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A comparative proteomic and glycoproteomic study of platelets from patients with diabetes and non-diabetic healthy individuals

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Declaration of Originality

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Subject of work: A comparative proteomic and glycoproteomic study of platelets from patients with diabetes and non-diabetic healthy individuals

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Ethics

This study was carried out under the guidelines of the Helsinki Protocol and the amendments' thereof. All blood collection was performed after informed consent was obtained from volunteering individuals. Ethical approval to conduct the study (reference: 358/2016), was obtained from the University of Pretoria's Faculty of Health Sciences Research Ethics Committee.

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Abstract

Keywords: Diabetes, Diabetic foot ulcers, Platelets, Glycation, Protein, SDS-PAGE, Mass spectrometry

Introduction: Diabetes mellitus is considered one of the four major non-communicable disease with the global prevalence of this disease nearly doubling over the past 40 years, making it one of the leading causes of morbidity and contributing to mortality.

Diabetes is characterized by elevated blood glucose levels due to insulin deficiency, resistance or a combination of both. The disease is associated with several complications, and some of these complications are attributed to glycation of proteins which impairs their function and stability. Diabetic foot ulcers (DFUs) are one of the most catastrophic and costly complications of diabetes. These foot ulcers do not naturally progress through the phases of the wound healing process and are therefore classified as chronic wounds. There is limited treatment availability for diabetic foot ulcers, and the current treatment strategies have been met with high rates of recurrence and failure. Therefore, there is need for more extensive research to be conducted to improve therapy and minimize the chance of developing complications. Blood platelets play a central role in initiation of wound healing and on account of this, the study was directed towards comparing platelet proteins from diabetic patients and non-diabetic healthy individuals to investigate the possible differences in protein expression and glycation. The aim was to characterize these proteins to further understand the role platelets play in impaired wound healing in DFUs and to provide a possible basis in developing targeted therapies.

Methods: All blood samples were tested for HbA1c levels which is an indicator of long term blood glucose levels. This was used as a screening tool to confirm participant status. After the screening test, non-stimulated platelets were isolated from whole blood of diabetic patients and non-diabetic healthy individuals, washed and the total protein complement separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gels were visualized using Stain-free™ imaging and then separately stained with Acqua (colloidal Coomassie blue), silver, Oriole™ or Periodic Acid-Schiff (PAS) stains for comparison. Following this, high performance

liquid chromatography (HPLC) coupled to fluorescence detection and western blotting were done to check for possible formation of advanced glycation end products (AGEs).

In-gel and in-solution trypsin digests of selected samples exhibiting protein band differences between the two groups were performed, followed by peptide sequencing using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Results and discussion: Gel electrophoresis results showed similarities in the general pattern of the protein mass fingerprint with subtle band differences identified between the two groups. Results from the PAS stain implied that there was no glycation of platelet proteins in diabetic patients, which led to the proposition that complex advanced AGEs may be forming. This was tested using HPLC with fluorescence detection of the trypsinized samples and the appearance of extra peaks from diabetic patient samples on the chromatograms obtained after HPLC analysis indicated that this was a possibility. Western blotting to confirm the formation of AGEs, showed similarities in the formation of AGEs between the two groups suggesting that there was no difference in AGE formation between diabetic patients and non-diabetic healthy individuals.

A few differences in platelet protein abundance were seen between the two groups when downstream LC-MS/MS analysis of the samples was done, which showed the superiority of the analysis technique over SDS-PAGE.

Conclusion: The study showed that there were no significant differences in glycation of proteins between the two groups which can possibly eliminate glycation as a potential cause of delayed healing of DFUs. However, LC-MS/MS analysis of samples identified proteins which had differences in abundance between the diabetic patients and non-diabetic healthy individuals, some of which are key proteins in the wound healing process. Therefore, based on these results, a proposition can be made that differences in the abundance of these proteins could be contributing to delayed wound healing of DFUs. Due to this, use of autologous platelet rich plasma from diabetic patients to promote healing of DFUs would result in little treatment enhancement as these platelets show proteomic differences that could provide an excess of undesirable proteins at the wound site. Thus, the study can be supplemented with

other studies to make a more substantiated conclusion and possibly develop therapies targeted at these proteins.

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Abbreviation List

°C	Degrees Celsius
µL	Microliter
µm	Micrometre
µg	Microgram
ng	Nanogram
%	Percent
βME	Beta mercaptoethanol
1D	One dimensional
ACN	Acetonitrile
ADF	actin depolymerizing factor
AGE	Advanced glycation end-product
bFGF	Basic fibroblast growth factor
$CaCl_2$	Calcium chloride
CML	Carboxymethyl lysine
CVD	Cardiovascular disease
DDA	Data-dependent acquisition
DFUs	Diabetic foot ulcers
dH_2O	Distilled water
DIA	Data-independent acquisition
DM	Diabetes mellitus
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FA	Formic acid
FGF	Fibroblast growth factor

GDM	Gestational diabetes mellitus
HCL	Hydrochloric acid
Hb	Haemoglobin
HGF	Hepatocyte growth factor
HILIC	Hydrophilic interaction chromatography
HMWK	High molecular weight kininogen
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin 1 beta
kDa	Kilodalton
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LMWK	low molecular weight kininogen
LOD	Limit of detection
MALDI	Matrix assisted laser desorption/ionization
MeOH	Methanol
MMPs	Matrix metalloproteinases
MAb	Monoclonal antibody
MS	Mass spectrometer
ms	Milliseconds
m/z	Mass-to-charge ratio
NaOH	Sodium hydroxide
NH_4HC0_3	Ammonium bicarbonate
PAF	Platelet activating factor
PAS	Periodic Acid Schiff
PBP	Platelet basic protein
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor

PFXII	Platelet coagulation factor XII (PFXII),
PRP	Platelet rich plasma
PSM	Peptide-to-spectrum matches
PTM	Post translational modification
ROS	Reactive oxygen species
rt	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SWATH-MS	Sequential Windowed Acquisition of All Theoretic Mass Spectra
SWR	Standard working reagent
TGF β	Transforming growth factor beta
TGF	Transforming growth factor
TNF α	Tumour necrosis factor alpha
TXA_2	Thromboxane A2
VEGF	Vascular endothelial growth factor
WBCs	White blood cells
WHO	World Health Organization

Chapter 1: Introduction and Literature review

1.1 Diabetes mellitus

1.1.1 Epidemiology of diabetes mellitus

Diabetes mellitus (DM), which is commonly referred to as diabetes, is one of the four major non-communicable diseases, that has been recognized as an important cause of premature death and disability (NCD Risk Factor Collaboration 2016; WHO 2016). According to the International Diabetes Federation approximately 415 million adults presented with diabetes in 2015, with 5 million deaths estimated to be directly caused by the disease (Ogurtsova *et al.* 2017). Two years later, approximately 4 million people aged between 20 and 79 years were estimated to die from diabetes (International Diabetes Federation 2017). This has negatively impacted the economy worldwide as losses are made due to direct medical costs and loss of work and wages (WHO 2016; WHO 2017). A rapid increase in the global prevalence of diabetes among adults over 18 years has been observed since 1980, rising from 4.7%, to 8.5% (NCD Risk Factor Collaboration 2016; WHO 2016). This rapid increase in prevalence has been observed particularly in middle and low-income countries, which account for 75% of adults with diabetes, hence the greatest burden of diabetes is falling on socially disadvantaged groups (International Diabetes Federation 2015; Zimmet *et al.* 2014). Figure 1 shows the global proportion (%) of premature death attributed to DM in the year 2017.

1.1.2 Pathology and classification of diabetes mellitus

Diabetes is characterized by insulin deficiency, resistance to insulin action or both. Owing to this, a deficient action of insulin on target tissues is experienced, causing abnormalities in carbohydrate, fat and protein metabolism. Consequently, blood glucose levels are elevated from the normal concentrations of 3.5 – 5.5 mmol/L (Guemes, Rahman and Hussain 2016) and the diseased patients may present with symptoms such as polyuria, polydipsia, polyphagia and weight loss (American Diabetes Association 2014; WHO 2016). Extreme elevation of the blood glucose levels can lead to a diabetic coma, which can cause permanent brain damage or death, if left untreated. Furthermore, since diabetic patients tend to have hypoglycaemia unawareness, the diabetic coma can also be induced by low blood glucose levels (Chiasson *et al.* 2003; Guest 1949). As the disease progresses, numerous complications develop, the major complications being retinopathy, nephropathy,

cardiovascular diseases and peripheral neuropathy (Harvey *et al.* 2012; WHO 2016). Diabetic retinopathy is responsible for moderate to severe visual impairment, which often leads to blindness in diabetes patients (Bourne *et al.* 2013). This occurs as a direct result of high blood glucose levels causing damage to the retinal capillaries, leading to capillary leakage and blockage (International Diabetes Federation 2017). Elevated blood glucose levels are also associated with increased intraocular pressure, which is the primary risk factor for glaucoma (Song, Aiello and Pasquale 2016). It has been estimated that the global prevalence of retinopathy in persons with diabetes is about 35% and the retinopathy rates are higher among people with type 1 diabetes, longer duration of diabetes, Caucasian populations and people of lower socioeconomic status (Yau *et al.* 2012). In addition to retinopathy, diabetic patients also experience renal disease due to nephropathy and the earliest marker of renal disease is albuminuria (International Diabetes Federation 2017). If the hyperglycaemic levels are not controlled, further development of renal disease occurs, resulting in a condition known as end-stage renal disease (ESRD) (Ghaderian *et al.* 2015). According to the *Global Report on Diabetes* published by the WHO, pooled data from 54 countries in 2014 showed that at least 80% of cases of ESRD were caused by diabetes, hypertension or a combination of both. The proportion of ESRD attributed to diabetes alone ranged from 12-55% (WHO 2016) (United States Renal Data System 2014). Diabetes patients are also prone to cardiovascular disease (CVD) such as hypertension and stroke, which often lead to death and disability (International Diabetes Federation 2017). The risk of CVD increases with rising plasma glucose levels, such that cardiovascular complications can be experienced before the glycaemic levels sufficient for diabetes diagnosis are reached (Danaei *et al.* 2006; Singh *et al.* 2013). The high glycaemic levels in blood tend to hyperactivate the coagulation system, thus increasing the risk of blood clots which may cause hypertension, myocardial infarction and stroke (Buttar, Li and Ravi 2005; International Diabetes Federation 2017). It has been observed that the rate of developing CVD is 2-3 times higher in adults with DM compared to those without DM, and the incidence increases with age (Sarwar *et al.* 2010). Peripheral neuropathy is the most common complication associated with DM, and this occurs due to damage of nerves in the periphery (International Diabetes Federation 2017) (Juster-Switlyk and Smith 2016). The lifetime prevalence of peripheral neuropathy is approximately 50% in the DM

population and is a leading cause for disability due to foot ulceration and amputation (Juster-Switlyk and Smith 2016).

The two major clinical classifications of the disease are Type 1 DM and Type 2 DM which account for 5-10% and 90-95% of the diabetic population respectively (American diabetes association 2004). Important to note is gestational diabetes (GDM) which occurs in pregnancy and is also on the rise (American Diabetes Association 2014).

In Type 1 DM, there is little, to no production of insulin often caused by an autoimmune destruction of the insulin producing beta cells in the pancreas. Beta cell destruction is extensive in some individuals, especially infants and children, and slow in others. This means a Type 1 DM patient who has rapid beta cell destruction relies on exogenous insulin for glycaemic control. Type 1 DM can also be idiopathic, where there is permanent insulinopenia but no evidence of autoimmunity towards the pancreatic beta cells. Type 1 DM was previously referred to as insulin-dependent or juvenile-onset diabetes because it was commonly diagnosed in young patients, but an increase in the adult incidence has been reported (American Diabetes Association 2014; National Diabetes Information Clearinghouse 2014).

Type 2 DM on the other hand is characterized by insufficient insulin production or lack of response to insulin by the target cells in the body, resulting in what is termed insulin resistance (Tuomi 2005). It is the more prevalent form of DM and can go unnoticed/undiagnosed for many years since the hyperglycaemia develops gradually and the clinical symptoms not severe in the early stages to be noticed. Although Type 2 DM is the most common form observed in adults, it can affect people of any age and the risk factors associated with it include physical inactivity, obesity, aging as well as a strong genetic predisposition (American Diabetes Association 2014) which is also the case with GDM. Pregnant women are diagnosed with GDM when their blood glucose levels are above normal but still below values diagnostic of diabetes. This is usually a temporary condition that subsides after the pregnancy but carries a long-term risk of Type 2 DM in the women as well as for their offspring (International Diabetes Federation 2015).

Even though extensive data at the molecular and cellular levels has been accumulated over the years, the mechanism of diabetes development and complications remain to

be elucidated. This has led researchers to conclude that more extensive research is of paramount importance to improve diagnosis, therapy and minimize chronic complications such as diabetic foot ulcers (Kharroubi and Darwish 2015).

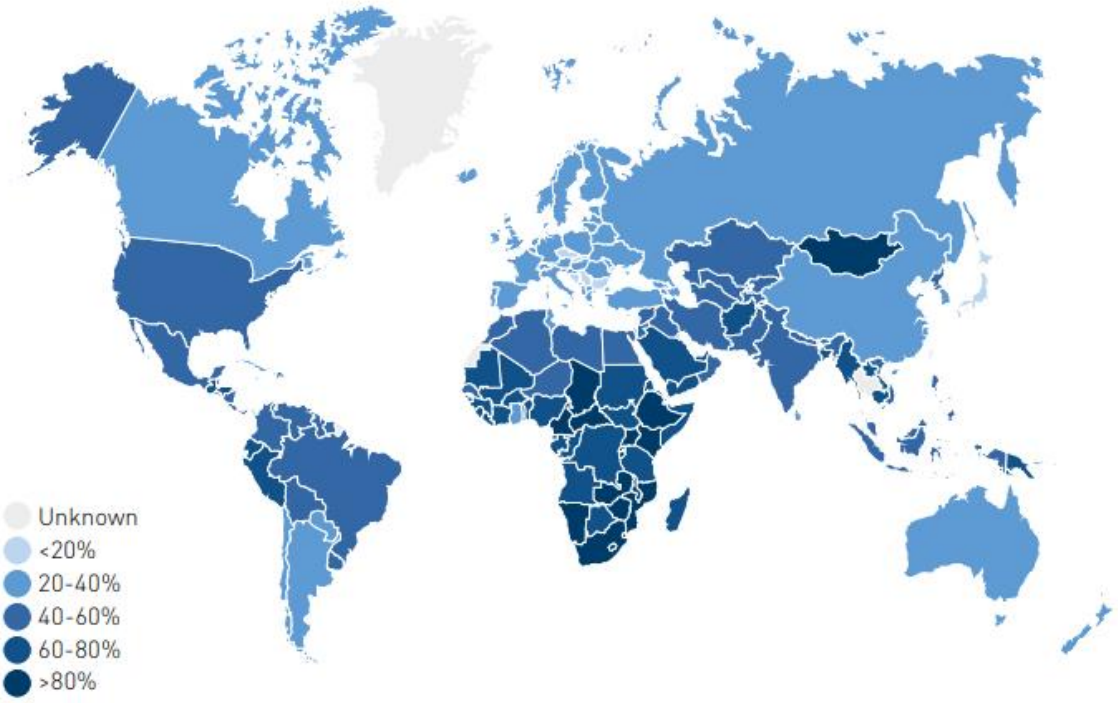


Figure 1: Global proportion of deaths attributed to diabetes that occurred in people under the age of 60 in 2017 (International Diabetes Federation, 2017)

1.2 Protein glycation in diabetes mellitus

1.2.1 Glycated haemoglobin as a diagnostic tool

Diagnostic tests that are employed for DM include measurement of fasting or random blood glucose levels, oral glucose tolerance testing and measurement of glycated haemoglobin (HbA1c test). Prior to conducting extensive research on glycated haemoglobin, it was only recommended for the determination of glucose control among people who had been diagnosed with DM, but now as recommended by the American Diabetes Association, it can be used as a diagnostic tool. The HbA1c test is not only useful for diagnosis but is also a gold standard that has been shown to reliably predict the risk of developing diabetic – related complications such as micro-vascular disease (Selvin *et al.* 2010). Haemoglobin (Hgb) which forms the basis of this test is a protein in red blood cells that carries oxygen from the lungs to the body's tissues and

returns carbon dioxide from the tissues back to the lungs. In a normal adult, the molecule is made up of four globulin chains that are interconnected, of which two are alpha-globulin chains while the other two are beta-globulin chains. When there are elevated glucose levels in the blood, Hb is glycated and the most reactive site is the N-terminal valine of the beta chain (HbA1c) which accounts for about 60% of all Hb bound glucose (Goldstein *et al.* 1986; Ito and Sanghera 2001). The test measures blood glucose levels over a 3-month period because haemoglobin resides in a red blood cell which has a half-life of approximately 120 days (Roohk and Zaidi 2008). Measurements of glycation from the HbA1c test are given in percentages as shown below:

•Normal HbA1c level = below 5.7% (39 mmol/mol)

•Pre-diabetic = between 5.7 – 6.4% (39 – 46 mmol/mol)

•Diabetic = above 6.5% (48 mmol/mol)

During the test, a blood sample can be taken from a vein or simply from a prick on the finger. The sample is then taken to a lab for analysis, which gives more accurate results, or analysed instantly using A1c test kits (Goldstein *et al.* 1986; Majek *et al.* 2010a). The principle on which this diagnostic tool works supports data that has been obtained from previous studies which show that non–enzymatic glycosylation of proteins occurs under conditions of hyperglycaemia (Blakytny and Harding 1992; Nakayama *et al.* 1999; Schleicher and Wieland 1986).

1.2.2 Advanced glycation end products

Glycosylation is a common post-translational modification (PTM) that is mediated by enzymes where a defined carbohydrate molecule is added to a predetermined region of the protein (Blakytny and Harding 1992). On estimate, 50 -70% of human proteins are glycosylated and this is to facilitate fundamental functions such as protein folding, stability, immunogenicity and other physiological processes (Dai *et al.* 2015). However, in instances where there is hyperglycaemia as is the case in DM, non-enzymatic glycosylation which is commonly referred to as glycation occurs. This is a random mechanism in the bloodstream where the reducing/ reactive carbonyl moiety of free sugars such as glucose, fructose and galactose covalently bond with proteins, lipids or nucleic acids thus creating glycated products (Ahmed 2005; Nakayama *et al.*

1999). Glycation is a starting point of a reaction known as the Maillard reaction, named after the leading pioneer of glycation research, Louis Camille Maillard (1878 – 1936). When proteins are glycated, non–enzymatic binding of a reducing sugar such as glucose/ fructose to free lysine or arginine NH2 residues in proteins takes place to form non-stable Schiff bases. The Schiff bases are further rearranged to form more stable products known as *Amadori* products (Maillard 1912; Nakayama *et al.* 1999; Paul and Bailey 1996; Rabbani and Thornalley 2012). As glycation progresses, the *Amadori* products undergo multiple dehydration and rearrangement stages, forming highly reactive products known as advanced glycation end-products (AGEs) (Nakayama *et al.* 1999; Wang *et al.* 2012). AGEs are structurally diverse (as shown in Figure 2), and they are classified according to the type of modified amino acid or sugar that is bound, as well as chemical characteristics. Examples include pentosidine and carboxymethyl lysine (CML) which are some of the most commonly characterized AGEs. CML is formed when *Amadori* products are oxidatively degraded or when there is a direct addition of glyoxal to lysine. Pentosidine formation occurs when lysine and arginine residues react with a pentose sugar (Arasteh *et al.* 2014; Rabbani and Thornalley 2012; Reddy *et al.* 1995). Auto fluorescence is one property which helps in identification of AGEs under *in vivo* or *in vitro* conditions (Ahmed 2005; Vigneshwaran *et al.* 2005) but this method is flawed in identifying non-fluorescent AGEs such as CML, which also happens to be one of the most prevalent AGEs (Ahmed, Thorpe and Baynes 1986).

Literature reports that at the time of DM diagnosis, AGE concentrations are elevated in serum, hence there is a close relation between AGE concentrations and HbA1c values (Jaisson *et al.* 2016). However, an increase in AGE formation has been observed during normal aging (Arasteh *et al.* 2014), and can also be accelerated by environmental factors such as diet and smoking (Cerami *et al.* 1997). Normal metabolic processes of the organism can produce the same AGEs, but at much lower rates (Fleming *et al.* 2011).

AGE formation usually results in impairment of protein function or stability. The process can contribute to development of organ disorders like renal insufficiency, vascular injury, retinopathy and peripheral neuropathy (Blakytny and Harding 1992; Nagai *et al.* 2014; Varo *et al.* 2005). As previously mentioned, peripheral neuropathy

in diabetic patients has a risk of DFUs and these DFUs result from an impaired healing process.

