Inflammation has been found to play a key role in atherosclerotic plaque formation as its markers have been seen in some studies. For example, in a study that was done on coronary artery disease involving human-based angiographic and immunohistological methods; Hansson (2005) found that immune cells dominate early atherosclerotic lesions and that their effector molecules accelerate progression of the lesions. Hansson (2005) states that the plaque is preceded by an accumulation of lipid-rich cells beneath the endothelium, and most of the cells found within this lipid bed are macrophages together with T-cells. These immune cells are then said to activate an immune response and release inflammatory cytokines. Immune cells (T-cells, antigen presenting cells and monocytes) are known to patrol various tissues, including atherosclerotic arteries, in search of antigens (Hansson, 2005). It is also stated in the same study that a T-cell infiltrate is always present in atherosclerotic lesions. Bastaa *et al.* (2004) carried out a study involving human subjects who were diagnosed with T2DM, and who had also been diagnosed with coronary artery disease as a complication of T2DM. The patients were reported to have developed coronary artery disease as a result of atherosclerotic plaques building up in and around the blood vessels of the heart. Immunohistochemical analyses of the patients' atherosclerotic lesions demonstrated diffuse extracellular, as well as dense intracellular, plaque-like depositions in macrophages and vascular smooth muscle cells. In another study focusing on the abdominal aorta of mice, it was revealed that atherosclerosis was another important complication in the formation of aneurysms, which were also linked to inflammation in the vessel wall underlying the plaques (Adiguzel *et al*., 2009). It is for this reason that the current study has paid special attention to this phenomenon by employing immunohistochemical techniques to label for inflammatory markers.

1.1.4 Collagen 8 presence in atherogenesis

It is pointed out that collagens, especially types 2 and 8, are dominant in vascular calcification as a result of atherosclerosis. It is stated that collagens form a major portion of the extracellular matrix in the atherosclerotic plaque and contribute to the strength and integrity of the plaque (Adiguzel *et al*., 2009). Under normal circumstances, Collagen type 8 alpha 2 (Col8A2) is a major component of the basement membrane of corneal endothelium; however, it can also be expressed as a matrix protein in a number of tissues undergoing active remodelling. This includes injured arteries in human atherosclerotic plaques (Hansen & Karsdal, 2016), but little is known about its function (Hansen & Karsdal, 2016). Col8A2 is said to be produced during arterial injury (Hansen *et al*., 2016), where it stimulates an increase in smooth muscle cell migration. It was found that antibodies against Col8A2

reduced attachment of smooth muscle cells by 77% (Hou *et al*., 2000). It is also stated that in the presence of Col8A2, smooth muscle cells change from a quiescent, to an active phenotype, characterised by proliferation, migration and synthesis of extracellular matrix (Adiguzel *et al*., 2009). This part of research is said to have received little attention. It is for this reason that the current study has focused on this identified gap in literature concerning collagen, as it forms an integral part in the atherosclerotic plaque structure.

As stated previously, a cascade of events are involved in atherogenesis (over and above hyperglycaemia), including inflammation and collagen production. It is therefore critical that the treatment options should incorporate anti-inflammatory and anti-collagen agents and mechanisms, as these are also implicated in the atherogenesis of T2DM.

1.1.5 Methods used to investigate atherosclerosis

Apart from the traditionally known treatment options for T2DM, methods of investigating the complications of this disease have been shown to be limited to certain routine techniques which are non-invasive, like angiography and radiography (Cogswell *et al.*, 2017). This can be attributed to the fact that the disease is prevalent in human beings and it would be unethical to employ invasive techniques in the human population for research purposes. However, the non-invasive methods of investigation fall short of conducting in-depth molecular analyses, and that might be hindering the breakthrough to better management and treatment. Previous investigations that were carried out around the topic of diabetes and its complications mainly employed x-rays and angiography, with very few studies focusing on other methods like immunohistochemistry and histology, which in their nature of directly studying implicated tissues are more invasive (Famakin, 2009). Some of these investigations established that stenosis was an important factor in ischeamic attacks. However, more recently, researchers have demonstrated that the degree of stenosis is no longer the sole predictor of vulnerable atherosclerotic lesions and many investigations into other factors such as plaque morphology, plaque components and intrinsic inflammation have revealed that intra-plaque factors are closely related to atherosclerotic plaque vulnerability (Ryu *et al*., 2014). For instance, a recent study that was carried out in human subjects established that important predictors of patients' vulnerability to catastrophic vascular events include both the degree of stenosis and the underlying plaque morphology, which can be detailed better by employing radiographic, histological and immunohistochemical imaging (Prabhakaran & Romano, 2012). It is for this reason that the current study employed histological and immunohistochemical techniques as methods of investigation instead of the routine radiography.

It is important also to examine whether the occurrence of atherosclerotic plaques is more likely to be consistently localized to any specific sites in the abdominal aorta. This is because Yu *et al*., (2017) found that the location of an atherosclerotic plaque in the basilar artery is a reliable tool for predicting whether or not the resulting stenosis is symptomatic; that is, whether or not the patient is more or less likely to experience a vascular event such as stroke. This indicates that further understanding of the likely locations and nature of plaques in atherosclerosis could assist in predicting the symptomatic nature of plaques that appear in radiographic material such as MRIs and CT scans. Furthermore, this information could be useful in studying radiographic materials by indicating the most likely sites of plaque occurrence, and thereby directing clinicians to more careful examination at possible implicated sites, and very importantly, to be able to predict to some degree the likelihood of vascular events based on plaque location and nature.

1.1.6 The Zucker Diabetic Sprague Dawley Rat as a model for Type 2 Diabetes study

Studying the effects and complications of T2DM in humans is both important and of interest, but acquiring such information in humans is somewhat impractical due to ethical concerns and would require long study duration (Choy *et al*., 2016). It is for this reason that animal models have been widely used to study the aetiology of diabetic complications. The Sprague Dawley (SD) and the Zucker Diabetic Sprague Dawley (ZDSD) rats have been used for this study.

The ZDSD is a rat breed that has been found to be a more translational model of diabetes, obesity and metabolic syndrome (Peterson *et al*., 2015). The ZDSD rats are susceptible to the development of obesity when fed a high fat diet; and are now recently used in diabetes studies to aid in the advancements of investigating the condition, and the development of drugs that could assist in control and treatment of the disease (Peterson *et al*., 2015). It is stated that the ZDSD rat develops T2DM in a manner that mirrors the development of the metabolic syndrome in humans; hence it is an ideal model for the study of this disease. With this rat model it is known that early feeding with a fatty diet (from 7 weeks of age) would result in the development of obesity; which is then followed by diabetes development; however, commencing these rats on a high fat diet later in life (around week 15 of age) results in the spontaneous development of diabetes, without obesity. For the purpose of this study, the ZDSD group was given a normal diet initially, and then was given a high fat diet at around 15 weeks of age, so as to develop the metabolic disease (T2DM) spontaneously without the development of obesity.

It has been demonstrated that insulin resistance is associated with elevated plasma leptin levels (Freitas-Lima *et al.*, 2015). Leptin, an adipose tissue-specific adipokine, is a key molecule that regulates appetite, energy expenditure and glucose metabolism (Richard, 2015). The adipokine has been shown to have an array of effects that may influence development of T2DM. The ZDSD rat has an intact leptin signalling pathway and more modest accumulation of body fat; similar to humans (Davidson *et al.*, 2014); and therefore, it is for this reason that the ZDSD was chosen for this study.

Given the burden of disease posed by T2DM and its complications, and the identified gap in literature, concerning atherosclerosis, inflammation and collagen involvement in the disease, it is necessary to focus on these factors and to employ a different approach in methods of investigation, using histological and immunohistochemical techniques instead of radiography alone.

1.2 Aims and objectives

1.2.1 Aim

The aim of this study was to examine the occurrence, location, histological and immunohistochemical nature of atherosclerotic plaques (if any) which occur as a result of T2DM in the abdominal aorta of the Zucker Diabetic Sprague Dawley (ZDSD) rat and compare these to the normal (non-diabetic) Sprague Dawley (SD) rat, and with the findings, suggest the development of improved treatment and management options, with the potential to control more than glucose levels solely.

1.2.2 Objectives

The main objectives of the study are to document the changes in the abdominal aorta in response to prolonged exposure to hyperglycemia, in order to examine whether this artery follows a similar atherogenic disease pattern as that previously documented in other atherosclerotic arteries. These objectives can be achieved in the following ways:

1. Determination of body mass, blood glucose levels, triglycerides levels and total cholesterol levels.
2. Using the Weigert's staining method; to document the elasticity of the arterial walls by comparing the morphology of the elastic fibres of diabetic subjects to those of non-diabetic subjects.

3. Using the Haematoxylin & Eosin staining method, to examine the thickness of the tunics in atherosclerotic walls of the abdominal aorta of diabetic animals, and compare this to non-diabetic animals.
4. Using Image J, quantify the differences in the tunics between diabetic and non-diabetic animals by measuring their wall thickness.
5. To investigate the presence of inflammation in the arterial walls by employing immunohistochemistry for the biomarker of activated platelets (CD 40).
6. To examine the presence of abnormal smooth muscle cell activity by immunolabelling for collagen VIII, typical of atherosclerotic arteries.
7. To examine whether diabetes disease progression notably alters the appearance of any of the atherosclerotic factors examined in the study by comparing the results of younger (at 20 weeks old) and older (at 28 weeks old) animals.
8. To examine the effects of feeding different diets (normal and high fat); by giving the one control group a normal diet and the other a high fat diet. The experimental groups were given a normal diet initially, and then changed to a high fat diet at week 15.

CHAPTER 2: MATERIALS AND METHODS

2.1 Permits and ethical issues

Ethical approval for the study was granted by the University of the Witwatersrand Animal Ethics Committee (2015/07/28/C).

2.2 Study design

An observational case-control approach was employed in this study, whereby the animals where divided into two groups; observed for 20 weeks, for the younger group, and 28 weeks for the older group, under a controlled environment.

2.3 Exclusion criteria

Animals that died spontaneously were excluded from the study. Only animals that lived until the predetermined age of termination were used in the study, which are 20 and 28 weeks respectively. The reason for exclusion of animals that died spontaneously was to avoid studying animals with an unknown cause of death.

2.4 Maintenance and monitoring of rats

A total of 24 specific pathogen-free Zucker Diabetic Sprague Dawley (ZDSD) male rats were used for the study as the experimental group. The diabetic rats were purchased from Preclinomics Laboratory (Indiana, USA). These animals were chosen since they are known to have a genetic predisposition for developing T2DM spontaneously. Normal Sprague-Dawley (SD) rats, a total of 24, were used as controls and were procured through the Central Animal Service Unit at the University of the Witwatersrand. The groups were further divided into two subgroups determined by age at time of sacrifice, namely 20 weeks old at sacrifice and 28 weeks old at time of sacrifice. For the purpose of the study, a total of 24 rats were used (12 SDs and 12 ZDSDs).

The animals were kept at the University of the Witwatersrand animal facility (Johannesburg, South Africa) and maintained under a pathogen-free, temperature controlled environment (22 +/-2 degrees Celsius) and relative humidity conditions (not measured) with a 12 hour light-dark cycle, running from 06:00 am to 18:00 pm. The animals were housed individually in acrylic cages, on wood shavings and were provided with water and a commercial diet (Purina 5008 and Purina 5SCA, USA). The diet was divided into normal (Purina 5008), given to the one control group (SDs up to week 28), and high fat (Purina 5SCA), given to the other control

group (SDs up to week 20). Both ZDSD groups were initially given a normal diet (Purina 5008), then given a high fat diet (Purina 5SCA) from 15 weeks of age. The body mass was measured weekly while the other parameters were measured monthly, until termination. A tabulation of the different rat groups and their allocated diets follows below.

Table 2.1: A tabulation of the different rat sub-groups indicating their ages and allocated diets.

Rat type	Rat age at termination	Group no.	No. of rats	Diet
SD	20 weeks	1a	6	High fat
SD	28 weeks	2a	6	Normal
ZDSD	20 weeks	1b	6	Normal till week 15 then high fat
ZDSD	28 weeks	2b	6	Normal till week 15 then high fat

2.4.1 Parameters measured to confirm or exclude T2DM

Parameters including body mass (g), glucose levels (mmol/l) (random, fasting and Oral Glucose Tolerance Test (OGTT)), cholesterol (mmol/l) and triglycerides (mmol/l) were taken to ascertain that the diabetic group indeed had diabetes, and the non-diabetic group had no diabetes. The weights were measured weekly and the rest of the parameters were measured monthly, until termination.

2.4.1.1 Body mass

The animals were weighed weekly, on the same day (Mondays), and around the same time of day (between 10am and 12 noon); in order to avoid confoundment by any fluctuations in body mass throughout the day. The first group of SD rats (destined for sacrifice at 20 weeks) was weighed weekly from week 10 up to week 20 of age. The average body mass in this group at week 10 was 458g (s= ±31.70). The second group of SD rats (destined for sacrifice at 28 weeks) was weighed from week 10 up to week 28. The average body mass in this group at week 10 was 303g (s= ±52.30). The difference in the starting body masses amongst the SD groups can be attributed to the diet given to these animals. The first group (SDs up to age 20 weeks) was given a high fat diet (Purina 5SCA) from acquisition up until termination,

whereas the second group (SDs up to 28 weeks) was on a normal diet (Purina 5008) throughout. The SD groups were given different diets in order to observe the effects of each diet on the body mass of the animals and whether the group on the high fat diet would develop obesity and/or diabetes.

Both ZDSD groups were initially given a normal diet (Purina 5008), then given a high fat diet (Purina 5SCA) from 15 weeks of age. The high fat diet was started at a later age (week 15) because the experimental group was intended to develop T2DM directly without developing obesity. Obesity was seen to lead to premature death in the ZDSD group that was fed a high fat diet from 7 weeks earlier in the study; hence a decision was taken to delay the development of obesity to 15 weeks, for this group. The first group of ZDSDs (destined for 20 week sacrifice) was weighed from week 10 up to week 20. The average body mass in this group at week 10 was 416g (s= ±4.5). The second group of ZDSDs (destined for 28 week sacrifice) was weighed from week 10 up to week 28. The average body mass in this group at week 10 was 404g (s= ±10.4). The ZDSD groups had similar starting weights as they were fed the same diet. **Table 2.2** and **Figure 2.1** illustrate the initial body masses between the four sub-groups, showing that three out of the four groups had almost the same starting weights (≥400g); with SDs (up to 28 weeks) having slightly lower starting weights (≥300g).

Table 2.2 A tabulation of the initial body mass (g) at 10 weeks amongst all rat groups. The first group of SDs had larger starting body masses compared to the second SD group, due to the first group of SDs being given a high fat diet from acquisition up until termination; whereas the second group of SDs was on a normal diet throughout. The two ZDSD groups had similar starting body masses as they were both given a normal diet initially (**Gr** = group, **wks** = weeks). ↓Shows low starting body masses.

Rat no.	SDs Gr1a 10w (up to 20 wks)	SDs Gr2a 10w (up to 28 wks)	ZDSDs Gr 1b 10w (up to 20 wks)	ZDSDs Gr 2b 10w (up to 28 wks)
1.	502	260↓	412	401
2.	462	273↓	412	400
3.	457	270↓	420	388
4.	421	358↓	412	418
5.	425	317↓	416	412
6.	483	340↓	422	402
Averages	**458** s = ±31.7	**303**↓ s = ±52.3	**416** s = ±4.5	**404** s = ±10.4

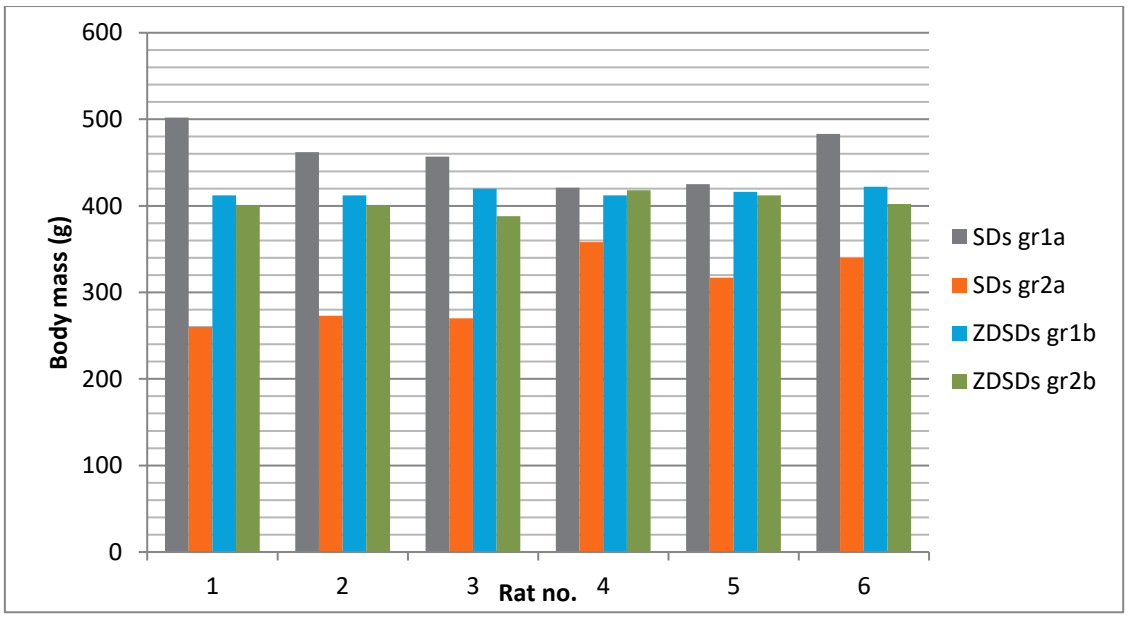


Figure 2.1 An illustration of the initial body mass (g) at 10 weeks amongst all rat groups. The first group of SDs had larger starting body masses (grey) compared to the second SD (orange) group. This is because the first group of SDs was given a high fat diet from acquisition up until termination, whereas the second group of SDs was on a normal diet throughout. The two ZDSD groups had similar starting body masses as they were both given a normal diet initially (blue and green).

2.4.1.2 Blood glucose levels

Blood glucose levels were measured every 2 weeks and monthly from week 12 up to termination, by means of a fasting glucose and an oral glucose tolerance test (OGTT) respectively. Glucose measurements were commenced at week 12 instead of week 10 in order to allow the animals to acclimatise to the controlled environment for at least 2 weeks before blood measurements could be taken. For the purpose of the study, only OGTTs are reported on. Blood was collected from the animals by pricking their tail ends and by squeezing gently until enough blood was seen and placed on the haemo-gluco test strip in the Accu-Check Perfoma® (Roche Diagnostics Corporation, South Africa) glucometer machine. In a normoglycaemic individual, the fasting blood glucose reading is between 3.9-5.5 mmol/l, followed by a 2 hour post prandial reading between 3.9-8.1 mmol/l, then a random reading between 3.9-6.9 mmol/l. For the purpose of this study, blood glucose levels equal to or above 9.0 mmol/l 2 hours postprandial, or fasting blood glucose levels above 6.0 mmol/l were regarded as hyperglycaemia but not necessarily as confirmation for diabetes. Fasting and 2 hour post-prandial blood glucose levels equalling or more than 16.7 mmol/l (300mg/dl) were considered to be confirmation for diabetes.

Firstly, the animals were fasted for about 8 hours prior to being given a 75g glucose solution by gavage. The food was removed from the cages the night before the OGTTs were due to be done. Fasting blood glucose (designated as 0 minutes) levels were measured first; thereafter the animals were given the glucose solution. After administration of the glucose solution, blood glucose levels were measured from each rat at intervals of 15, 30, 60 and 120 minutes respectively (**Table 2.3**; **Figure 2.2**), using an Accu-chek Performa® glucometer that was calibrated before use. The blood glucose readings amongst the SD groups showed to be within normal (non-diabetic) ranges (≤ 8 mmol/l) at week 12; whereas in the ZDSD group, traces of hyperglycaemia were already noted (**Table 2.2**, **Figure 2.2** & **Figure 2.3**).

Table 2.3: A tabulation of the average initial blood glucose levels (mmol/l) amongst all rat groups when studied at age 12 weeks. ↑ shows an increase in blood glucose levels.

Rat group	0 min	15 min	30 min	60 min	120 min
SDs gr 1a	5.8	6.5	6.8	6.2	7.0
SDs gr 2a	5.0	6.7	7.3	7.2	6.2
ZDSDs gr 1b	7.7↑	7.7	12.5	14.8	11.1↑
ZDSDs gr 2b	6.6↑	7.1	10.2	11.5	9.0↑

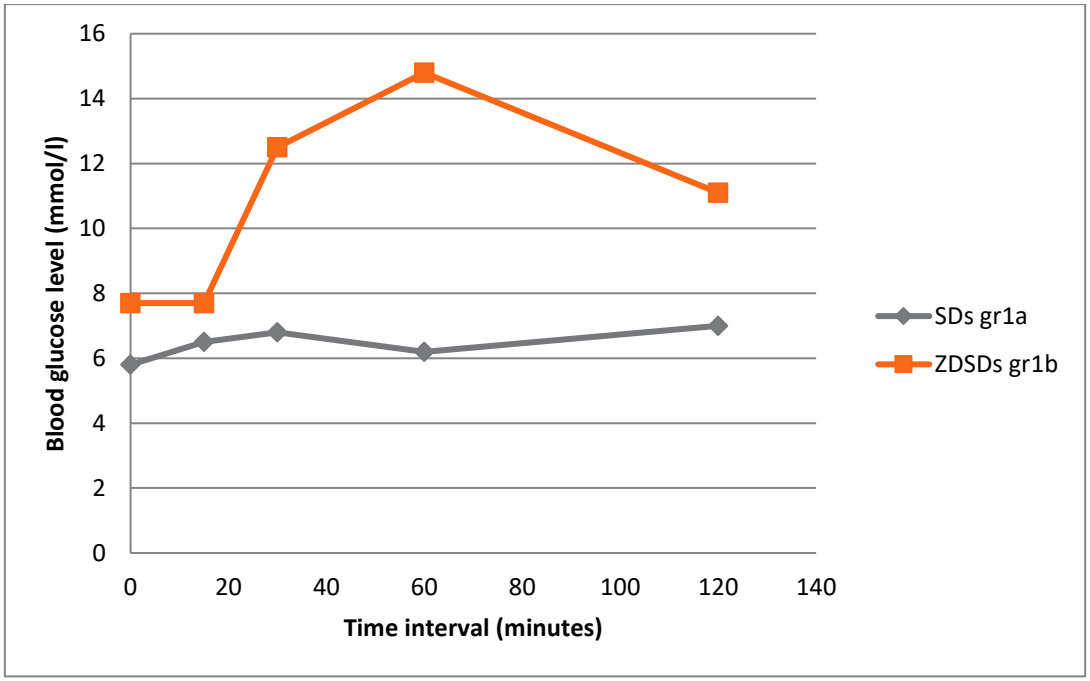


Figure 2.2: An illustration of OGTT (mmol/l) readings amongst SDs and ZDSDs (first group up to 20 weeks) when studied at age 12 weeks. The blood glucose readings amongst the SD groups showed to be within normal (non-diabetic) ranges (≤ 8 mmol/l) at week 12; whereas in the ZDSD group, traces of hyperglycaemia were already noted (> 8mmol/l).

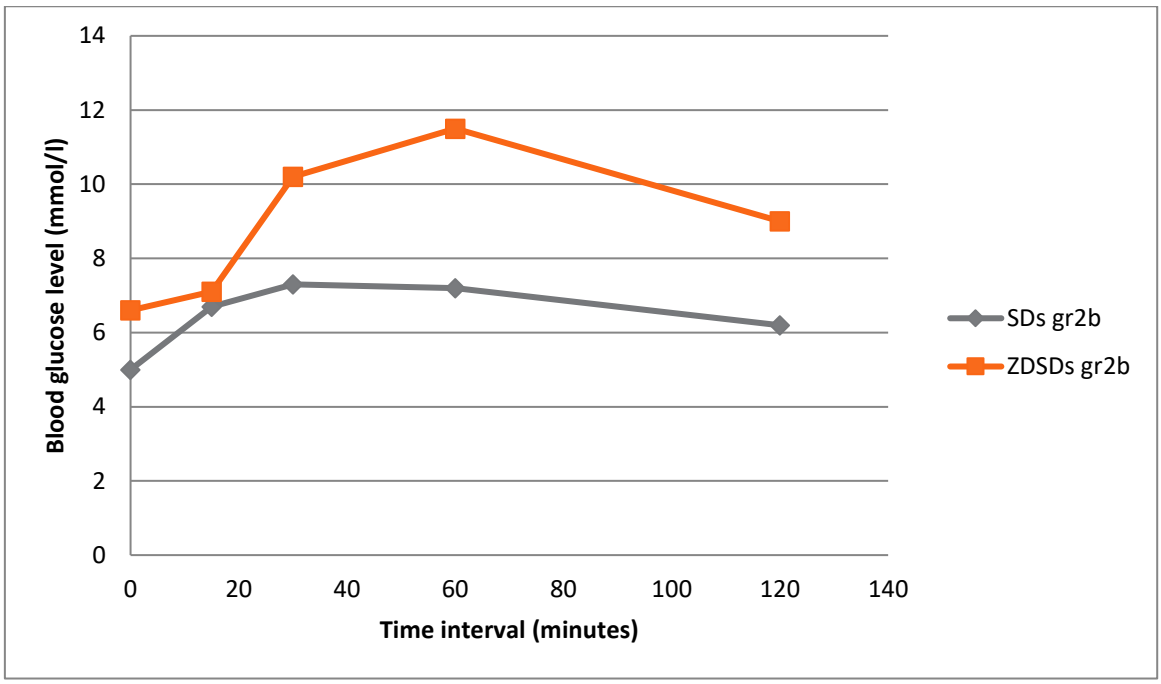


Figure 2.3: An illustration of OGTT readings amongst SDs and ZDSDs (second group up to 28 weeks) when studied at age 12 weeks. The SD group shows normal blood glucose readings (< 8mmol/l) while the ZDSD group demonstrates elevated blood glucose levels (> 8mmol/l) given a normal diet, demonstrating a genetic predisposition to diabetes mellitus regardless of the diet.

2.4.1.3 Cholesterol and triglycerides

Cholesterol and triglycerides were measured concurrently with the OGTTs, and were done monthly, to minimise distress from tail pricking of animals for blood collection. Both the cholesterol and triglycerides levels were measured using Accu-Chek Performa® (Roche Diagnostics Corporation, South Africa) machines specific for cholesterol and triglycerides respectively, and were calibrated before each use. For the purpose of this study, only total cholesterol was measured, with normal ranges being less than 5.5 mmol/l (Fletcher, 2017). Values equal to or greater than 6.0 mmol/l were regarded as hypercholesterolaemia (Fletcher, 2017). Total cholesterol levels were measured from both the SD and ZDSD groups, but for the purpose of this section, only total cholesterol levels belonging to the ZDSD groups were reported on. This is due to the fact that the SD groups had undetectable ranges (<2.0 mmol/l) on the Accu-Chek Performa® throughout testing, and as a result were assumed to be within normal ranges. Triglyceride levels were measured from both the SD and ZDSD groups. Normal triglycerides levels were considered to be less than 1.7 mmol/l (WHO, 2017).

2.4.2 Sacrifice and perfusion of rats

At each sacrifice (20 weeks old for group1 animals and 28 weeks for group 2 animals), the animals were euthanized using Euthapent (Abbott, USA) (with the active agent being Pentobarbital), which was given intraperitoneally, according to the animal weight (1ml per 300-500g and 1.2ml for weights greater than 500g); and perfused using transcardial perfusion, with a perfusion machine, at a rate of 200ml/min, for 2 minutes each.