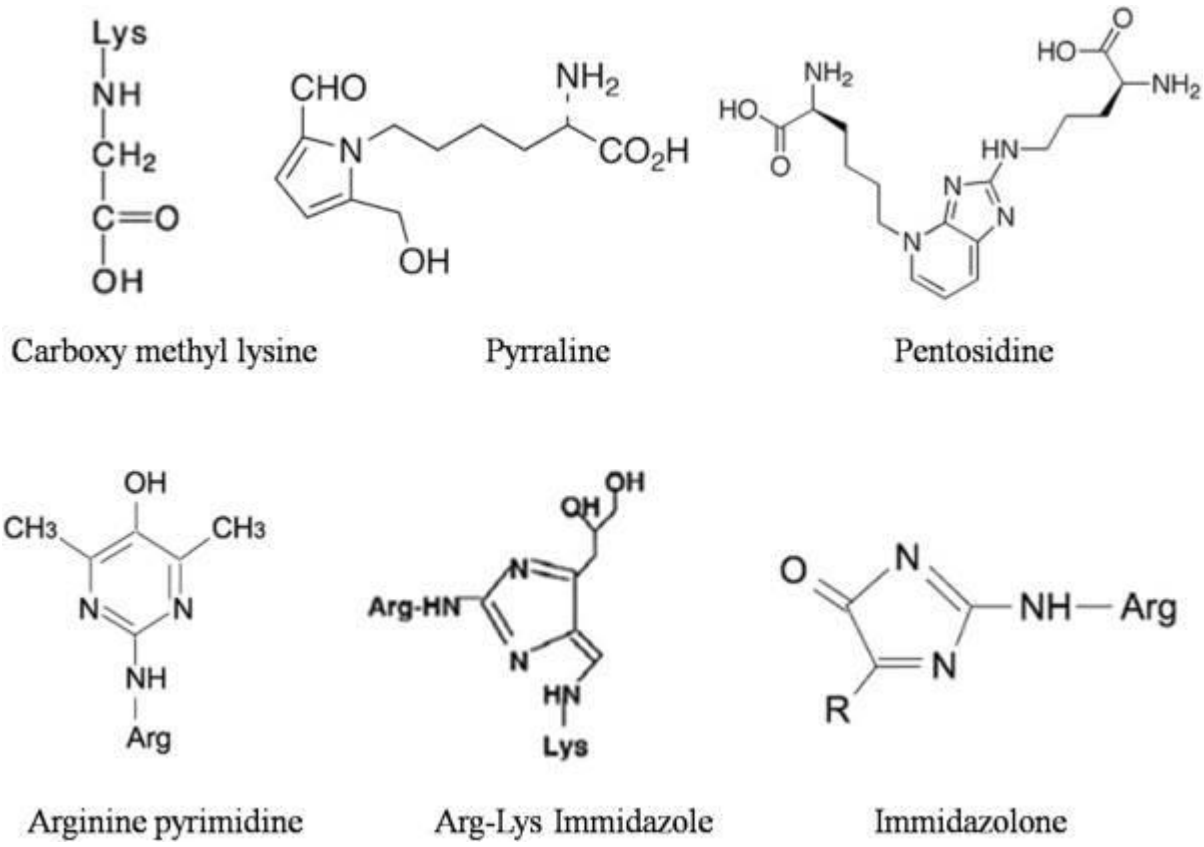


Figure 2: Chemical structures of several possible different AGE products formed from sugars and the two amino acids lysine and arginine that have terminal side chain amine groups (Arasteh et al. 2014) (with permission).

1.3 Diabetic foot ulcers

DFUs are one of the most catastrophic and costly diabetic complications, which is also accompanied by limited treatment availability (Bruhn-Olszewska *et al.* 2012). The major associated symptoms of DFUs at a macroscopic level are neuropathy and ischaemia resulting from angiopathy and hyperglycaemia-induced metabolic changes (Bakker *et al.* 2012; Bruhn-Olszewska *et al.* 2012). Since 50% of the DM population has peripheral neuropathy especially in the extremities, which is a known risk for developing foot ulcers, this is of great concern in the health sector. Amputation of the lower extremities is often the final outcome of diabetic foot ulcers (Bakker *et al.* 2012; Edmonds 2006) and has been estimated to be required in approximately 15% of

patients with diabetic foot ulcers where healing cannot be induced (Edmonds 2006; Pemayun *et al.* 2015). Peripheral neuropathy causes the patient to lose sensitivity in the extremities of the limbs, and minor trauma due to pressure, contusions or cuts can initiate DFUs. As DM progresses, neuropathy can develop to *Charcot* neuropathy which is characterized by foot deformities and limited joint mobility. This often leads to abnormal biomechanical loading of the foot and formation of skin calluses. Consequently, the abnormality of the biomechanical loading is potentiated, and subcutaneous haemorrhage is experienced (Bakker *et al.* 2012; Bruhn-Olszewska *et al.* 2012; Edmonds 2006). Due to the loss of sensitivity to pain, there is poor protection of the wounds by the patients, and mechanical stress is repetitively applied. This causes continued tissue destruction and possibly enlargement of the wounds, with a high risk of susceptibility to infection (Edmonds 2006). This sequence of events leading to a complicated diabetic foot ulcer is illustrated in Figure 3. An infected foot ulcer can be limb or life-threatening owing to potential for gangrene and sepsis, therefore in most cases it is promptly amputated (Bruhn-Olszewska *et al.* 2012; Cavanagh *et al.* 2005a). To prevent foot or leg amputations or reduce the severity of amputations, new treatment strategies have been implemented, with more still under trial to test for effectiveness (Adeghate *et al.* 2017; Cianfarani *et al.* 2013; Tecilazich, Dinh and Veves 2013; Tian *et al.* 2014).

1.4 Treatment of DFUs

Treatment of diabetic foot ulcers focuses on wound management and treatment often requires long-term hospitalization and frequent out-patient visits (Löndahl *et al.* 2010). The main treatments that are in use are wound dressings, wound debridement and off-loading for lower grade foot ulcers, and broad-spectrum antibiotics coupled with limited or complete amputation for higher grades (Cavanagh *et al.* 2005b; Chadwick *et al.* 2013; Eldor *et al.* 2004). Wound dressings are designed to create a moist wound environment and absorb exudates to support progression towards wound healing. Debridement on the other hand is aimed at removing ischemic and necrotic tissue, which is susceptible to infection and contamination by foreign bodies. It may be a once-off procedure, or it may need to be ongoing for maintenance of the wound bed. There are many methods of debridement used in the management of DFU's including surgical/sharp, larval, ultrasonic, autolytic and hydro-surgery (Chadwick *et al.* 2013). Correct debridement has the potential to convert a chronic into an acute wound, which

can then progress through the normal stages of wound healing (Velnar, Bailey and Smrkolj 2009). Off-loading or pressure relief on ulcers is aimed at even distribution of plantar pressures to avoid areas of high pressure that will prevent or delay healing. The methods used for this include bed rest, wheel chair mobility, crutch-assisted gait, total contact casts (TCCs), half shoes and removable cast walkers (Wu *et al.* 2008).

The most studied and the gold standard for offloading is the TCC which is minimally padded and moulded carefully to the shape of the foot. The TCC reduces pressure at the wound site by 84 – 92%, but it is not suitable for ischemic ulcers, since frequent wound inspection and daily dressing changes are not possible (Brem, Sheehan and Boulton 2004). To supplement these mainstay treatment approaches, current research has been focused on development of new wound therapies (Cianfarani *et al.* 2013).

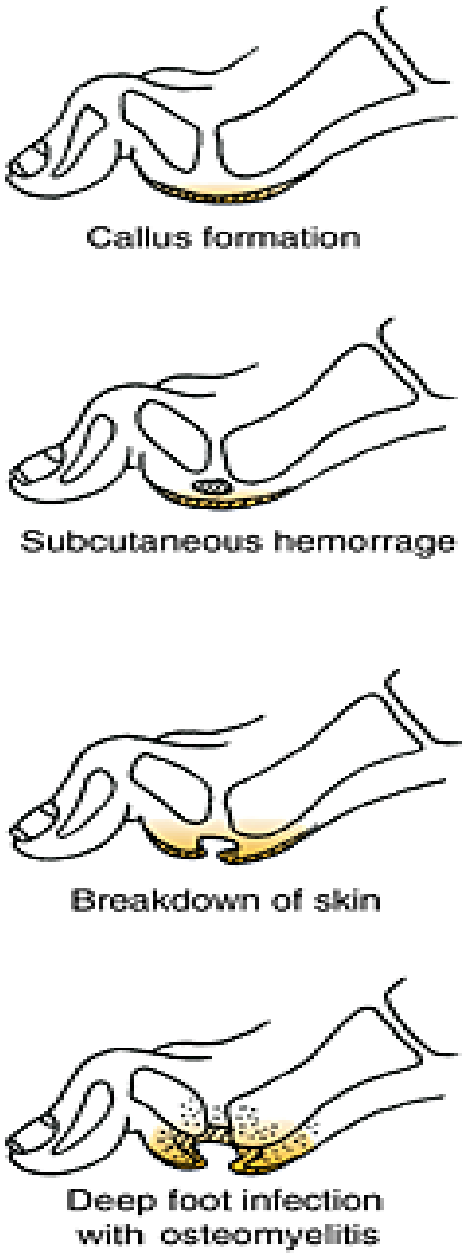


Figure 3: Progression of a diabetic foot ulcer due to repetitive stress (Bakker et al. 2012) (with permission).

New treatment strategies include the use of hyperbaric oxygen therapy (HBOT), topical growth factors, bioengineered skin equivalents, adipose tissue derived stem cells (ASCs) or bone marrow-derived stem cells and platelet rich plasma (PRP). Apart from HBOT, these treatments rely on cytokines and chemokines for initiation of the wound healing process and these are mostly derived from platelets (Blumberg *et al.* 2012; Cavanagh *et al.* 2005b; Cianfarani *et al.* 2013; Nurden *et al.* 2008b). HBOT is

still under research and it has an antimicrobial effect which is due to an increase in oxygenation of hypoxic wound tissue, thus enhancing neutrophil killing ability, angiogenesis, fibroblast activity and collagen synthesis. However, it is thought to work best as an adjunctive therapy rather than to be used independently (Löndahl *et al.* 2010).

Although these treatment strategies have been put in place, a high rate of recurrence of DFUs has been identified and the rate of foot/leg amputations is on the rise (Cianfarani *et al.* 2013). In addition to amputations which have the potential to be life threatening, DFUs have become globally clinically burdensome because they have impaired healing and usually become chronic wounds. These chronic wounds require increased amounts of financial resources for their management (Edmonds 2006; Falanga 2005; Guo and DiPietro 2010a; International Diabetes Federation 2017). Figure 4 shows the areas of the foot that are prone to ulceration in diabetic patients with peripheral neuropathy.

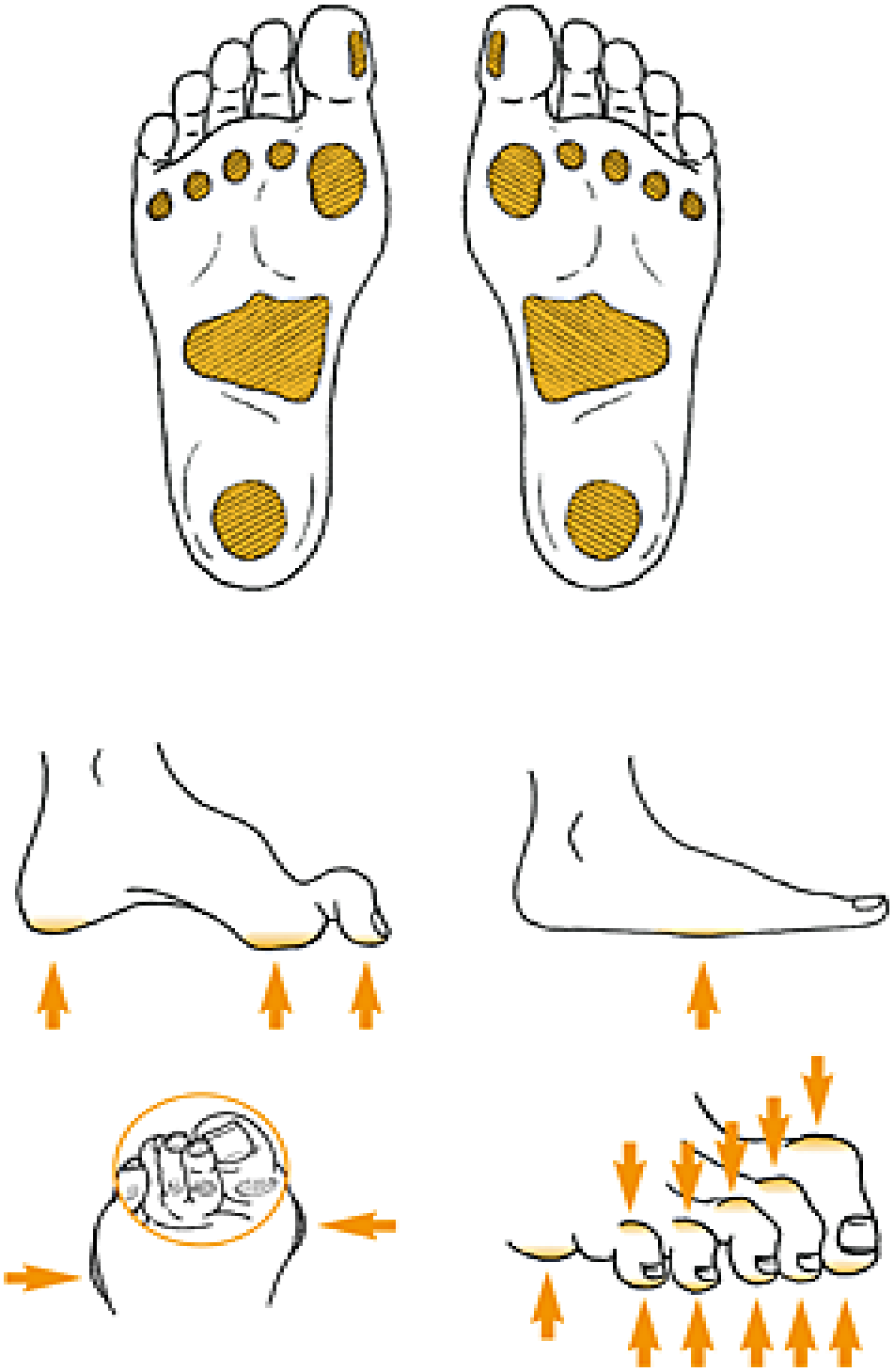


Figure 4: Areas of the foot that are prone to ulceration in diabetic patients with peripheral neuropathy (Bakker et al. 2012) (with permission).

1.5 The wound healing process

1.5.1 Acute wounds as a wound healing model

Acute wounds form the basis of the wound healing model. They are the most common wounds and heal in less than four weeks, with the least amount of energy. A well-organized healing process of four sequential but overlapping phases namely haemostasis (coagulation), inflammation, migration-proliferation and remodelling is characteristic of acute wounds. Owing to this well organised healing process leading to predictable tissue repair, acute wounds are therefore used as a model for wound healing (Demidova-Rice, Hamblin and Herman 2012; Spear 2013). The different stages of the process are shown in Figure 5.

1.5.2 Haemostasis (Coagulation)

Haemostasis lasts for about 30 - 40 minutes and the principal aim of this stage is to prevent severe loss of blood and provide a matrix for required cells that serve critical functions needed in the later phases of healing (Velnar *et al.* 2009). When injury has occurred, platelets and blood components adhere to the exposed endothelium and other extracellular matrix (ECM) components. This contact stimulates the platelets to undergo a change in shape, accumulate, aggregate and degranulate, releasing many different protein, lipid and metabolite based factors such as transforming growth factor (TGF) β_1, platelet-derived growth factor (PDGF) and platelet-activating factor (PAF) (Bryant and Nix 2015). The different factors released during the degranulation of the involved platelets form a clot matrix to ward off bacteria, control bleeding, provide wound coverage, and act as a provisional matrix for cell migration in subsequent phases. Thrombin is produced and initiates transformation of fibrinogen to fibrin which forms a mesh of fibrous protein, stabilizing platelets at the site of injury, thus aiding in formation of a clot. The damaged blood vessels also constrict to limit blood loss and this is mediated by thromboxane A_2 (TXA_2) and prostaglandin 2-α, released by damaged cells and activated platelets (Bryant and Nix 2015). The cytokines that are released by the platelets at the site of injury as well as histamine produced by the mast cells also lead to oedema and vascular leakage which increase production, migration and attraction of inflammatory cells at the wound site. This sequence of events enables and stimulates the next overlapping wound healing phase which is inflammation

(Blakytny and Jude 2006; Bruhn-Olszewska *et al.* 2012; Falanga 2005; Spear 2013; Velnar *et al.* 2009; Vinik *et al.* 2001).

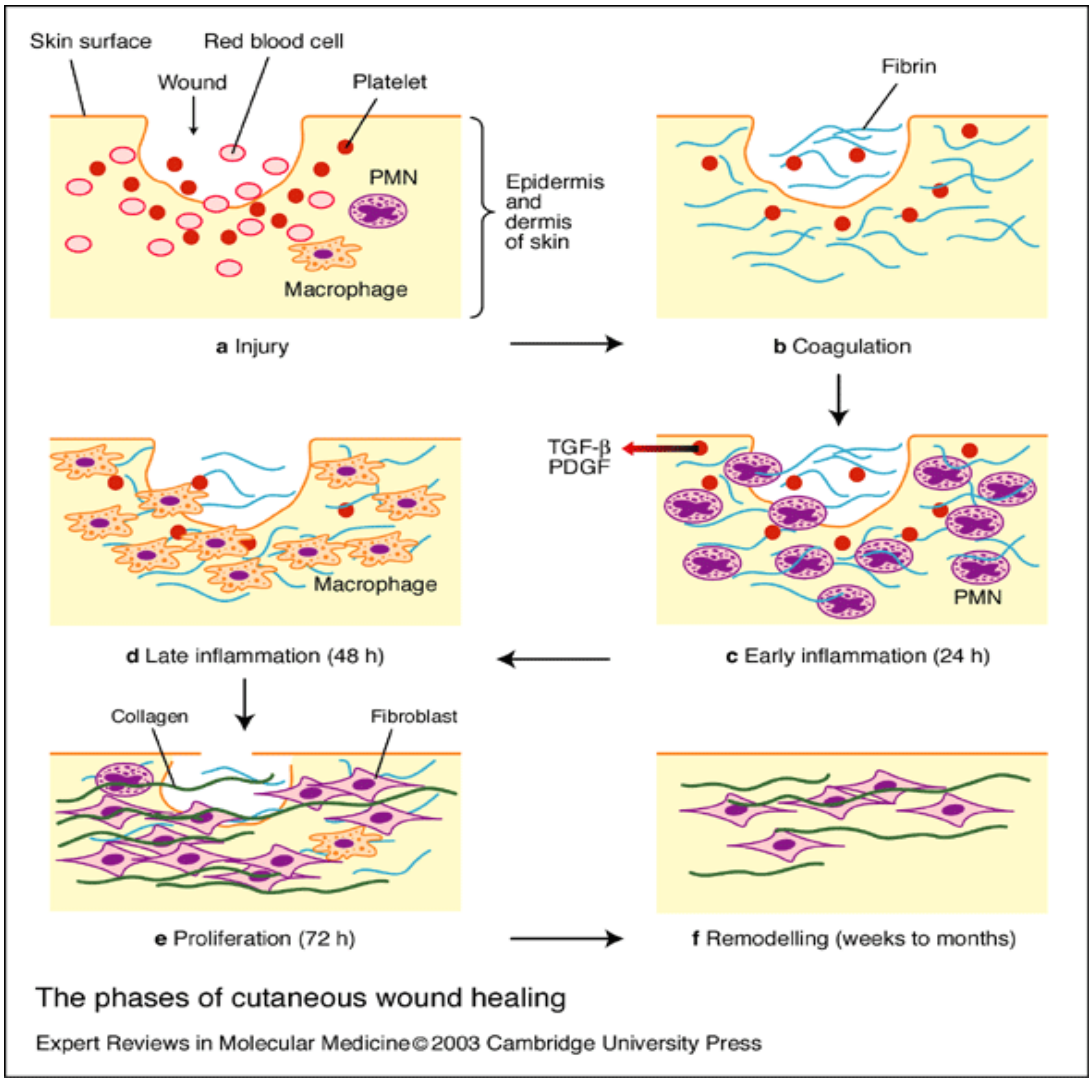


Figure 5: The sequential but overlapping stages of the wound healing process (a) Immediately following cutaneous injury, blood elements and vasoactive amines exude from locally damaged blood vessels within the dermis. Vascular permeability is temporarily increased to allow neutrophils, platelets and plasma proteins to infiltrate the wound. (b) Haemostasis/coagulation then occurs as platelets aggregate with fibrin (c) Platelets release several factors which attract neutrophils to the wound, signalling the beginning of inflammation. (d) After 48 hours, macrophages replace neutrophils as the principal inflammatory cell. (e) The proliferation phase begins at about 72 hours as fibroblasts begin to synthesize collagen. (f) Collagen crosslinking and reorganisation occur for months after injury in the remodelling phase of repair (Beanes *et al.* 2003) (with permission).

1.5.3 Inflammation

In normal wound healing this phase takes up to four days after the wound healing has initiated. The primary aim of this phase is to establish an immune barrier against invading microorganisms and it is divided into two sub-phases, namely early and late inflammatory phases (Velnar *et al.* 2009).

During the early inflammatory phase, cytokines released by platelets activate the complement cascade and initiate molecular events, leading to infiltration of the wound site by neutrophils, primarily to prevent infection. During haemostasis, blood vessels constrict to prevent blood loss, but the reverse occurs in this early inflammatory phase, where there is vasodilation and increased capillary permeability. This permits plasma and blood cells to move into the wound bed through a process known as diapedesis (Bryant and Nix 2015). In this way, the neutrophils are attracted to the site and adhere to the injured vascular endothelial cell walls where they defend the host against infection through phagocytosis of foreign material and dead cells. They destroy the foreign material and bacteria by releasing proteolytic enzymes and reactive oxygen species (ROS). Apoptosis of the neutrophils occurs upon completion of their task, and they are eliminated to the wound surfaces as slough, allowing progression to the next phase of healing (Guo and DiPietro 2010b; Velnar *et al.* 2009).

In the late phase of inflammation, macrophages make a major contribution to the repair process. The chemo-attractive agents released by platelets and neutrophils initiate the interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF α) cascade. This cascade attracts circulating monocytes to reside at the site of injury as tissue macrophages after undergoing phenotypic changes. The macrophages continue the process of phagocytosis and their advantage over neutrophils is that they have a longer lifespan than neutrophils, and can function at a lower pH (Bryant and Nix 2015; Velnar *et al.* 2009). Macrophages function as important regulatory cells (Bryant and Nix 2015; Falanga 2005) by providing a reservoir of potent tissue growth factors and mediators such as fibroblast growth factor (FGF) which stimulates fibroblast migration and proliferation, connective tissue synthesis to form the early granulation tissue and for angiogenesis. The granulation tissue begins the process of contraction. Macrophages also release cytokines and vasodilator prostaglandins to attract and release other immune cells such as T lymphocytes, which secrete additional cytokines and destroy viruses and foreign cells. Another vital function of macrophages is the

production of collagenase and elastase to assist with breakdown of dead tissue and mediate transition from the inflammatory phase to the proliferative phase. However, it is important to note that if there is no proper regulation, the inflammatory mediators produced by macrophages and neutrophils during this phase can cause extensive tissue damage and degradation of growth factors needed for normal function of proliferative cells such as fibroblasts. A consequence of such an occurrence would be prolongation of the inflammatory phase in the wound healing process (Bryant and Nix 2015; Guo and DiPietro 2010b; Spear 2013).

1.5.4 Migration-proliferation

This stage usually lasts for about two weeks and it is characterized by epithelialization, angiogenesis, granulation, tissue formation and collagen III deposition. Epithelial cells at the edge of the wound, fibroblasts and myofibroblasts migrate towards the wound gap to form a protective barrier under the chemo-attractive influence of TGF-β and PDGF (Falanga 2005; Spear 2013; Velnar *et al.* 2009). This replaces the provisional matrix formed during haemostasis. Migration requires up-regulation of binding sites (integrin receptors) on the cell membrane which is essential because fibroblasts migrate by maintaining attachment to one binding site while extending lamellipodia in search of another potential binding site. When the fibroblast is able to bind to a new site, it releases the original attachment and moves in the direction of the wound bed (Bryant and Nix 2015). Once fibroblasts are in the wound bed area they proliferate profusely and express matrix proteins, hyaluronic acid (to protect cells against free radicals and proteolytic damage), fibronectin, proteoglycans and Types I and III collagen. Many fibroblasts differentiate into their myofibroblast phenotype, facilitating wound contraction. Abundant ECM accumulates, further supporting cell migration (Velnar *et al.* 2009). Secretion of matrix metalloproteinases (MMPs) is also involved and these partially degrade the initial clot fibrous network and ECM at the wound site to create more space for migrating cells. Angiogenesis is initiated at an early stage of the proliferation phase stimulating capillary sprouts from surrounding edges to migrate into the wound area and a microvascular network composed of many new capillaries is formed to supply nutrients and oxygen to the proliferating cells. Hypoxia acts as a stimulus to angiogenesis, but if it persists it has the potential to interfere with endothelial cell proliferation and new vessel formation (Bryant and Nix 2015). Keratinocyte growth factors are also expressed by fibroblasts causing keratinocytes

to migrate and proliferate at the wound edges where they form tight junctions with the nerves of the skin. The keratinocytes also serve as a barrier that prevents toxins and pathogens from entering the body (Blakytny and Jude 2006; Bruhn-Olszewska *et al.* 2012; Falanga 2005; Spear 2013).

1.5.5 Remodelling

This dual process of synthesis and degradation lasts from Day 8 to several months or years after the initial wounding. A new epithelium is developed, and final scar tissue is formed during this phase. The intracellular matrix matures, and collagen III is replaced with collagen I where the collagen bundles increase in diameter, while hyaluronic acid and fibronectin are degraded. Collagen I is deposited, progressively increasing the tensile strength of the wound in proportion to collagen present. The deposition of collagen bundles is initially disorganized but overtime the new collagen matrix becomes more oriented and cross-linked. MMPs which are quite significant during migration and proliferation are also down-regulated by inhibitory factors such as TIMP1 to control their activity. This is to stop further degradation and ensure accumulation of the new matrix. Fibroblast and macrophage density is further reduced via apoptosis. Capillary growth is halted once the immune cell density decreases, maintaining the established blood flow to the area and consequently decreasing the metabolic activity at the wound site (Velnar *et al.* 2009). The process continues until the scar tissue has gained complete reorganisation which can have up to approximately 80% of the skins original strength. It will never be as strong as the original skin and will always be at risk of injury and breakdown, therefore it should be protected especially in stretch and load bearing areas (Spear 2013). The scar tissue is also relatively stiff in comparison with normal tissue and this is because the new matrix lacks elastin which provides elasticity to uninjured skin. A problem that can occur in this stage of wound healing is hypertrophic scarring and keloid formation because of an imbalance between matrix synthesis and breakdown. In both these cases there is excessive production of extracellular matrix involving both collagen and keratin that can result in thick protruding shiny surfaced scar tissue at the wound area (Robles and Berg 2007).

Failure of a wound to heal can occur in cases when an excess of MMPs are released, which then breaks down the newly formed fibrinogen, collagen, and keratin and this in turn interferes with synthesis and deposition of new matrix proteins and

glycosaminoglycans, thereby limiting the binding sites of fibroblasts and keratinocytes. This in turn limits the space filling effects that these cells can provide while enabling excessive tissue breakdown, thus causing the wound not to effectively close (Bryant and Nix 2015).

1.6 Chronic wounds

Chronic wounds fail to progress through and complete all stages of normal wound healing and usually persist for longer than four weeks without signs of healing. These wounds do not repair in an orderly and timely manner, as the healing process is incomplete and disturbed by several factors including hypoxia, necrosis and extracellular matrix breakdown as well as excess inflammatory cytokines. This causes the wound to be in a continuous state of inflammation, creating a cascade of inflammatory tissue responses and an over-abundance of phagocytic cell-infiltration that prolong a non-healing state. Chronic wounds also have increased production of MMPs which destroy components of the ECM and damage growth factors and their receptors (Blakytny and Jude 2006; Bryant and Nix 2015; Spear 2013; Velnar *et al.* 2009). DFUs are regarded as a chronic wound mainly because they appear to be locked in the inflammatory phase and do not naturally progress to the proliferation and maturation phases of the wound healing process, in a similar manner to any other chronic wound. In addition, growth factors, cytokines and chemokines released by keratinocytes, fibroblasts, endothelial cells, macrophages and platelets have been found at low levels in DFUs which further contributes to impaired wound healing. These growth factors and mediators are important for the initiation and transition to successive phases of inflammation (Blakytny and Jude 2006; Bruhn-Olszewska *et al.* 2012). Platelets release a significant amount of these growth factors which influence every phase of the wound healing process also in addition to initiation of haemostasis which makes them vital components in healing of DFUs.

1.7 The role of platelets and their importance in wound healing

Platelets are small anucleate particles (about 2-5 μm) derived from megakaryocytes in the bone marrow and they play a key role in the haemostasis phase of wound healing due to their ability to form aggregates. Their ability to release anti-inflammatory cytokines, inflammatory mediators and growth factors, also helps to regulate the inflammatory and coagulant pathways. This is made possible by the presence of

mRNA and spliceosome components for mRNA processing, as well as the translational machinery for protein synthesis (Anfossi *et al.* 2010; Beaulieu *et al.* 2014). Platelets are maintained in a resting state when circulating in blood (Majek *et al.* 2010b; Zeiler, Moser and Mann 2014) and they find clinical application in cancer, blood diseases, trauma and cardiac surgery where they are often administered as platelet rich plasma (PRP) transfusions or as direct topical applications of the PRP, as is the case in anti-aging creams (Springer *et al.* 2009). In disease states such as diabetes or when injury has occurred, they are seen to exhibit hyperactivity and therefore circulate in an activated form which also amplifies inflammation (Santilli *et al.* 2015; Vinik *et al.* 2001). This activation is attributed to adenosine diphosphate, insulin resistance and glycation due to hyperglycaemia (Beck *et al.* 2017; Suslova *et al.* 2015). Insulin prevents aggregation and adhesion of platelets but due to a decrease in the number of insulin receptors on platelets in Type 2 DM, the inhibitory effect of insulin is not exerted (Suslova *et al.* 2015).

Following vascular injury, platelets adhere to exposed sub-endothelial tissue, become activated, secrete mediators that promote haemostasis and tissue repair and influence processes such as angiogenesis, inflammation and immune responses. The mediators released by platelets include IL-1β, TGF, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1) and platelet-derived serotonin, which leads to initial vasoconstriction (Kieffer *et al.* 1987; Nurden *et al.* 2008a). The exact role of platelets in impaired wound healing in diabetic patients is yet to be clearly defined. Different research studies have identified changes to platelets or platelet components due to diabetes. These changes are discussed below.

- Some studies suggest that glycoprotein 1b, a platelet membrane receptor comprised of alpha and beta chains that participate in the initial adhesion to the sub-endothelium through binding to von Willebrand factor is down regulated. This implies that platelet aggregation is decreased, and wound healing is delayed (Avila *et al.* 2012; Springer *et al.* 2009). However the majority of literature states that hyperglycaemia causes platelets to be activated and aggregate more readily than those in healthy individuals (Vinik *et al.* 2001; Zeiler *et al.* 2014) which could suggest that the proper function is not exerted even though aggregation occurs as expected in diabetic patients.

- In a study conducted by Kutti *et al.,* lower mean plasma levels of β-thromboglobulin, platelet factor 4 and TXA_2 were reported in diabetic patients. These agents are responsible for platelet activation and aggregation, resulting in clot formation and they are also chemotactic for neutrophils and fibroblasts. Therefore, as a consequence, the haemostasis and inflammatory phases of wound healing would be affected (Kutti *et al.* 1986).
- In another study, platelets from patients with DM were seen to have diminished sensitivity to the natural anti-aggregating agents, prostacyclin (PGI_2) and nitric oxide (NO) (Santilli *et al.* 2015). However, the number of PGI_2 receptors is not decreased in DM, suggesting that the defect could be downstream of the receptor. This reduced sensitivity to anti-aggregating agents would imply an increase in aggregation. Although platelet aggregation is a necessary requirement for the wound healing process, hyper-activation results in amplification of inflammatory cytokines in the wound, which is one of the causes of impaired wound healing in DFUs (Velnar *et al.* 2009).
- In DM platelet antioxidant levels are reduced (Devangelio *et al.* 2007) and there is an increase in expression of TGF-β genes and their proteins. Researchers speculate that TGF-β is involved in the progression of diabetic nephropathy (Garud and Kulkarni 2014).
- AGEs that are formed in DM generate reactive oxygen species (ROS) directly through receptors for advanced glycation end-products (RAGE) which are expressed in most cells including the surface membranes of platelets. This ROS generation exacerbates inflammation and stimulates pro-coagulant agents. In normal circumstances, soluble RAGE (sRAGE), a splice variant of RAGE circulates in plasma and tissues. sRAGE decreases AGE binding to cellular RAGE thus providing protection against RAGE-mediated pathogenesis. However low levels of sRAGE have been observed in DM, and since sRAGE is expressed in all tissues or cells that express RAGE, there is a possibility that it could also be found in low levels in platelets (Devangelio *et al.* 2007; Fuentes, Rojas and Palomo 2014; Gawlowski *et al.* 2009; Oliveira *et al.* 2013).

These findings directly or indirectly affect the wound healing process of DFUs, but despite the accumulation of extensive data at the molecular and cellular levels, many questions remain and the development of diabetes complications such as DFUs is still

not fully understood (Bruhn-Olszewska *et al.* 2012; Kharroubi and Darwish 2015). Therefore, as suggested by Kharroubi *et al*, there is need for more extensive research to improve therapy and minimize the chance of chronic complications (Kharroubi and Darwish 2015).

1.8 Scope of the study

1.8.1 Study motivation

The implication of the increase in prevalence of DM, particularly in middle and low income areas, is that the occurrence of DFUs also increases in these socially disadvantaged groups (International Diabetes Federation 2015). DFUs reduce the quality of life of diabetic patients and in about 15% of cases result in lower extremity amputations (Bakker *et al.* 2012; Edmonds 2006; Saad Setta *et al.* 2011). Patients with DFUs also pose an economic burden on the health system due to the large amounts of time, professional help and financial resources needed for their management (Falanga 2005; Guo and DiPietro 2010a).

The current treatments of DFUs have been met with high rates of recurrence and failure (Cianfarani *et al.* 2013). The global approach of health sectors in management of DFUs, relies mainly on prevention, patient and staff education and close monitoring of patients with foot ulcers in an attempt to reduce amputation rates (Bakker *et al.* 2012). However, in underdeveloped economies, there are problems of staff and resource shortages, therefore this is not always possible to assess which factors contribute the most to treatment failure or recurrence of the DFUs (Guell and Unwin 2015).

On the other hand, surgical treatments such as tenotomy and tendon lengthening have been reported to be good approaches to prevent foot ulcer recurrence. The limitation of these surgical approaches is that they may cause secondary ulcerations and other complications, albeit claims of their success (Cavanagh *et al.* 2005a). Having observed the limitations of the conventional therapies, researchers have done a lot of work to develop new therapies to improve healing of DFUs. These new therapies, however have been seen to work best as adjuncts, and none have shown that they can be used independently (Löndahl *et al.* 2010).

Therefore, with the knowledge of the above-mentioned limitations, this study was directed towards looking at factors at molecular levels to see if there are any

differences in platelet protein expression and glycation, with the aim of characterizing these proteins to form a basis in development of targeted therapies for DFUs.

1.8.2 Analysis techniques

Several different techniques were used from early sample preparation to the protein comparisons between platelet proteins of diabetic patients and healthy individuals. Flow cytometry was done on early platelet enriched samples to check for potential platelet activation of the collected samples, while high performance liquid chromatography (HPLC) with fluorescence detection and western blotting were done to identify possible formation of AGE products. For proteomic analysis, one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) combined to different staining techniques, and liquid chromatography tandem mass spectrometry (LC-MS/MS) of selected protein gel bands and in-solution protein digests was done.

1.8.2.1 Flow cytometry

Flow cytometry analysis was performed on the samples to check for platelet activation, since the goal was to have minimum activation of these platelets. This was to prevent loss or premature activation of proteins such as growth factors which are degranulated when platelets are activated (Nurden *et al.* 2008a; Sadoul 2015). However, a degree of activation was expected in the samples from diabetic samples, as it has been reported in literature that some of the platelets are normally activated in diabetes (Santilli *et al.* 2015; Vinik *et al.* 2001).

Flow cytometry analysis of the samples was done using the surface marker analysis approach. P-selectin (also known as CD62P) which is the gold standard and widely used marker of platelet activation was targeted (Michelson 1996; Michelson *et al.* 2001). P-selectin is expressed on the surface of platelets when they are activated (Michelson *et al.* 2001) therefore flow cytometry analysis of platelet proteins to check for platelet activation makes use of monoclonal antibodies (MAbs) that target this antigen to measure the extent of activation (Michelson 1996). The advantage to this approach is that the antibodies bind to activated and not resting platelets, which makes it easy to distinguish between activated and resting platelet dominant samples (Michelson 1996). To be certain that the samples were indeed made up of platelets, a MAb targeting the GPIb-IX-V complex (CD42) which is expressed on the surfaces of both resting and activated platelets was also used in combination with the P-selectin

MAb. However, surface expression of the GPIb-IX-V complex is decreased when platelets are activated (Michelson 1996), implying that the P-selectin MAb would be the dominating antibody in identifying activated samples when the MAbs are used in combination.

Other studies have made use of aggregation tests such as the light transmission aggregation (LTA) test to measure platelet activation. The test works on the principle that platelets aggregate when activated. In this method, light transmission is measured through a cuvette filled with PRP. When platelets are not aggregated, the PRP sample is turbid and light transmission is decreased. In contrast, light transmission is increased in an aggregated PRP sample as it is less turbid (Paniccia *et al.* 2015; Yardumian, Mackie and Machin 1986). Although the LTA test would be sufficient to provide a semiquantitative measure of platelet activation, it requires large sample volumes (Yardumian *et al.* 1986) which makes flow cytometry more suitable for platelet activation analysis because it requires much smaller sample volumes.

1.8.2.2 AGE identification

AGE determination is a complex and time-consuming procedure due to the diversity of these compounds and lack of a “gold standard” technique for detection and measurement (Ashraf *et al.* 2015; Kalousova *et al.* 2002). Nevertheless, several techniques can be employed for AGE identification (Kalousova *et al.* 2002) and for this study, HPLC with fluorescence detection of typsinised proteins and western blotting of unhydrolysed proteins were used.

Reverse-phase HPLC with fluorescence detection is a technique that has been traditionally and is still widely used for AGE identification and quantification due to the fluorescence introduced into the protein structure on formation of the AGE products at the lysine and arginine residues (Munch *et al.* 1997). An example of where this technique has been applied is in the determination of HbA1c levels, which is a common early glycation product that is often monitored as a measure of long-term glucose concentrations in the serum (Ashraf *et al.* 2015). Advantages of using reverse-phase HPLC are that it gives clean chromatograms and the retention time for selected peptides are very stable. Like any other analytical method, HPLC analysis also has limitations which include long run times and time-consuming sample preparation meaning that it is not applicable for high throughput screening analysis. Another limitation is that several AGE products, such as the pentosidine derivative does not

have natural fluorescence. For this reason, some researchers would resort to alternative AGE-identification techniques such as enzyme-linked immunosorbent assay (ELISA) or western blotting (Ashraf *et al.* 2015; Kalousova *et al.* 2002) both of which require selective antibodies.

The principle by which western blotting works, is immune-detection of proteins post-electrophoresis and the ability to detect proteins that are of low abundance makes this technique particularly powerful (Kurien and Scofield 2006). Western blotting allows identification of specific proteins within a complex mixture (Mahmood and Yang 2012) which is why it has found application in processes such as AGE-identification (Zarina, Zhao and Abraham 2000). However, the sensitivity and reproducibility of western blotting in AGE-identification has been reported to be questionable by other researchers. This is due to the difficulty in characterizing the specificity of the antibody since some of the epitopes on the glycated protein may not be accessible for interaction with the antibody (Ashraf *et al.* 2015).

1.8.2.3 Proteomic analysis

The proteome, which is defined as the collective group of proteins expressed by a given cell or tissue, represents actual contributors to cellular function. In contrast, the genome represents potential cellular-function contributors and due to this, a huge shift from genome to proteome research has been observed over the years for a complete understanding of molecular functions of living systems (Kinter and Sherman 2005b). Several techniques can be applied for proteomic research but for purposes of this study, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled to different staining techniques and LC-MS/MS were the techniques used for sample analysis to identify possible protein differences between diabetic patients and non-diabetic healthy individuals.

One dimensional (1D) SDS-PAGE was used for separation and qualitative characterization of platelet proteins. This technique separates proteins by size which allows a degree of quantitation of proteins in complex mixtures (Brunelle and Green 2014). The separation is carried out on gels formed from polymerized acrylamide and the operational parameters of these gels can be altered to optimize specific separations. For instance, a range of pore size on the gel can be created by varying the acrylamide content. A most commonly used gel has an acrylamide content

between 4-20% and this is referred to as a gradient gel, which allows better resolution of separated proteins. A higher acrylamide content on the gel would mean that the pore size is smaller, which favours movement of smaller proteins, with little or no movement of larger proteins, while a lower acrylamide content would result in larger pores that allow movement of the larger proteins. During SDS-PAGE, smaller proteins move through the gel more quickly than larger proteins allowing for the separation of proteins within a sample (Brunelle and Green 2014; Kinter and Sherman 2005a; Laemmli 1970). Prior to separation, proteins are solubilised and denatured in sodium dodecyl sulphate (SDS) which is an anionic detergent. A uniform negative charge per protein mass is also added to the proteins by SDS, which allows their migration towards the anode in an electric field (Dunn and Bradd 1993). The rate of migration or mobility of the charged proteins will also be dependent on the magnitude of the charge on the protein and the differences in mobility will bring about the separation. The use of different staining techniques aids in visualization and qualitative characterization of the protein bands as different stain molecules bind to different moieties of the proteins (Kinter and Sherman 2005a).

SDS-PAGE is a favoured tool in proteomic research as it is a robust and reliable technique whereby the sample requirements are tolerant of the variety of methods used for protein sample preparation. However, the major challenge of SDS-PAGE lies in the fact that what appears as a single band is in most cases still a very complex mixture of proteins, the inability to identify individual proteins and to quantitate significance of protein changes that may be observed. Consequently, the general approach that is taken in proteomic research, where SDS-PAGE is involved, is to use the electrophoresis experiments to separate, enrich and characterize the biological system of interest and select, based on the electrophoretic separation, specific bands of interest in representative gels for sequencing and identification using mass spectrometry (Kinter and Sherman 2005a).