2.4.3 Extraction of abdominal aorta and post fixation

Following perfusion, the abdominal aorta was removed carefully with surgically clean instruments and placed in formaldehyde until ready for dissection and processing; according to standard protocols suitable for histology (Bancroft & Stevens, 1996) and immunohistochemistry (Abcam, 2018; Sigma 2018). The abdominal aorta was then sectioned into three parts (A, B, & C) (**Figure 2.4**). Part A was designated as the proximal 1/3 of the artery just inferior to the emergence of the superior mesenteric artery and medial to the renal arteries. Part B was the intermediate 1/3 of the artery inferior to the renal arteries and medial to the gonadal/genital arteries. Part C was the distal 1/3 of the artery superior to and including a small segment of the two common iliac arteries. For the purpose of the study, only parts A and C were studied.

The tissue was placed in a tissue processor (Thermo Scientific, Citadel 2000) for dehydration and wax embedding (Tissue-Tek Cryo Console, Miles Scientific) such that they were ultimately embedded into tissue blocks which were then sectioned into a 1 in 8 series (1=H&E, 2= Weigert's, 3&4= CD40 (positive & negative), 5&6= Col8A2 (positive & negative), 7&8= spares) of 5 μm sections using the Microtome (Jung Biocut, Leica (Pty)Ltd). To prepare for further processing, the sections were mounted on 20mm by 50mm silane coated glass slides, labelled appropriately, and allowed to dry overnight in an incubator before any staining was done.

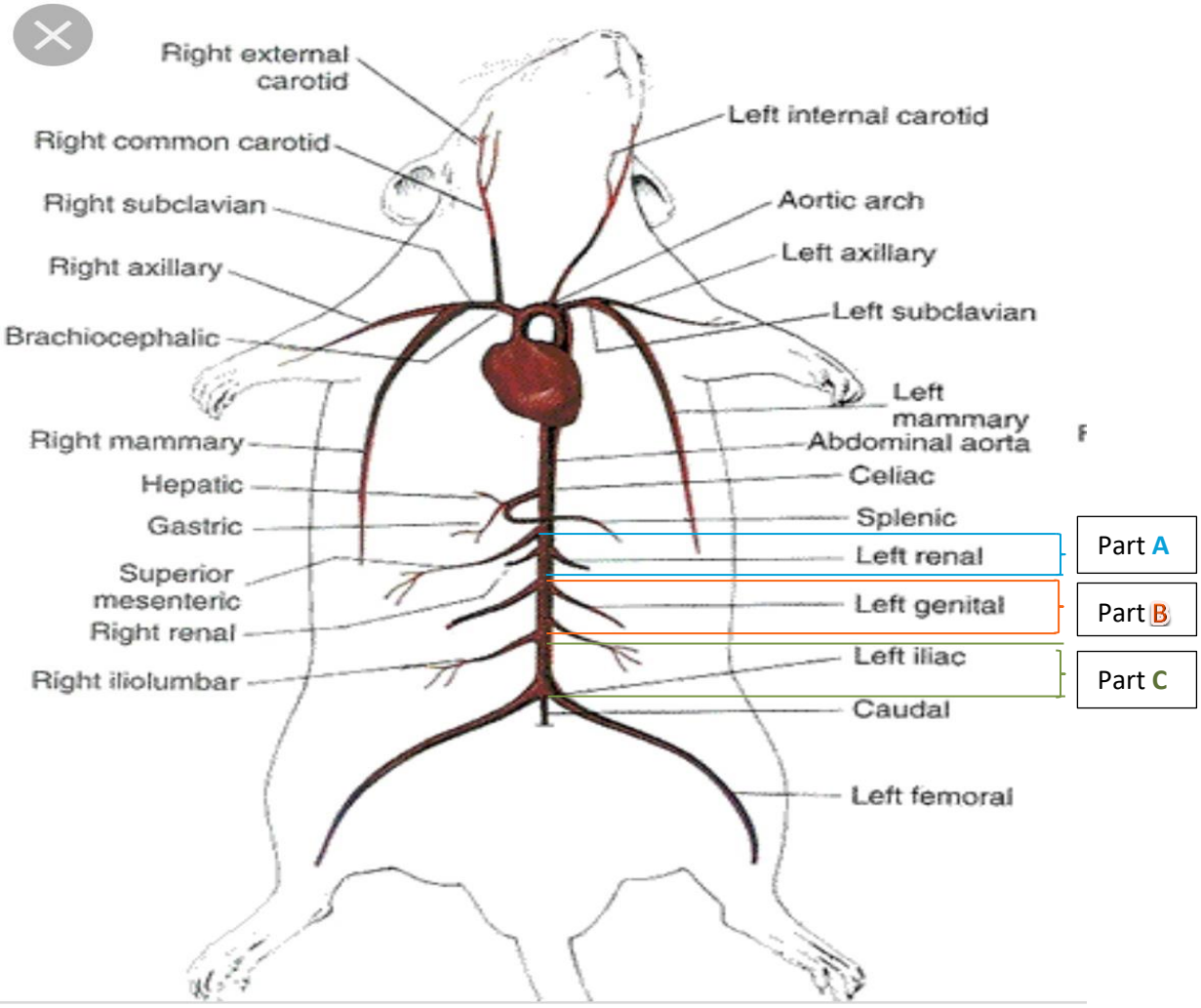


Figure 2.4: An illustration of the segments/parts of the abdominal artery used in the study. **Part A** is the proximal 1/3 of the artery just inferior to the emergence of the superior mesenteric artery and medial to the renal arteries. **Part B** is the intermediate 1/3 of the artery inferior to the renal arteries and medial to the gonadal/genital arteries. **Part C** was the distal 1/3 of the artery superior to and including a small segment of the two common iliac arteries. (Picture adopted from "http://www.utm.edu/staff/rirwin/pubblic_html/RatAnat.htm.Virtual Rat Dissection).

2.5 Tissue staining

Staining of the tissue was performed in the University's laboratory, School of Anatomical Sciences, under the supervision and guidance of a qualified laboratory technician, Mrs Hassiena Ali. The necessary staining protocols were strictly adhered to. The stains included Weigert's elastic stain (Bancroft & Stevens, 1996), Haematoxylin & Eosin (Bancroft & Stevens, 2016) and immunohistochemistry for Cluster of Differentiation 40 (Abcam, UK) and Collagen type 8 Alpha 2 (Col8A2) (SIGMA Aldrich, USA).

2.5.1 Procedure for Weigert's elastic stain

One in every 8 sections in the series was de-waxed and hydrated (see appendix for steps). The sections were then treated with 1% Potassium permanganate for 5 minutes. The section slides were then dipped into 2% Oxalic acid until colourless. They were then placed in Weigert's elastic tissue stain for 20 minutes. Following that, the sections were washed in tap water for 5 minutes, and then differentiated in acid alcohol until the elastic fibres were distinct. The sections were then washed in tap water for 5 minute before being stained in haematoxylin for 2 minutes. Washing of sections in tap water for 5 minutes followed, and then dipped in Scott's tap water for 5 minutes. The sections were then washed in tap water for 5 minutes, and then stained with Eosin for 2 minutes. The sections were then washed until Eosin was out; dehydrated and cover-slipped using Entellan mounting medium (Merck,USA) (Bancroft & Stevens, 1996)

2.5.2 Procedure for Immunohistochemistry (CD40 and Col8A2)

Immunohistochemistry staining was carried out over 3 consecutive days for each run. Two sections for CD 40 and two for collagen 8 alpha 2 (Col8A2) were used from every series of 8 sections in the study. The Anti-CD40 antibody (ab13545) was purchased from Abcam while the Anti-Col8A2 antibody (HPA049788) was purchased from Sigma.

<u>DAY 1</u>

To de-wax, the slides were placed in 2 changes of xylene for 10 minutes each then transferred to 100% alcohol, for 2 changes of 1 minute each. They were then placed through 95% and 70% alcohols respectively for 1 minute each, and then hydrated to tap water for 5 minutes. The slides were then immersed in a Citrate buffer solution (pH6) overnight in a water bath, at 60˚C for antigen retrieval.

<u>DAY 2</u>

The slides were removed from the water bath and allowed to reach standard room temperature, then rinsed in Phosphate Buffered Saline (PBS) for 3 changes of 5 minutes each. Endogenous peroxidase activity was blocked by incubating sections in 1% Hydrogen peroxide (H2O2) solution in methanol at standard room temperature for 15-30 minutes. Rinsing was done in 300ml of PBS for 3 changes of 5 minutes each. A 100µl of blocking buffer (5% Normal goat serum in PBS) was added onto the slide sections and incubated in a humidified chamber to ensure no dehydration, at room temperature for 30 minutes; after which the access blocking buffer was drained from the slides. A 100µl of diluted primary

antibody (1µl Anti-Col8A2 in 19µl PBS and 1µl Anti-CD40 in 999µl PBS) was added to the sections on the slides and incubated in a humidified chamber to ensure no dehydration, at 4˚C overnight.

DAY 3

Slides were allowed to reach standard room temperature for 20 minutes and then washed in 300ml PBS for 3 changes of 5 minutes each. Then 100µl of appropriately diluted biotinylated secondary antibody (Vector labs,USA) (1µl in 999µl PBS) was added to the sections on the slides and incubated in a humidified chamber to ensure no dehydration, at standard room temperature for 30 minutes. Slides were washed again in 300ml PBS for 3 changes of 5 minutes each, then 100µl appropriately diluted ABC (Vectastain, USA) kit was applied to the sections and incubated in a humidified chamber to ensure no dehydration, at standard room temperature for 30 minutes (kept protected from light). Again the slides were washed in 300ml PBS for 3 changes of 5 minutes each. A 100µl solution of Diaminobenzidine (DAB) (SIGMA Aldrich) (freshly made just before use: 0.05% DAB - 0.015% H_2O_2 in PBS) was added to the sections to reveal the colour of antibody staining, allowing the colour development for about 5 minutes until the desired colour intensity was reached. Slides were then washed in running tap water for 5 minutes and dehydration of tissue slides through 4 changes of alcohol (70%, 95%, 100% and 100%) followed for 1 minute each. Finally, clearing the tissue slides in 3 changes of xylene and cover slipped using mounting solution (Permount, Merck, USA) was carried out. The colour of the antibody staining in the tissue sections was viewed under a Carl Zeiss light microscope (ABCAM, SIGMA Aldrich).

2.5.3 Procedure for H&E stain

To de-wax, the slides were placed in 2 changes of xylene for 10 min each then transferred to 100% alcohol, for 2 changes of 1 min each. They were then placed through 95% and 70% alcohols respectively for 1 min each, and then hydrated to tap water for 5 minutes.
For Hematoxylin staining; sections were placed for 3 minutes in Hematoxylin and then rinsed in deionized water. Then 5 minutes in running tap water (to allow the stain to develop), then dipped for 8 to12 times in 1% acid ethanol to de-stain. Subsequently they were rinsed twice for 1 minute in tap water, then once for 2 minutes in deionized water. For Eosin staining; the sections were placed once for 30 seconds in Eosin, thrice in 95% ethanol for 5 minutes each time, then thrice in 100% ethanol for 5 minutes each time, then finally into xylene thrice, for 15 minutes each time. Slides were then cover slipped using Permount (Merck, USA).

After each staining procedure was completed, data analysis followed, whereby all the slides were viewed under the Carl Zeiss light microscope (Zeiss, Gerneva) in order to record and compare the results between diabetic and non-diabetic animals (Bancroft & Stevens, 1996)

CHAPTER 3: RESULTS

This study describes the atherogenic effects of type 2 diabetes mellitus (T2DM) on the abdominal aorta of a diabetic rat model, the Zucker Diabetic Sprague Dawley (ZDSD) rat, and compares that to the aortic anatomy in a normal non-diabetic rat model, the Sprague Dawley (SD) rat. The study also examines T2DM disease progression, with a specific focus on the abdominal aorta's histology, and quantifies it using histological and immunohistochemical techniques. For the most part, both the experimental and control groups exhibited similar characteristics as those described in previous studies. However, various exceptions were observed in both species. As indicated by the presence of CD40 antigens, inflammation was confirmed to be present in significant amounts, especially in the diabetic ZDSD group. The marked presence of Col8A2 indicated significant arterial injury in the ZDSD group, evidenced by increased wall thickness, suggestive of abnormal smooth muscle cell activity. The results are discussed broadly under each measured parameter; that is, the weights, glucose levels, cholesterol and triglycerides, and all the histological methods including H&E and Weigert's stain for elastic fibres and immunohistochemistry for CD40 and Col8A2.

3.1. Body mass

The two animal groups (ZDSDs and SDs), which were further divided into two sub-groups each, were weighed weekly from age week 10 up to age week 20 for the first sub group (group 1) and from age week 10 up to age week 28 for the second sub group (group 2). Fluctuations in body mass over the weeks were observed in both animal groups. SD rat groups 1a and 2a were given different diets from acquisition/birth, in order to observe the effects of a high fat diet on a normal rat as compared to a diabetes prone rat. SD rat group 1a was given a high fat diet whereas SD rat group 2a was given a normal diet (Purina 5008), and the body masses were observed to check the effects of each diet. The first SD group (age week 10 up to week 20) showed a steady increase in body mass on a high fat diet (**Table 3.1**, **Figure 3.1**). The average body mass in this group at week 10 was approximately 458g and 600g at week 20, showing a 23% increase in body mass. The second group of SDs (week 10 up to 28) also showed a steady increase in body mass on a normal commercial diet (Purina 5008) (**Table 3.3**; **Figure 3.2**). The average body mass in this group at week 10 was around 303g and 525g at week 28, showing a 41% increase in body mass. The first SD group demonstrated higher starting body masses compared to the second SD group, due to the high fat diet the animals were fed; although this phenomenon changed over time, whereby the second group of SDs

(on normal diet) proved to have a steady gain in body mass, demonstrating that the rate of body mass increase was attributable to age rather than the type of diet. The increase in body mass in the first group demonstrated that a high fat diet does ultimately result in obesity without necessarily the development of diabetes in the absence of genetic predisposition, since none in this group developed diabetes by the time of termination.

The first group of ZDSDs (age week 10 up to week 20) initially showed a slow increase in body mass, then followed by a decrease in body mass when compared to the SDs; given the high fat diet (**Table 3.2**; **Figure 3.1**). This phenomenon is attributed to the genetic nature of this rat model which was found to develop diabetes spontaneously on a high fat diet without being obese. The average body mass in this group at week 10 was approximately 416g and 463g at week 20, showing a 10% increase in body mass, which was significantly less than the 23% average gain in body mass observed in the SD group of similar age and diet. The initial trend in this group was of an increasing pattern, and then from week 17, the body mass started to show a decreasing pattern as illustrated in **Table 3.2** and **Figure 3.1.** The decrease in body mass observed from week 17 up to week 20 can be attributed to the development of the disease (T2DM), as most, if not all of the animals in this group were confirmed to have developed T2DM by 16 weeks of age (**Table 3.9**). The second group of ZDSDs was weighed from week 10 up to week 28. This group showed a steady increase in body mass, given a high fat diet from acquisition (6 weeks old) (**Table 3.3**, **Figure 3.2**). The average body mass in this group at week 10 was approximately 404g and 530g at week 28, showing a 25% increase in body mass. The gain in body mass in this group proved to be much smaller than the 41% weight gain observed in the SD group at similar age and on a lower fat diet.

Table 3.1: A tabulation of the weekly body mass (g) of the SDs (control group 1a) from week 10 up to week 20 (on a high fat diet). ↑ shows an increase in body mass and ↓shows a decrease in body mass (**W**= week). The overall trend in this group shows a steady increase in body mass.

Rat no	W10	W11	W12	W13	W14	W15	W16	W17	W18	W19	W20
1	502	536↑	564↑	568↑	585↑	602↑	625↑	616↓	623↑	645↑	650↑
2	462	494↑	517↑	521↑	539↑	557↑	575↑	568↓	577↑	595↑	586↓
3	457	478↑	508↑	519↑	534↑	549↑	562↑	575↑	585↑	609↑	597↓
4	421	448↑	487↑	494↑	513↑	532↑	548↑	548↑	554↑	568↑	561↓
5	425	447↑	475↑	470↑	490↑	510↑	538↑	539↑	543↑	569↑	574↑
6	483	514	539↑	534↓	557↑	580↑	604↑	604	621↑	640↑	643↑

Table 3.2: A tabulation of the weekly body mass (g) of the ZDSDs (experimental group 1b) from week 10 up to week 20 (on high fat diet). ↑ shows an increase in body mass and ↓shows a decrease in body mass (**W**= week). The initial trend in this group is of increasing pattern, and then from week 17, the body masses start to show a ↓decreasing pattern.

Rat no.	W10	W11	W12	W13	W14	W15	W16	W17	W18	W19	W20
1	412	434↑	456↑	482↑	503↑	524↑	537↑	532↓	493↓	439↓	483↑
2	412	446↑	470↑	486↑	509↑	523↑	538↑	534↓	497↓	489↓	476↓
3	420	446↑	462↑	488↑	511↑	515↑	537↑	534↓	513↓	502↓	487↓
4	412	438↑	460↑	486↑	497↑	516↑	532↑	537↑	512↓	448↓	430↓
5	416	442↑	456↑	486↑	507↑	529↑	542↑	533↓	521↓	505↓	483↓
6	422	446↑	468↑	488↑	501↑	517↑	509↓	485↓	418↓	440↑	420↓

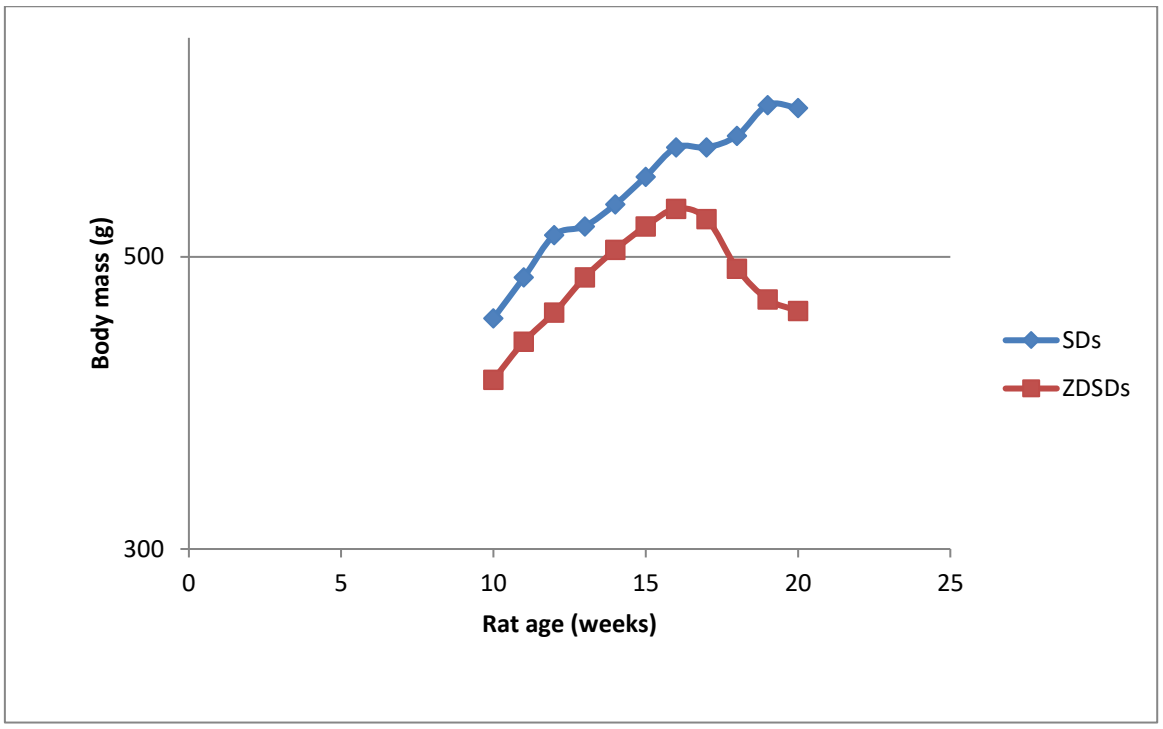


Figure 3.1: An illustration of the progression in average body mass (g) amongst the SDs and ZDSDs from 10 up to 20 weeks of age. The SD (control) group shows a steady increase in body mass from week 10 up to week 20, while the ZDSD group shows an initial increase in body mass from week 10 up to week 17, then a steady decrease in body mass is observed from week 17 up to week 20.

Table 3.3: A tabulation of the weekly body mass (g) of the SDs (control group 2a) from age week 10 up to week 28 (on normal diet). ↑ shows an increase in body mass and ↓shows a decrease in body mass (**W**= week). The overall trend in this group shows a steady increase in body mass.

Rat no.	W 10	W 11	W 12	W 13	W 14	W 15	W 16	W 17	W 18	W 19	W 20	W 21	W 22	W 23	W 24	W 25	W 26	W 27	W 28
1	260	280 ↑	311↑	337↑	353 ↑	373 ↑	379 ↑	389↑	401↑	429↑	428↓	441↑	454 ↑	467↑	471↑	467↓	481 ↑	496↓	**507 ↑**
2	273	311 ↑	334↑	359↑	379 ↑	396 ↑	405 ↑	416↑	421↑	425↑	428↑	441↑	457 ↑	485↑	488↑	469↓	476 ↑	482↑	**492 ↑**
3	277	305 ↑	338↑	358↑	377 ↑	393 ↑	398 ↑	396↓	411↑	434↑	443↑	454↑	465 ↑	478↑	479↑	482↑	497 ↑	505↑	**516 ↑**
4	358	387 ↑	414↑	435↑	454 ↑	467 ↑	476 ↑	463↓	478↑	499↑	493↓	511↑	498 ↓	513↑	521↑	529↑	537 ↑	548↑	**533 ↓**
5	317	357 ↑	387↑	409↑	428 ↑	447 ↑	443	455↑	432↓	443↑	421↓	445↑	471 ↑	499↑	507↑	517↑	522 ↑	528↑	**536 ↑**
6	340	383 ↑	412↑	434↑	452 ↑	472 ↑	476 ↑	487↑	469↓	473↑	459↓	489↑	503 ↑	522↑	540↑	544↑	566 ↑	576↑	**566 ↓**

Table 3. 4: A tabulation of the weekly body mass (g) of the ZDSDs (experimental group 2b) from week 10 up to week 28 (on high fat diet). ↑ shows an increase in body mass, ↓shows a decrease in body mass and ↔ shows no change in body mass(**W**= week). This group shows an initial increase in body mass up to week 21, and then fluctuations in body mass are noted thereafter.

Rat no.	W 10	W 11	W 12	W 13	W 14	W 15	W 16	W 17	W 18	W 19	W 20	W 21	W 22	W 23	W 24	W 25	W 26	W 27	W 28
1	401	418 ↑	448 ↑	468 ↑	471 ↑	492 ↑	495 ↑	501 ↑	506 ↑	506 ↔	520 ↑	538 ↑	534 ↓	535 ↑	538 ↑	543 ↑	540 ↓	526 ↓	**530**↑
2	400	426 ↑	450 ↑	472 ↑	451 ↓	432 ↓	491 ↑	518 ↑	537 ↑	552 ↑	525 ↓	549 ↑	548 ↓	563 ↑	571 ↑	577 ↑	575 ↓	581 ↑	**568**↓
3	277	305 ↑	338 ↑	358 ↑	377 ↑	393 ↑	398 ↑	396 ↓	411 ↑	434 ↑	443 ↑	454 ↑	465 ↑	478 ↑	479 ↑	482 ↑	497 ↑	505 ↑	**516**↑
4	358	387 ↑	414 ↑	435 ↑	454 ↑	467 ↑	476 ↑	463 ↓	478 ↑	499 ↑	493 ↓	511 ↑	498 ↓	513 ↑	521 ↑	529 ↑	537 ↑	548 ↑	**533**↓
5	317	357 ↑	387 ↑	409 ↑	428 ↑	447 ↑	443 ↓	455 ↑	432 ↓	443 ↑	421 ↓	445 ↑	471 ↑	499 ↑	507 ↑	517 ↑	522 ↑	528 ↑	**536**↑
6	340	383 ↑	412 ↑	434 ↑	452 ↑	472 ↑	476 ↑	487 ↑	469 ↓	473 ↑	459 ↓	489 ↑	503 ↑	522 ↑	540 ↑	544 ↑	566 ↑	576 ↑	**566**↓

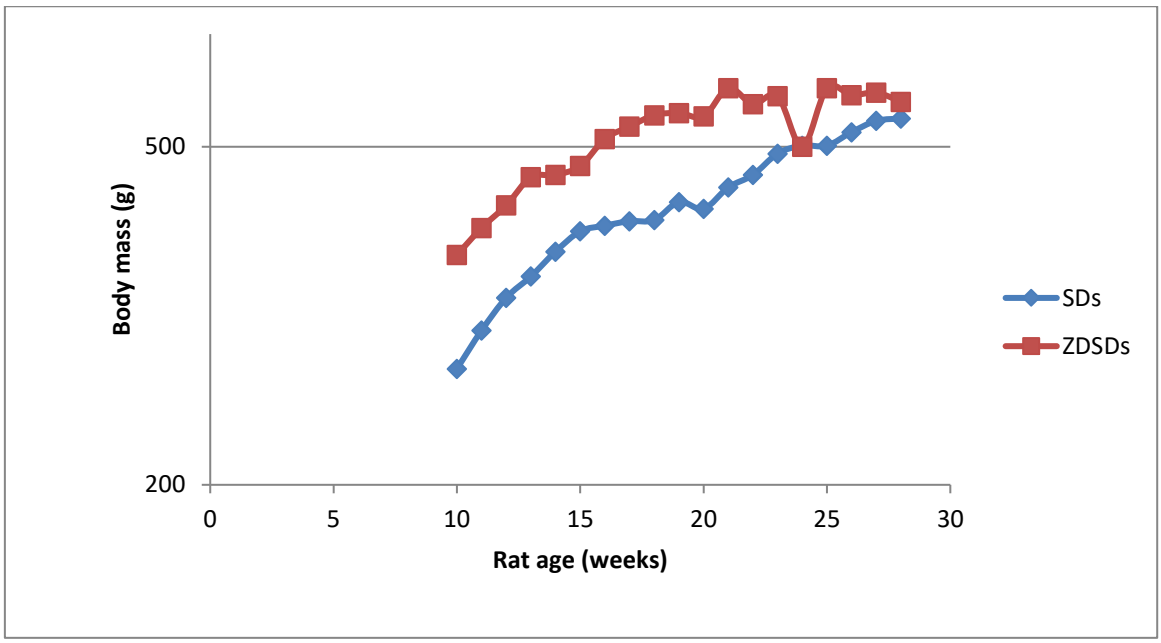


Figure 3.2: An illustration of the progression in average body mass (g) amongst the SDs and ZDSDs from 10 up to 28 weeks of age. The SD group shows a steady increase in body mass from week 20 up to week 28, while the ZDSD group shows an initial increase in body mass at week 20 and 21, and then fluctuations in body mass are noted thereafter. At 22-24 weeks, a decrease in body mass was noted for the ZDSD group.

3.2 Oral glucose tolerance test readings

Blood glucose levels were measured 2 weekly (fasting blood glucose) and every 4th week (OGTTs) from week 12 up until termination in both the SD and ZDSD groups, but only OGTTs are reported on. The blood glucose measurements were taken at five intervals; starting with fasting blood glucose (designated as 0 minutes), then at 15, 30, 60 and 120 minutes respectively. In a normoglycaemic individual, the fasting blood glucose reading is between 3.9-5.5 mmol/l, followed by a 2 hour post prandial reading between 3.9-8.1 mmol/l, then a random reading between 3.9-6.9 mmol/l. For the purpose of this study, blood glucose levels equal to or above 9.0 mmol/l 2 hours postprandial, or fasting blood glucose levels above 6.0 mmol/l were regarded as hyperglycaemia but not necessarily as confirmation for diabetes. Blood glucose levels (fasting and 2 hour postprandial) equalling or greater than 16.7 mmol/l were regarded as confirmation for diabetes. The two SD sub-groups proved to be non-diabetic at each glucose-testing session as the average blood glucose levels remained below 9 mmol/l each time. The average fasting blood glucose reading in the first SD sub-group (up to 20 weeks) at week 16 was 4.9 mmol/l (s= ±0.42), then 5.4 mmol/l (s= ±1.02) at 15 minutes, 6.7 mmol/l (s= ±1.65) at 30 minutes, 6.9 mmol/l (s= ±1.03) at 60 minutes and 7.1 mmol/l (s=

± 1.24) at 120 minutes. The average fasting blood glucose reading in the same group at week 20 was 4.0 mmol/l (s= ±0.60), then 7.3 mmol/l (s= ±0.66) at 15 minutes, 7.2 mmol/l (s= ±1.03) at 30 minutes, 7.1 mmol/l (s= 0.71) at 60 minutes and 6.9 mmol/l (s= 0.60)s at 120 minutes. These readings were confirmation that this sub-group was free of T2DM at the time of termination (**Table 3.5; Figure 3.2**).