The introduction of ionization techniques such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) which are suitable for protein and peptide analyses have resulted in a rapid growth in the use of MS in proteomic research. MS analysis is superior over the other proteomic techniques, because of its high sensitivity, rapid speed of analysis, ability to

characterize PTMs and generation of a large amount of information for each experiment (Kinter and Sherman 2005b). For a bottom-up approach the protein is first digested into peptides by a chemical or an enzyme such as trypsin (Gundry *et al.* 2009). Following this, the LC-MS/MS techniques are used to analyse the protein digests and characterize the peptides in the digests in a manner that identifies the source protein in a database (Kinter and Sherman 2005b). Gel electrophoresis has been the standard method to separate and purify proteins prior to digestion and LC-MS/MS analysis (Gundry *et al.* 2009). The merit of this technique lies in its inexpensiveness and simplicity in removal of interfering contaminants. However, the resolving power of the technique is generally insufficient to separate individual proteins of similar molecular weights in a complex sample. Another common challenge of the gel-based technique is poor recovery of proteins and digests from the gel matrix (Jafari *et al.* 2012). Due to these limitations, non-selective approaches such as the in-solution digestion while using hydrophilic interaction chromatography (HILIC) sample clean up method have been developed. The method integrates sample clean-up and digestion using magnetic multi-mode HILIC microparticles for solid phase extraction, followed by on-bead tryptic digestion (Stoychev *et al.* 2017). The advantage to this method is that it provides robust and reproducible sample purification without compromising sample recovery (Jafari *et al.* 2012). In this study, both methods were applied with the objective that the strengths of one method would compensate for the drawbacks of the other.

1.9 Study aim

The main focus of this study was the identification of possible proteomic and glycoproteomic differences between platelets from diabetic patients and healthy individuals to help further understand the role of platelets in impaired wound healing of diabetic foot ulcers in diabetic patients.

1.10 Study objectives

- Perform HbA1c test on all participants to confirm the hyperglycaemic status of diabetic patients and normal glucose levels for the non-diabetic healthy volunteers who were participants in this study
- To confirm the non-activated status of the platelets from all participants using flow cytometry and known general platelet and activated platelet markers

- To separate and compare platelet proteins using SDS-PAGE and different staining procedures to identify differences, especially for protein glycation from diabetic patients and healthy individuals
- To perform proteomic analysis on a selection of bands from SDS PAGE and sequence the peptides using LC-MS/MS to identify relevant proteins by searching a protein database
- To perform global proteomic analysis of platelets from selected participants to assess differences between diabetic patients and healthy individuals

Chapter 2: Materials and Methods

2.1 Sample collection

2.1.1 Participant recruitment and screening

Ethical approval for this study was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria for recruitment and blood sample collection from volunteers (Approval number: 358/2016, see Appendix). Participants from diabetic patients and non-diabetic healthy individuals who were older than 25 years were asked to participate in the study. Each group consisted of 30 participants and they were relatively matched one group to the other according to age and gender as shown in the patient information list in the Appendix. Recruitment of diabetic patients was carried out at the Steve Biko Academic Hospital (SBAH) podiatry unit and diabetic clinic, whilst non-diabetic healthy individuals were recruited from the University of Pretoria, Faculty of Health Sciences. Information regarding the research project and the risks and benefits was clearly explained and discussed with prospective participants before signing informed consent forms that were obtained from all the participants prior to collection of blood samples. Identity of the participants was kept confidential and samples were referred to by means of coding letters that identified gender and diabetic status and sequence number only. A questionnaire and the HbA1c test with the same coding was used to confirm participants' long-term glucose status. The HbA1c test was performed using the A1CNow® kit from Polymer Technology Systems, Inc (Indianapolis, USA). At the time of recruitment, information from patient files was also used for screening and recorded to determine if there were other conditions present that may influence reporting of results. Table 1 shows the inclusion and exclusion criteria used in participant screening.

Table 1: Participant inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Type 2 DM patients or non-diabetic healthy individuals	Type 1 DM patients
Type 2 DM patients should have DFUs or a high risk of developing DFUs	Diabetic patients should not be on any medication for diseases not associated with DM.
HbA1c level below 5.7% (39 mmol/mol) for healthy individuals	
HbA1c level above 6.5% (48 mmol/mol) for diabetic patients	
Participants should be aged between 25 – 65 years	
Participants must understand the information and sign their own informed consent form	

2.1.2 Platelet isolation and flow cytometry analysis

Materials

i) Phosphate buffered saline (PBS)

A mass of 9.23 g of FTA Haemagglutination buffer (pre-formulated phosphate buffered saline, PBS) purchased from BD Biosciences (San Jose, USA) was dissolved in 1 litre of dH_2O as per product instructions and stored at 4°C.

ii) Antibodies

Fluorescently conjugated monoclonal antibodies against CD42a conjugated to FITC and CD62P conjugated to PE were used for flow cytometry analysis of the isolated platelets to determine the extent of activation. The antibodies were from BioLegend® (San Diego, CA, USA) and stored at 4°C.

Method

A total volume of 16 ml of venous blood was collected into two glass 8 ml sodium citrate CPT™ Vacutainer® tubes (BD Biosciences, San Jose, USA) from the volunteers. The blood was centrifuged at 1800 *x g* for 20 minutes at 18°C as per the supplier's protocol and the PRP buffy coat layer isolated and transferred to a 50 ml centrifuge tube. A single wash step was carried out, where PBS at room temperature (rt: 22-23°C) was added to the 50-ml mark of the tube. Following this, the tube was centrifuged at 1500 *x g* for 10 minutes at 18°C. The supernatant was discarded, and the soft platelet pellet resuspended in 3 ml of PBS. All work from this step on was done on ice to prevent activation of the platelets. Flow cytometry analysis was done on an aliquot of the platelet samples to check for activation. To this end, 5 µl of each of the antibodies, CD42a FITC and CD62P PE was added to a 5-ml round-bottom Falcon™ tube and incubated with 100 µl of the platelet suspension for 10 minutes in the dark. When the incubation period had lapsed, 700 µl of flow cytometer sheath fluid (Beckman Coulter, Brea, USA) was added to the tube and analysis done on a Cytomics FC500 flow cytometer (Beckman Coulter, Brea, USA).

2.2 Bicinchoninic acid assay (BCA) for protein quantitation

Materials

i) Reagent A

One-gram bicinchoninic acid disodium salt hydrate (Sigma-Aldrich, St Louis, USA), 2 g sodium carbonate, 0.16 g sodium tartrate, 0.4 g sodium hydroxide (NaOH), all from Merck (Darmstadt, Germany) and 0.95 g sodium bicarbonate (Sigma-Aldrich, St Louis, USA) were dissolved in a final volume of 100 ml with dH_2O, adjusted to a pH of 11.25 with 10 M NaOH and stored at 4°C.

ii) Reagent B

A solution of 0.4 g cupric sulphate (Rochelle Chemicals, Johannesburg, RSA) in 10 ml dH_2O was prepared and stored at 4°C.

iii) PBS

PBS was prepared as described in Section 2.1.2 (i).

iv) Protein standards

A 2.5 mg/ml stock solution of bovine serum albumin (BSA), purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) was prepared by dissolving 5 mg of BSA in 2 ml of PBS. From the stock solution, a range of protein standards were prepared and made up to a final volume of 1 ml with PBS. The concentrations were as follows: 0.1 mg/ml, 0.2 mg/ml, 0.25 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 1 mg/ml, 2 mg/ml and 2.5 mg/ml.. A fresh stock solution and protein standards were prepared each time the assay was done.

Method

The BCA assay was performed to determine the protein concentration of the platelet samples. The prepared protein standards were added in triplicate to the wells of a round-bottomed 96-well plate at a volume of 5 µl. The same volume of each platelet samples and PBS (blank) were also added to the plate. Reagents A and B were mixed at a ratio of 50:1 respectively to make a standard working reagent (SWR). The SWR was added to each well at a volume of 250 µl to give a final volume of 255 µl. The plate was covered with foil and placed on a shaker for two minutes to mix the contents. The plate was then incubated at 60°C for 30 minutes, after which it was cooled to room temperature and analysed using a Synergy 2 plate reader (Bio-Tek Instruments Inc, Vermount, USA) using a wavelength of 562 nm. GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA) was used to create a standard curve from the absorbance vs protein standards concentration and perform linear regression analysis to determine the protein concentration of the samples. After the BCA assay an aliquot was taken from each of the samples and diluted such that the final protein concentration was 1 mg/ml and a volume of the cOmplete® protease inhibitor (Roche Diagnostics, Mannheim, Germany) added. These aliquots were quickly mixed then stored at -80°C.

2.3 One dimensional SDS-PAGE

Materials

i) Laemmli buffer

A pre-prepared solution of 2x Laemmli sample buffer was purchased from Bio-Rad Laboratories Inc, (Berkely, USA) and stored at room temperature. β-mercaptoethanol (βME), purchased from Sigma-Aldrich (St Louis, USA) was added to the sample buffer as a reducing agent at a ratio of 50 µl βME to 950 µl sample buffer.

ii) Protein mass standards

Unstained Precision Plus Protein™ Standards which cover a mass range of 10-250 kiloDaltons (kDa) were purchased from Bio-Rad Laboratories Inc, (Berkely, USA) and stored at -20°C.

iii) 10x Tris/Glycine/SDS buffer

The 10x concentration of electrophoresis running buffer was purchased from Bio-Rad Laboratories Inc, (Berkely, USA) and stored at room temperature. To make 900 ml of a 1x working solution with a pH of 8.3, 90 ml of the concentrate was added to 810 ml of deionised water and mixed thoroughly.

iv) Precast gels (Stain-free™)

Pre-manufactured 4-20% acrylamide gradient gels with 10-wells, (Mini-PROTEAN® TGX Stain-free™ Gels) were purchased from Bio-Rad Laboratories Inc, (Berkely, USA), stored at 4°C and used according to the product instructions.

v) Acqua stain (Colloidal Coomassie Blue stain

The Acqua colloidal Coomassie Blue stain was purchased from Vacutec (Johannesburg, RSA) and stored at 4°C. This is a rapid staining version of Coomassie stain that does not require extensive washing of the gel post staining.

vi) Oriole™ Fluorescent Gel Stain

Oriole™ Fluorescent stain was purchased from Bio-Rad Laboratories Inc, (Berkely, USA) and stored at ambient temperature.

vii) Periodic acid–Schiff (PAS) stain

The Pierce Glycoprotein Staining Kit, purchased from ThermoFisher Scientific (Rockford, IL, USA) was used to perform the PAS stain. Preparation of the kit contents as well as additional reagents was done as follows:

- 3% Acetic acid: To make 1 litre, 30 ml of glacial acetic acid purchased from Merck (Darmstadt, Germany) was mixed with 970 ml deionised water and stored at RT.
- 50% Methanol (MeOH): 250 ml of methanol purchased from Sigma-Aldrich (St Louis, USA) was mixed with 250 ml deionised water and stored at rt.
- Oxidizing solution: 250 ml of 3% acetic acid was added to the oxidizing reagent of the kit and mixed until all material was completely dissolved. The solution was stored at RT.
- Reducing solution: 250 ml of deionised water was added to the reducing reagent of the kit, mixed until all material was completely dissolved and stored at RT.
- Horseradish peroxidase positive control: The vial contents were reconstituted with 0.5 ml of deionised water to produce a 2 mg/ml solution and stored as 100 µl aliquots at -20°C. The positive control was diluted to 1 mg/ml with Laemmli sample buffer before loading into the wells of the gel.
- Soybean trypsin inhibitor negative control: The vial contents were reconstituted with 0.5 ml of deionised water to produce a 2 mg/ml solution and stored as 100 µl aliquots at -20°C. The negative control was diluted to 1 mg/ml with Laemmli sample buffer before loading into the gel wells.

viii) Silver stain

The silver stain was done based on the method developed in the ‘80s Merril *et al.* (1981). Preparation of reagents for the silver stain was done as follows:

- Fixing solution: 40% methanol, 10% acetic acid and 50% dH_2O. Methanol and acetic acid were purchased from Merck (Darmstadt, Germany)
- Wash solution: 30% ethanol in dH_2O. 96% ethanol was purchased from Sigma-Aldrich (St Louis, USA).
- Reductant: 200 mg sodium thiosulfate (Merck, Darmstadt, Germany) was dissolved in 1 litre of dH_2O.

- Silver stain: 2 g of silver nitrate (Merck, Darmstadt, Germany) was dissolved in 1 litre of dH_2O containing 200 µl of formaldehyde (Merck, Darmstadt, Germany). The bottle containing the stain was wrapped with aluminium foil to prevent light exposure.
- Developer: 30 g of sodium carbonate and 5 mg sodium thiosulfate were dissolved in 1 litre of dH_2O containing 500 µl formaldehyde. All reagents were purchased from Merck (Darmstadt, Germany).
- Stop solution: 5% acetic acid in dH_2O. Acetic acid was purchased from Merck (Darmstadt, Germany).

Method

Samples were diluted at a 1:1 ratio with Laemmli sample buffer and boiled at 100°C for 5 minutes. The samples were cooled to ambient temperature and centrifuged in a microcentrifuge (Beckman Coulter) at 16 000 *g* for 5 minutes to remove any insoluble material. The Stain-free™ precast gels in their cassettes were rinsed with deionised water, stripped of the sealing strips and inserted into a Mini-PROTEAN electrophoresis tank from Bio-Rad Laboratories (Berkely, USA). Running buffer (1x concentration) was poured into the top reservoir module and the tank. The protein mass standards were pipetted into the first and last wells of the gels at a volume of 10 µl, and selected samples into the remainder of the wells at a volume corresponding to a mass of 10 µg of protein. The samples were loaded in duplicate. For the gels that were stained with the PAS stain, the protein mass standard in the last well (well number 10) was replaced with the positive and negative controls, which were pipetted at a volume of 5 µl per control. The electrophoresis tank was connected to a Hoeffer PS300-B power source and electrophoresis was carried out at 60 volts for the first 40 minutes. The voltage was taken up to 200 volts for the rest of the run. On completion of the run, the gel cassettes were cracked open, the gels removed and placed on a Stain-free™ imaging tray (Bio-Rad Laboratories Inc, Berkely, USA) and Stain-free™ imaging done on a Gel Doc™ EZ Imager also from Bio-Rad Laboratories Inc, (Berkely, USA). The software used for gel imaging was the Bio-Rad™ Image Lab 5.2.1 software. Coomassie (Acqua), Silver, Oriole™ and PAS staining was done on individual gels as described below, and imaging of all the differently stained gels was done using the Gel Doc™ EZ Imager from Bio-Rad with the appropriate imaging tray.

- **Coomassie (Acqua) stain**: Enough stain to cover the gels was added and the gels were gently agitated for 1 hour. The gels were washed once with deionised water, placed on a White light imaging tray (Bio-Rad Laboratories Inc, Berkely, USA) and imaged.
- **Oriole™ fluorescent stain:** Enough stain to cover the gels was poured into the gel containers, which was then covered completely with aluminium foil. The gel was gently agitated for 90 minutes and washed once with deionised water prior to placement on an Ultraviolet imaging tray (Bio-Rad Laboratories Inc, Berkely, USA) for imaging.
- **Silver stain:** The gels were fixed in fixing solution for 30 minutes then washed three times with wash solution, for 20 minutes per wash. Following this, the gels were placed in the reductant for one minute and washed 3 times with dH_2O for 30 seconds per wash. Silver nitrate stain was added to the gels for 15 minutes, followed by three 1-minute wash steps in dH_2O. The developer solution was added to the gels, and they were left in the solution until bands became visible. The reaction was terminated by adding the stop solution and the gels were washed three times in dH_2O for 1 minute per wash. The gels were placed on a White light imaging tray (Bio-Rad Laboratories Inc, Berkely, USA) and imaged.
- **PAS stain:** The gels were fixed for 30 minutes in 50% methanol and washed twice by gently agitating with 3% acetic acid for 10 minutes. The gels were transferred to 25 ml of oxidizing solution and gently agitated for 15 minutes. Following this, the gels were washed twice by gently agitating with 3% acetic acid for 5 minutes, transferred to 25 ml of the glycoprotein staining reagent and gently agitated for 15 minutes. The gels were then transferred to 25 ml of the reducing solution and gently agitated for 5 minutes. The gels were washed extensively with 3% acetic acid and deionised water, then imaged on a White light imaging tray (Bio-Rad Laboratories Inc, Berkely, USA) and imaged.

2.4 HPLC analysis for AGE identification

Materials

i) 5.5 mM Calcium chloride ($CaCl_2$) in 25 mM ammonium bicarbonate (NH_4HCO_3)

This was prepared by dissolving 0.1 g of NH_4HCO_3 and 0.03 g of $CaCl_2$ in 50 ml of deionised water.

ii) Trypsin

The trypsin kit containing a resuspension buffer and 20 µg sequencing grade modified trypsin was purchased from Promega (San Diego, CA, USA). A volume of 200 µl of the resuspension buffer was added to a vial containing the 20 µg trypsin. The vial was slowly vortex mixed to dissolve the trypsin which was then placed on ice. Aliquots of 20 µl of the stock were prepared and stored at -20°C. To make a final working concentration of 10 ng/µl trypsin, 20 µl of the stock trypsin was added to 180 µl of 5.5 mM $CaCl_2$ in 25 mM NH_4HCO_3

iii) Mobile phase A

Mobile phase A was made up of 0.1% formic acid (FA) in deionised water. Formic acid was of mass spectrometer grade purchased from Sigma-Aldrich (St Louis, USA).

iv) Mobile phase B

Mobile phase B was made up of 0.1% FA in 100% acetonitrile (ACN). MS-grade acetonitrile was purchased from Romil (Cambridge, USA).

v) 25% ACN

This was prepared by adding 2.5 ml of MS-grade ACN (Romil, Cambridge, USA) to a graduated measuring cylinder and making it up to 10 ml with deionised water.

Method

A volume of 25 µl of the final working trypsin solution was added to 100 µl of each of the platelet samples. The samples were incubated overnight at 37°C and dried completely in a vacuum drier. Prior to HPLC analysis, 50 µl of 25% ACN was added to the dried samples to solubilize them. The samples were vortex mixed and centrifuged in a Beckman Coulter microcentrifuge at 16 000 *g* for 10 minutes. The supernatant was used for HPLC analysis. The HPLC system used comprised of a SIL-20A HT autosampler, LC-20AB solvent delivery unit, DGU-20A$_5$ prominence degasser and an RF-10A $_{XL}$ fluorescence detector (all from Shimadzu) coupled to a Phenomenex Gemini C18 column (100 x 2.1 mm, 3.0 µm). A gradient elution program was used where the initial conditions were 95% mobile phase A and 5% mobile phase B and run for 1 minute. The program was run from 5-45% over 25 minutes, after which the gradient was quickly pushed up to 60% for 30 seconds, then dropped to 5%. Re-equilibration was done over an 8-minute period. The injection volume was 10 µl and the flow rate 250 µl/minute. Fluorescence was measured at 330 nm (excitation) and 420 nm (emission) wavelengths, which are in the range of the fluorescence spectrum of AGEs (Munch *et al.* 1997). The Shimadzu LC Solutions software was used for system control and data analysis.