Table 3.5: A tabulation of the SD (group 1a) rats' OGTT (mmol/l) readings at week16 of age. Normoglycaemia is observed in the majority of the animals in this group. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. Only 1 out of the 6 rats demonstrates hyperglycaemia at 120 minutes.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	5.3	5.2	5.4	6	6.2
2	5.1	5.3	7	6.6	6
3	4.4	4	5.3	5.7	6.2
4	4.7	6	5.5	7.5	7.4
5	5.4	4.8	9.5	7.9	9.2↑
6	4.5	7.1	7.5	8.2	7.7
Average	**4.9** s= ±0.42	**5.4** s= ±1.02	**6.7** s= ±1.65	**6.9** s= ±1.03	**7.1** s= ±1.24

The average fasting blood glucose reading in the second SD sub-group (up to 28 weeks) at week 16 was 5.8 mmol/l (s= ±0.56), then 8.1 mmol/l (s= ±1.36) at 15 minutes, 8.3 mmol/l (s= ±1.25) at 30 minutes, 7.5 mmol/l (s= ±0.67) at 60 minutes and 6.3 mmol/l (s= ±0.89) at 120 minutes. The average fasting blood glucose reading in the same group at week 28 was 5.2 mmol/l (s= ±0.51), then 7.1 mmol/l (s= ±1.55) at 15 minutes, 7.5 mmol/l (s= ±0.80) at 30 minutes, 7.0 mmol/l (s= ±0.75) at 60 minutes and 6.6 mmol/l (s= ±0.39) at 120 minutes.

These readings were confirmation that this sub-group was also free of T2DM at the time of termination (**Table 3.6; Figure 3.3**).

Table 3.6: A tabulation of the SD (group 2a) rats' OGTT (mmol/l) readings at week16 of age. Normoglycaemia is observed in the majority of the animals in this group. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. 3 out of the 6 rats demonstrate fasting blood glucose levels above 6 mmol/l but these return to normoglycaemia at 120 minutes.

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Rat ID	0 min	15 min	30 min	60 min	120 min
1	5.3	7.0	7.8	7.6	5.5
2	6.5↑	6.9	6.7	6.7	5.7
3	5.4	7.2	7.7	8.2	6.7
4	6.2↑	9.0	10.0	7.5	7.7
5	6.4↑	10.3	9.6	8.4	6.9
6	5.2	8.6	8.0	6.9	5.6
Average	**5.8** s= ±0.56	**8.1** s= ±1.36	**8.3** s= ±1.25	**7.5** s= ±0.67	**6.3** s= ±0.89

The two ZDSD sub-groups were confirmed to have developed T2DM from week 16, as significantly elevated blood glucose measurements were noted from week 16 up until termination. The spontaneous development of T2DM following the commencement of a high fat diet at week 15 of age in the ZDSD confirmed the predisposition of these animals to the metabolic disease. The average fasting blood glucose reading in the first ZDSD sub-group (up to 20 weeks) at 16 weeks was 11.2 mmol/l (s= ±5.96), then 13.9 mmol/l (s= ±8.35) at 15minutes, 17.4 mmol/l (s= ±5.97) at 30 minutes, 19.3 mmol/l (s= ±5.10) at 60 minutes and 21.4 mmol/l (s= ±4.33) at 120 minutes. At 20 weeks of age, the average fasting blood glucose reading in the first ZDSD sub-group (up to 20 weeks) was 18.7 mmol/l (s= ±5.94), then 20.6 mmol/l (s= ±5.85) at 15 minutes, 27.3 mmol/l (s= ±4.52) at 30 minutes, 27.1 mmol/l (s=

±3.91) at 60 minutes and 26.1 mmol/l (s= ±2.63) at 120 minutes. These readings were confirmation that this sub-group had T2DM at the time of termination. The average fasting blood glucose reading in the second ZDSD sub-group at week 16 (up to 28 weeks) was 7.8 mmol/l (s= ±1.04), then 9.2 mmol/l (s= ±1.41) at 15minutes, 11.8 mmol/l (s= ±0.91) at 30 minutes, 13.2 mmol/l (s= ±2.73) at 60 minutes and 16.2 mmol/l (s= ±2.14) at 120 minutes. These readings were confirmation that this sub-group had T2DM at the time of termination (**Table 3.7, Table3.8, Figure 3.3, and Figure 3.6**).

Table 3.7: A tabulation of the ZDSD (group 1b) rats' OGTT (mmol/l) readings at week 16 of age. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. All the animals in this group demonstrate hyperglycaemia and were confirmed to be diabetic at week 16 of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	11.4↑	13.1	17.2	21.4	24.8↑
2	8.3↑	9.7	17.6	18.8	19.2↑
3	7.3↑	10.3	10.9	13.2	14.6↑
4	9.2↑	8.5	12.4	13.9	19.8↑
5	7.9↑	11.3	18.4	22.3	24.3↑
6	23.0↑	30.7	27.9	26.4	25.9↑
Average	**11.2** s= ±5.96	**13.9** s= ±8.35	**17.4** s= ±5.97	**19.3** s= ±5.10	**21.4** s= ±4.33

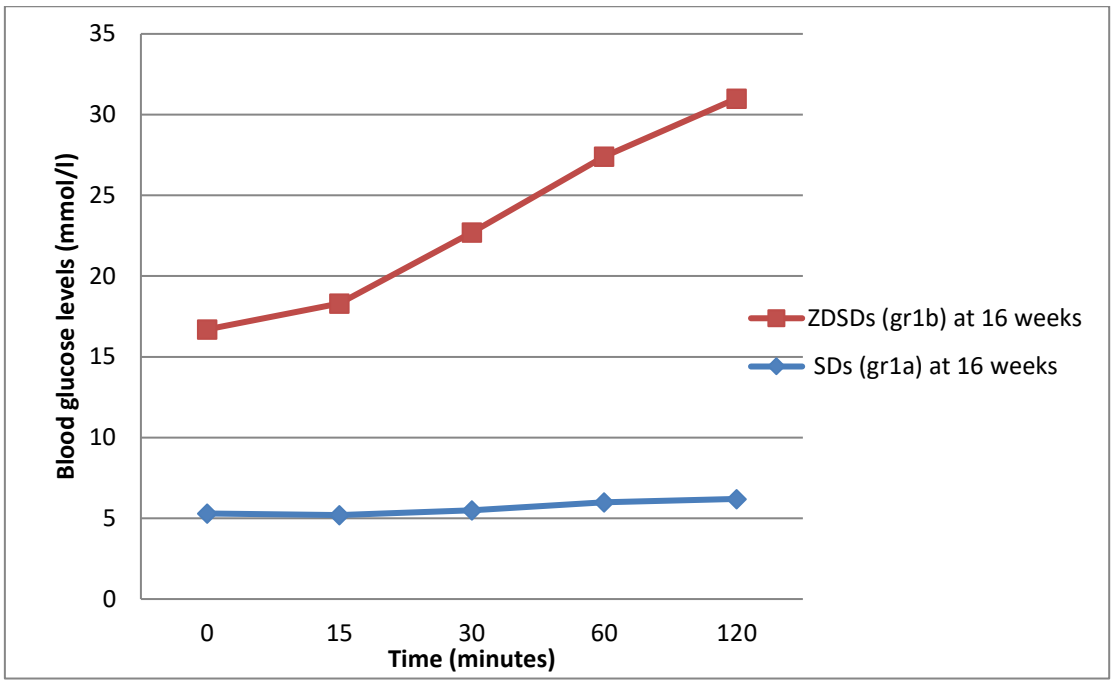


Figure 3.3: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (first group up to 20 weeks) at 16 weeks of age. The SD group shows normoglycaemia (blue line) while the ZDSD group demonstrates hyperglyacaemia (red line). The SD group was non- diabetic while the ZDSD group was confirmed to be diabetic at week 16 of age.

Table 3.8: A tabulation of the ZDSD (group 2b) rats' OGTT (mmol/l) readings at week 16 of age. The majority of animals in this group demonstrate hyperglycaemia. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. This group was confirmed to be diabetic at week 16 of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	9.4↑	7.1	11.9	10.2	15.0↑
2	6.7↑	9.3	11.9	14.6	17.7↑
3	8.5↑	9.5	12.6	15.4	16.5↑
4	8.0↑	10.8	13.0	15.7	17.9↑
5	6.8↑	8.2	10.4	9.4	12.5↑
6	7.4↑	10.6	11.5	14.2	17.8↑
Average	**7.8** s= ±1.04	**9.2** s= ±1.41	**11.8** s= ±0.91	**13.2** s= ±2.73	**16.2** s= ±2.14

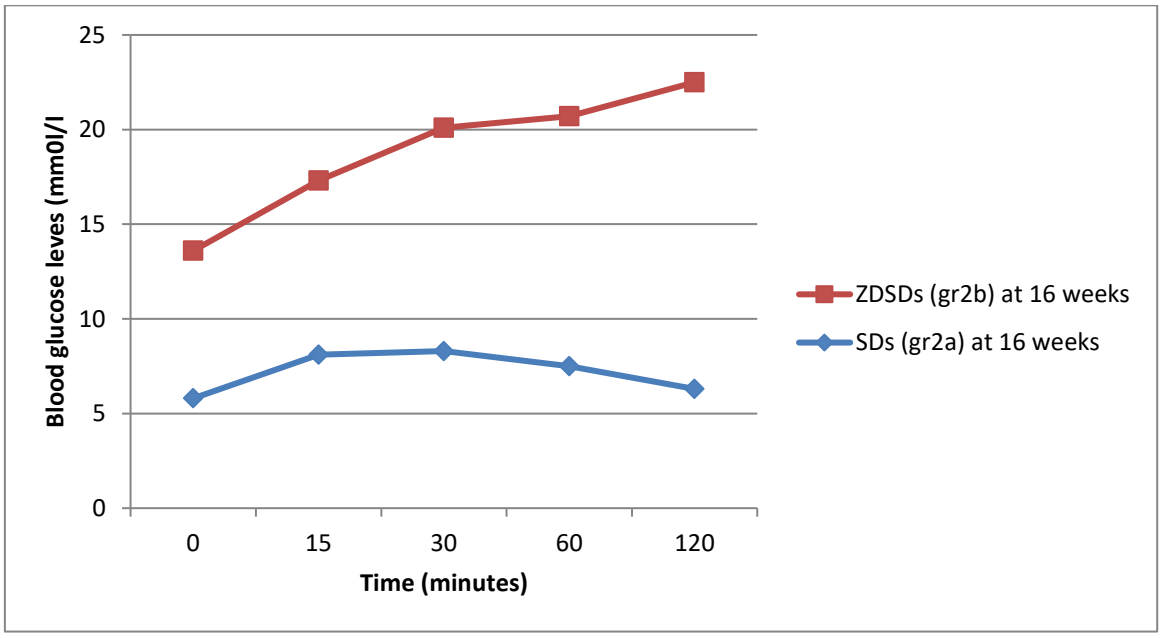


Figure 3.4: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (second group up to 28 weeks) at 16 weeks of age. The SD group (blue line) shows normoglycaemia while the ZDSD group (red line) demonstrates hyperglycaemia (diabetic).

Table 3.9: A tabulation of the SD (group 1a) rats' OGTT (mmol/l) readings at 20 weeks of age. This group demonstrates normoglycaemia and was confirmed to be non-diabetic at week 20 of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	4.7	7.8	8.5	6.5	7.1
2	3.2	8.2	6	8.4	6.1
3	4.2	6.5	6.7	6.8	6.2
4	3.6	6.8	6.7	7.3	7.6
5	4.1	7.2	7	7.3	7.3
6	4.7	7.8	8.5	6.5	7.1
Average	**4.0** s= ±0.60	**7.3** s= ±0.66	**7.2** s= ±1.03	**7.1** s= ±0.71	**6.9** s= ±0.60

Table 3.10: A tabulation of the ZDSD (group 1b) rats' OGTT (mmol/l) readings at week 20 of age. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. This group demonstrates hyperglycaemia and all the animals were confirmed to be diabetic at 20 weeks of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	23.6↑	24.4	32.6	24.7	22.8↑
2	24.0↑	22.8	27.7	29.8	28.3↑
3	21.5↑	21.2	24.4	29.5	25.5↑
4	9.9↑	9.6	20.8	22.1	24.3↑
5	20.6↑	26.1	32.0	32.1	30.0↑
6	12.7↑	19.9	26.2	24.3	25.6↑
Average	**18.7** s= ±5.94	**20.6** s= ±5.85	**27.3** s= ±4.52	**27.1** s= ±3.91	**26.1** s= ±2.63

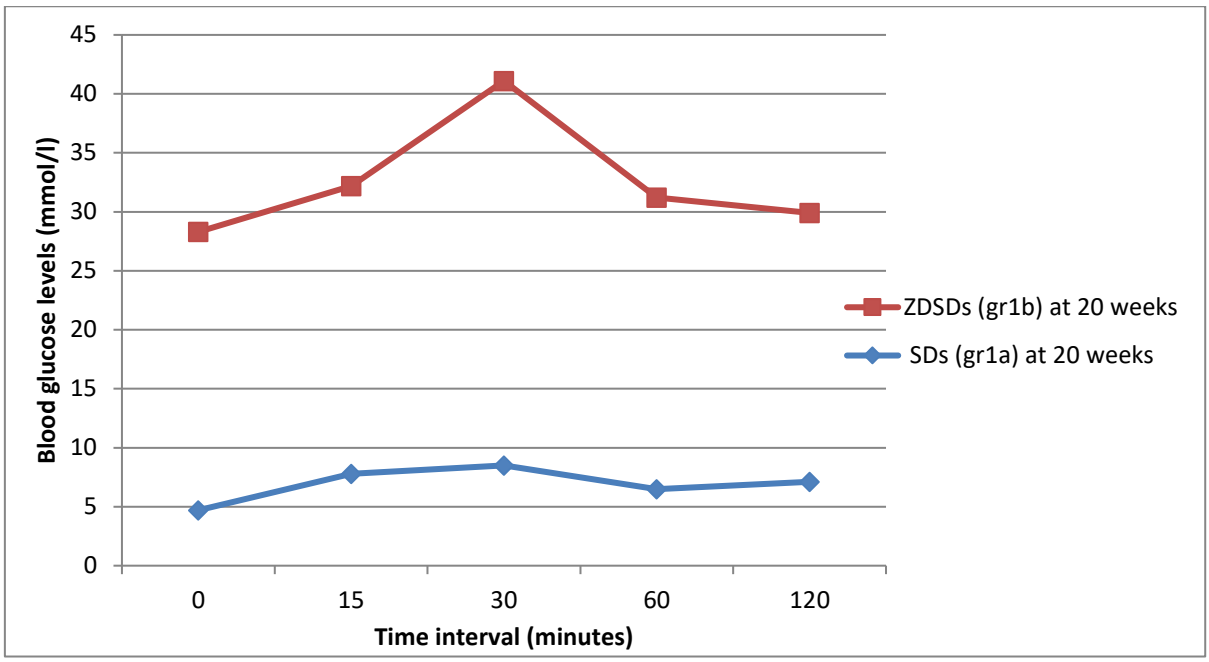


Figure 3.5: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (first group up to 20 weeks) at 20 weeks of age. The SD group shows normoglycaemia while the ZDSD group demonstrates hyperglycaemia (diabetic).

Table 3.11: A tabulation of the SD (group 2a) rats' OGTT (mmol/l) readings at week 20 of age. The majority of animals in this group demonstrate normoglycaemia. This group was confirmed to be non-diabetic. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. Only 1 out of 6 rats demonstrates a slightly elevated fasting blood glucose which returns to normoglycaemia at 120 minutes.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	4.8	6.5	6.9	6.8	6.9
2	4.6	6.8	6.8	6.9	6.7
3	4.9	8.2	7.6	7.8	6.3
4	4.6	6.4	6.7	7.1	6.1
5	7.2↑	10.3	8.2	7.8	6.1
6	6.0	10.9	9.4	8.4	6.4
Average	**5.3** s= ±1.04	**8.2** s= ±1.98	**7.6** s= ±1.05	**7.5** s= ±0.63	**6.4** s= ±0.32

Table 3.12: A tabulation of the ZDSD (group 2b) rats' OGTT (mmol/l) readings at week 20 of age. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. This group demonstrates hyperglycaemia and all the animals were confirmed to be diabetic at 20 weeks of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	6.4↑	9.2	9.3	14.0	16.5↑
2	6.5↑	6.8	8.3	12.3	13.8↑
3	6.1↑	8.9	9.8	11.0	15.2↑
4	7.6↑	8.0	10.5	14.7	18.0↑
5	6.2↑	9.4	11.6	10.7	12.9↑
6	7.0↑	9.8	12.0	15.2	18.5↑
Average	**6.6** s= ±0.56	**8.7** s= ±1.10	**10.2** s= ±1.40	**12.9** s= ±1.92	**15.8** s= ±2.25

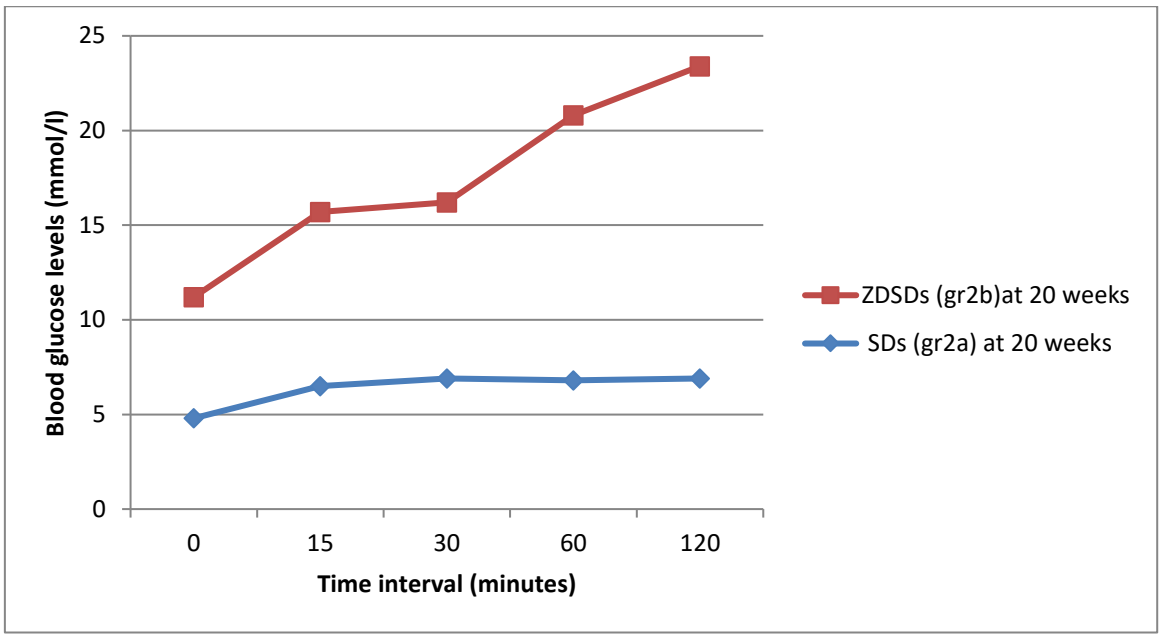


Figure 3.6: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (second group up to 28 weeks) at 20 weeks of age. The SD group shows normal blood glucose readings while the ZDSD group demonstrates elevated blood glucose levels (hyperglycaemia).

Table 3.13: A tabulation of the SD (group 2a) rats' OGTT (mmol/l) readings at week 24 of age. The majority of animals in this group demonstrate normoglycaemia. This group was confirmed to be non-diabetic.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	5.3	7.4	7.4	7.3	6.0
2	4.9	6.4	7.4	7.2	6.1
3	5.0	7.4	7.1	7.4	6.7
4	4.8	7.3	7.5	8.5	6.5
5	4.6	5.4	6.2	6.7	6.2
6	4.8	8.7	9.0	8.9	7.2
Average	**4.9** s= ±0.23	**7.1** s= ±1.10	**7.4** s= ±0.90	**7.6** s= ±0.84	**6.4** s= ±0.45

Table 3.14: A tabulation of the ZDSD (group 2b) rats' OGTT (mmol/l)readings at week 24 of age. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. This group demonstrates hyperglycaemia and all the animals were confirmed to be diabetic at 24 weeks of age.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	6.9↑	13.0	12.6	14.7	14.0↑
2	8.2↑	12.7	14.6	15.6	17.4↑
3	7.7↑	10.9	11.6	16.5	16.5↑
4	9.0↑	14.0	14.3	19.0	18.1↑
5	6.1↑	10.2	9.0	12.5	15.7↑
6	6.9↑	14.2	16.4	17.7	20.0↑
Average	**7.4** s= ±1.04	**12.5** s= ±1.62	**13.0** s= ±2.60	**16.0** s= ±2.29	**16.9** s= ±2.06

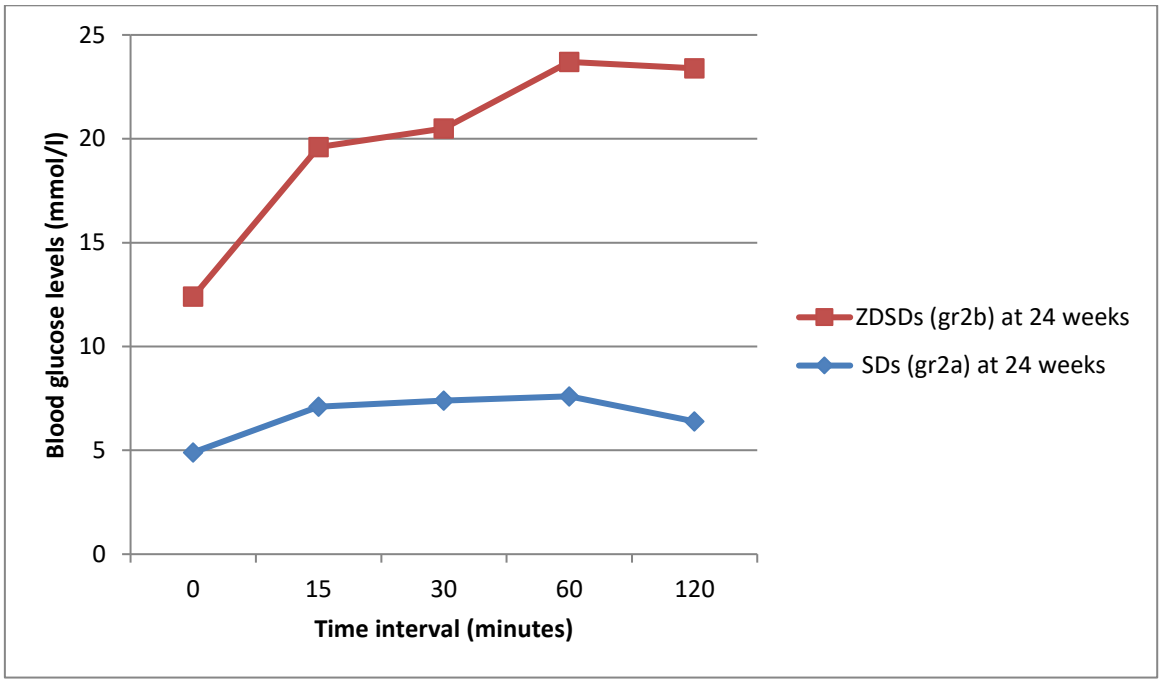


Figure 3.7: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (second group up to 28 weeks) at 24 weeks of age. The SD group shows normal blood glucose readings while the ZDSD group demonstrates elevated blood glucose levels.

Table 3.15: A tabulation of the SD (group 2a) rats' OGTT (mmol/l) readings at week 28 of age. The majority of animals in this group demonstrate normoglycaemia. This group was confirmed to be non-diabetic.

Rat ID	0 min	15 min	30 min	60 min	120 min
1	4.7	5.8	6.6	5.9	7.3
2	5.3	6	7.4	7	6.7
3	4.6	5.9	6.8	6.5	6.2
4	5	6.7	7.5	6.9	6.5
5	6.0	8.6	8.0	7.6	6.4
6	5.3	9.4	8.8	8.0	6.4
Average	**5.1** s= ±0.51	**7.0** s= ±1.55	**7.5** s= ±0.80	**6.9** s= ±0.75	**6.5** s= ±0.39

Table 3.16: A tabulation of the ZDSD (group 2b) rats' OGTT (mmol/l) readings at week 28 of age. ↑ shows an increase in blood glucose levels (hyperglycaemia) above 9.0 mmol/l 2 hours postprandial or fasting blood glucose above 6.0 mmol/l. This group demonstrates hyperglycaemia and all the animals were confirmed to be diabetic at 28 weeks of age.

Rat ID	**0 min**	**15 min**	**30 min**	**60 min**	**120 min**
1	5.6	9.3	10.9	13.7	14.9↑
2	5.3	7.3	8.9	9.0	10.5↑
3	6.5↑	9.2	9.5	10.4	14.4↑
4	6.8↑	7.0	11.6	16.1	14.7↑
5	6.0	7.9	11.5	7.0	8.9
6	6.3↑	9.7	7.4	10.4	14.4↑
Average	**6.0** s= ±0.57	**8.4** s= ±1.05	**9.9** s= ±1.66	**11.1** s= ±3.27	**12.9** s= ± 2.58

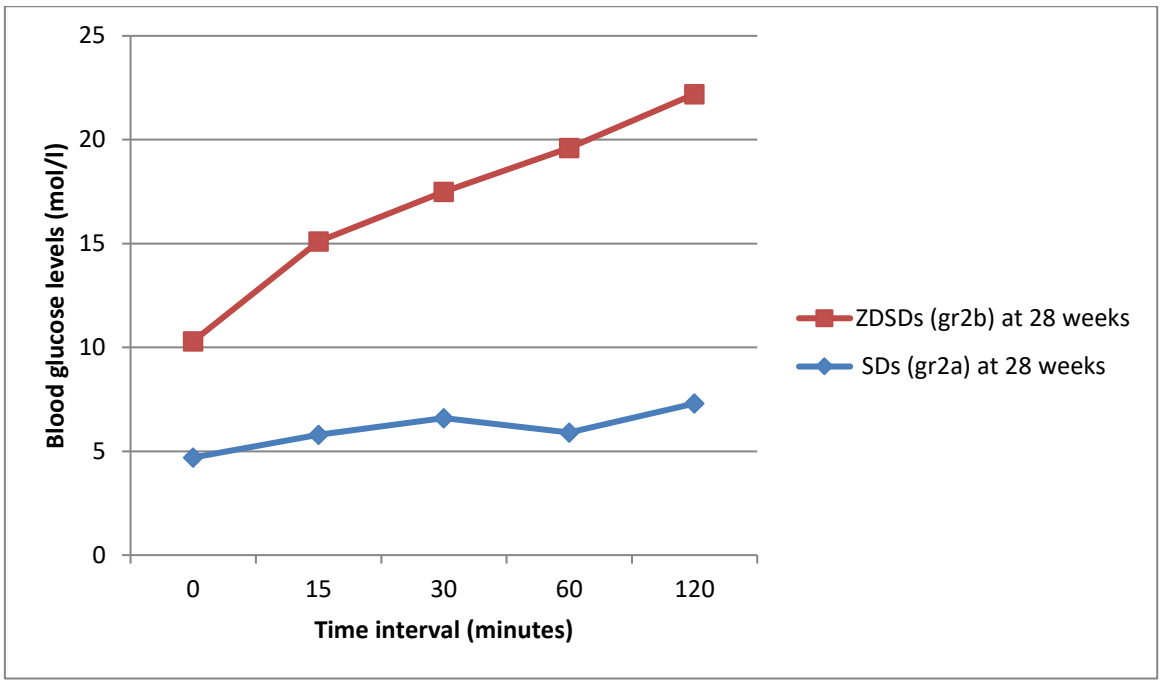


Figure 3.8: An illustration of the average OGTT (mmol/l) readings amongst SDs and ZDSDs (second group up to 28 weeks) at 28 weeks of age. The SD group shows normal blood glucose readings (normoglycaemia) while the ZDSD group demonstrates elevated blood glucose levels (hyperglycaemia).