2.5 Western blotting for AGE identification

Materials

i) **Precast gels**

Pre-manufactured 4-20% acrylamide gradient gels with 10-wells, (Mini-PROTEAN® TGX Stain-free™ Gels) were purchased from Bio-Rad Laboratories Inc, (Berkely, USA), stored at 4°C and used according to the product instructions.

ii) **Trans-Blot® Turbo™ transfer pack (Midi format, 0.2 µm PVDF)**

The PVDF membranes in semi-dry transfer pack format were purchased from Bio-Rad Laboratories Inc, (Berkely, USA) and used according to the product instructions.

iii) 1x Tris-buffered saline (TBS)

A stock solution of 10x TBS was initially prepared by dissolving 24 g of Tris-HCL, 5.6 g Tris base and 88 g sodium chloride (all from Merck, Darmstadt, Germany) in 900 ml of dH_2O water. The pH was adjusted to 7.6. After this, dH_2O was added to the solution to give a final volume of 1 L and it was stored at rt. A one litre solution of 1x TBS was prepared fresh by adding 100 ml of the stock solution to 900 ml dH_2O.

iv) Washing buffer (TBST)

The wash buffer comprised of 0.05% of Tween-20 (Sigma-Aldrich, St Louis, USA) in 1x TBS prepared as described above. This was stored at 4°C.

v) Stripping buffer

The stripping buffer was made by dissolving 15 g glycine, 1 g SDS and 10 ml Tween-20 in 800 ml dH_2O, adjusting the pH to 2.2 and bringing the volume up to 1 L with dH_2O.

vi) PBS

PBS was prepared as described in Section 2.1.2 (i).

vii) 3% BSA

To prepare 1 L of the solution, 30 g of BSA (Santa Cruz Biotechnology, Dallas, Texas, USA) was dissolved in 1 litre of 1x TBS prepared as described above.

viii) Blocking buffer

This was made up from 3% BSA in TBS by adding 500 µl Tween-20 per litre solution. The buffer was stored at 4°C and was only stable for two weeks.

ix) Primary antibody: Rabbit anti-human AGE polyclonal antibody

The anti-AGE antibody was purchased from Abcam (Cambridge, UK) and stored at -20°C in 10 µl aliquots. The antibody was diluted 1:1000 in 3% BSA prior to use.

x) Loading control: β-Actin (N-21) rabbit polyclonal antibody

The antibody against β-Actin was used as the loading control, and was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). It was also diluted 1:1000 in 3% BSA prior to use.

xi) Secondary antibody: Goat anti-rabbit IgG-HRP

The secondary antibody was purchased from Elabscience (Houston, Texas, USA) and diluted 1:2000 prior to use in 3% BSA.

xii) Clarity™ ECL Western Blotting Substrate (200 ml)

The substrate which is compatible with any HRP conjugate was purchased from Bio-Rad Laboratories Inc, (Berkely, USA). The substrate was comprised of 100 ml Clarity™ western peroxide reagent and 100 ml Clarity™ western luminol/enhancer reagent. The two reagents were mixed in a 1:1 ratio to make an 800 µl solution for analysis of each membrane. ECL reagent was prepared fresh just before analysis.

xiii) Positive control: Glycated BSA

In vitro AGE induction was done following a method adapted from Munch *et al*. (1997). BSA was incubated (final concentration: 10 mg/ml) in phosphate buffer, with glucose (final concentration: 500 mM) at 60°C in the dark. A small volume of only 1 ml of the AGE solution was prepared. Preparation of the individual reagents was done as follows:

- Glucose (Sigma-Aldrich, St Louis, USA) was made as a 1 M glucose solution by dissolving 1.8 g in 10 ml deionised water.
- Sodium phosphate buffer (0.2 M) was made by dissolving 356 mg of sodium phosphate dibasic dihydrate (**A**) (Sigma-Aldrich, St Louis, USA) in 10 ml deionised water and dissolving 276 mg of sodium phosphate monobasic monohydrate (**B**) (Sigma-Aldrich, St Louis, USA) in a separate 10 ml deionised water. To make 5 ml of the final buffer, 3.85 ml of (**A**) was mixed with 1.15 ml of (**B**).
- BSA (Santa Cruz Biotechnology, Dallas, Texas, USA): 400 µl of 40 mg/ml BSA was prepared in deionised water.
- These reagents were combined in a ratio of 500 µl Glucose solution, 250 µl phosphate buffer and 250 µl of the BSA solution and incubated for 30 days in the dark at 60ºC.

Method

Western blot analysis was done following the procedure by Zhao *et al.* (Zhao *et al.* 2010). Gels were run as explained in Section 2.2 and after the run, gel proteins were transferred to the membranes of the transfer packs using the Trans-Blot® Turbo™

Semi-Dry Transfer system, under a seven-minute mixed program (Bio-Rad Laboratories Inc, Berkely, USA). The gels were scanned under the Stain Free conditions to confirm efficient blotting from the gels. The membranes were then blocked in the blocking buffer for 1 hour with gentle agitation at rt. Following this, the membranes were each incubated with 10 ml of the diluted primary anti-AGE antibody overnight at 4°C with gentle agitation. The membranes were then washed 5 times with the washing buffer for 5 minutes per wash. The diluted secondary antibody was added to the membranes at a volume of 10 ml per membrane, and they were incubated for 1 hour at RT with gentle agitation. The 5 wash steps as described above were repeated, and the membranes were incubated for 5 minutes with the ECL substrate. Band detection was done using a ChemiDoc™ imager and analysed with the Image Lab 6.0 software from Bio-Rad Laboratories Inc, (Berkely, USA). After this, the membranes were incubated in stripping buffer at RT for 5 minutes and the stripping process was repeated for a second time. The membranes were then washed twice with PBS for 10 minutes per wash and twice with the wash buffer for 5 minutes per wash. Following this, the membranes were blocked in the blocking buffer for 1 hour with gentle agitation at RT and then incubated with the diluted loading control overnight at 4°C. The membranes were washed and incubation with the diluted secondary antibody for 1 hour at RT followed. Another series of five wash steps was done, ECL added to the membranes and β-actin band detection done using the ChemiDoc™ imager as described for the anti-AGE antibodies.

2.6 Mass spectrometric analysis of proteins

2.6.1 Sample preparation for In-solution and In-gel digests

Materials

i) 10% Sodium dodecyl sulphate (SDS)

This was prepared by dissolving one gram of SDS (Sigma-Aldrich, St Louis, USA) in 10 ml of dH_2O and sonicating to dissolve any undissolved particles.

Method

The aliquots of platelet protein stored at -80°C in the presence of a protease inhibitor cocktail were thawed and SDS added to a final concentration of 3% in solution. The samples were vortex mixed and sonicated for 5 minutes. The samples were left to stand for 1 minute and centrifuged at 16 000 *g* for 5 minutes. The individual supernatant was collected for downstream in-gel or in-solution digestions and to perform the BCA assay for protein concentration determination as described in Section 2.2.

2.6.2 Protein in-gel digests

Materials

i) 50 mM Ammonium bicarbonate (NH_4HC0_3)

This was prepared by dissolving 0.2 g of NH_4HCO_3 (Sigma-Aldrich, St Louis, USA) in 50 ml of deionised water. The solution was stored at 4°C and used within two weeks.

ii) 5.5 mM Calcium chloride ($CaCl_2$) in 25 mM (NH_4HCO_3)

This was prepared as described in Section 2.4 (i).

iii) 50 mM NH_4HC0_3 in 50% MeOH

To prepare this solution, 0.2 g of NH_4HC0_3 was dissolved in 25 ml of deionised water and 25 ml of 100% MS-grade MeOH (Romil, Cambridge, USA). The solution was stored at 4°C and used within two weeks.

iv) 25 mM NH_4HC0_3 in 50% ACN

To prepare 2ml of solution, 1 ml of 50 mM NH_4HC0_3 prepared in (i) was added to 1 ml of 100% MS-grade ACN (Romil, Cambridge, USA). The solution was stored at 4°C and used within two weeks.

v) 75% ACN

To make 10 ml of ACN, 7.5 ml of ACN was added to a graduated measuring cylinder and made up to 10 ml with deionised water. This was stored at 4°C and used within two weeks.

vi) Trypsin

Protein sequencing grade trypsin was prepared as described in Section 2.4 (ii).

vii) Dithiothreitol (DTT)

A 1 M stock solution was prepared by dissolving 0.154 g DTT (Sigma-Aldrich, St Louis, USA) in 1 ml of deionised water. A 10 mM DTT working solution was prepared by adding 10 µl of the stock DTT to 495 µl of 50 mM NH_4HC0_3 and 495 µl of deionised water. DTT was always prepared fresh before use.

viii) 55 mM Iodoacetamide (IAA) in 25 mM NH_4HC0_3

A volume of 1 ml of IAA (Sigma-Aldrich, St Louis, USA) was prepared by dissolving 10.2 mg IAA in 500 µl of 50 mM NH_4HC0_3 and 500 µl deionised water. This was also prepared fresh before use.

Method

SDS-PAGE was performed on the samples prepared in Section 2.6.1 as described in Section 2.3 except that instead of using Stain-free™ gels, standard 10-well 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels purchased from Bio-Rad Laboratories Inc, (Berkely, USA) were used. The gels were stained with the Acqua stain and after imaging bands of interest were cut out using a new, sharp scalpel blade. The gel slices were diced into small pieces (>1 x 1 mm) and placed into 0.5 ml Protein Lo-Bind Eppendorf tubes (Merck, Darmstadt, Germany). To de-stain the gel pieces, 200 µl (or

enough to cover the gel pieces) of 50 mM NH4HC03 in 50% MeOH was added and the Eppendorf tubes were vortex mixed for 20 minutes. The supernatant from this was discarded and the de-staining procedure was repeated using 100 µl (or enough to cover) of 75% ACN that was added to the gel pieces and they were vortex mixed for 20 minutes. The supernatant was again discarded, and the gel pieces were vacuum dried for 10 minutes at 30°C. After drying the gel pieces, a sequential reduction and alkylation step was done. Approximately 25 µl (or enough to cover) of 10 mM DTT in 25 mM NH_4HCO_3 was added to each dried gel piece which was then vortex mixed for 1 minute and centrifuged at 75 *g* for 2 minutes to collect the gel at the bottom of the tube. The tubes were incubated at 60°C for 1 hour in a shaking dry-block thermomixer, allowing the reduction reaction to proceed. Following this, the tubes were chilled to RT and 500 µl of 100% ACN was added. The tubes were incubated for 10 minutes at RT and the supernatant was discarded. Approximately 25 µl (or enough to cover) of 55 mM IAA in 25 mM NH_4HCO_3 was added to the gel pieces, quickly mixed and the reaction allowed to proceed in the dark for 20 minutes at rt. The supernatant was discarded, and the gel pieces were washed with a 1:1 ratio of 25 mM NH_4HCO_3 and deionised water (100 µl total volume). The supernatant was discarded, and the gel pieces were dehydrated with 100 µl (or enough to cover) of 25 mM NH_4HC0_3 in 50% ACN, vortex mixed for 5 minutes and centrifuged at 75 *g* for 2 minutes. The supernatant was discarded, and the dehydration step was repeated. The gel pieces were vacuum dried for 20 minutes at 30°C. The Eppendorf tubes with the gel pieces were placed on ice and 25 µl (or enough to cover) of the trypsin working solution (10 ng/µl) was added. After 30 minutes, another 10 µl (or equal volume to first addition) was added if the trypsin had been absorbed by the gel pieces. The gel pieces were rehydrated on ice for a further 90 minutes and centrifuged at 75 *g* for 2 minutes. A further amount of 25 mM NH_4HCO_3 as needed to cover the gel pieces was added to keep them wet during enzyme cleavage. After this, the Eppendorf tubes were centrifuged at 75 *g* for 2 minutes and incubated overnight in a shaking dry-block thermomixer at 37°C.

For peptide extraction, the Eppendorf tubes were centrifuged at 75 *g* for 1 minute and the aqueous digest solution (referred to as **Extract A)** from each tube was transferred into a clean 0.5 ml Protein Lo-Bind tube. To the gel pieces, 50 µl (or enough to cover) of 50% ACN/5% formic acid was added and the tubes were vortex mixed for 30

minutes. The tubes were centrifuged at 75 *g* for 1 minute and sonicated for 5 minutes. The new aqueous extract was added to **Extract A.** The extraction process was repeated to allow for maximum peptide extraction. Lastly, the combined digest supernatant solution was vacuum dried to complete dryness for 7 hours and stored at -20°C until LC-MS/MS analysis could be done.

2.6.3 Protein in-solution digests

Materials

i) MagReSyn HILIC Beads/Microparticles

MagReSyn HILIC Beads were purchased from ReSyn Biosciences (Edenvale, RSA).

ii) 1% SDS

A volume of 1 ml of 1% SDS solution was prepared by adding 25 µl of a 40% SDS stock (Sigma-Aldrich, St Louis, USA) to 975 µl of deionised water.

iii) 1 M DTT

A volume of 100 µl solution was prepared fresh by dissolving 15.4 mg of DTT (Amresco, USA) in 100 µl of deionised water.

iv) 1M IAA

A volume of 100 µl solution was prepared freshly by dissolving 18.5 mg of IAA (Sigma-Aldrich, St Louis, USA) in 100 µl of 1% SDS.

v) Trypsin

The trypsin kit containing a resuspension buffer and 20 µg sequencing grade modified trypsin was purchased from Promega (San Diego, CA, USA). An accurate volume of 200 µl of the resuspension buffer was added to a single vial containing the 20-µg trypsin. The vial was very gently vortex mixed to dissolve the trypsin and placed on ice. Aliquots of 20 µl of the stock were prepared in Lo-Bind tubes and stored at -20°C.

vi) MS-grade ACN

This was purchased from Romil through MicroSep (Jhb, South Africa).

vii) HILIC equilibration buffer (10 ml)

The buffer was made up of 15% ACN in 100 mM Ammonium acetate (NH_4Ac), pH 4.5.

viii) HILIC binding buffer

This was made up of 30% ACN in 200 mM NH_4Ac, pH 4.5.

ix) HILIC wash buffer

The wash buffer was made up of 95% ACN in 100 mM Ammonium acetate (NH_4Ac), pH 4.5.

x) Digestion buffer

The buffer was made up of 50 mM NH_4HC0_3 pH 8.0, as described in Section 2.6.2 (i).

Method

An amount of 20 µg protein of each of the samples prepared in Section 2.6.1 was used for in-solution digestion using trypsin and the automated system loaded with the HILIC ReSyn beads. The volumes of the samples corresponding to 20 µg protein were topped up with MS-grade water to give a final volume of 96 µl. This was then treated with 1 µl of 1 M DTT that was added, and the samples were incubated at RT for 30 minutes. Following this, 3 µl of 1 M IAA was added and the samples incubated at RT for 30 minutes. DTT and IAA were added to the samples to give final concentrations of 10 mM (1:99 dilution) and 30 mM (3:97 dilution) respectively. Sample clean-up was done using the HILIC 96 deep-well plate setup as shown in the Figure 6. Reagents and samples were plated from row G to row A as follows:

Row G: 10 µl of completely resuspended 20 mg/ml HILIC beads was added and topped up to 200 µl with equilibration buffer to wash off the bead storage buffer and to ensure that the protein:bead ratio was 1:10.

Row F: 500 µl of the equilibration buffer was added.

Row E: 100 µl of the alkylated and reduced sample and 100 µl of the binding buffer was added to give a 1:1 dilution of the processed protein per sample.

Row D: 200 µl of 95% ACN was added for the first wash.

Row C: 200 µl of 95% ACN was added for the second wash.

Row B: Row B was left empty.

Row A: 180 µl of the digestion buffer was plated and 20 µl of the trypsin stock was added.

Following plating of the samples and reagents, automated protein clean-up and digestion was done on a KingFisher™ Duo magnetic handling station (ThermoFisher Scientific, Rockford, IL, USA). The system which has a 12-pin robotic magnet head with disposable plastic comb prevents sample cross-contamination during binding, mixing and transfer steps between wells. The KingFisher™ Duo Peltier block (row A) was used for protein digestion at 37°C. The washed HILIC beads were collected from Row G, transferred to Row F for equilibration, and thereafter transferred to Row E for protein binding. Contaminants were removed by two successive washes in Rows D and C. Digestion proceeded for 4 hours in Row A (Peltier system). The HILIC beads were then removed from the digested protein mixture back to the storage position in Row G. Figure 7 shows the workflow of the program. Peptide–containing supernatant from Row A was recovered, transferred to new 0.5 ml Lo-Bind tubes and vacuum dried to complete dryness. The dried samples were stored at -80°C for LC-MS/MS analysis.

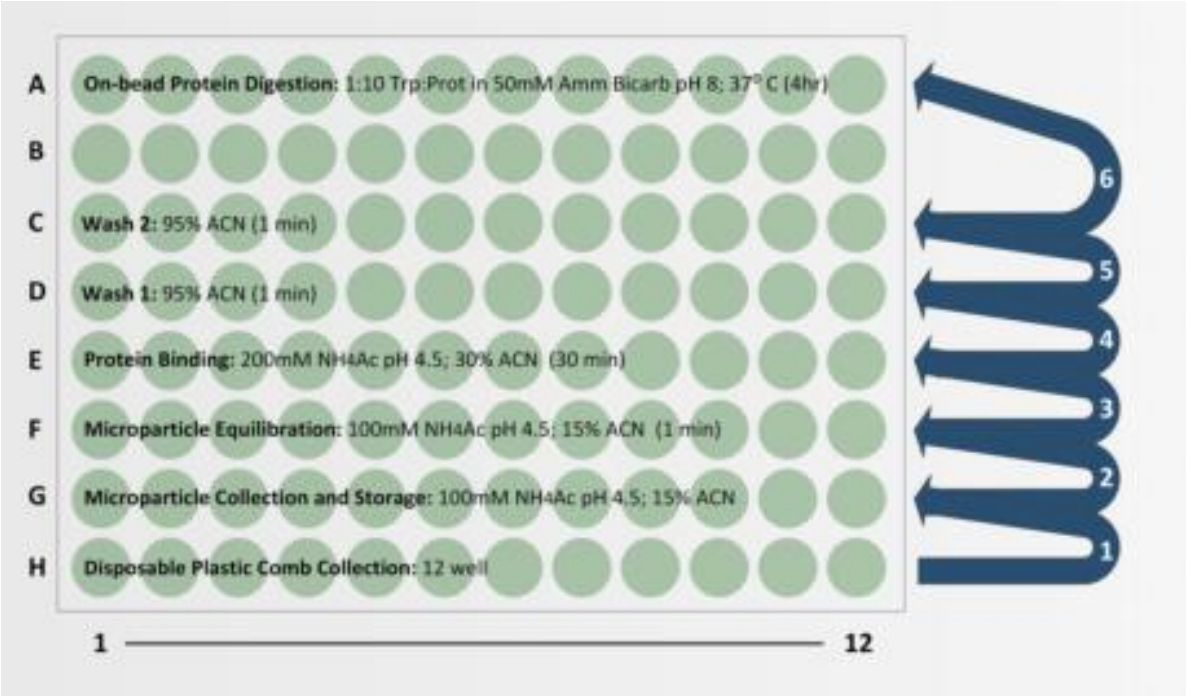


Figure 6: Plate layout of the HILIC sample clean-up method. Reproduced from www.resynbio.com (with permission).

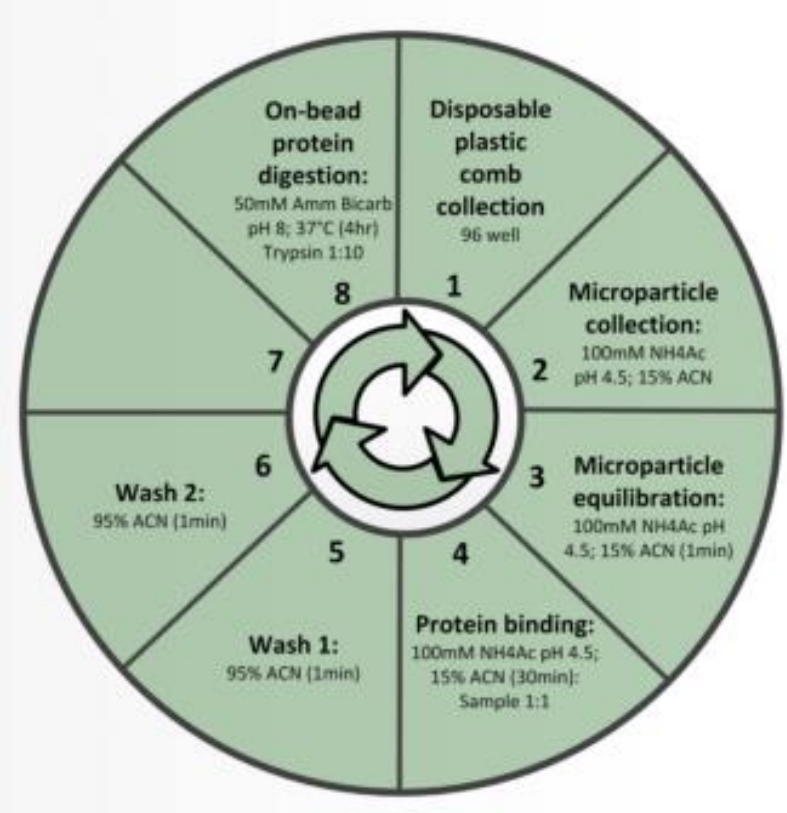


Figure 7: Workflow of the HILIC sample clean-up method. Reproduced from www.resynbio.com (with permission).

2.6.4 LC-MS/MS analysis

Materials

i) 2% ACN/0.2% Formic Acid (**FA)**

To make 1 L of the solution, 20 ml of MS-grade ACN (Waters, Massachusetts, USA) and 2 ml of mass spectrometer grade Formic Acid (Sigma-Aldrich, St Louis, USA) were added to 978 ml of MS-grade water.

ii) Mobile phase A (0.1% FA)

To make a 1 L solution, 1 ml of mass spectrometer grade FA was added to 999 ml of MS-grade water.

iii) Mobile phase B (80% ACN/0.1%)

To make 1 L, 800 ml of ACN was added to 1 ml of mass spectrometer grade FA and made up to 1 L with MS-grade water.

Methods

The individual dried protein digest samples were resuspended in 20 µl of the 2% ACN/0.2% FA solution and vortex mixed for 1 minute. Following this, the samples were centrifuged for 14 000 *g* for 10 min and supernatants transferred to new 0.5 ml inserts in autosampler vials. Sample analysis was performed using a Dionex™ Ultimate 3000 RSLC system coupled to an AB Sciex TripleTOF® 6600 mass spectrometer. A volume of 8 µl (~8 µg total protein) from each sample was loaded on an Acclaim® PepMap C18 trap column (100 µm × 2 cm) and inline desalted for 2 min at 15 µl/minute using 2% ACN/0.2% FA. Trapped peptides were gradient eluted and separated on an Acclaim® PepMap C18 RSLC column (300 µm × 15 cm, 3 µm particle size) using a flow-rate of 8 µl/minute with a gradient of 12-60% solution **B** over 60 min (**A**: 0.1% FA; **B**: 80% ACN with 0.1% FA). The 6600 TripleTOF mass spectrometer was operated in positive ion mode. For Data-dependent acquisition (DDA) runs, precursor (MS) scans were acquired from m/z 400-1500 (2+-5+ charge states) using an accumulation time of 250 ms followed by 50 fragment ion (MS/MS) scans, acquired from m/z 100-1800 with 25 ms accumulation time each. For Data-independent acquisition (DIA) runs, precursor scans were from m/z 400-900 followed by 63 fragment ion scans of 9 Da fixed window width and 1 Da overlap between windows, acquired from m/z 100-1800 with 25 ms accumulation time per window.

2.6.5 Database searching of mass spectra

For the in-gel digest peptide samples, spectral data from DDA runs (healthy individual control and diabetic patient samples) was analysed using SearchGui software version 3.2.20 (Compomics, Belgium (Vaudel *et al.* 2011)), with X!Tandem, MS-GF+ and Comet as the search engines. The spectral data was searched against a Uniprot/Swissprot human non-redundant proteome database supplemented with sequences of common protein contaminants. Decoy sequences were created by reversing the sequence orders in the target database in Search GUI. The following search parameters were applied: parent mass tolerance error of 30 ppm; fragment

mass tolerance error of 0.1 Da; up to two trypsin missed cleavages per peptide; cysteine carbamidomethylation as a fixed modification; methionine oxidation and N-terminal acetylation as variable modifications. A false discovery rate of 1% was used at the peptide-to-spectrum matches (PSM) and protein level inferences. A minimum of two unique peptides was required for protein identification. Peptide Shaker v 1.16.15 (Compomics, Belgium (Vaudel *et al.* 2011)), was used for visualization and further analysis.

For in-solution digest peptide samples, the acquired DDA runs (3x pooled control and 3x pooled patient samples) were searched using MaxQuant (Cox and Mann 2008). The search database included the human proteome from Swiss-Prot, common contaminants and internal retention time standards were searched using the same search engines as above. A minimum peptide length of 7 amino acids and a maximum of 2 missed cleavages were allowed. A 1% false discovery rate cut-off was applied at both the PSM and protein level. Quantitative analysis of DIA runs was performed using Spectronaut™ (Biognosys, USA (Bruderer *et al.* 2015)). A spectral library was generated using the searched DDA output and the spectral library generation functionality of Spectronaut™ with default parameters. DIA spectra were matched to the generated spectral library for peptide identification with quantification being performed on the fragment ion level.

Chapter 3: Results and discussion

3.1 Platelet isolation and flow cytometry analysis

The goal of the simplified method for platelet isolation was to have minimum platelet activation as possible. Platelets are known to be very fragile particles and are prone to activation (Ford, Graham and Rickwood 1990), therefore, to prevent or minimize platelet activation, whole blood from the volunteers was drawn relatively slowly into collection tubes that had citrate as an anticoagulant, which helps prevent platelet activation prior to use. In addition to that, an optimized buffy coat layer method with isolation procedures adapted from previous protocols that claim minimal platelet activation (Amable *et al.* 2013; Dhurat and Sukesh 2014) was followed. A buffy coat layer, which is rich in PRP was successfully obtained after differential centrifugation of whole blood from all the sixty participants recruited for the study as shown in Figure 8. Literature has reported that the buffy coat also contains white blood cells (WBCs), but to remove or decrease the WBCs from the samples, faster acceleration and longer centrifugation time periods were used (Perez *et al.* 2014).

It was also observed that three of the samples from the thirty diabetic patients had a tinge of red in both the platelet poor plasma and the buffy coat as depicted in Figure 9. This was thought to be caused by haemolysis which is consistent with observations reported in previous studies that type 2 DM patients have a significantly higher mechanical erythrocyte fragility in comparison to non-diabetic individuals (Lippi *et al.* 2012).

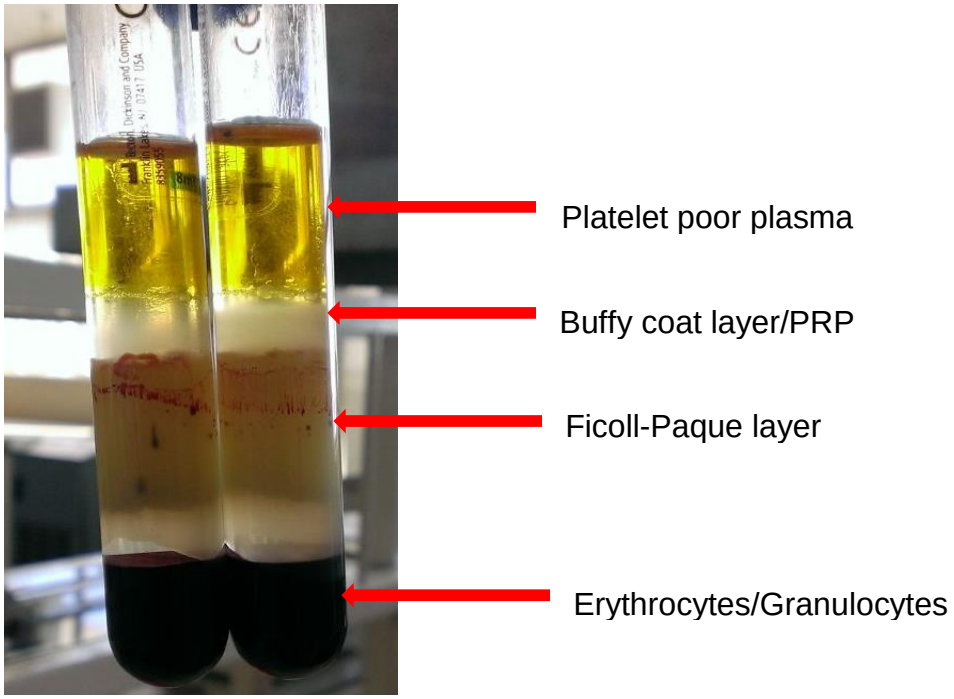


Figure 8: Isolation of buffy coat/PRP from whole blood. The buffy coat/PRP was isolated and washed once in PBS, the resulting pellet from the wash step resuspended in PBS and flow cytometry analysis done to check for platelet activation. Arrows indicate the different layers after separation of whole blood.

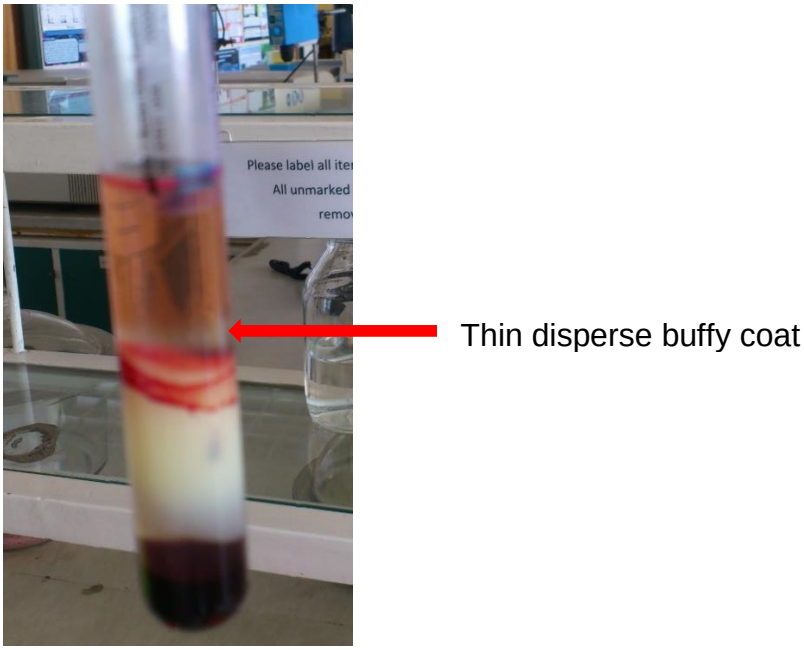
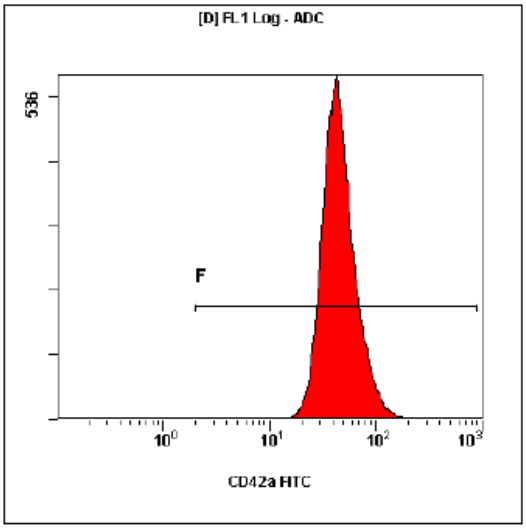


Figure 9: Red tinge formed in samples from 3 diabetic patients. A thin buffy coat layer was obtained in these samples as indicated by the arrow.

After isolation and purification of platelets from the PRP, flow cytometry analysis was done on the platelet pellets resuspended in PBS to check for activation, which was used to determine the success of the isolation procedure. The results showed that none of the thirty samples from the non-diabetic healthy individuals were activated, whilst in the diabetic patients, twenty-four of the thirty samples showed slight activation of about 3% on average. Figures 10 and 11 are representations of the flow cytometry results obtained.

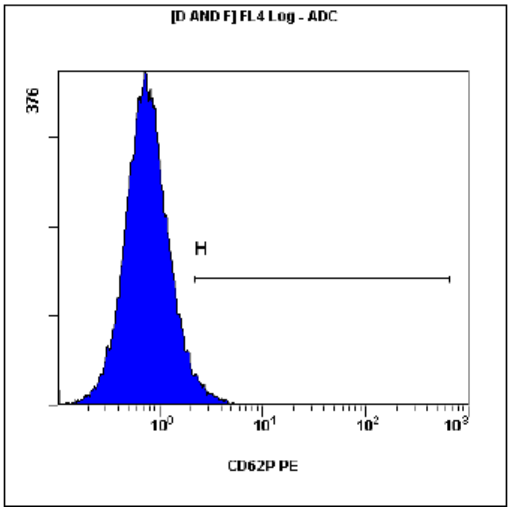
Therefore, it can be concluded that the protocol used for the isolation of platelets did show no or minimal activation which would then indicate that the second objective had been achieved. Activation of the platelets observed in twenty-four of the diabetic patients was not a surprising result, since platelets of some diabetes patients are known to be in an activated form while circulating *in vivo*, and if not already activated, they are highly prone to activation with minimal mechanical force being applied (Santilli *et al.* 2015). This fragility and ease of activation was already reported in the 1990's where the isolation methods involving centrifugation can be a harsh treatment that induces activation especially when pelleting is done during the washing procedure due to the high fragility of diabetic patient platelets (Ford *et al.* 1990). Additionally, limited platelet activation observed in some of the diabetic patients could have been caused by medications such as aspirin which hinder the activation process (Ornelas *et al.* 2017; Pulcinelli *et al.* 2004; Schror 1997) as shown in the patient information list in the Appendix.

(a)



[D] FL1 Log					
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	47334	94.67	100.00	48.6	###
F	47151	94.30	99.61	48.7	###

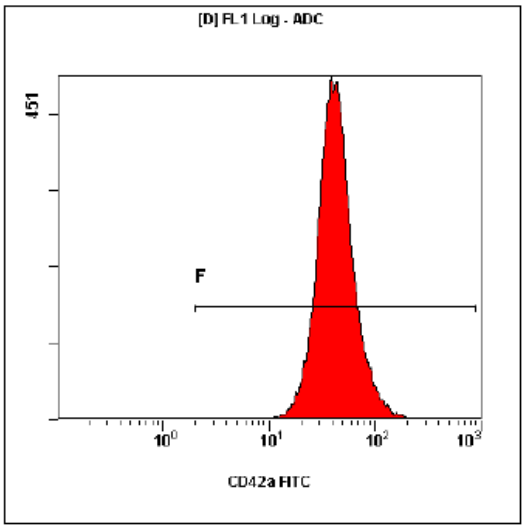
(b)



[D AND F] FL4 Log					
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	47151	94.30	100.00	0.895	###
H	1624	3.25	3.44	3.39	###

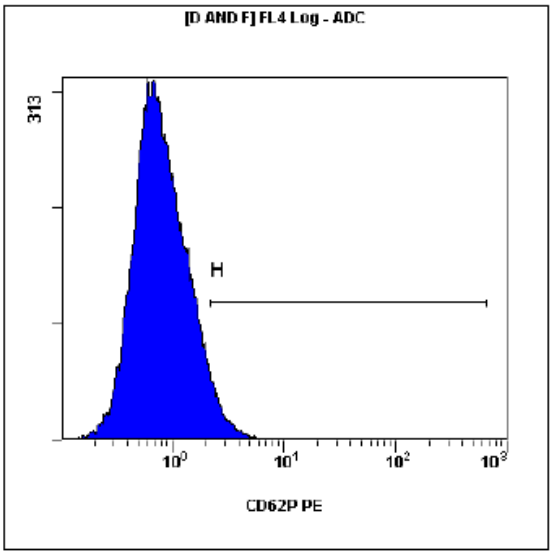
Figure 10: Flow cytometry histograms of washed platelets from non-diabetic healthy individual. Histograms depict (a) CD42a expression for platelet identification and (b) CD62P for platelet activation.

(a)



[D] FL1 Log					
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	44469	88.94	100.00	46.5	###
F	44199	88.40	99.39	46.8	###

(b)



[D AND F] FL4 Log					
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	44199	88.40	100.00	0.957	###
H	2184	4.37	4.94	3.1	###

Figure 11: Flow cytometry histograms of washed platelets from diabetic patient. Histograms depict (a) CD42a expression for platelet identification and (b) CD62P for platelet activation. Platelet activation of about 4.8% was observed for this patient.

3.2 Proteomic analysis: SDS-PAGE

The BCA assay was used to determine the protein concentrations of the platelet samples and the concentrations of the different samples were normalized to 1 mg/ml by appropriate dilution. After performing a few pilot gel electrophoresis runs, it was determined that 10 µg of protein was the optimum protein load for easily visualized and well-defined protein bands with clear resolution for the size and thickness of the gel. The amount of protein loaded onto the gel as well as the mass fingerprint obtained was similar to that observed in the study conducted by Yu *et al*. where a proteome comparison between the rat and human platelets was done (Yu *et al.* 2010).

For each of the 60 participants, individual gel runs were performed for every different imaging technique but for reporting of results in this thesis, a gel representing the overall protein mass fingerprint for each visualization technique was used, and a comparison made for the same patients. These representative results of the total sample size are shown in Figures 12 – 16.

By using the Bio-Rad special Stain-free™ gels it was possible to perform Stain-free™ imaging of the gels, as illustrated in Figure 12, prior to visualization with the different staining techniques. This technique works on the principle of fluorescent detection of aromatic amino acid residues such as tryptophan and tyrosine in the protein. These residues are modified by a trihalo compound present in the pre-cast gel to generate a fluorescence signal following brief ultraviolet (UV) irradiation (Rivero-Gutierrez *et al.* 2014). The advantage of this technique is that it gives higher resolution with low background since it makes use of fluorescence for visualization (Ladner *et al.* 2006) but it has a limited sensitivity requiring between 20-50 ng of protein for detection . Therefore to allow visualization of lower concentrations of protein, the gels were stained with the Acqua stain which is one formulation of the many colloidal Coomassie blue stains available (Kinter and Sherman 2005a).

Visualization of protein bands using the Acqua stain works on the same principle as the traditional colloidal Coomassie stains, but the difference lies in the greater sensitivity exhibited by the Acqua stain where the LOD can be down to less than 10 ng. The greatest advantage of this stain is that bands can be visualised in about 15 minutes to 1 hour, and no de-staining is required since only the bands are stained with minimal or no staining of the background (Bulldog Bio Inc 2017). In contrast to Stain-

free™ imaging which relies on fluorescent detection of aromatic amino acid residues, Acqua stain binds to the basic amino acid (arginine, lysine and histidine) and the hydrophobic aromatic residues of proteins (Eschenbruch and Bürk 1982), the implication being higher sensitivity where more protein bands can be detected as more than one type of amino acid residue is targeted. This explains the differences in appearance of protein bands observed between Stain-free™ and Acqua-stained gel images, with more bands being observed on the Acqua-stained gel, particularly in the low molecular weight protein region (15 – 20 kDa). This difference in targeted protein moieties between the two visualization techniques can also be seen at about 60 kDa, where the band on the Acqua-stained gel is more distinct than on the Stain-free™ image. This band is thought to be albumin, which contains many basic amino acids (Routledge 1986) and would thus be detected more clearly with the Acqua stain.

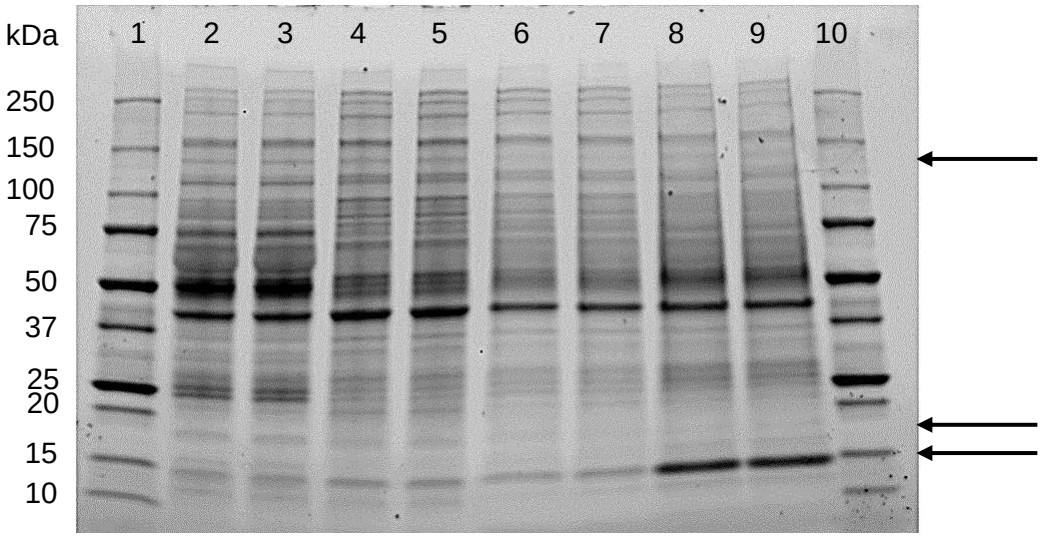


Figure 12: Stain-free ™ imaging of SDS-PAGE performed on washed PRP from 2 healthy individuals (Lanes 2-5) and 2 diabetic patients (Lanes 6-9). 10 µg of protein from each sample was loaded onto the gel. Lanes 1 and 10 are protein mass standards ranging from 10-250 kiloDaltons (kDa). Band differences are indicated with arrows.

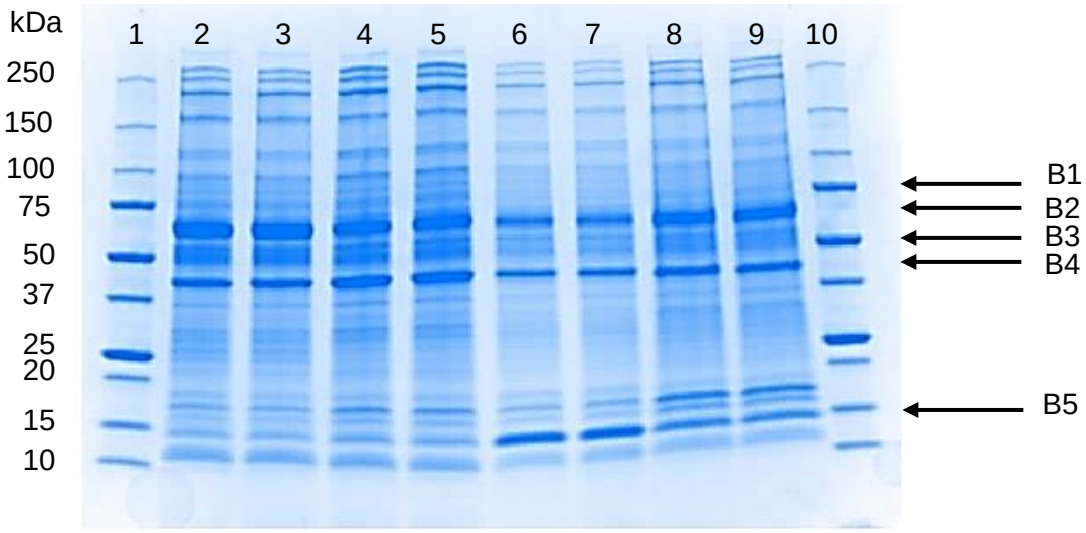


Figure 13: Acqua (Coomassie) stained gel. SDS-PAGE performed on washed PRP from 2 healthy individuals (Lanes 2-5) and 2 diabetic patients (Lanes 6-9).10 µg of protein from each sample was loaded onto the gel. Lanes 1 and 10 are the protein mass standards ranging from 10-250 kiloDaltons (kDa). Band differences are indicated with arrows. The bands indicated are those bands selected to be excised and digested with trypsin. The different fractions were identified as B1- B5.