3.3 Total cholesterol and triglyceride levels

Total cholesterol and triglyceride levels were measured from the animals at week 12, 16, 20, 24 and 28 respectively, but for the purpose of data analysis in this section, total cholesterol and triglyceride levels only at week 12, 16 and at termination (20 weeks for group 1 and 28 weeks for group 2) were reported on. Normal total cholesterol levels were considered to be less than 5.5 mmol/l. Values equal to or greater than 6 mmol/l were regarded as hypercholesterolaemia. Total cholesterol levels were only reported on for the ZDSD groups (**Table 3. 17)** as the SD groups were assumed to have normal levels due to their total cholesterol levels being below detectable (less than 2.0mmol/l) on the Accu-Chek ® cholesterol machine each time on testing. The triglyceride levels were reported for both the SD and ZDSD groups (**Table 3. 18 & Table 3.19)**.

The ZDSDs proved to have normal total cholesterol levels at all ages, as none of them demonstrated TC levels above 5.5 mmol/l (**Table 3. 17, Figure 3.9)**. The first group of ZDSDs (20 weeks) demonstrated an average total cholesterol level of 4.1 mmol/l (s= ±0.95) at 12 weeks; 4.2mmol/l (s= ±0.28) at 16 weeks and 4.0 mmol/l (s= ±0.26) at 20 weeks. The

second group of ZDSDs (28 weeks) showed an average of 4.5 mmol/l (s= ±2.24) at 12 weeks, 4.2 mmol/l (s= ±0.10) at 16 weeks, and 3.9 mmol/l (s= ±0.34) at 28 weeks. **Table 3.17** and **Figure 3.9** tabulate the total cholesterol levels between the ZDSD groups, demonstrating normal total cholesterol levels at all ages.

Table 3.17: A tabulation of the total cholesterol levels (mmol/l) amongst the ZDSDs (groups 1b & 2b). **Low** values are those that were undetectable and considered to be equal to or less than 2,0 mmol/l. **A**. is total cholesterol levels at 12 weeks, **B**. is total cholesterol levels at 16 weeks and **C**. at 20/28 weeks. All total cholesterol levels were found to be normal (<5.5 mmol/l) amongst this group.

A.

Rat no.	ZDSDs group 1b	ZDSDs group 2b
1	4.26	**Low**
2	4.32	**Low**
3	**Low**	**Low**
4	4.29	4.13
5	4.29	4.39
6	4.19	**Low**
Mean	**4.1** s= ±0.95	**4.5** s= ±2.24

B.

Rat no.	ZDSDs group 1b	ZDSDs group 2b
1	4.0	4.34
2	4.16	4.13
3	3.93	4.19
4	4.13	4.24
5	4.16	4.06
6	4.57	4.11
Mean	**4.2** s= ±0.28	**4.2** s= ±0.10

C.

Rat no.	ZDSDs group 1b	ZDSDs group 2b
1	4.19	4.03
2	4.32	4.24
3	4.03	4.21
4	3.88	4.13
5	3.98	4.24
6	4.44	4.32
Mean	**4.0** s= ±0.26	**3.9** s= ±0.34

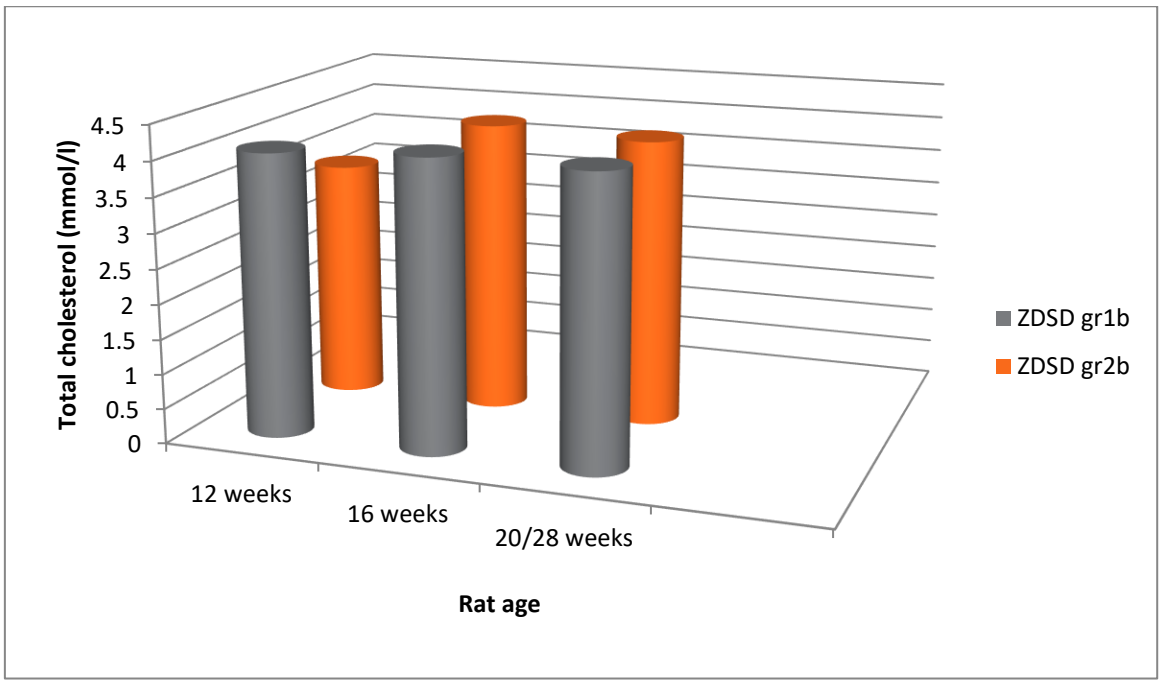


Figure 3.9 An illustration of the average total cholesterol levels (mmol/l) amongst the ZDSDs at week 12, 16 and at termination (week 20 for group 1 and week 28 for group 2). The total cholesterol levels are observed to be within normal ranges (<5.5 mmol/l) in both groups, at all the ages.

Triglyceride levels were measured in both the SD and ZDSD groups. Normal triglyceride levels were considered to be less than 1.7 mmol/l. The first SD group was found to have overall normal triglyceride levels while their ZDSD counterparts had significantly increased triglyceride levels, following the commencement of a high fat diet at week 15. The first SD group had an average of 1.30 mmo/l (s= ±0.36) at 12 weeks, 1.74 mmol/l (s= ±0.52) at 16 weeks and 1.61 mmol/l (s= ±0.96) at termination (20 weeks). The first ZDSD group had an average of 1.68 mmol/l (s= ±0.72) at 12 weeks, 2.95 mmol/l (s= ±1.11) at 16 weeks and 1.71 mmol/l (s= ±0.47) at termination (20 weeks). **Table 3.18** and **Figure 3.10** demonstrate triglyceride levels amongst the first group of SDs and ZDSDs.

Table 3.18: A tabulation of the triglyceride levels (mmol/l) amongst the first group of SDs and ZDSDs. **Low** values are those that were undetectable and considered to be equal to or less than 0, 5 mmol/l. ↑ shows an increase in triglyceride levels (above 1.7 mmol/l). **A**. is triglyceride levels at 12 weeks, **B**. is triglyceride levels at 16 weeks and **C**. at termination (20 weeks).

A.

Rat no.	SDs (1a) (12 weeks)	ZDSDs(1b) (12 weeks)
1	1.57	1.97↑
2	1.23	1.87↑
3	1.58	1.66
4	1.37	2.69↑
5	1.00	1.38
6	1.09	**Low**
Mean	**1.31** s= ±0.36	**1.68** s= ±0.72

B.

Rat no.	SDs (1a) (16 weeks)	ZDSDs (1b) (16 weeks)
1	1.57	1.52
2	1.86↑	2.16↑
3	2.21↑	2.54↑
4	1.84↑	4.13↑
5	0.80	3.05↑
6	2.16↑	4.33↑
Mean	**1.74** s= ±0.52	**2.95** s= ±1.11

C.

Rat no.	SDs (1a) (20 weeks)	ZDSDs (1b) (20 weeks)
1	2.62↑	1.70
2	0.48	2.40↑
3	1.51	1.82↑
4	1.76↑	2.25↑
5	1.69	1.55
6	1.61	2.42↑
Mean	**1.61** s= ±0.96	**1.71** s= ±0.47

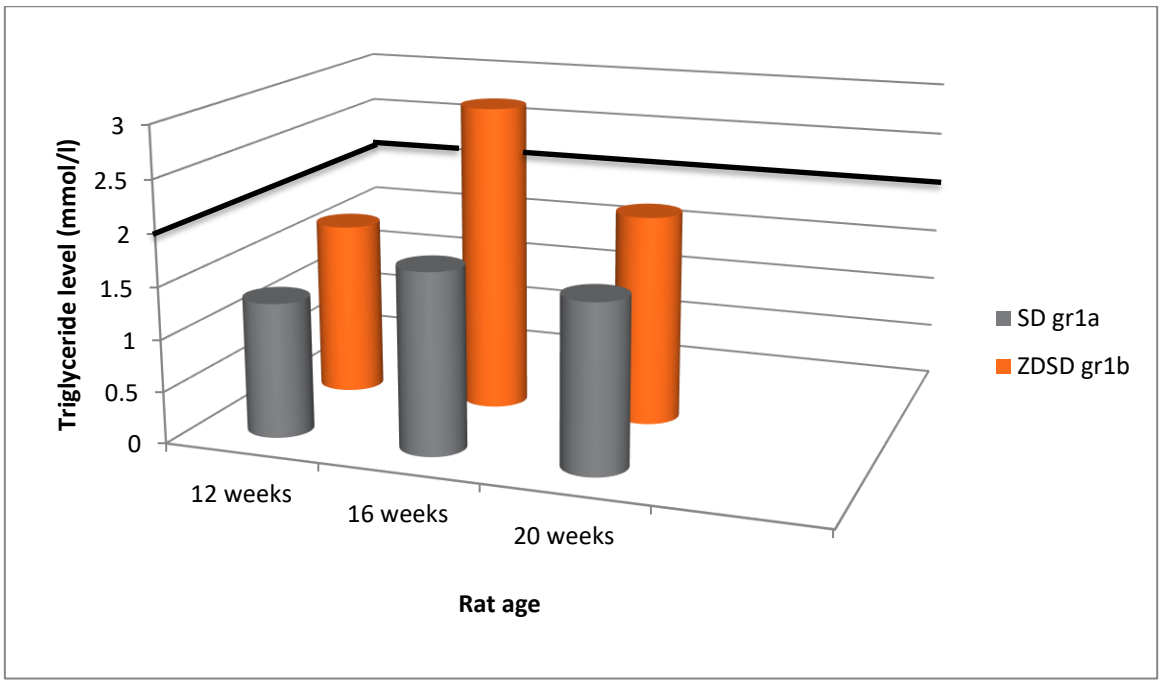


Figure 3.10 An illustration of the average triglyceride levels (mmol/l) amongst the first group of SDs and ZDSDs. The triglyceride levels are observed to be within normal ranges (<1.7 mmol/L) amongst the SDs while they are elevated (>2.0 mmol/l- solid black line) in the ZDSD group at week 16.

The second SD group also demonstrated normal triglyceride levels at all the ages. The average triglyceride level was observed to be 1.33 mmol/l (s=± 0.10) at 12 weeks, 1.01 mmol/l (s= ±0.37) at 16 weeks and 1.18 mmol/l (s= ±0.38) at termination (28 weeks). In contrast to the first ZDSD group, the second ZDSD group proved to have normal triglyceride levels at all the ages; with an average triglyceride level of 0.94 mmol/l (s= ±0.60) at 12 weeks, 1.17 mmol/l (s= ±0.74) at 16 weeks and 1.71 mmol/l (s= ±0.33) at 28 weeks. **Table 3.19** and **Figure 3.11** illustrate the triglyceride levels (mmol/l) amongst the second group of SDs and ZDSDs.

Table 3.19: A tabulation of the triglyceride levels (mmol/l) amongst the second group of SDs and ZDSDs. **Low** values are those that were undetectable and considered to be equal to or less than 0, 5 mmol/l. ↑ shows an increase in TG levels above 2.0 mmol/l. **A**. is the TG levels at 12 weeks, **B**. is the TG levels at 16 weeks and **C**. at termination (28 weeks).

A.

Rat no.	SDs (2a) (12 weeks)	ZDSDs (2b) (12 weeks)
1	1.35	**Low**
2	1.29	**Low**
3	1.44	**Low**
4	1.37	1.61
5	1.18	**Low**
6	1.36	2.00↑
Mean	**1.33** s= ±0.10	**0.94** s= ±0.68

B.

Rat no.	SDs (2a) (16 weeks)	ZDSDs(2b) (16 weeks)
1	0.93	**Low**
2	1.12	1.77↑
3	**Low**	**Low**
4	1.01	2.34↑
5	1.36	1.03
6	1.15	0.89
Mean	**1.01** s= ±0.37	**1.17** s= ±0.74

C.

Rat no.	SDs (2a) (28 weeks)	ZDSDs (2b) (28 weeks)
1	1.31	1.97↑
2	1.64	2.11↑
3	1.06	1.27
4	1.33	1.61
5	Low	1.89↑
6	1.22	1.44
Mean	**1.18** s= ±0.38	**1.71** s= ±0.33

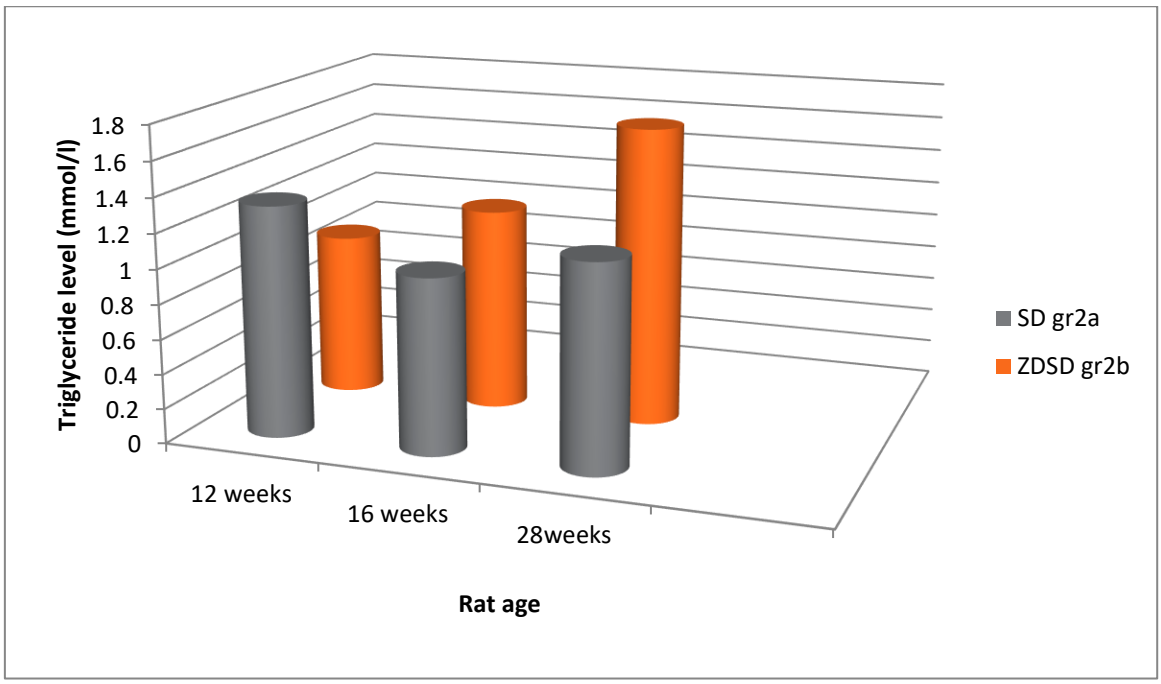


Figure 3.11 An illustration of the triglyceride levels (mmol/l) amongst the SDs and ZDSDs. Triglyceride levels are observed to be within normal ranges (<1.7 mmol/l) for both the SD and ZDSD groups at all ages.

3.4 Haematoxylin & Eosin stain

The Haematoxylin & Eosin (H&E) is a routine general stain used in the study of cell morphology mostly in the field of Anatomical Sciences (Ross & Pawlina, 2006). The Haematoxylin component stains the nuclei blue-black; while the Eosin component stains the cytoplasm and elastic fibres pink (Ross & Pawlina, 2006). In the current study, the H&E stain was purely employed to depict the morphology and size of the arterial wall structures in the non-diabetic abdominal aorta and compare these to that of the diabetic abdominal aorta. Several features of the arterial wall were studied, including characteristics of each tunic; intima, media and adventitia, the overall arterial and luminal shape, and the arterial wall thickness. **Figure 3.12** is a cross section through the abdominal aorta of a non-diabetic rat, illustrating the normal arterial wall and the three tunics. Tunica intima, the thinnest of all tunics, and is in proximity to the lumen, is depicted by a continuous, intact and slightly convoluted appearance of the internal elastic lamina. Tunica media, immediately after tunica intima, is the thickest of all the tunics, and is depicted by +/- 6 layers of smooth muscle cells with oval/flattened nuclei; each layer of cells separated by elastic lamellae. Tunica adventitia which is the last tunic and follows immediately after tunica media, is depicted by the loose arrangement of connective tissue elements.

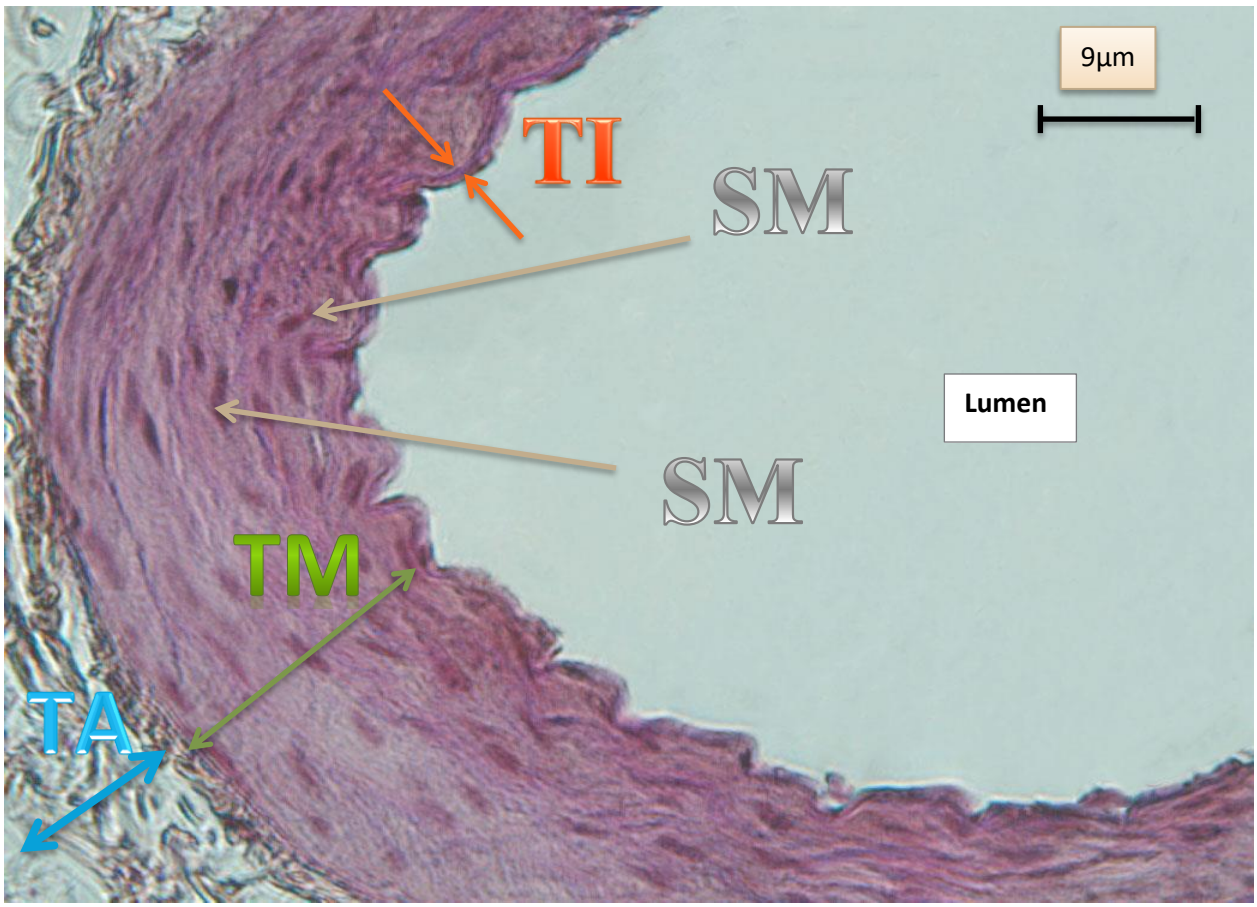


Figure 3.12: A photomicrograph (H&E) depicting the arterial wall in a normal, non-diabetic rat, with illustration of the three tunics. **TI:** Tunica intima (orange arrows): note the continuous and slightly convoluted appearance of this innermost layer. **TM:** Tunica media (green arrow): this tunic contains +/- 6 layers of smooth muscle cells with oval/flattened nuclei, each layer separated by elastic lamellae. **TA:** Tunica adventitia (blue arrow): this tunic possesses a loose arrangement of connective tissue elements (x40 objective). **SM:** smooth muscle cells with oval nuclei.

The tunica intima in the ZDSD group was found to be discontinuous, straight and broken when compared to the SD group, which had an intact, slightly convoluted and continuous tunica intima. The tunica media in the SD group had an overall normal arrangement of smooth muscle cells with an average of six layers of individual smooth muscle cells resting upon five elastic lamellae. The tunica adventitia in the SD group appeared to be normal, consisting of connective tissue elements primarily. The ZDSD group was found to demonstrate an abnormal mixture of tunica adventitia and tunica media, as the smooth muscle cell of the tunica media were mixed with the connective tissue of the tunica adventitia. When observing the overall shape of the arteries, the ZDSD group showed to have smaller lumina, with the arterial walls proliferating towards the lumen. In contrast, the SD group demonstrated

normal arterial shape with patent lumina. With regards to the arterial wall thickness, the ZDSD group demonstrated significantly thicker walls when compared to their SD counterparts; at both Part A and Part C of the artery.

3.4.1 Characteristics of each tunic in the arterial wall

As previously stated, the arterial wall consists of three layers or tunics, viz, the tunica intima, the tunica media, and the tunica adventitia.

3.4.1.1 Tunica intima

In the normal arterial wall, the tunica intima possesses a prominent, intact and slightly convoluted internal elastic lamina, and is the thinnest tunic amongst all the three tunics. When the tunica intima was observed in the non-diabetic groups, it was found to have an internal elastic lamina which was intact, continuous and slightly convoluted, as that described under normal circumstances, as 10 out of the 12 non-diabetic (SD) arteries displayed these characteristics (>80%). **Figure 3.13** demonstrates the tunica intima in a non-diabetic (SD at 20 weeks) artery.

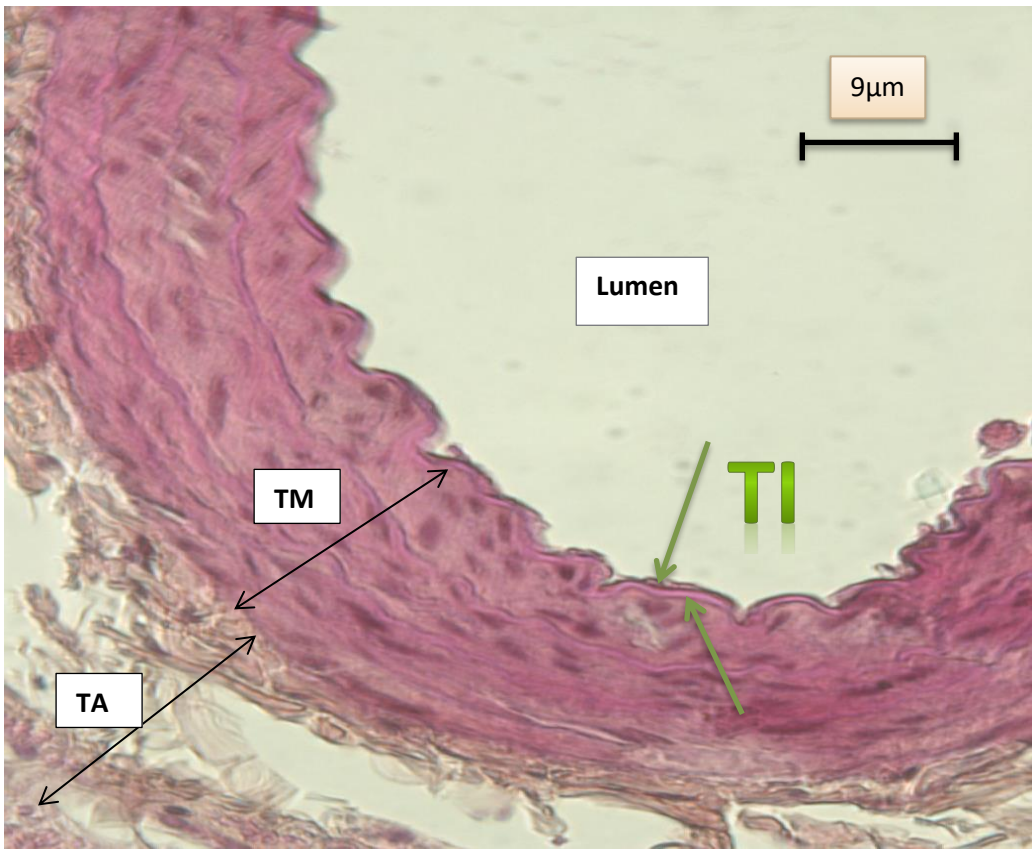


Figure 3.13: An illustration (H&E) of the tunica intima (TI) (green arrows) in an artery of a non-diabetic rat (SD at 20 weeks). The tunica intima has a prominent, intact and slightly convoluted internal elastic lamina. TM= tunica media and TA= tunica adventitia (x40 objective).

The tunica intima in the diabetic arteries was observed to follow a different pattern to that described in normal arteries. In the diabetic group, almost all the arteries (>80%) had a tunica intima that was initially observed to have a prominent internal elastic lamina, which appeared less convoluted than that documented for non-diabetic arteries, and then disappears, and is discontinuous; confirming the presence of a disease process in this group. **Figure 3.14** depicts the changes seen in the tunica intima of a diabetic artery.