To achieve greater sensitivity use was made of the highly sensitive silver stain and the Oriole™ fluorescent stain for identification of other protein bands. For this study, the acidic method which makes use of weakly acidic silver nitrate as a silvering agent, with development in an alkaline formaldehyde solution was followed (Steinberg 2009). The silver stain is claimed to be approximately 100-fold more sensitive than the Acqua stain (Coomassie stain) and this is partially due to its ability to rapidly immobilize proteins in the gel (Kinter and Sherman 2005a). This makes it a more suitable staining procedure when the amount of sample is limited. In contrast to the Acqua stain, the silver stain binds to the acidic protein residues more efficiently than the basic residues (Chevallet, Luche and Rabilloud 2006) which explains the difference in band appearance between Figures 13 and 14. More bands and darker staining were observed on the silver-stained gels, which was an expected result since band detection is also attributed to staining of macromolecules such as glycoproteins, lipoproteins and nucleic acids (Dzandu *et al.* 1984; Wang, Li and Li 2007). However, the silver stain is prone to saturation resulting in negative staining of some bands and formation of a background which can mask proteins (Eschenbruch and Bürk 1982; Kinter and Sherman 2005a) though it is highly praised for its sensitivity. This masking effect is seen with some of the denser protein bands in the 37 - 75 kDa mass range in

Figure 14. Due to the possibility of over saturation, the band development reaction of the silver stain had to be stopped before the less intense bands appeared on the gel (Chevallet *et al.* 2006). This is seen in Figure 14 where protein bands below the 37 kDa protein standard were not clearly detected. To allow a distinct appearance of these bands, the silver-stained gels would need to be immersed in the developer solution for an extended period, but the implication of this would be continued band development of the higher molecular weight or more intense proteins, resulting in oversaturation. Therefore, to compensate for these limitations, the Oriole™ stain was also incorporated as a visualization technique to provide a sensitivity of the same order or greater than that of silver (Ladner *et al.* 2006; Steinberg 2009), without over-saturation or background staining as shown in Figure 15. The Oriole™ stain allowed clear visualization of all the protein bands, with the more intense bands appearing to be brighter but not over saturated.

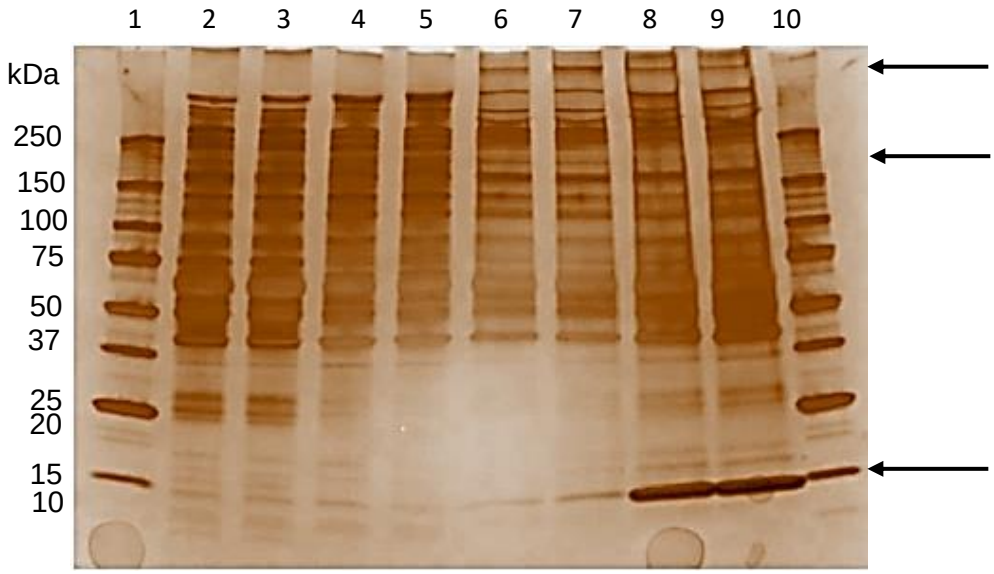


Figure 14: Silver stained gel. SDS-PAGE performed on washed PRP from 2 healthy individuals (Lanes 2-5) and 2 diabetic patients (Lanes 6-9). 10 µg of protein from each sample was loaded into the gel wells. Lanes 1 and 10 are the protein standards ranging from 10-250 kiloDaltons (kDa). Band differences are indicated with arrows.

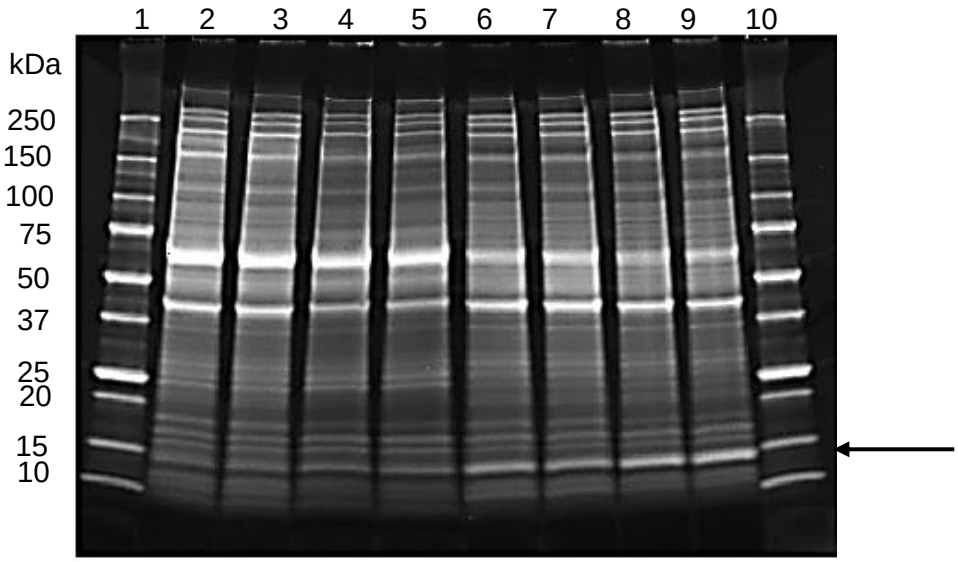


Figure 15: Oriole™ stained gel. SDS-PAGE performed on washed PRP from 2 healthy individuals (Lanes 2-5) and 2 diabetic patients (Lanes 6-9). 10 µg of protein from each sample was loaded into the gel wells. Lanes 1 and 10 are the protein standards ranging from 10-250 kiloDaltons (kDa). Band differences are indicated with arrows.

Due to the above-mentioned imaging techniques targeting different protein moieties for visualization, differences in the appearance of proteins were observed for the same patients from one visualization technique to the other. For instance, on the Stain-free™ gels, at about 18 kDa, bands were visible in lanes 2-5 (non-diabetic healthy individual samples) but these bands were almost invisible in lanes 6-9 of the diabetic patient samples. Comparing this observation to the Acqua, silver and Oriole™ stained gels, at the same molecular mass (18 kDa), bands could be observed in both groups which shows that Stain-free™ imaging was not sensitive enough to pick up the bands in the diabetic patients. This is seen again at about 120 kDa where Stain-free™, Acqua and silver imaging show bands to be missing in the diabetic patients but when compared to the Oriole™ stained gel, the bands are visible in both groups. The appearance of bands on the silver stained gels is also different when compared to the other visualization techniques, where the bands appear to be stacked with no spaces between them, revealing that the stain is very sensitive to pick up protein bands which are not picked up by the other visualization techniques. In the 10-20 kDa mass range, not a lot of bands are detected on the Stain-free™ gels but more bands are detected on the Acqua and Oriole stained gels. These additional bands can also be detected after silver staining of gels, but due to method optimization to prevent oversaturation

of the higher molecular weight proteins, the reaction was stopped before they could develop. Therefore, these observations make the simultaneous use of the different imaging techniques very important as it allows a broad comparison of proteins between samples due the ability of a more sensitive imaging technique to detect proteins missed by a less sensitive one. However, these differences in sensitivity between the imaging techniques show that there is a loophole in making conclusions about protein differences between groups based on these results only. This is because each imaging technique will target specific moieties and only those targeted proteins will be visualized. In this study, only Stain-free ™ imaging and three staining techniques as described above were used for protein comparisons between diabetic patients and non-diabetic healthy individuals, but there are several other stain-based imaging techniques such as the Sypro Ruby stain which have better sensitivity to detect proteins that were not detected by the techniques used in this study. However, it would not be feasible to use all the available staining techniques to identify the protein differences, therefore LC-MS/MS analysis of in-solution samples was an important step to determine possible platelet protein differences between diabetic patients and non-diabetic healthy individuals as it allows identification of individual proteins and shows whether they differed in expression between the two groups.

Analysis of the gels with the different imaging techniques showed similarities between the control healthy individuals and the diabetic patients in the general pattern, with subtle band differences between diabetic patients and non-diabetic healthy individuals as indicated by the arrows on the gel images. The major difference between the two groups was seen at about 15 kDa were a more distinct band, thought to be haemoglobin was observed in the diabetic patients, particularly those that had a tinge of red in their PRP. This proposition was derived from a study conducted by Lippi *et al*. where it was observed that Type 2 diabetes patients have high erythrocyte mechanical fragility in comparison to non-diabetic healthy individuals (Lippi *et al.* 2012). Therefore, if haemolysis had occurred, PRP from diabetic patients would have been contaminated with haemoglobin subunits which fall in the 15-17 kDa mass range. The platelet basic protein (PBP) (Mass: 13.89 kDa) which is cleaved into peptides that act as chemo-attractants and activators of neutrophils could also have been represented in that band. Since 90% of the diabetic patients recruited for the study had DFUs, this would not have been a surprising result. DFUs are said to be caught

in the inflammatory phase of the wound healing process, where neutrophils play a major role (Blakytny and Jude 2006), therefore it would make sense to observe an increase in the expression of the PBP that promotes activation of the neutrophils. A conclusion as to the identity of the proteins in the different bands cannot be done using SDS-PAGE data alone, and the observed differences between the groups highlighted the technical variance making it difficult to claim significant differences between the platelet proteins of diabetic patients and non-diabetic healthy individuals. This inability to conclude that there are any differences led to the next objective of the study, which was to assess possible differences in protein glycation between the two groups. Gels were run for each of the samples from diabetic patients and non-diabetic patients and these gels were stained with the PAS stain to see if there were any protein glycation differences between the two groups. The gel shown in Figure 16 is a representation of the overall result obtained for all the samples.

Patients suffering from diabetes show poor blood glucose concentration control with a classic post-prandial hyperglycaemia that can conceivably lead to non-enzymatic glycation of proteins and blood components. As wound healing is initially controlled through the activity of platelets it was reasoned that there could be a long-term effect on platelet function when there are some of the key proteins altered through glycation which in turn results from elevated blood glucose levels. To detect possible protein glycation where glucose is involved, the PAS stain was used. The mechanism by which this stain works is that carbohydrate groups with terminal hydroxyl groups are easily converted to aldehyde groups that rapidly react covalently with active amine moieties through a Schiff's base producing mechanism and can form highly coloured products such as with acid or basic fuchsin (Zhou *et al.* 2014). The reaction, when carried out on an SDS-PAGE gel, produces magenta bands where sugar-based glycated proteins are present (Bhattacharjee, Das and Mandal 2004). From the observations made, as shown in the gel image in Figure 16, a distinct magenta band was detected in the lane containing the positive control, but only very faint bands were detected in the platelet protein sample lanes despite following the staining protocol that required less than 30 µg of sample protein to be loaded onto the gel. This result indicated that the stain was working as the positive control could be detected in all the gels where this control was applied. By implication, insignificant amounts of glycated proteins were formed in the platelets due to hyperglycaemia. The faint bands were

more visible in the lanes containing samples from the non-diabetic healthy individuals, which was contrary to the expectation of detecting more glycated proteins in the diabetic patients' samples since they have elevated levels of circulating glucose.

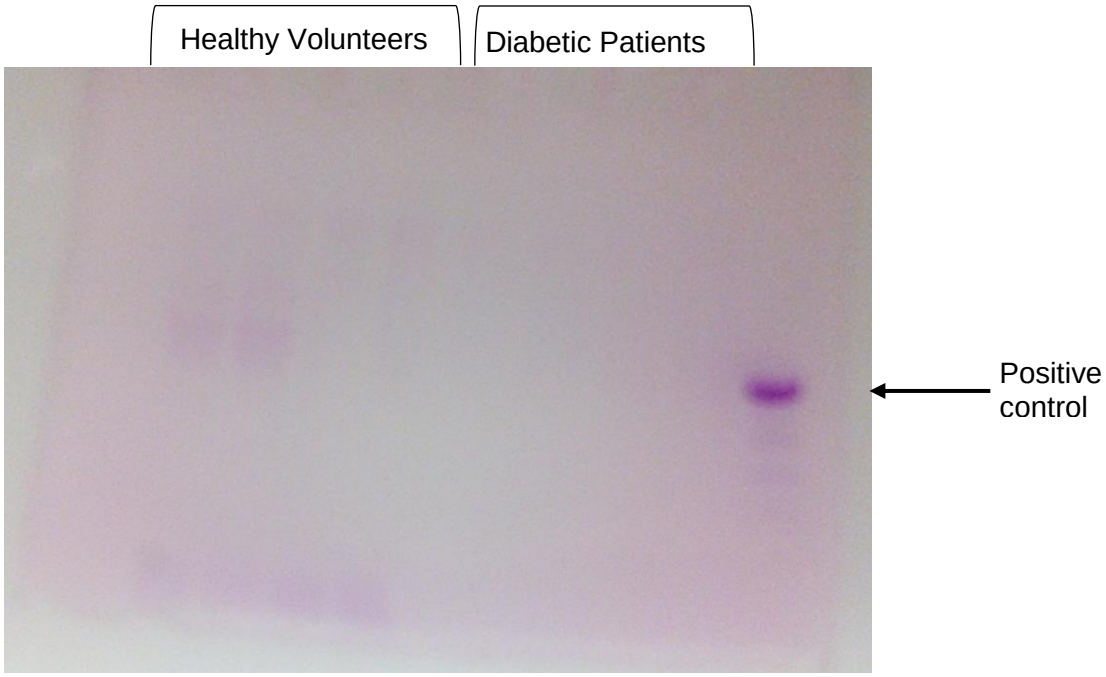


Figure 16: Photograph of PAS stained gel. The left and right sides of the gel indicated loaded samples from non-diabetic healthy individuals and diabetic patients respectively. The sample volumes loaded into the wells corresponded to a protein concentration of 1 mg/ml. Horseradish peroxidase was used as the positive PAS control and is shown by the arrow.

Based on the reports made in literature that glycation of proteins often leads to formation of intermediate residues that are rearranged to form AGE products (Nakayama *et al.* 1999; Wang *et al.* 2012) that are not reactive towards the PAS stain technique, a proposition was made that significant glycation of proteins from diabetic patients could have occurred, but that these proteins were then further processed to form AGE products. This led to the next objective which was to compare AGE formation between the two groups.

3.3 AGE Identification

The first step in AGE-identification involved HPLC analysis coupled to fluorescence detection of three representative samples from each group. As indicated by the chromatograms in Figures 17 and 18, similar peaks were obtained between the two groups suggesting that a similar amount of AGEs was present in the samples.

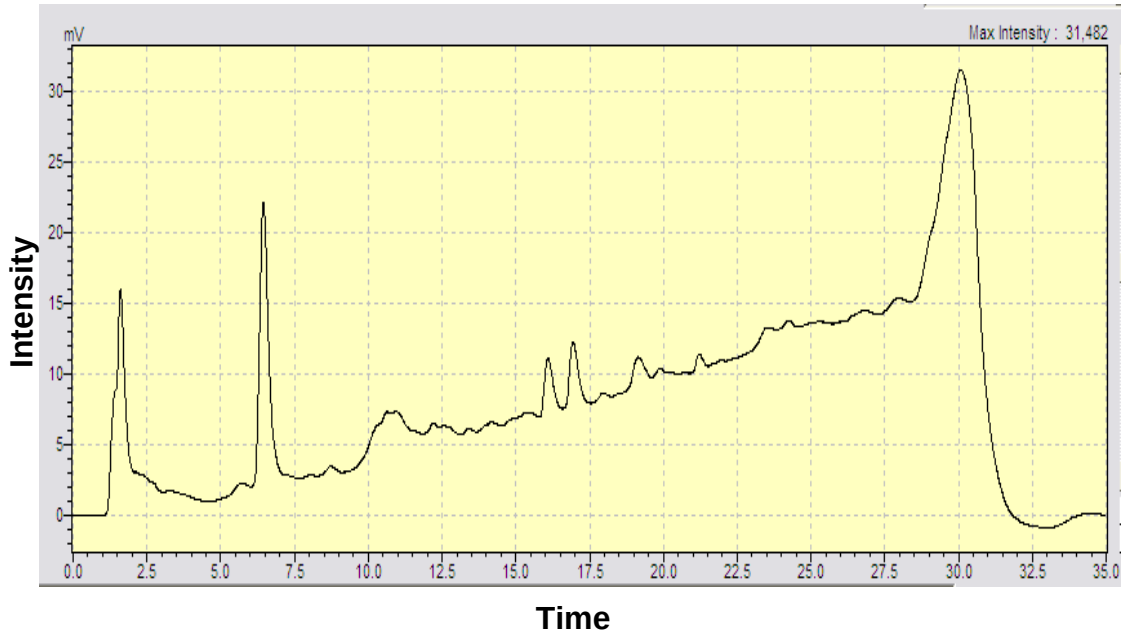


Figure 17: HPLC chromatograms of samples from non-diabetic healthy individuals. Excitation wavelength: 330 nm; Emission wavelength: 420 nm

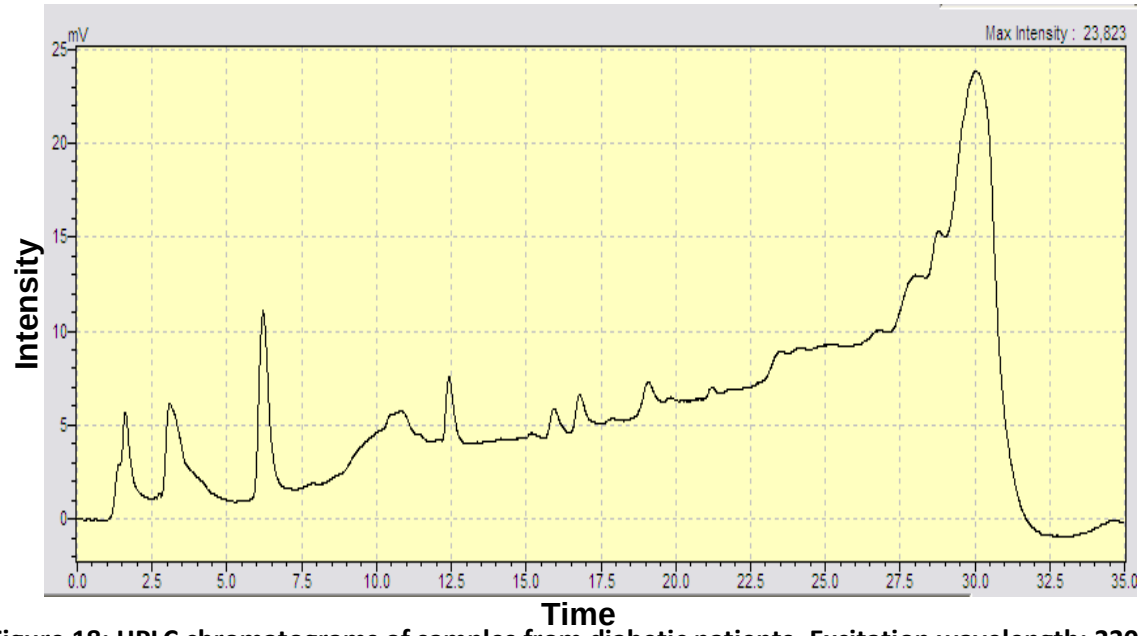


Figure 18: HPLC chromatograms of samples from diabetic patients. Excitation wavelength: 330 nm; Emission wavelength: 420 nm

A question that may arise from this observation is why possible AGE-product peaks would be identified in samples from healthy individuals. A possible explanation to this would be that, although AGE products are largely formed as a consequence of disease, exogenous AGE forming compounds are commonly found in “modern” foods, particularly processed foods, and these contribute to the total pool of AGEs in the body (Wang *et al.* 2012), therefore peaks showing classical fluorescence associated with

AGE products would be identified on the chromatograms from healthy individuals' samples too. There were subtle differences observed in the chromatograms at retention times of 3, 12.5, 28 and 28.5 minutes, where extra peaks which could have been additional AGE products due to hyperglycaemia in the diabetic patients' samples were observed. Nonetheless, a major drawback lay in the non-selective detection method used for the identification of possible AGEs. The fluorescent detection method used meant that some of the non-fluorescent AGEs like CML (Gkogkolou and Böhm 2012) would not be detected and accounted for, and these might have represented a major proportion of the AGEs in the samples from diabetic samples. Therefore, to account for these limitations and to assess whether formation of AGE products was significantly increased in diabetic patients, western blot analysis of all the 60 protein samples from both diabetic patients and non-diabetic healthy individuals was done with the use of antibody that specifically targeted the AGE component of any protein. Figure 19 is a representation of the results obtained from all the protein samples.