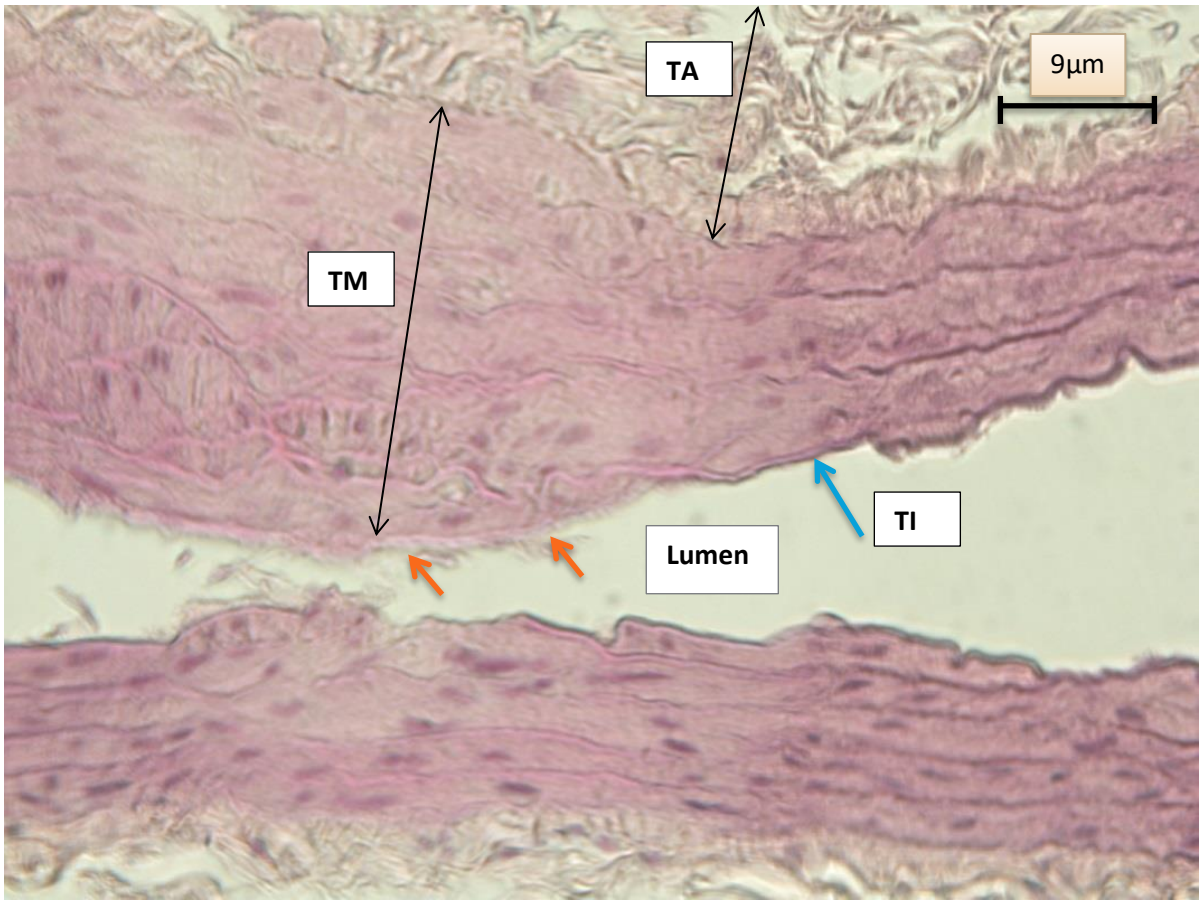


Figure 3.14: An illustration (H&E) of the tunica intima (blue arrow) in an artery of a diabetic rat (ZDSD at 20 weeks). The tunica intima has a prominent internal elastic lamina which initially appears straight and prominent (blue arrow) and then disappears (orange arrows), demonstrating a disease process in this artery (x40 objective).

3.4.1.2 Tunica media

The tunica media is the thickest of the three tunics in large/elastic arteries like the abdominal aorta, and begins posteriorly to the internal elastic lamina and terminates anteriorly to the external elastic lamella. The tunica media consists of an arrangement of smooth muscle cells that are separated by elastic lamellae. The smooth muscle cells are spindle shaped and contain elongated nuclei, in the normal non-diabetic artery. The tunica media in the non-diabetic arteries was observed to follow the normal morphology described above, with 2 out of the 12 arteries (<20%) deviating from the norm. **Figure 3.15** depicts the normal arrangement of the tunica media in a normal (non-diabetic) artery as observed during data analysis.

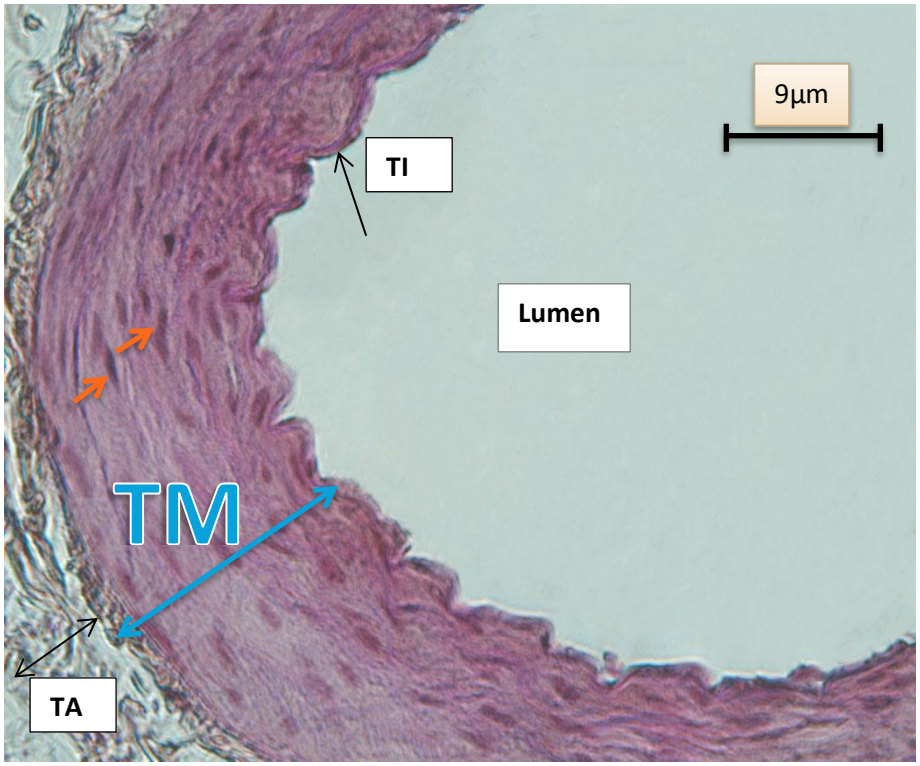


Figure 3.15: A photomicrograph (H&E) of a cross section through an artery of a non-diabetic rat, depicting the tunica media (TM) (blue arrow). The smooth muscle cells are spindle shaped and contain elongated, oval shaped nuclei (orange arrows) in the normal (non-diabetic) artery. TI= tunica intima and TA= tunica adventitia (x40 objective).

In the diabetic group, the tunica media was observed to have an atypical appearance from the norm. The smooth muscle cells in the diabetic group (ZDSDs) were found to be haphazardly arranged and seemingly disrupted, while others were loosely arranged. Some appeared to be

clustered together as opposed to those observed in **Figure 3.15**. The elastic lamellae were observed to be broken and discontinuous (**Figure 3.16**) while the smooth muscle cell nuclei were noted to have transformed from the elongated spindle shape, to a smaller, rounder shape; confirming the occurrence of a disease process in these arteries. **Figure 3.16**, **Figure 3.17, Figure 3.18** and **Figure 3.19** depict the changes that were observed in the tunica media of diabetic arteries.

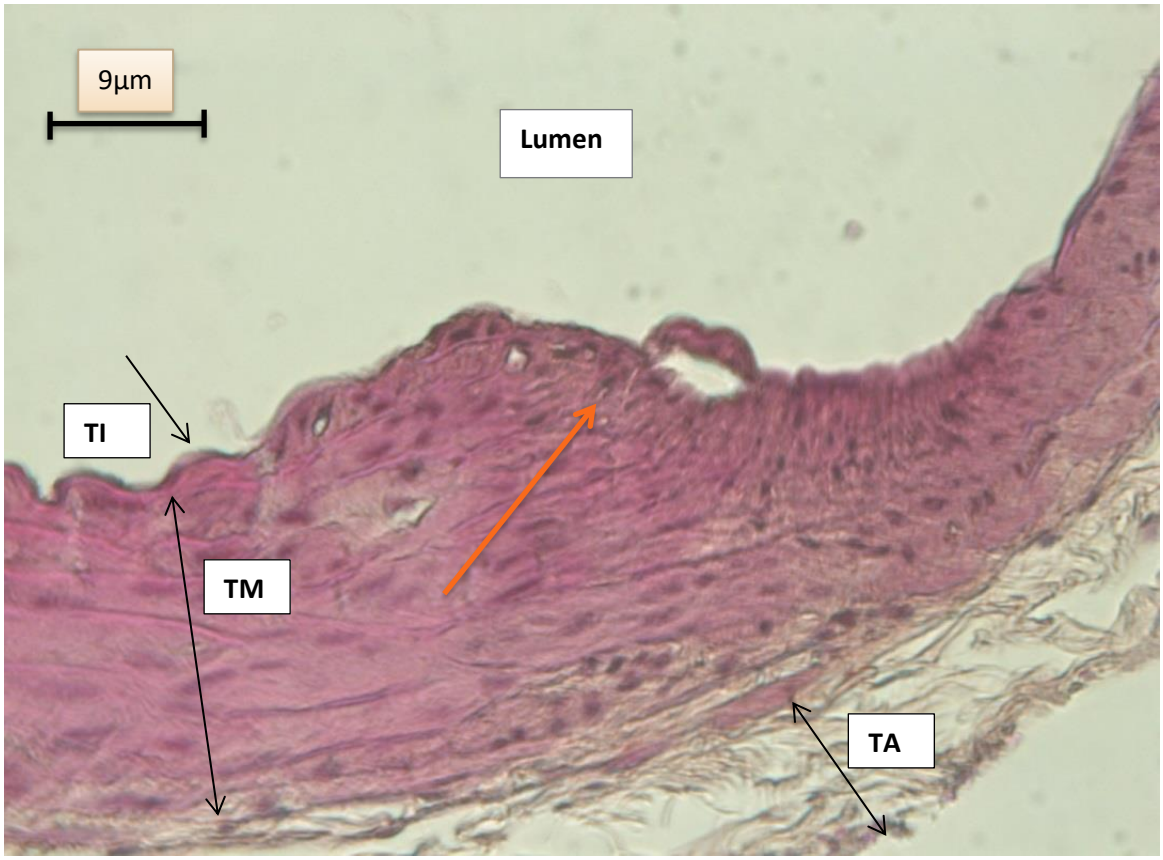


Figure 3.16: A photomicrograph (H&E) of a cross section through an artery of a diabetic rat (ZDSD at 28 weeks). The smooth muscle cells are haphazard in arrangement and appear clustered together (orange arrow), confirming the occurrence of a disease process in this artery. TI=tunica intima, TM= tunica media and TA=tunica adventitia (x40 objective).

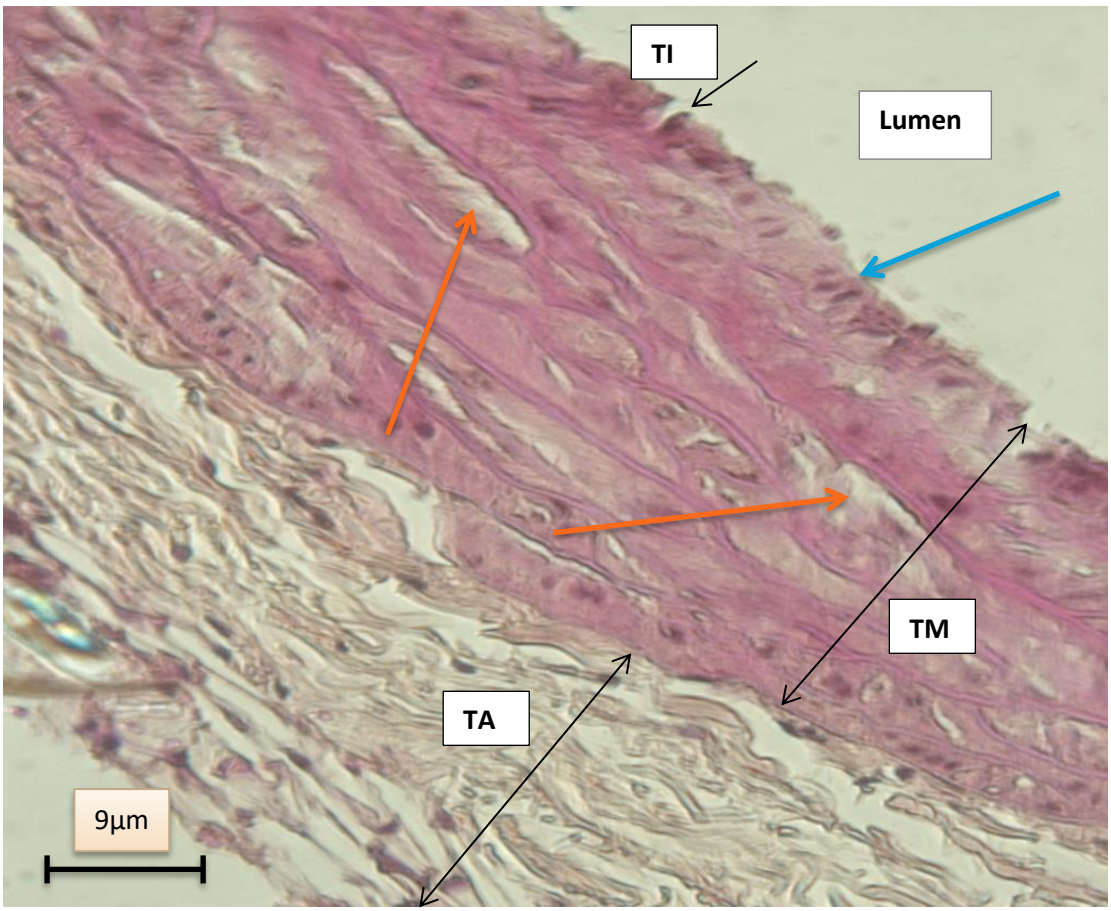


Figure 3.17: A photomicrograph (H&E) of a cross section through an artery of a diabetic rat (ZDSD at 28 weeks). The smooth muscle cells in the tunica media (TM) are loosely arranged (orange arrows) and nuclei appear rounder, smaller and clustered together (blue arrow), confirming the occurrence of a disease process in this artery. TI= tunica intima and TA= tunica adventitia (x40 objective).

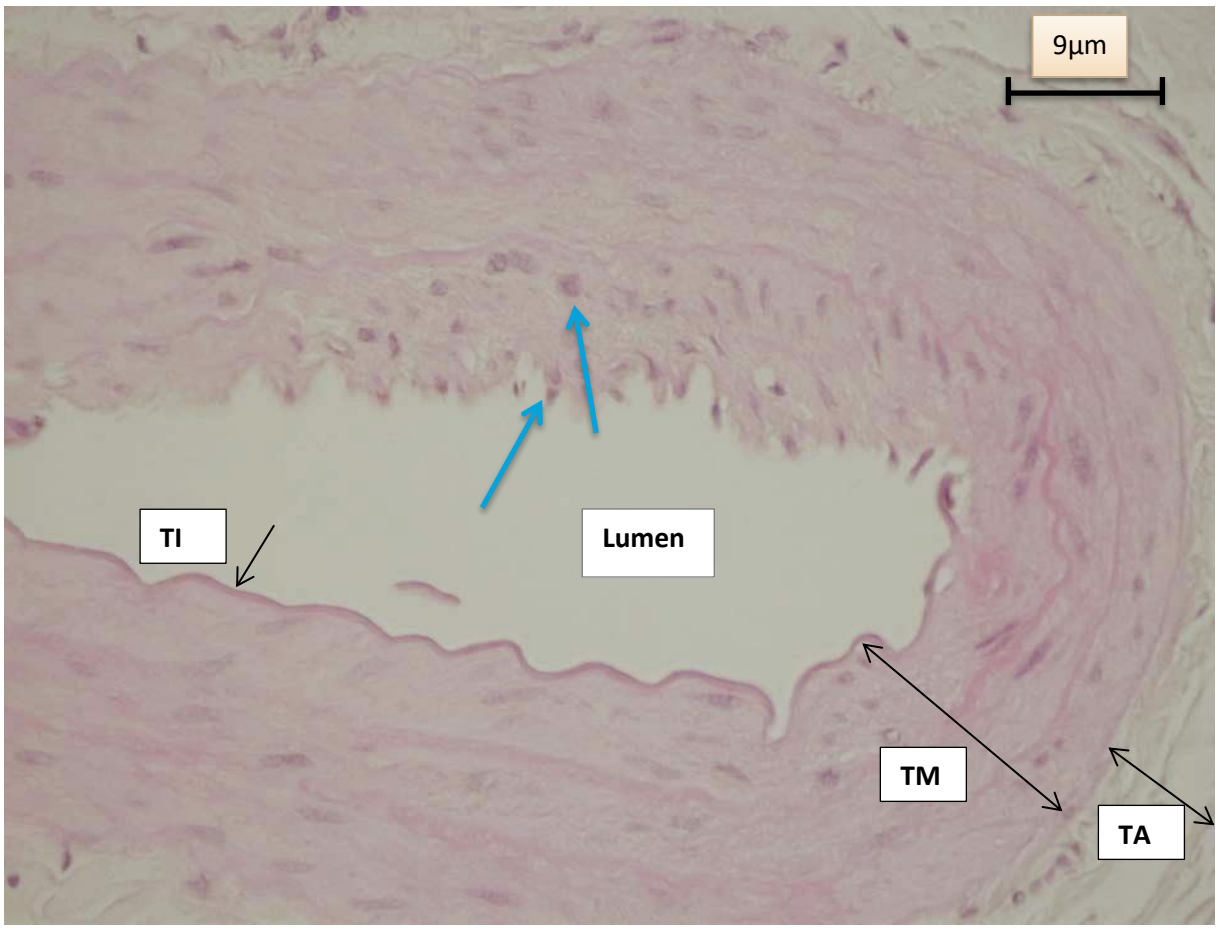


Figure 3.18: A photomicrograph (H&E) depicting the arterial wall morphology in the abdominal aorta of a diabetic rat. The smooth muscle cells appear to have migrated towards the lumen as their nuclei appear rounder and clumped together (blue arrows). TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

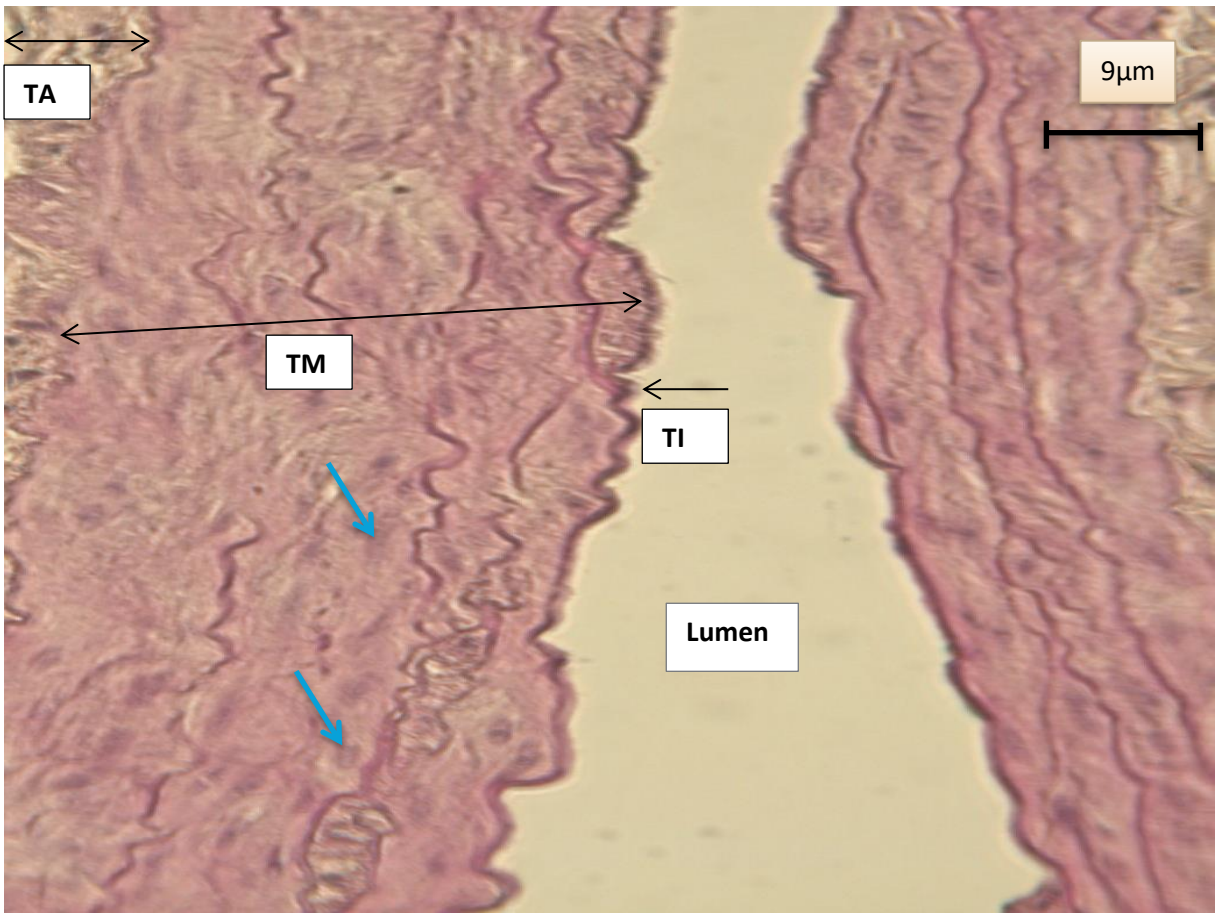


Figure 3.19: A photomicrograph (H&E) depicting a cross section through an artery of a diabetic rat (ZDSD at 28 weeks). The tunica media (TM) demonstrates a disruption in the normal smooth muscle cell layer arrangement with the nuclei haphazardly arranged and absorbing little stain (blue arrows). TI= tunica intima and TA= tunica adventitia (x40 objective).

3.4.1.3 Tunica adventitia

The tunica adventitia is the outermost of all the tunics and consists mainly of loosely arranged connective tissue elements. The tunica adventitia was observed to have a normal arrangement in most of the non-diabetic arteries and is illustrated in **Figure 3.20**.

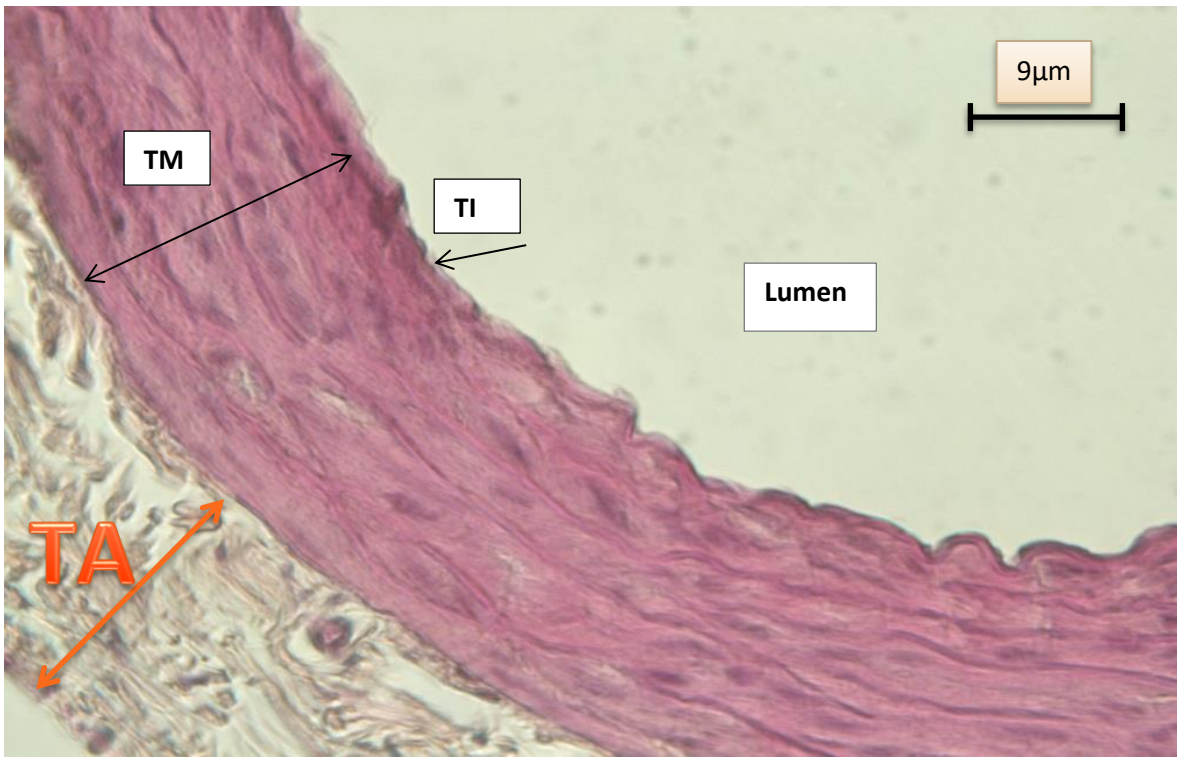


Figure 3.20: A photomicrograph (H&E) of a cross section through an artery of a non-diabetic rat (SD at 20 weeks), depicting the tunica adventitia (TA). The orange arrow shows the loose arrangement of the connective tissue in the tunica adventitia. TI= tunica intima and TM= tunica media (x40 objective).

Some of the diabetic arteries were found to have a normal tunica adventitia while some abnormalities were noted in others. In some diabetic arteries, the tunica adventitia was observed to be mixed with the tunica media. The connective tissue of the tunica adventitia appeared to have migrated to the tunica media; giving the tunica media an abnormal appearance of a mixture of smooth muscle cells and connective tissue. This abnormal arrangement was evident of a pathological process occurring at the arterial wall, noting that the section was taken from a diabetic subject. **Figure 3.21** is a photomicrograph of a cross section through a diabetic artery (ZDSD at 20 weeks), depicting an abnormal tunica adventitia.

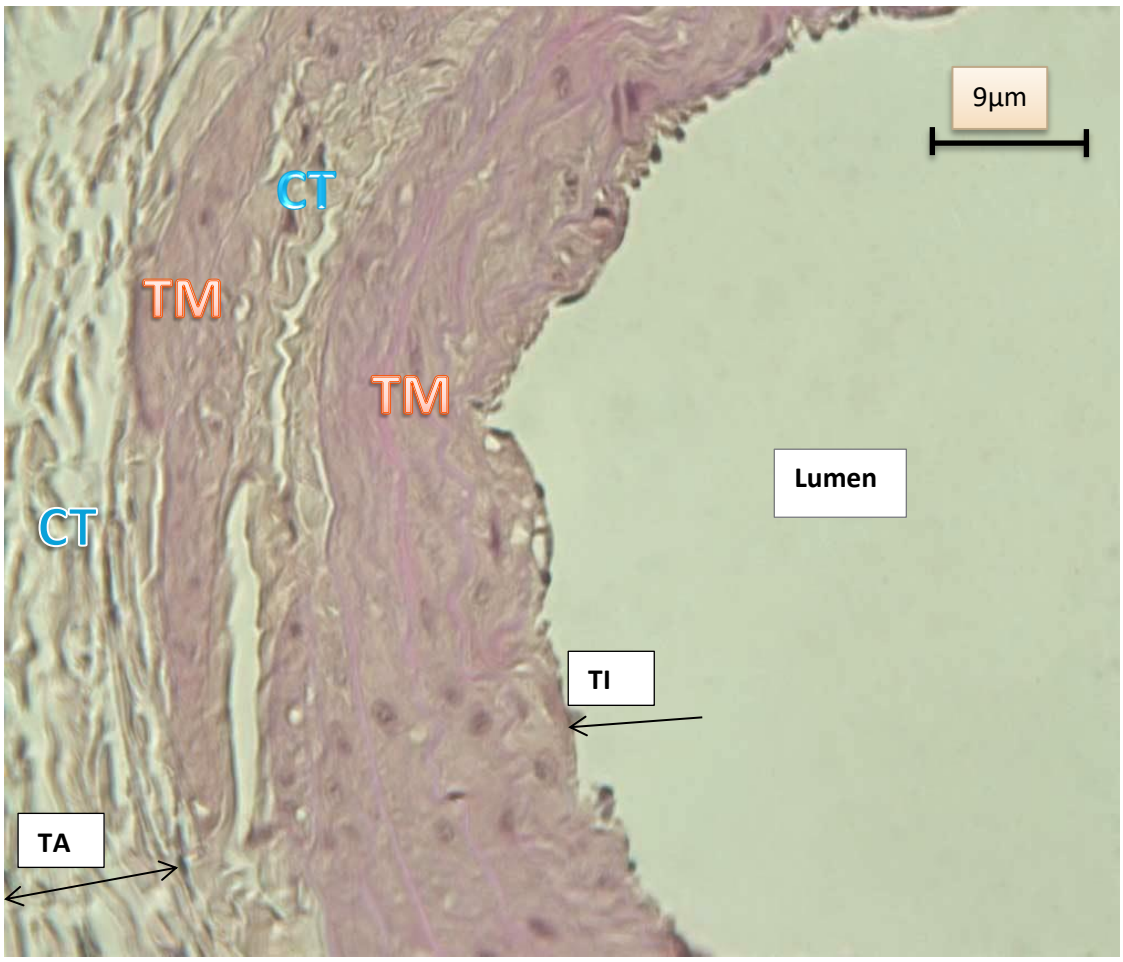


Figure 3.21: A photomicrograph of a cross section through a diabetic artery (ZDSD at 20 weeks), depicting tunica adventitia (TA) mixed with tunica media (TM). The connective tissue (CT) of the tunica adventitia is seen apparently migrating into the tunica media, giving the tunica media an abnormal appearance of a mixture of smooth muscle cells and connective tissue (x40 objective).

3.4.2 Overall arterial and luminal shape

The normal (non-diabetic) artery possesses an almost oval shape. The non-diabetic group was mostly observed to have a normal oval shape of the artery and a patent lumen. Photographs depicting the overall arterial and luminal shape were taken at x10 objective in order to reveal the entire artery. **Figure 3.22** depicts the normal shape of the artery and lumen in a non-diabetic animal.

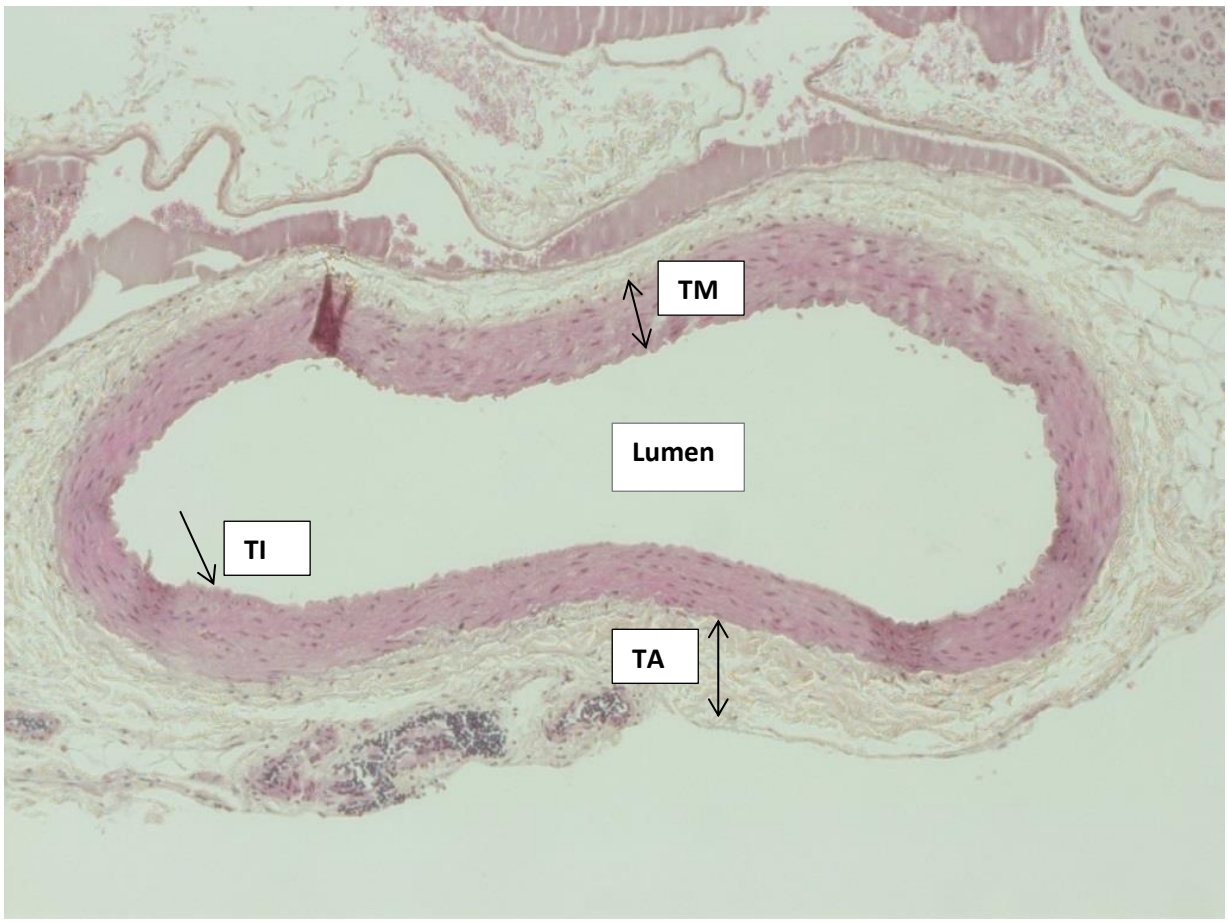


Figure 3.22: A photo micrograph of an H&E stain depicting an artery in a non-diabetic rat (SD at 28 weeks), with a normal patent lumen. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x10 objective).

In contrast to the non-diabetic arteries, the diabetic arteries were found to have an oval shape but with a reduced luminal patency, where slight to almost complete closure of the lumen is observed. **Figure 3.23** and **Figure 3.24** demonstrate the arterial and luminal shape observed in the diabetic group.

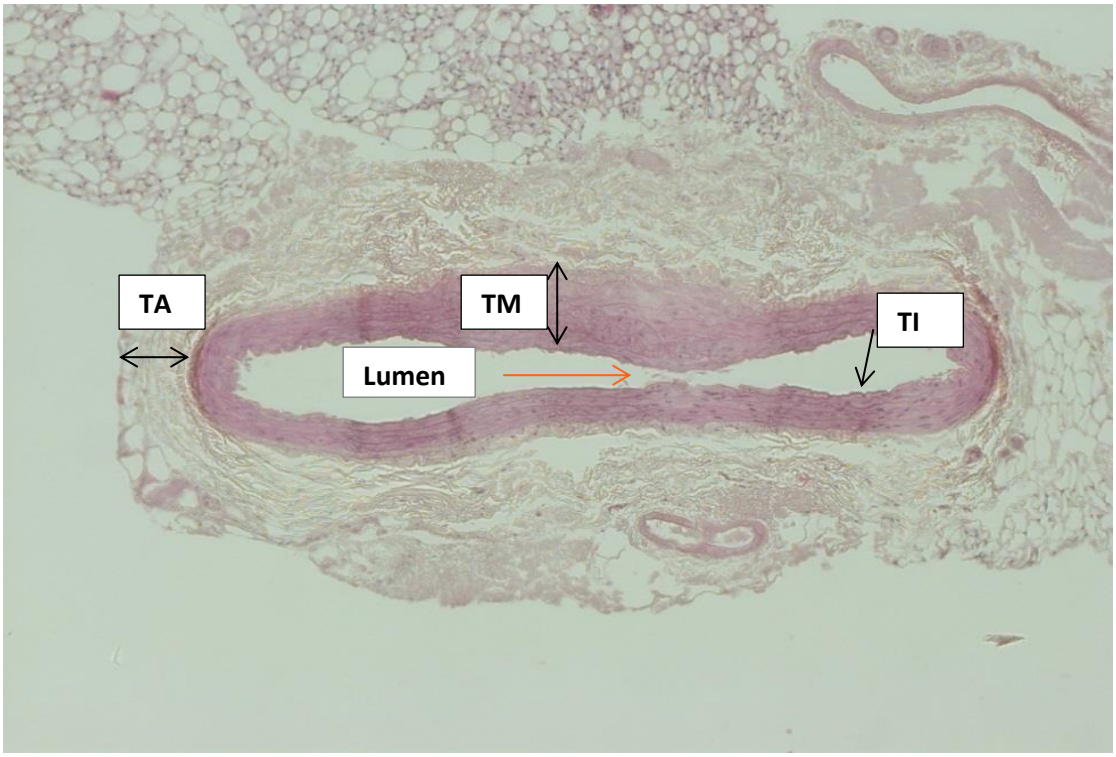


Figure 3.23: A photomicrograph (H&E) depicting a cross section through an artery of a diabetic rat (ZDSD at 28 weeks). A significant reduction of the lumen (orange arrow) is noted in this artery when compared to its non-diabetic counterpart. TM= tunica media and TA= tunica adventitia (x10 objective).

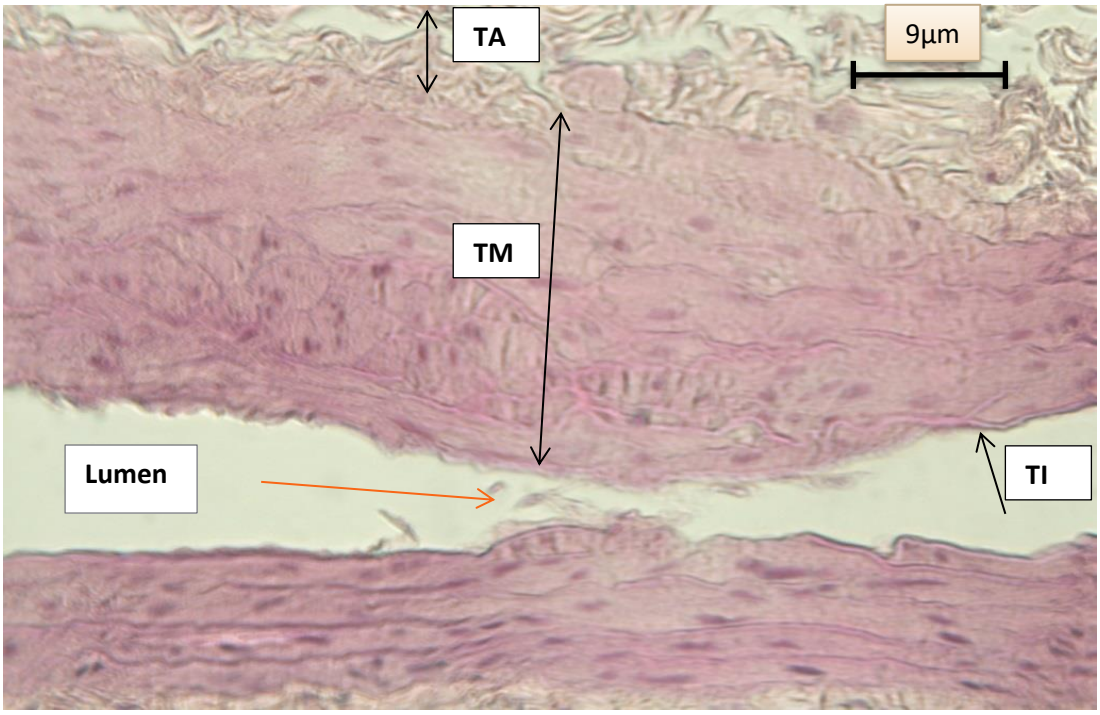


Figure 3.24: A photomicrograph (H&E) depicting the overall arterial wall structure and lumen in an artery of a diabetic rat (ZDSD) at 20 weeks of age. This artery depicts a reduction

in the size of the lumen (orange arrow) when compared to its non-diabetic counterpart. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

3.4.3 Measuring arterial wall thickness

The arterial wall thickness was measured from forty eight (48) H&E section slides, with one measurement per slide. The slides were viewed under the Carl Zeiss microscope, at various objectives (x5, x10 and x40). Pictures of the sections were taken using the Axiocam, in both the SD (group 1 and 2) and ZDSD (group 1 and 2) groups. For the purpose of this study, only pictures taken at x40 objective were measured. The application Image J ® was employed for measuring the arterial wall thickness, from the luminal aspect of the tunica intima to the internal border of the tunica adventitia (**Figure 3.25**). The scale measurement from Image J was calibrated in pixels and then converted into micrometers (µm) using an Olympus Objective Micrometer ruler (Olympus, Japan); which was viewed under the microscope at different objective (5x, 10x and 40x). The scale bar was measured to be approximately 39 pixels per µm at x40 objective, given that only pictures viewed under the same objective were measured. **Figure 3.25** illustrates the borders used to calculate wall thickness, which was found to be approximately 9.0µm thick in the non-diabetic (SD) groups.

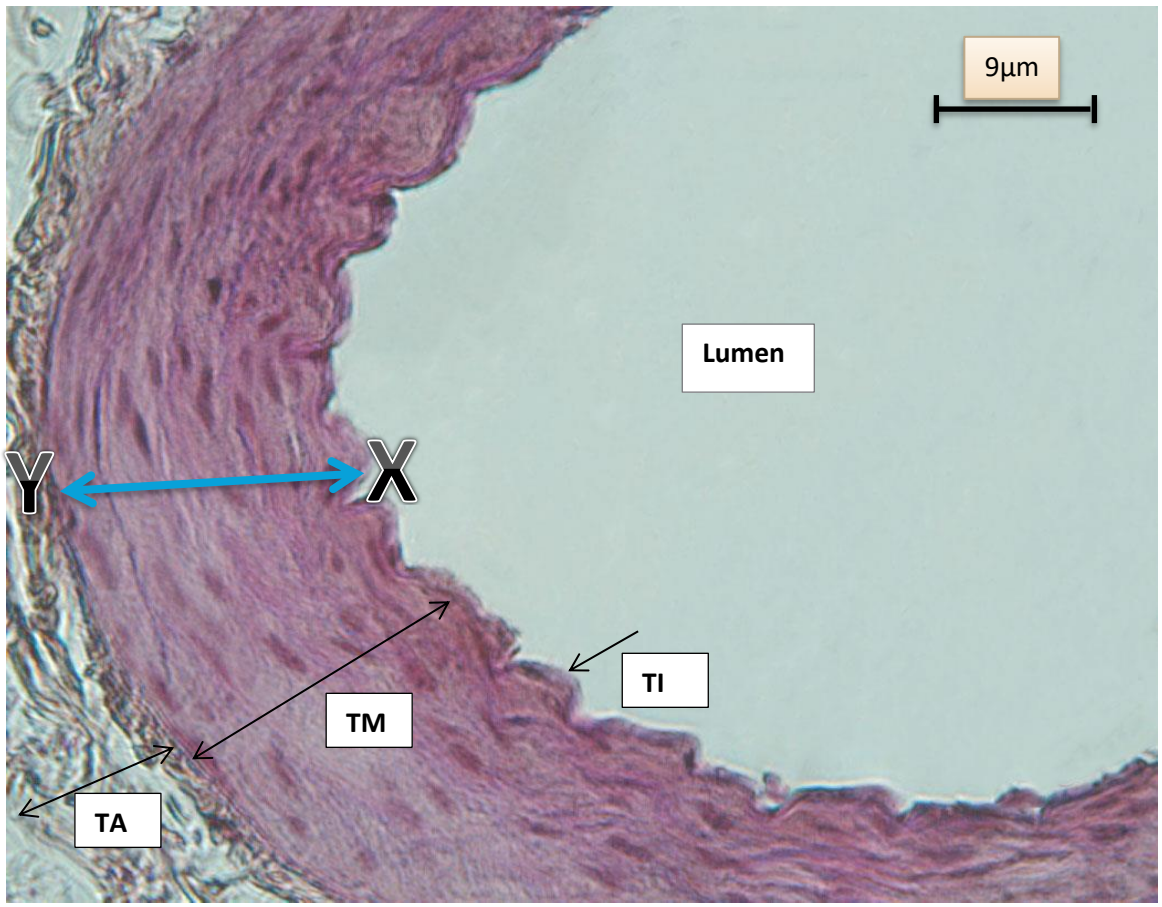


Figure 3.25 An H&E photomicrograph of a cross section through the abdominal aorta, depicting the wall thickness; from the luminal aspect of the tunica intima (**X**) to the internal border of the tunica adventitia (**Y**). Normal wall thickness in the non-diabetic (SD) group is~ 9µm. TI= tunica intima, TM= tunica media and TA=tunica adventitia (x40 objective).

The first segment of the artery (Part A) was measured initially and the last segment (Part C) followed. For Part A, the average wall thickness in the first SD group (up to 20 weeks) was found to be approximately 9.5µm (s= ± 1.25) **(Table 3.20)**. The ZDSD counterparts (Part A at 20 weeks) were found to have thicker arterial walls, with an average arterial wall thickness of approximately 10,8 µm (s= ± 2.24); demonstrating an approximate 12% difference in wall thickness when compared to the SD group**. Table 3.20** is an illustration of the different wall measurements and **Figure 3.26** demonstrates the different trends that were found between these groups at Part A of the artery.

Table 3.20: A tabulation of the arterial wall thickness (µm) (Part A of the artery) amongst the SD (**A**) and ZDSD (**B**) groups at 20 weeks of age. ZDSDs demonstrate thicker arterial walls (mean= 13.1 µm ±2.24) when compared to SDs (mean= 9.2 µm ±1.25).

A.

Rat no.	Wall thickness
SD 1	9.9
SD 2	7.7
SD 3	8.8
SD 4	10.0
SD 5	10.9
SD 6	8.0
Mean	**9.2** s= ±1.25

B.

Rat no.	Wall thickness
ZDSD 1	16.8
ZDSD 2	12.2
ZDSD 3	10.5
ZDSD 4	13.3
ZDSD 5	14.3
ZDSD 6	11.5
Mean	**13.1** s= ±2.24

For the second SD group (up to 28 weeks), at Part A of the artery, the average wall thickness was found to be approximately 8.4 µm (s= ±1.38) (**Table 3.21**) when compared to the ZDSD counterparts, with an average wall length of approximately 13.3 µm (s= ±3.42); demonstrating thicker arterial walls in these diabetic animals ($p<0.05$). **Table 3.21** and **Figure 3.26** illustrate these findings.

Table 3.21: A tabulation of the arterial wall thickness (µm) at Part A amongst the SD (**A**) and ZDSD (**B**) groups at 28 weeks of age. ZDSDs demonstrate thicker arterial walls (mean= 13.3 µm, s= ± 3.42) compared to SDs (mean= 8.4 µm, s= ± 1.38).

A.

Rat no.	Wall thickness
SD 1A	6.1
SD 2A	6.9
SD 3A	8.2
SD 4A	8.4
SD 5A	9.4
SD 6A	9.4
Mean	**8.4** s= ±1.38

B.

Rat no.	Wall thickness
ZSDS 1A	13.6
ZDSD 2A	16.8
ZDSD 3A	14.0
ZDSD 4A	14.5
ZDSD 5A	19.4
ZDSD 6A	16.0
Mean	**13.3** s= ±3.42

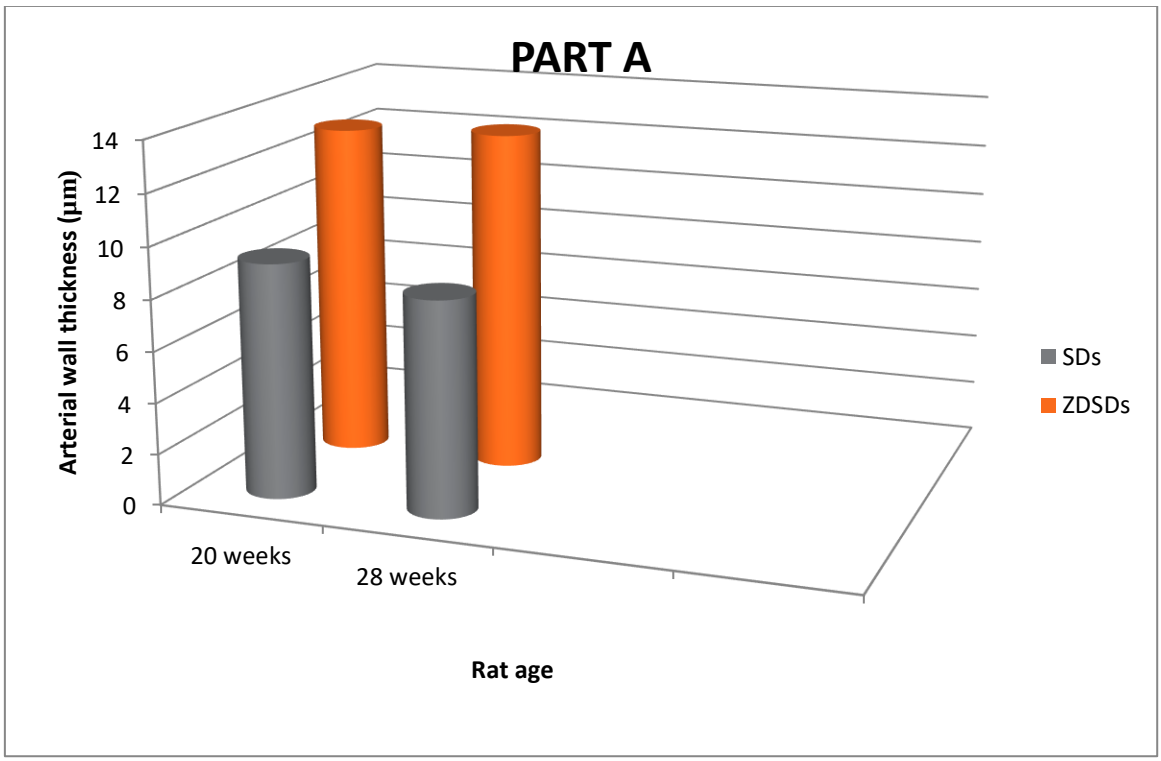


Figure 3.26: An illustration of the average arterial wall thickness (µm) at **Part A** of the artery, amongst the SD and ZDSD groups at week 20 and week 28 of age. The ZDSD group demonstrates thicker arterial walls when compared to the SD group.

The second segment of the artery (Part C) in the first SD group (up to 20 weeks) was found to be approximately 9.5µm (s= ±1.31) thick **(Table 3.22)** when measured; proving to have slightly thicker walls when compared to Part A of the artery. The ZDSD counterparts (Part C at 20 weeks) were found to have thicker arterial walls, with an average arterial wall thickness of approximately 12,8µm (s= ±3.55) , demonstrating an approximate 35% difference in wall thickness ($p<0.05$). **Table 3.22** and **Figure 3.27** illustrate the wall thickness amongst these two groups.

Table 3.22: A tabulation of the arterial wall thickness (µm) at Part C of the artery amongst the SD (**A**) and ZDSD (**B**) groups at 20 weeks of age. ZDSDs demonstrate thicker arterial walls (mean= 12.8 µm ± 3.55) when compared to SDs (mean= 9.5 µm ± 1.31).

A.

Rat no.	Wall thickness
SD 1C	11.0
SD 2C	7.5
SD 3C	8.7
SD 4C	10.0
SD 5C	10.2
SD 6C	8.5
Mean	**9.5** s= ±1.31

B.

Rat no.	Wall thickness
ZSDS 1C	10.9
ZDSD 2C	15.9
ZDSD 3C	14.8
ZDSD 4C	17.7
ZDSD 5C	13.7
ZDSD 6C	17.4
Mean	**12.8** s= ±3.55

For Part C of the artery, in the second SD group (up to 28 weeks) of animals, the average wall thickness was found to be approximately 8.8 µm (s= ±1.53) when compared to the ZDSD counterparts, which were found to have an average wall thickness of approximately 14.6 µm (s= ±2.91); demonstrating significantly thicker ($p<0.05$) arterial walls in these diabetic

animals. **Table 3.23** and **Figure 3.27** illustrate the arterial wall thickness between these two groups.

Table 3.23: A tabulation of the arterial wall thickness (µm) (Part C) amongst the SD and ZDSD groups at 28 weeks of age. ZDSDs demonstrate thicker arterial walls (mean= 14.6 µm ± 2.91) compared to SDs (mean= 8.8 µm ±1.53).

A.

Rat no.	Wall thickness
SD 1C	8.1
SD 2C	7.7
SD 3C	9.0
SD 4C	11.7
SD 5C	8.4
SD 6C	7.6
Mean	**8.8** s= ±1.53

B.

Rat no.	Wall thickness
ZSDS 1C	14.3
ZDSD 2C	12.9
ZDSD 3C	10.8
ZDSD 4C	19.5
ZDSD 5C	14.7
ZDSD 6C	15.6
Mean	**14.6** s= ±2.91

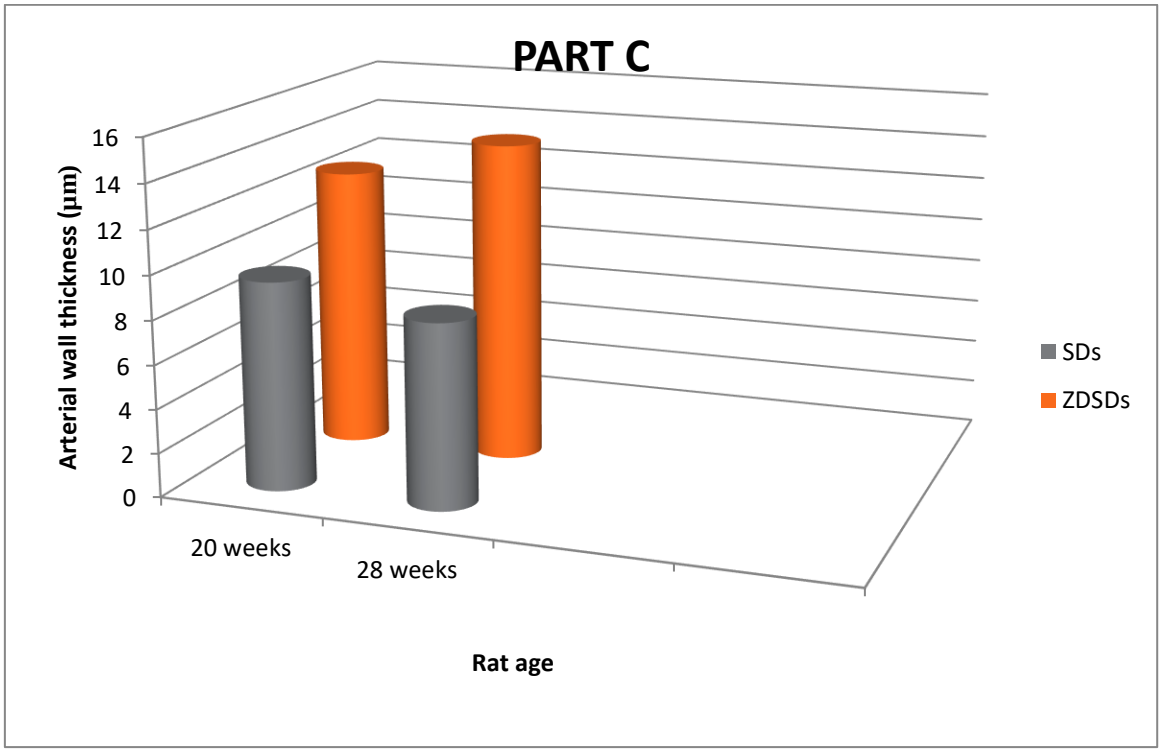
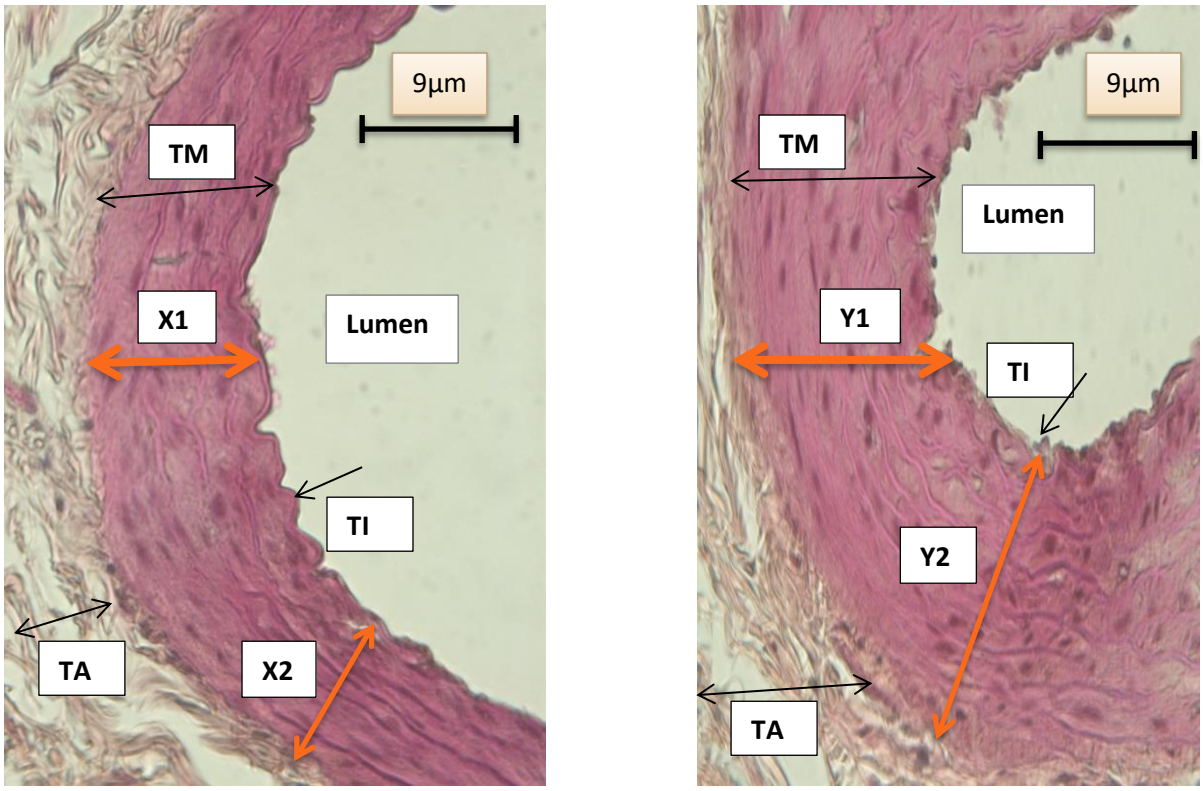


Figure 3.27: An illustration of the arterial wall thickness (µm) at **Part C** of the artery, amongst the SD and ZDSD groups at 20 weeks of age. The ZDSD group demonstrates thicker arterial walls when compared to the SD group.

Both Parts A and C of the artery proved to have significantly thicker walls in the ZDSD groups; compared to their SD counterparts. **Figure 3.28** depicts the actual wall thickness differences that were observed.



A. **B.**

Figure 3.28: An illustration of the arterial wall thickness amongst the SD (**A**) and ZDSD (**B**) groups. **A.** Demonstrates normal arterial wall length in a non-diabetic rat (**X1**= 9.3 µm) and **B.** demonstrates a thicker arterial wall (**Y1**= 13.1- 18 µm) in a diabetic rat. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

3.5 The Weigert's elastic stain

The Weigert's stain is particularly used to illuminate the elastic lamellae embedded in between the smooth muscle cell layers in the walls of large (elastic) arteries. The abdominal aorta is an example of an elastic artery and was used in this study. The number and integrity

of elastic lamellae, together with smooth muscle cell layer number (each layer containing one smooth muscle cell layer) in the tunica media; was of interest with this particular stain.

3.5.1 Number of elastic lamellae within the abdominal aorta's tunica media

The Weigert's elastic stain gives the elastic lamellae a blue-black appearance between the smooth muscle cells, as each layer of smooth muscle cells rests upon an elastic lamella. The non-diabetic arteries (SD groups) were found to have about 6 layers of smooth muscle cells, stacked within 5 concentric and continuous elastic lamellae within the tunica intima and tunica media combined. **Figure 3.29** is an illustration of the smooth muscle cells and the elastic lamellae they rest upon.

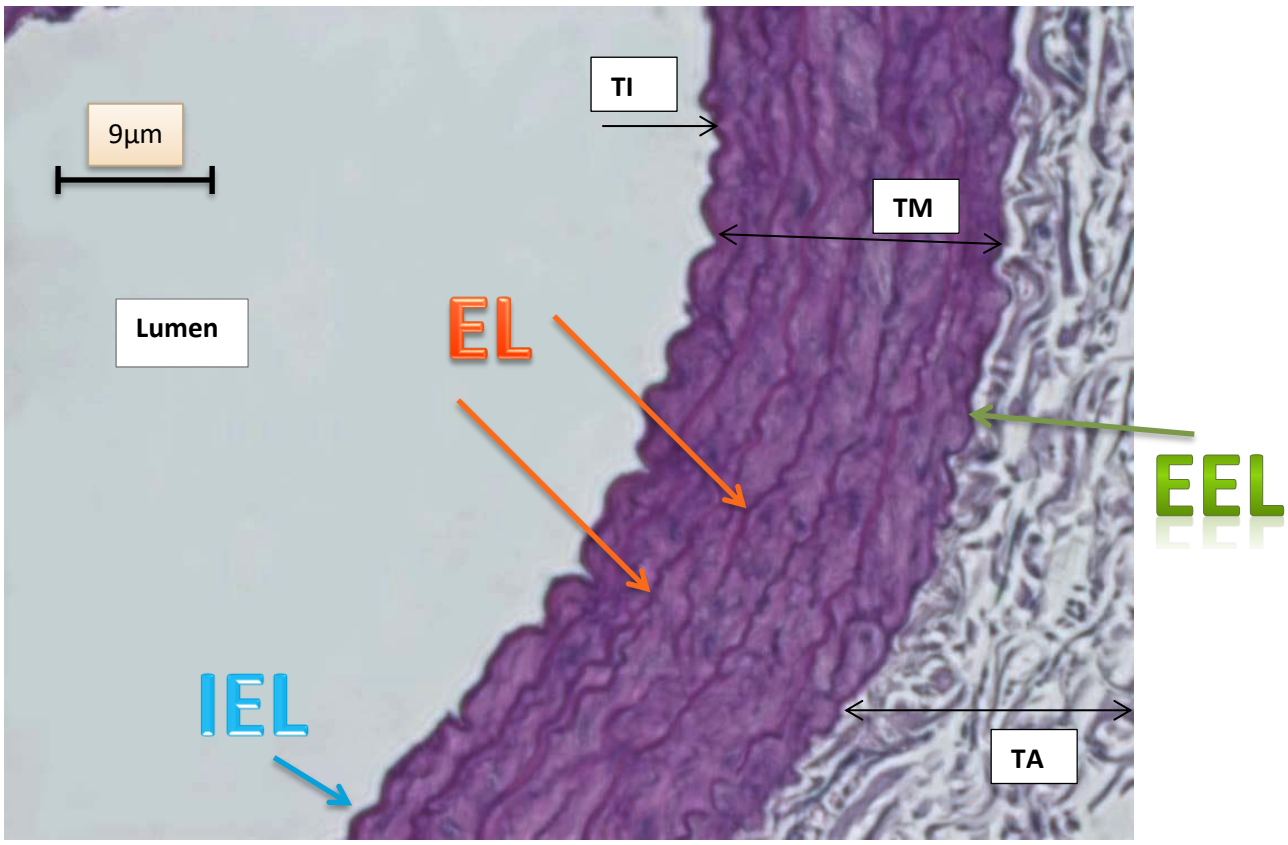


Figure 3.29: A photomicrograph depicting a Weigert's elastic stain in an abdominal aorta of an SD (non-diabetic) rat at 20 weeks of age. Note the slightly convoluted appearance of the internal elastic lamella (IEL) (blue arrow) and the external elastic lamella (EEL) (green arrow). The tunica media (TM) possesses +-5 intact and continuous elastic lamellae (EL) (orange arrows), where the smooth muscle cells rest upon. TI= tunica intima and TA= tunica adventitia (x40 objective).

The diabetic arteries (ZDSD groups) on the other hand were found to have between 2 and 4 layers of smooth muscle cells and the elastic lamellae they rest upon were not as clearly defined as those in the SD groups. The elastic lamellae appeared to be discontinuous, non-concentric and did not absorb the Weigert's stain as effectively, compared to their non-diabetic counterparts. The smooth muscle cells were arranged haphazardly and were not clearly seen to be resting on the elastic lamellae when compared to the SDs. **Figure 3.30** and **Figure 3.31** illustrate the smooth muscle cells and elastic lamellae in some of the diabetic arteries.

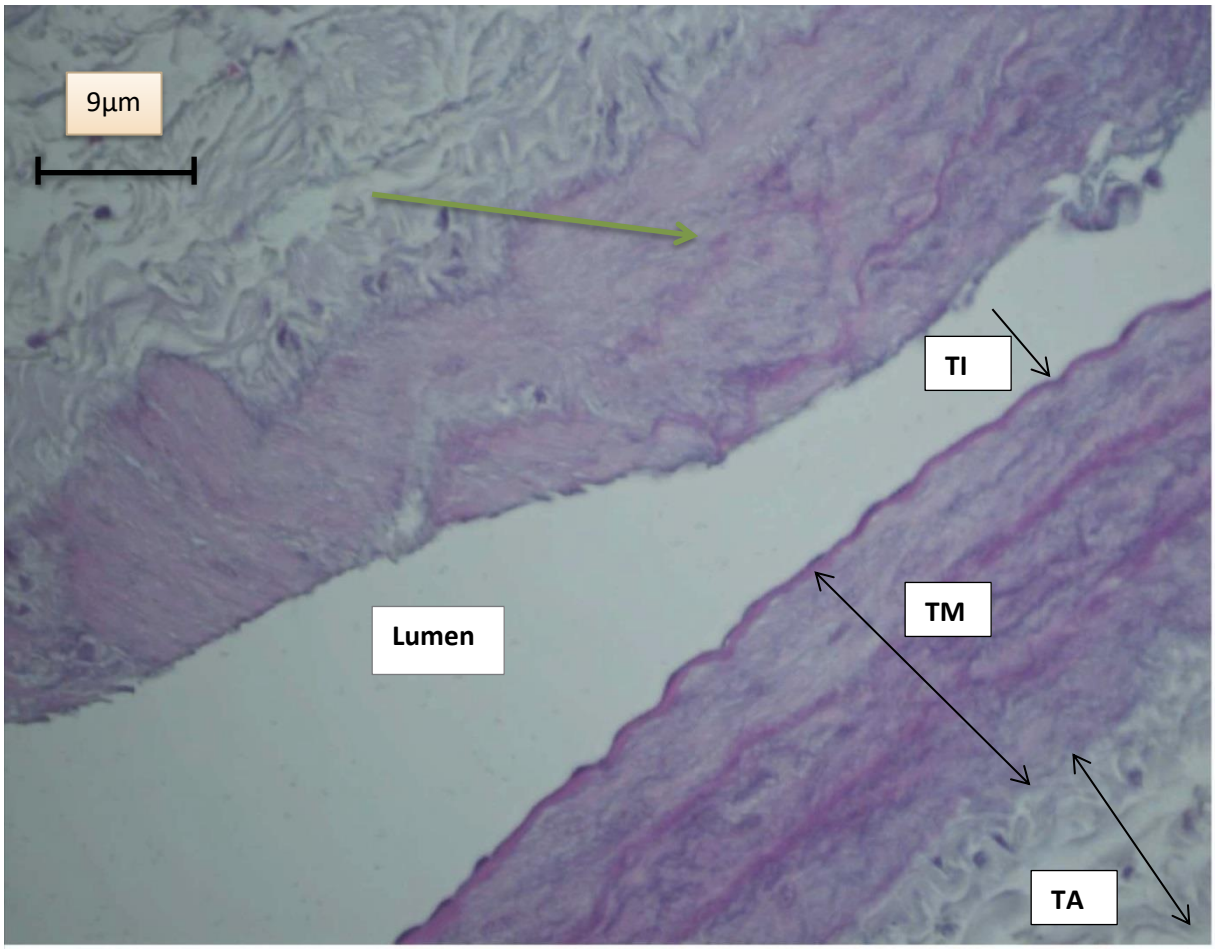


Figure 3.30: A photomicrograph depicting a Weigert's elastic stain in the abdominal aorta of a ZDSD (diabetic) rat at 20 weeks of age. Elastic lamellae (+-2) appear to be discontinuous, non-concentric (green arrow) and did not absorb the Weigert's stain when compared to their non-diabetic counterparts. The internal and external elastic lamellae are not well defined. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

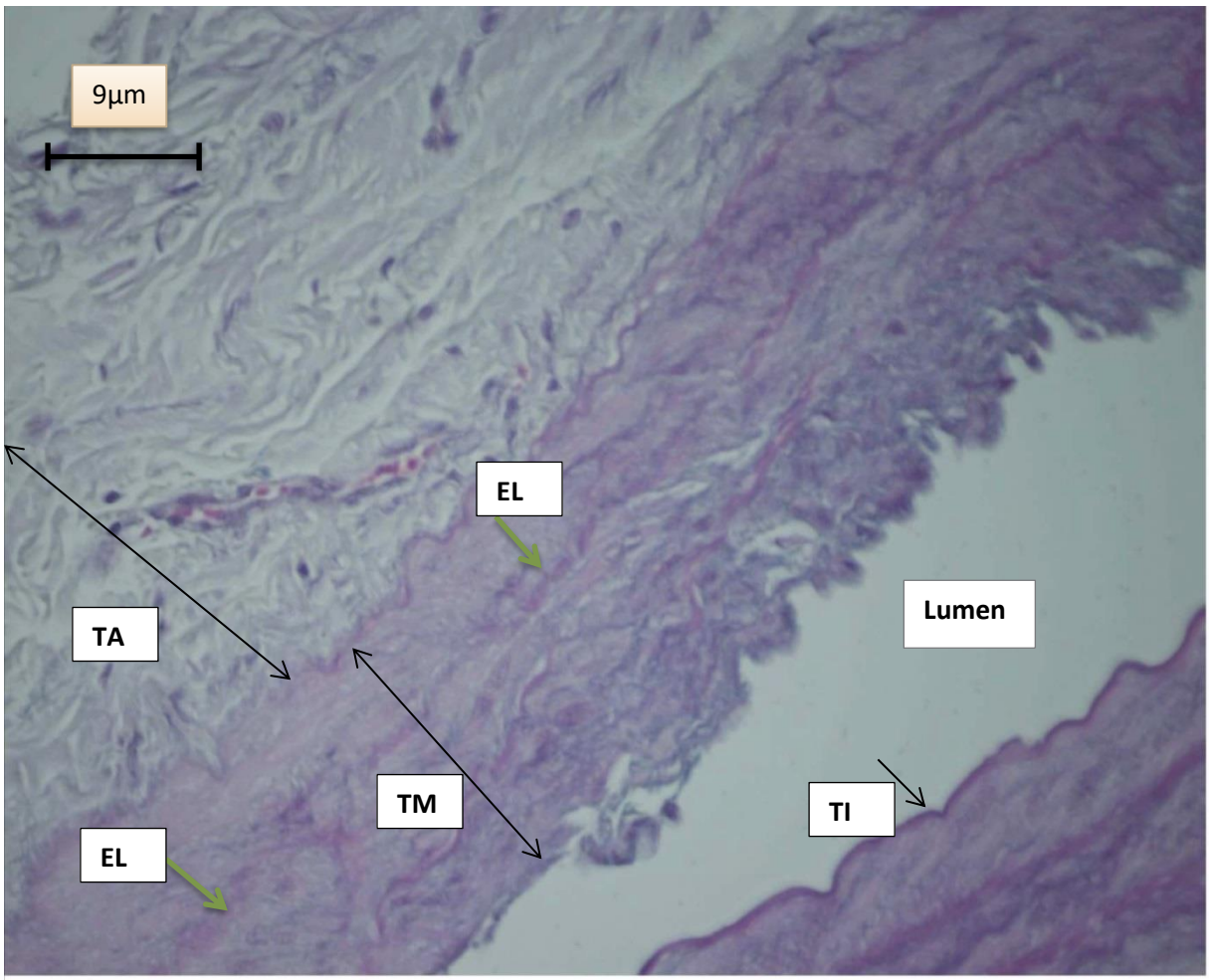


Figure 3.31: A photomicrograph depicting a Weigert's elastic stain in an abdominal aorta of a ZDSD (diabetic) rat at 20 weeks of age. Elastic lamellae (EL) (+-2) appear to be discontinuous, non-concentric (green arrows) and did not absorb the Weigert's stain when compared to their non-diabetic counterparts. The internal and external elastic lamellae are not well defined. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

3.6 Immunohistochemistry for Cluster of differentiation 40

The current study, in effort to further investigate some aspects of inflammation, employed immunohistochemical methods to reveal for Cluster of differentiation 40 (CD40). CD40 is one of the Immuno-globulin G (IgG) antigens produced during an immune response and is an inflammation marker. During analysis of these sections, it was noted that antigens for CD40 were present in both the diabetic and non-diabetic groups, to some degree. The non-diabetic group was found to have some CD40 antigens, especially in the tunica adventitia; but to a small degree, as depicted by the presence of a brown colour in the positively stained sections. **Figure 3.32A** and **Figure 3.32B** is an illustration of the CD40 immunohistochemistry stain in the non-diabetic artery.

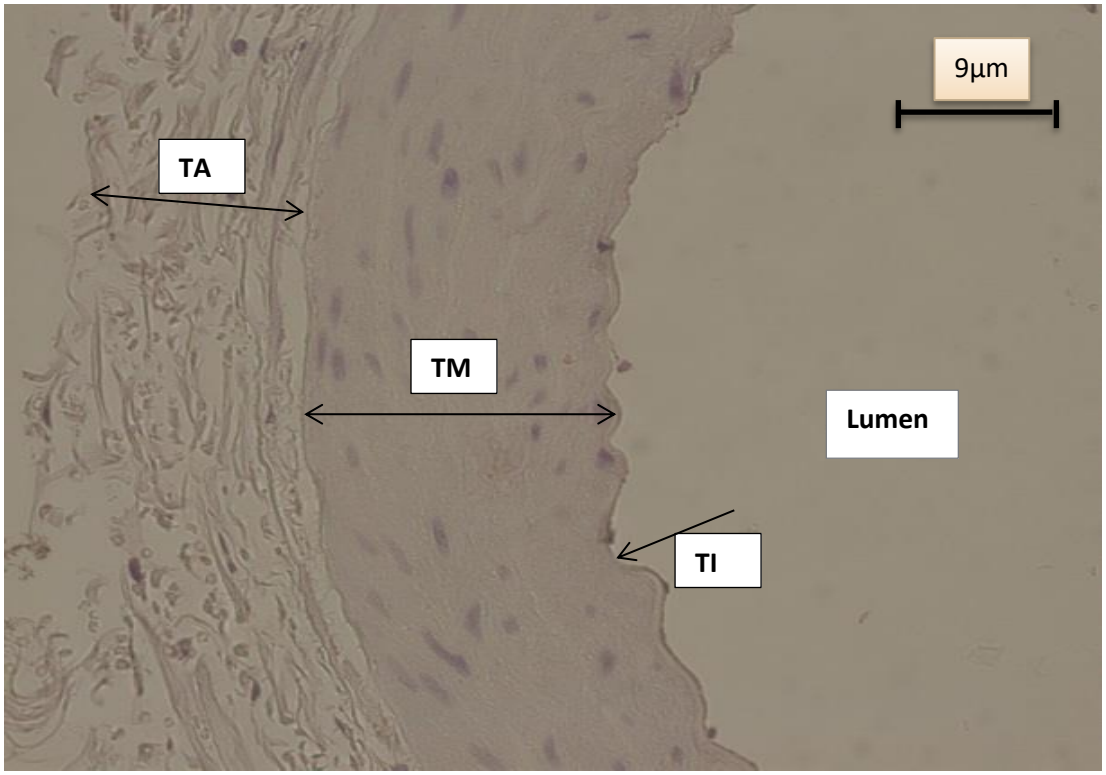


Figure 3.32A: A photomicrograph depicting a negative CD40 immunohistochemistry stain in the abdominal aorta of an SD (non-diabetic) rat at 20 weeks of age. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

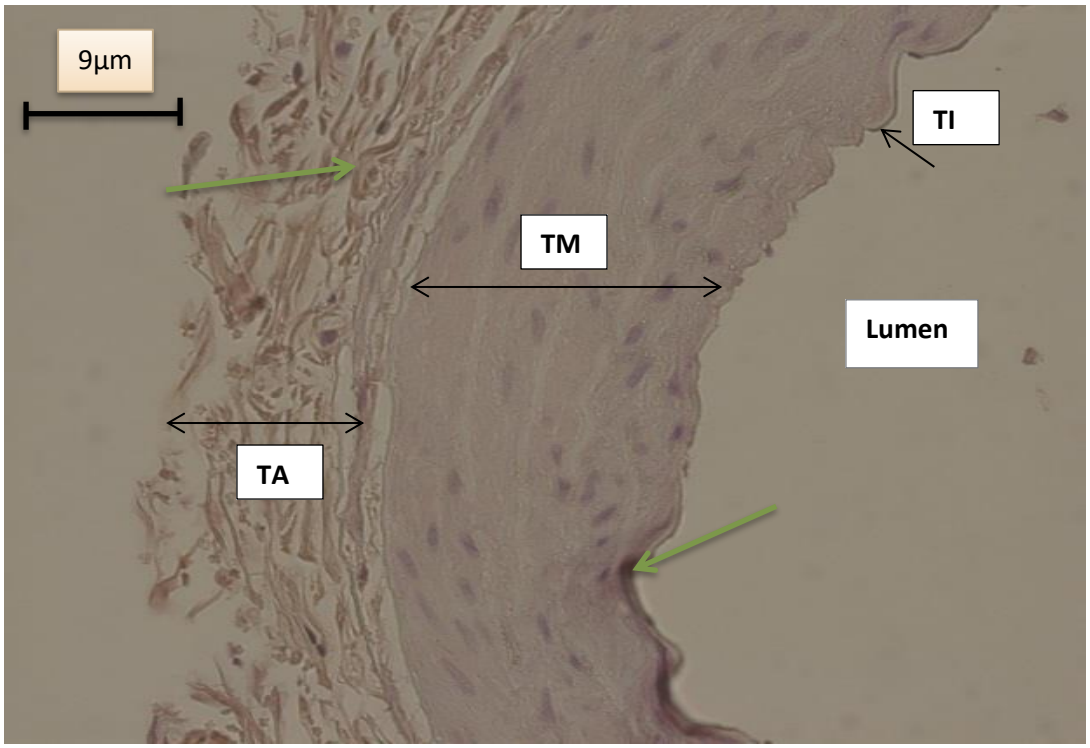


Figure 3.32 B: A photomicrograph depicting a positive CD40 immunohistochemistry stain (green arrows) in the abdominal aorta of an SD (non-diabetic) rat at 20 weeks of age. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

The diabetic arteries were also observed to possess CD40 in a manner similar to that seen in the non-diabetic group in some sections, especially in the diabetic group at 20 weeks. The CD40 was not as pronounced in the ZDSD group at 20 weeks, while some sections appeared to have a significant positive stain, especially in the older group (28 weeks). **Figure 3.33A and Figure 3.33B** illustrate the minimal CD40 presence observed in some of the diabetic arteries while **Figure 3.34** depicts sections with intense CD40 presence in the tunica adventitia of the ZDSDs at 28 weeks, as evidenced by the dark brown colour.

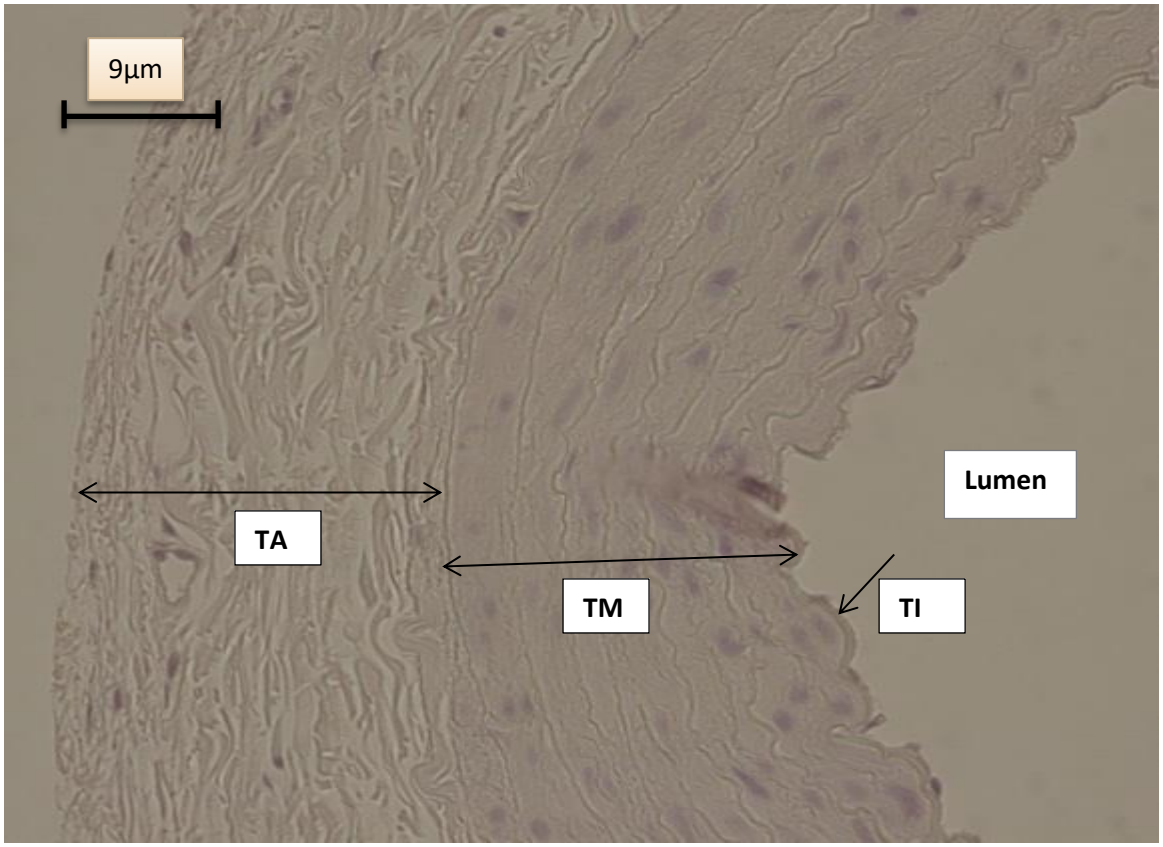


Figure 3.33A: A photomicrograph depicting a negative control for CD40 immunohistochemistry stain in the abdominal aorta of a ZDSD (diabetic) rat at 20 weeks of age. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

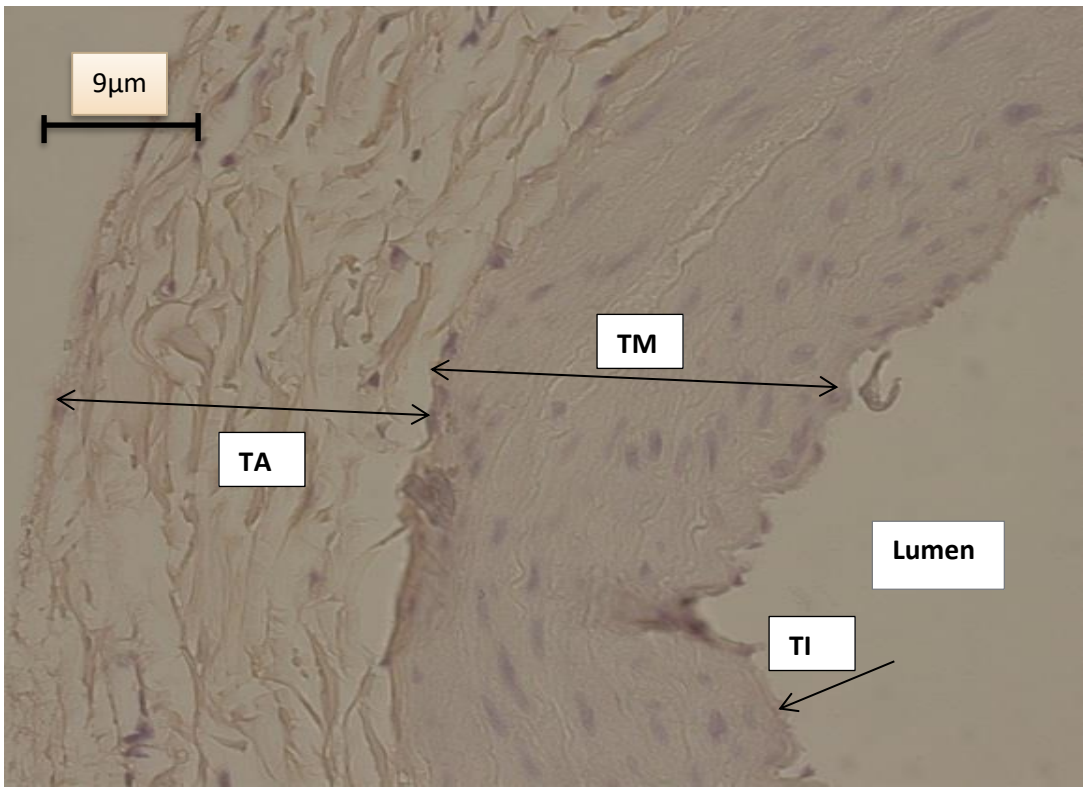


Figure 3.33B: A photomicrograph depicting a positive CD40 immunohistochemistry stain in the abdominal aorta of a ZDSD (diabetic) rat at 20 weeks of age. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

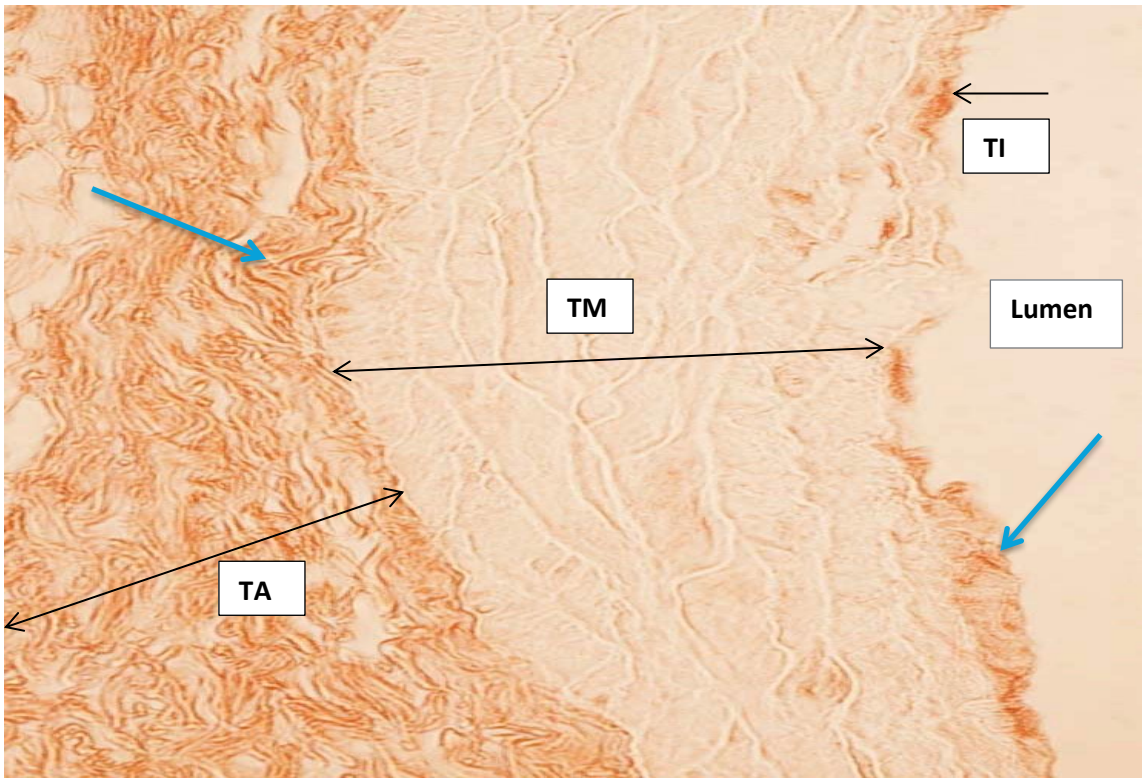


Figure 3.34: A photomicrograph depicting an intense positive stain for CD40 immunohistochemistry (blue arrows) in the abdominal aorta of a ZDSD (diabetic) rat at 28 weeks of age, suggesting inflammation in this artery. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

3.7 Immunohistochemistry for Collagen type 8 alpha 2 (Col8A2)

The presence of collagen in the atherosclerotic plaque is thought to contribute to the plaque's integrity by providing structural stability and strength; and research is still lacking in this field. The antibody used for this purpose was specific for human tissue (rat-specific antibody was unobtainable at the time of research) and for this reason; the result might have been negatively affected since the tissue used in the study was from rat models. The non-diabetic arteries appeared to have some presence of Col8A2 in the tunica adventitia (TA) and tunica intima (TI), as depicted by presence of a brown colour, denoting a positive stain. The diabetic arteries, especially those at 28 weeks, appeared to have an increased presence of Col8A2, in all the tunics of the arterial wall, as depicted by the presence of a brownish colour, denoting a positive stain. **Figure 3.35** and **Figure 3.36** depict a Col8A2 negative control and positive stain respectively, seen in some of the non-diabetic arteries while **Figure 3.37** and **Figure 3.38** depict a Col8A2 positive stain seen in some of the diabetic arteries.

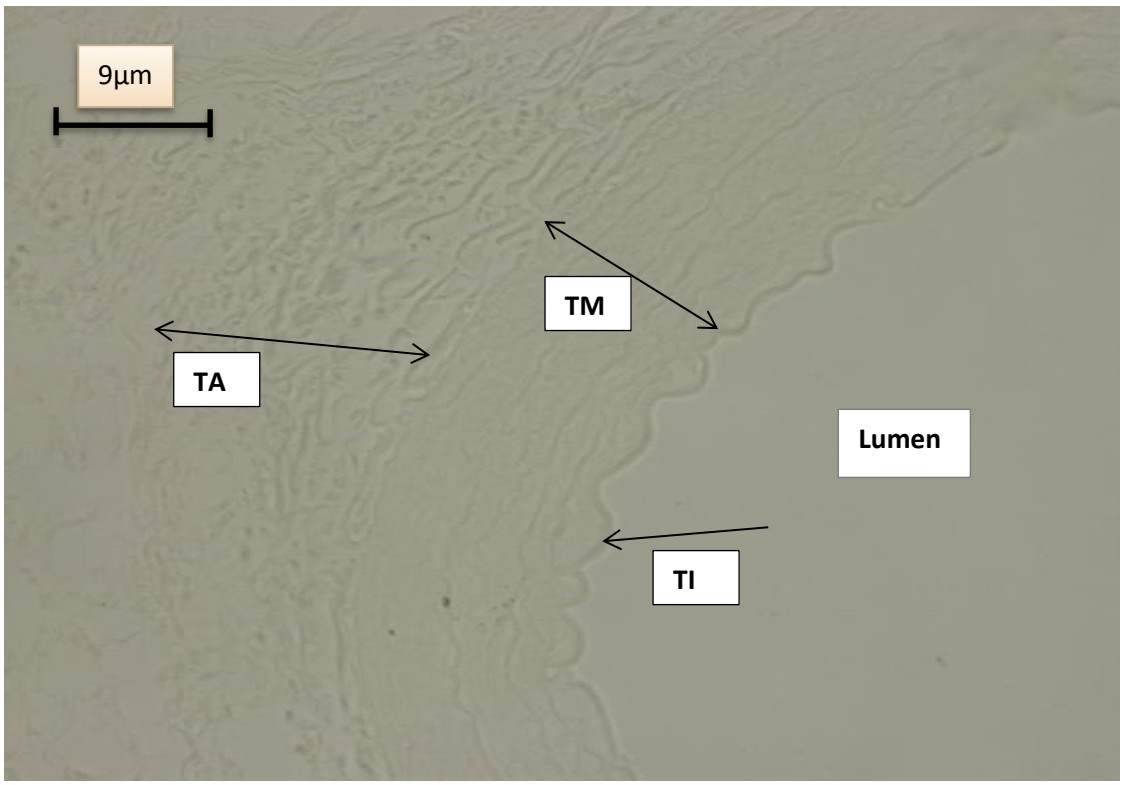


Figure 3.35: A photomicrograph of an artery (SD) depicting a negative Col8A2 antibody stain at 28 weeks of age. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

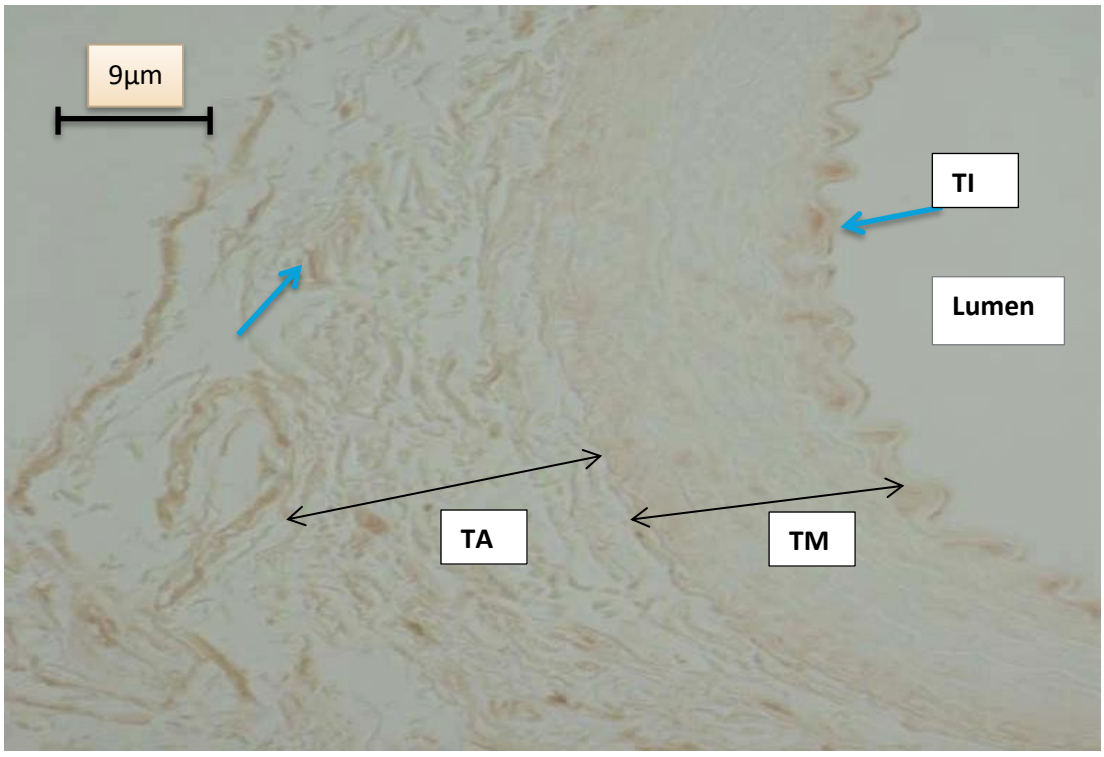


Figure 3.36: A photomicrograph of an artery (SD) depicting a positive Col8A2 antibody stain at 28 weeks of age. Blue arrows show a positive stain in the tunica intima, tunica media and

tunica adventitia. TI= tunica intima, TM= tunica media and TA= tunica adventitia (x40 objective).

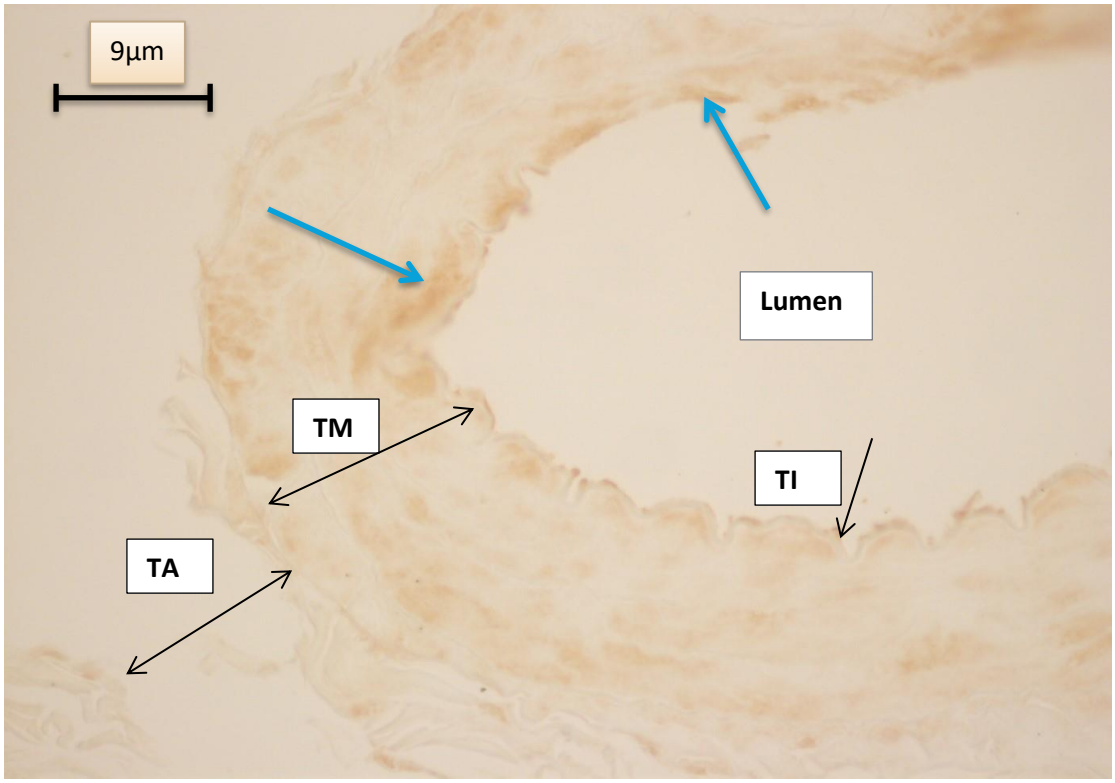


Figure 3.37: A photomicrograph of an artery in a diabetic rat (ZDSD at 20 weeks of age) depicting a positive Col8A2 antibody stain (blue arrows) in the tunica intima (TI) and tunica media (TM). TA= tunica adventitia (x40 objective).

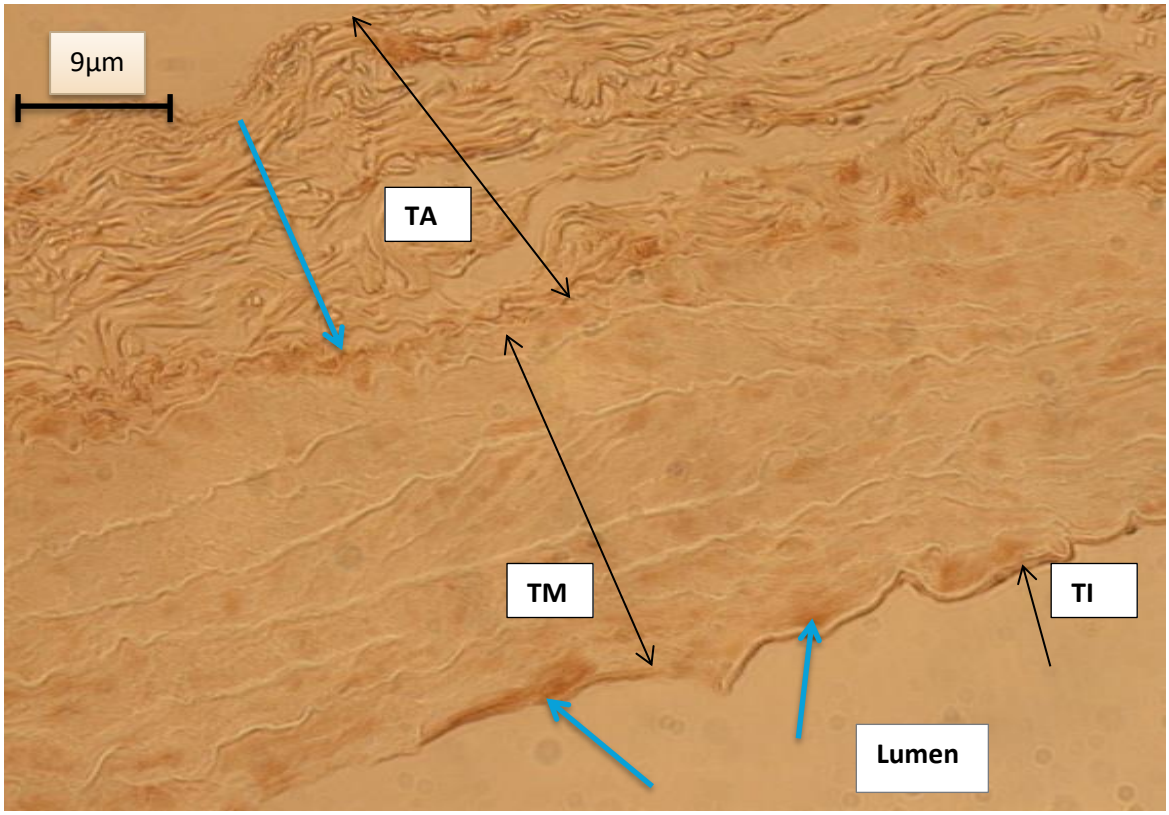


Figure 3.38: A photomicrograph of an artery in a diabetic rat (ZDSD at 28 weeks of age) depicting a positive Col8A2 antibody stain (blue arrows) in the tunica media (TM) and tunica adventitia (TA). TI= tunica intima (x40 objective).

CHAPTER 4: DISCUSSION

Type 2 Diabetes Mellitus is currently among the ten leading causes of death globally and presents both a tremendous burden of disease upon economies and compromise to daily lives in adults; it is estimated to afflict approximately 9% of South Africa's population over 30 years of age. Chief among the challenges faced in combating Type 2 Diabetes is the lack of understanding of the microscopic anatomy and physiological mechanisms involved in the disease. Diabetes leads to several secondary morbidities which may compromise quality of life and even lead to death. Atherosclerosis is one such example and was studied in more detail in the current work. The mechanisms involved in the atherogenic process are still not fully understood, and this work endeavours to unveil more detail on the characteristics of this disorder. The objectives of the current study were therefore to examine the vessel wall of the rat aorta in the diabetic and non-diabetic disease scenario with respect to its thickness, its general structural appearance and composition, and to note any evidence of plaques within the wall.

The analyses undertaken in the current study revealed several findings of interest. Firstly, the two animal groups (SDs and ZDSDs) appeared to have followed the same characteristics as those described in previous studies regarding the diets given to the animals and the development of the metabolic syndrome; with some exceptions noted. T2DM was confirmed in the ZDSD rats by a fasting blood glucose reading greater than 7.0mmol/l, as suggested by the WHO (2017). Secondly, the presence of inflammation was observed to some extent, as evidenced by the presence of CD40 antigens. T2DM was seen to have resulted in dyslipidaemia in the ZDSD group, where increased plasma triglycerides (TGs) were observed but cholesterol levels were seen to fall within normal ranges. The narrowing of arteries (stenosis) was observed in the diabetic subjects, suggestive of an atherosclerotic process; as described by Aronson & Rayfield (2002).

4.1 Body mass

The two animal groups were given different diets and the effects of these diets were evident at the time of termination. The first SD group (20 weeks) was given a high fat diet whereas the second group (28 weeks) was given a normal diet. The first SD group demonstrated to have developed obesity with the high fat diet whereas the second group had normal body mass. The ZDSD groups had similar starting body masses as they were fed the same normal diet initially and then fed a high fat diet later. The ZDSD groups were not obese at the time of termination

as they demonstrated body mass loss attributed to the development of T2DM. Initially the ZDSD groups had been earmarked to be given the same diets as the SD groups; i.e. the fatty diet for group 1 and the normal diet for group 2; however, it was established early on in the study that the ZDSD animals given the high fat diet at the start, tended to gain body mass too rapidly and died spontaneously before reaching the first week of termination (Week 20). The protocol was then adjusted to avoid this extreme body mass gain as these animals are not to be genetically prone to obesity. The decision was therefore taken to give the high fat diet to the ZDSD animals starting at week 15 rather than birth as had been done for the SD rats.

4.2 Type 2 diabetes mellitus

It is stated that the ZDSD rat develops T2DM in a manner that mirrors the development of the metabolic syndrome in humans and hence it was chosen for this study. It is also known that early fat feeding (from 7 weeks of age) in the ZDSD rats would result in the development of obesity; which is then followed by diabetes occurrence; however, commencing these rats on a high fat diet results in the spontaneous development of diabetes, without necessarily developing obesity.The SD rats proved to be free of T2DM despite having been given a normal (group 2) and a high fat diet (group 1); while the ZDSD rats developed T2DM in a manner that followed the classical metabolic syndrome described above. Hamed (2016) suggested that diabetes mellitus is characterised mainly by obesity and hyperglycaemia, however, this statement, especially regarding the obesity was found to be arguable to a certain degree. The ZDSDs were diagnosed to have developed T2DM around week 16 of age but were never noted to have developed obesity at any stage prior to their termination dates. These results demonstrate that, commencing the ZDSD rats on a high fat diet results in the spontaneous development of diabetes, but not necessarily the development of obesity prior to diabetes.

4.3 Dyslipidaemia in T2DM

As suggested by Mooradian (2009), T2DM resulted in dyslipidaemia in the ZDSD group, where increased plasma triglycerides were observed but cholesterol levels were seen to fall within normal ranges. The SD group which was given a normal diet demonstrated to have normal triglyceride levels while the other SD group (on high fat diet) demonstrated increased triglyceride levels. This suggested that cholesterol levels do not necessarily increase in the case of diabetes, but triglycerides do as evidenced by normal cholesterol levels in all groups diabetic and non-diabetic; yet increased triglyceride levels in the diabetic groups as opposed

to the non-diabetic groups. The SD group given the high fat diet did show an elevated level of triglycerides, and this is to be expected when consuming a diet high in fat.

4.4 Atherosclerosis

Adiguzel et al. (2009) suggested that arteries in atherosclerosis will exhibit, among other features, a reduced luminal diameter (stenosis) due to a combination of changes including deposition of plaques within the tunica intima. This was seen in the diabetic group, especially in the older animals (28 weeks), where an almost complete closure of the lumen was observed in this group when viewed in H&E sections. Yu et al. (2017) found that the location of an atherosclerotic plaque in the basilar artery is a reliable tool for predicting whether or not the resulting stenosis is symptomatic or not. The location of the atherosclerotic plaques in this study was mainly found in Part C of the arteries, where increased wall thicknesses, together with the presence of increased CD40 were observed. This finding is in line given that part C of the artery is closer to the major bifurcation into the common iliac arteries, meaning that thermodynamic forces are stronger in this part than in part A, the more proximal part of the aorta. The increased turbulence of blood in and around part C of the artery makes it more prone to developing these plaques (Prado *et al.*, 2008).

4.5 Cluster of Differentiation 40 presence and inflammation

Bastaa, Schmidt & Caterina, (2005) suggested that atherosclerotic lesions demonstrated diffuse extracellular, as well as dense intracellular, plaque-like depositions in macrophages and vascular smooth muscle cells. Their findings were confirmed in the current work, as immunohistochemical staining for Cluster of Differentiation 40 revealed positive staining, mainly in the connective tissue of the tunica adventitia and in the tunica intima. The positive staining revealed the presence of inflammation within the abdominal aorta which serves as evidence for atherosclerotic plaques at various stages of development.

4.6 Collagen type 8 alpha 2 presence

Collagen type 8 alpha 2 presence is said to be produced during arterial injury, where it stimulates an increase in smooth muscle cell migration and is expressed as a matrix protein in a number of tissues undergoing active remodelling, including injured arteries in human atherosclerotic plaques. This notion was confirmed in the current work as the presence of Collagen type 8 alpha 2 presence was noted in exaggerated amounts, especially in the diabetic group at 28 weeks of age.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Concluding remarks

It is noted that the diagnosis of T2DM results in complications that were previously discussed by other researchers and that the gap for improved treatment and management strategies exists. Given the burden posed by this metabolic condition, further research in the field is still highly needed to combat the costs of treating this disease with ultimate poor prognoses.

In conclusion, the present study demonstrated characteristics of diabetic atherosclerosis that are consistent for the most with documented and published literature. These include a reduced luminal diameter, thickened arterial walls, activated smooth muscle cells, disrupted elastic lamellae and the presence of inflammation and injury. These findings, more especially the presence of inflammatory markers and collagen 8, present a possibility of new approaches to treatment if these factors could be manipulated or exploited. For example, if the inflammatory process could be prevented chemically, plaques might perhaps be hindered from developing or slowed down at the least.

5.2 Recommendations

The field of diabetic research has developed various treatment options for the metabolic disorder, all of which manipulate the state of hyperglycemia to reduce the advancement and symptoms of diabetes. However, more is possible to do now that we know some of the mechanisms involved in the disease such as inflammation, arterial injury and smooth muscle cell activation and migration; which would reduce the occurrence of potentially fatal secondary morbidities such as atherosclerosis. These mechanisms could be exploited to avoid the development of such secondary conditions that complicate lives so severely and commonly.

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Solutions used in the study

1. **2% 3-aminopropyltriethoxysilane (APES) (for coating glass slides)**
2. **Neutral buffered formalin**
3. **10mM Sodium Citrate buffer pH6**
4. **1M Phosphate buffered saline (PBS) pH 7.4**
5. **3.3 Diaminobenzidine (DAB) working solution**
6. **De-waxing and hydrating steps**

1. **APES solution for coating glass slides**

-2ml Silane added into 98ml Acetone (100ml)

Steps:

- Immerse slides in APES for 30 minutes
- Rinse in 2x washes of acetone (x10 dips each)
- Rinse in distilled water (x10 dips)
- Leave to dry overnight at 37˚C
- Store at 4˚C for +/- 2 months

2. **Neutral buffered formalin for routine fixation**
-37-40% Formalin (100ml)

Steps:

- Sodium di-hydrogen phosphate 3.5g
- Di-sodium hydrogen phosphate 6.5g
- Distilled water 90ml

3. **10mM Sodium Citrate buffer pH6 for antigen retrieval**
-1000ml solution

Steps:

- Tri-sodium citrate (di-hydrate) 2.9g
- Distilled water 1000ml
- 0.05% Tween 20 (500µl)
- Mix to dissolve sodium citrate and adjust pH to 6 with 1M HCl
- Cover slides in solution and leave in water bath overnight at 60˚C

4. **1M Phosphate buffered saline (PBS) pH 7.4**
-2000ml solution

Steps:

- NaCl 16g
- Na2HPO4 3.5g
- KCl 0.4g
- KH2PO4 0.4g
- Distilled water 2000ml

5. **3.3 Diaminobenzidine (DAB) working solution**
   -Mix in a new bijou bottle

   Steps: add

   a)
   - 1mg DAB (0.001g)
   - 2ml Tris HCl 0.05M

   b) in a separate tube: add

   - 29µl distilled water
   - 1µl hydrogen peroxide

- Add 20µl of solution **b)** to DAB solution **a)**

6. **De-wax and hydrate prodecure**

   Steps:
   - Immerse slides in xylene for 20 minutes (x2)
   - Immerse slides in 100% ethanol for 1 minute (x2)
   - Immerse slides in 95% ethanol for 1 minute
   - Immerse slides in 70% ethanol for 1 minute
   - Wash in running tap water for 5 minutes

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ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/07/28/C

APPLICANT: Dr R Ndou

SCHOOL: School of Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: Bone integrity and health in type 2 diabetes using a rat model (Zucker Fatty Rat)

Number and Species

20 Male Zucker Fatty Rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/07/28. This approval remains valid until 2017/08/13.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 21st August 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 21 August 2015

cc: Supervisor: N/A
Director: CAS

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