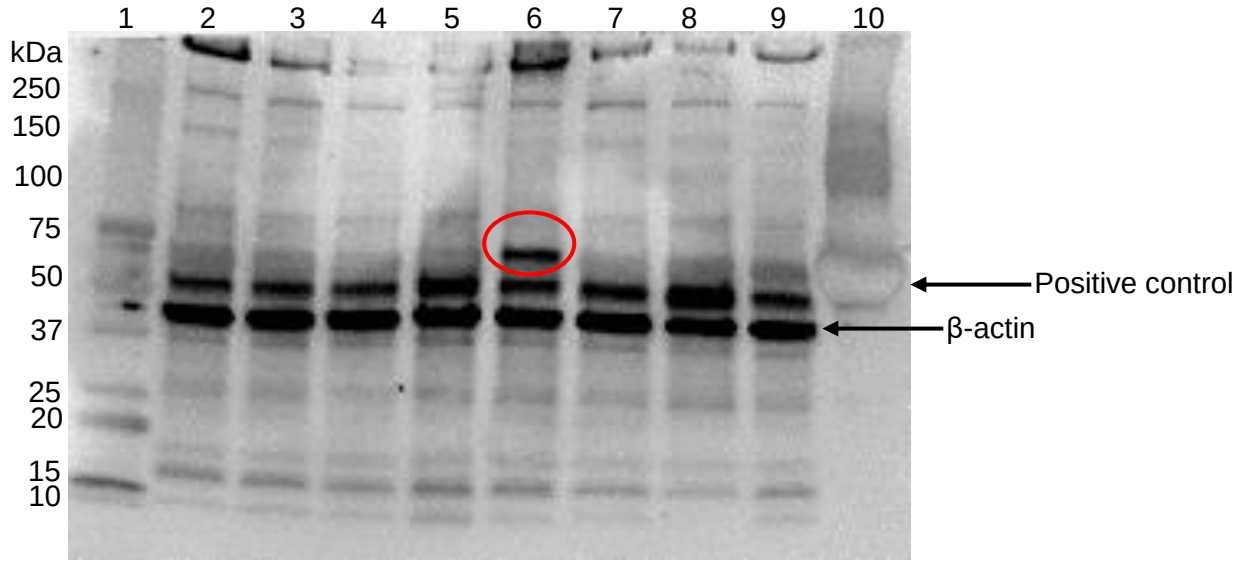


Figure 19: Western blot analysis of protein samples from healthy individuals (Lanes 2-5) and diabetic patients (Lanes 6-9). Lane 1 represents the Precision Plus Protein™ All Blue Prestained protein standards ranging from 10-250 kDa. Lane 10 represents glycated BSA which was used as the positive control. β-Actin was used as the loading control. Circle indicates band difference.

Glycated BSA was used as the positive control and this was made following the method by Munch *et al*. (1997). An interesting observation was made where the positive control appeared as a smear with a huge bulge at about 60 kDa. This was

initially thought to be caused by sample overloading, and after method optimisation, 5 µg/L was seen to be the lowest amount of glycated protein that could be loaded on the gel to give a signal when the membrane was probed with the anti-AGE antibody. However, this amount of protein still caused smearing and bulging. This observation could possibly have been a result of the heterogeneity of glycosylated protein which can change the mass of the original protein by several thousand Daltons thus affecting migration on the gel. Glycosylated proteins are also known to bind less SDS, which makes them less charged and thus run as higher molecular weights. Therefore, these effects combined could have been the cause of the smearing and bulging (Roy and Kumar 2014).

A common practice when performing western blot analysis is to make use of a loading control to ascertain that the same amount of protein was loaded on the gels. Housekeeping proteins which have a substantial expression level and that are unaffected by the experimental conditions are generally used as loading controls (Rivero-Gutierrez *et al.* 2014). For this study, beta-actin chain (β-actin) was selected as the housekeeping protein and from the results shown in Figure 19, the amount of protein loaded in the gel wells was relatively consistent. The blot confirmed the results obtained from HPLC analysis that AGEs were present in both the diabetic patients and the non-diabetic healthy individuals' samples. The intensity of the western blot bands representing the AGES on the membrane was similar between the two groups, the only difference being in lane 6, where one diabetic patient had a distinct band at about 70 kDa. This could account for the appearance of the extra peaks on the HPLC chromatograms of the same diabetic patient. Therefore, these results suggested that no significant differences in AGE formation could be observed between the diabetic patients and non-diabetic healthy individuals. The expectation was to observe increased glycation of platelet proteins in the diabetic patients since hyperglycaemia is known to increase the chances of protein glycation (Singh *et al.* 2014) and previous studies have also shown that platelets from diabetic patients are glycated to a greater extent than those from diabetic patients (Watala *et al.* 2005; Yatscoff *et al.* 1987). A possible explanation to these results would be that the oral hypoglycaemic drugs which the diabetic patients were taking, particularly metformin and pioglitazone were preventing or reducing the levels of protein glycation. Information obtained from the patient records showed that about 70% of the patients were taking either metformin or

pioglitazone and these hypoglycaemics have been reported to have moderate inhibitory effects on early stages of protein glycation (Rahbar and Figarola 2003; Rahbar *et al.* 2000). Furthermore, as mentioned earlier, diabetes patients tend to have comorbidities such as cardiovascular disease and consequently they also take CVD medications such as aspirin which help with blood thinning. It was also determined from the patient records that the 30% of diabetic patients not taking metformin or pioglitazone had CVD and were on chronic aspirin therapy. Aspirin has also been reported to inhibit the glycation process by acetylation of free amino acids, thus blocking the attachment of reducing sugars (Crompton, Rixon and Harding 1985; Rao, Lardis and Cotlier 1985; Urios, Grigorova-Borsos and Sternberg 2007). However, use of more sensitive techniques such as enrichment of glycated protein followed by mass spectrometry analysis would help validate the results obtained from HPLC and western blot analysis to determine if protein glycation occurred similarly between diabetic patients and non-diabetic healthy individuals (Zhu *et al.* 2017).

3.4 Proteomic analysis: LC-MS/MS

SDS-PAGE analysis provided qualitative results of how platelet proteins were expressed in both the diabetic patients and the non-diabetic healthy individuals. Important to note is that SDS-PAGE does not allow identification of individuals proteins and each band on the gel is often comprised of multiple proteins (Kinter and Sherman 2005a). Consequently, in a bid to identify the individual proteins that were differentially expressed between the two groups and correlate their function to the wound healing process, LC-MS/MS analysis from both in-gel and in-solution digests of representative samples including three non-diabetic healthy individuals and three selected samples from diabetic patients showing protein band differences was conducted.

The global platelet proteome is still largely unknown and vast research studies have been and are still being conducted to explore this treasure chest, since platelets have been implicated in several disease conditions such as vascular diseases. The reasoning that has been given by many researchers concerning this extensive research of the platelet proteome, is that knowledge of global changes in platelet proteins may contribute to a broader understanding of disease, thus aiding in development of therapies (Santilli *et al.* 2015; Yu *et al.* 2010). Proteins identified in this study were compared to lists compiled by Maynard *et al.* (2007), Yu *et al.* (2010), Burkhart *et al*. (2012) and Trugilho *et al.* which is the most recent of the three studies (Burkhart *et al.* 2012; Maynard *et al.* 2007; Trugilho *et al.* 2017; Yu *et al.* 2010). This was to confirm that indeed platelet proteins were analysed and from the results obtained from Spectronaut™ and Peptide Shaker, the identified proteins were comparable to those listed in the above-mentioned studies.

3.4.1 Data analysis of in-gel digests

Separate gels were run with six representative samples including three non-diabetic healthy individuals and three selected samples from diabetic patients showing protein band differences. These samples were run on standard 10-well 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels which were then stained with the Acqua stain for band visualization. The gels from which the bands of interest would be excised for in-gel trypsinization were stained with the Acqua stain as this stain is suitable for downstream MS analysis. Since the Acqua stain is a form of the Coomassie stain, de-staining of the excised bands prior to LC-MS/MS analysis was necessary owing to the

stain's ability to penetrate and bind with low affinity to the gel matrix (Steinberg 2009). The silver stained gels could not be used due to silver staining technique introducing protein crosslinking, hence its incompatibility with mass spectrometric analysis (Gundry *et al.* 2009). The Oriole™ stain is also MS-compatible (Ladner *et al.* 2006), but since it is not a visible stain unless exposed to UV irradiation, it could not be used for downstream MS analysis either. The amount of protein loaded on the gels used for in-gel digests was normalized to ensure that the same amount of protein from each sample group was loaded onto the gel. Select bands, which despite appearing to have different intensities were excised and trypsinized for LC-MS/MS analysis as shown on the Acqua-stained gel in Figure 13. A combination of X!Tandem, MS-GF+ and Comet search engines in SearchGUI was used for analysis of the spectral data since it has been observed that multiple search engines increase the coverage of protein identification (Yu *et al.* 2010). Further analysis was done using Peptide Shaker to visualize the proteins which were identified by matching the peptide fragment generated by tandem MS analysis with spectra from a Uniprot/Swissprot human, non-redundant proteome database supplemented with sequences of common protein contaminants. A total of 500 proteins were identified from the excised gel-bands where 335 were validated and 165 were doubtful. This number of proteins identified supports the notion that one band on an SDS-PAGE gel does not necessarily represent a single protein. Figures 20 is a typical overview of results obtained from Peptide Shaker.

Figure 20: An overview of typical results obtained from Peptide Shaker. The overview shows the proteins and peptides identified, peptide spectrum matches, the spectrum and ions view as well as the protein sequence coverage. The color codes of the sequence coverage: Green – validated peptides, Yellow – peptides that were doubtful, Red – peptides not validated, Blue – peptides carrying a variable modification such as oxidation.

The excised bands formed the fractions for each of the sample representatives. Since five select bands were excised from each lane from six representative samples, including three diabetic and three non-diabetic healthy individuals, the total number of fractions for analysis was 30. These fractions were then compared to each other to determine if there was a difference in protein abundance between the two groups. It has been reported that a higher absolute concentration of individual peptides results in more MS/MS spectra that can be confidently sequenced, leading to a higher spectral count for a protein that has greater abundance (Zhu, Smith and Huang 2010). Consequently, spectral count was used to assess possible differences in protein abundance. Other parameters that could have also been used for quantitation are 'the

number of identified peptides' and 'protein sequence coverage', but spectral count has been reported to show the greatest linear correlation to protein abundance (Old *et al.* 2005; Zhang *et al.* 2006). Spectral count was therefore the preferred method for measuring protein abundance to determine possible differences between diabetic patients and non-diabetic healthy individuals. The proteins had to be 1.5-fold up or down-regulated for the protein differences to be considered significant. Table 2 represents the major differences observed, where the protein abundance of seven platelet specific proteins differed between diabetic patients and non-diabetic healthy individuals.

Table 2: Platelet proteins of in-gel trypsinized bands showing differences in abundance between diabetic patients and non-diabetic healthy individuals.

Accession number	Protein name	Fold change
Q9Y490	Talin-1	1.9
P37840	Alpha synuclein	3.0
P01024	Complement C3	2.1
P02647	Apolipoprotein A-I	3.0
P01042	Kininogen-1	2.0
P60981	Destrin	2.0
O15511	Actin-related protein 2/3 complex subunit 5	N/A (Protein only in DB patients)

Of the proteins exhibiting differences between the two groups, talin-1 and apolipoprotein A-I were found to be more abundant in the non-diabetic healthy individuals, while complement C3, alpha-synuclein, kininogen-1 and destrin were more abundant in the diabetic patients by a fold change more than 1.5. In addition to this, actin-related protein 2/3 complex subunit 5 was observed only in the diabetic patients. The functions of these proteins were deduced from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO), which are a collection of databases that deal with genomes, biological pathways, diseases, drugs, chemical substances and enable functional interpretation. The observed differences were correlated to the wound healing process to assess whether they can potentially influence it.

Previous studies have shown that talin-1 is a critical platelet protein for haemostasis and thrombosis, as it binds to and activates integrins which mediate platelet adhesion and aggregation. The platelets are also dependent on these activated integrins for retraction of the fibrin clot, which promotes wound healing (Haling *et al.* 2011; Petrich *et al.* 2007). Therefore, a loss of, or a low abundance of this protein, as was the case in the samples from the diabetic patients in this study would result in impaired integrin associated platelet activation, meaning that the DFUs would not contract, and as they remain exposed, becoming susceptible to infection. DFUs are captured in the inflammatory phase of the wound healing process (Blakytny and Jude 2006), and it is likely that a low abundance of talin-1 could contribute to this, since inflammatory cells are continually attracted into the non-contracting open wound to fight off foreign material that may cause infection.

Apolipoprotein is a protein with diverse functionality, but with regards to platelet function, it is known for its anti-aggregation and anti-clotting effects (Mangaraj, Nanda and Panda 2016). In this study, this protein was seen to be in lower abundance in the diabetic patients and it would make sense to observe these low levels of the protein indicating inhibited platelet aggregation and clot formation to facilitate closure of the open foot ulcers. Nevertheless, impaired wound healing of DFUs still occurs, even though there appears to be down-regulation of apolipoprotein, as observed in this study. This phenomenon can be explained by the fact that apolipoprotein is known to inhibit TNF α and IL-1β which are key initiators of the inflammatory phase but can also inhibit tissue repair by prolonging this phase (Cubedo *et al.* 2015; Rapala 1996; Ritsu *et al.* 2017). Therefore, if there are low levels of apolipoprotein in the diabetic patients, TNF α and IL-1β are not inhibited fully and the consequence of this would be continuation of the inflammatory phase with inhibited progression to the later stages of wound healing. This is in agreement to what has been reported by other researchers that DFUs are stationary in the inflammatory phase of the wound healing process (Blakytny and Jude 2006).

Complement C3 has for a long time been known to be a plasma protein that translocates to the cell surface of platelets, but recent studies have shown that this protein is also platelet-borne (Arbesu *et al.* 2016). A greater abundance of this protein was seen in the diabetic patients than in the non-diabetic healthy individuals, which could be the result of low grade inflammatory processes common to diabetic patients

who are generally infection prone. Complement C3 which plays a central role in both the classical and alternative complement pathways is essential for activation of the complement system to ward off infiltrating infectious agents. However, the complement system comprises of several other proteins with immunological and inflammatory properties (Arbesu *et al.* 2016), therefore the implication of higher levels of complement C3 could result from constant activation of the inflammatory phase, thus inhibiting tissue repair.

Kininogen-1 which was also found to be in high abundance in the diabetic patients could also be contributing to the constant inflammation of DFUs. This protein is a precursor to high molecular weight kininogen (HMWK), low molecular weight kininogen (LMWK) and bradykinin, the latter of which is an inflammatory mediator (Ho *et al.* 2000; Renne 2012). When tissue injury or wounding occurs, the contact-kinin system is activated by the platelet coagulation factor XII (PFXII), which triggers cleavage of kininogen-1 to form HMWK, LMWK and subsequent release of the pro-inflammatory peptide mediator, bradykinin (Langhauser *et al.* 2012). Though bradykinin has a very short half-life *in vivo* (Renne 2012), high levels of kininogen-1 would result in a constant supply of this inflammatory molecule, which could also play a role in delayed wound healing of DFUs.

Another very important observation to note was the greater abundance of alpha synuclein in the diabetic patients than the healthy individuals. Alpha synuclein acts as a negative regulator for alpha granule release in platelets (Park *et al.* 2002). The platelet alpha granules contain mediators that promote tissue repair such as platelet factor 5 and fibrinogen (Kieffer *et al.* 1987; Schmaier *et al.* 1983), therefore a higher expression of alpha synuclein would lead to a reduced degranulation of these granules causing lower concentrations of the mediators that promote healing.

Destrin which is also known as the actin depolymerizing factor (ADF) forms a part of the major actin proteins which regulate the platelet-actin assembly reaction. The reaction occurs during the production of platelets from megakaryocytes, where actin filaments are organised into a rigid cytoplasmic scaffold by filamin A, giving rise to the discoid platelet shape. The reaction is important for the formation of platelets of the normal size as well as preventing fragility and poor circulation of platelets (Falet 2017). However, a study conducted on destrin-null mice showed that the mice still had a

normal platelet count, normal platelet size and a normal platelet-actin assembly reaction following activation (Bender *et al.* 2010). The authors of that publication suggested that the other actin regulatory proteins could be filling in the gap created by low concentrations of destrin in the platelet-actin assembly reaction, making the effect of destrin absence negligible through redundancy mechanisms. This is consistent with the observation made in this study where the actin-related protein 2/3 complex subunit 5 was observed in the diabetic patients only. This protein could have been expressed to compensate for the low levels of destrin, since it has a similar function.

The results discussed above could indicate that that the protein differences can potentially directly or indirectly influence the wound healing process, which can contribute to the delayed wound healing observed in diabetic patients. However, due to the small sample size used for the LC-MS/MS proteomics it cannot be concluded that these protein differences as observed between the diabetic patients and non-diabetic healthy individuals were a true reflection of the difference in proteome status as it might have been mere coincidence. A much larger cohort of the 60 participants used in this study should be analysed to give a clearer picture of whether these protein differences were significant and reproducible between the two groups.

To verify the assumptions made from the SDS-PAGE results, where the distinct band was thought to be the platelet basic protein or haemoglobin, the number of spectra for these proteins was compared between the two groups as shown in Figures 21 and 22 to see if any differences in protein abundance could be determined. The expectation was to observe greater abundance of the platelet basic protein in the diabetic patients due to its reported role in the inflammatory phase of the wound healing process of DFUs. However, the non-diabetic healthy individuals exhibited greater abundance of this protein, which implied that the platelet basic protein might not have contributed to the dominant band seen at about 15 kDa on the gels. Following this, the abundance of haemoglobin based on spectral counting was compared between the two groups, and it was observed that one of the male diabetic patients had a much greater abundance of the haemoglobin subunit beta (Figure 23). Since this occurred in one patient in a group of 3 selected patients, it might have been due to an increased fragility of the erythrocytes with occult plasma haemoglobin showing a greater abundance which then showed up as a dominant band at about 15 kDa when separated by SDS-PAGE. This is not a surprising result since diabetic patients have high erythrocyte

mechanical fragility as reported in literature (Lippi *et al.* 2012) which could easily have led to contamination of the samples with the haemoglobin subunit during sample collection and initial centrifugation.

In addition to these propositions, the dominant band could have also been due to alpha synuclein (Mass: 14.45 kDa) which was observed to be more abundant in the diabetic patients than in the non-diabetic healthy individuals as previously mentioned.

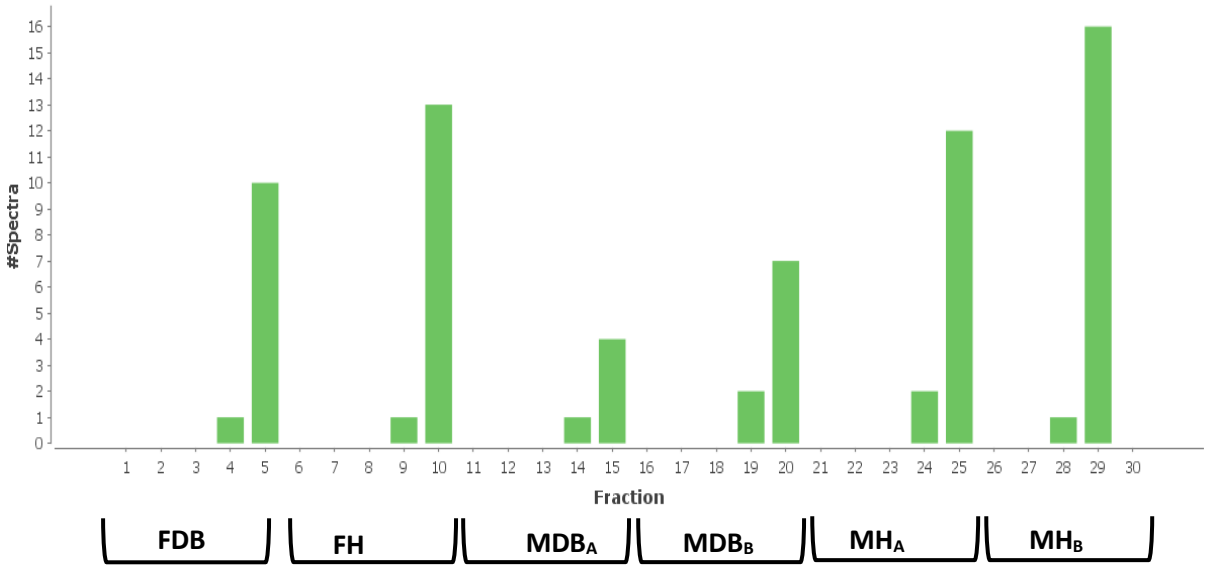


Figure 21: Number of spectral counts for the "platelet basic protein" in the five protein bands fractions excised from the individual lanes of an SDS-PAGE gel comparing platelet proteins from both the diabetic and non-diabetic healthy individuals. Each of the fractions FDB (female diabetic), FH (healthy female), MDB$_A$ (male diabetic A), MDB$_B$ (male diabetic B), MH$_A$ (healthy male A) and MH$_B$ (healthy male B) had five sub-fractions (numbered) representing the five excised bands cut from each lane.

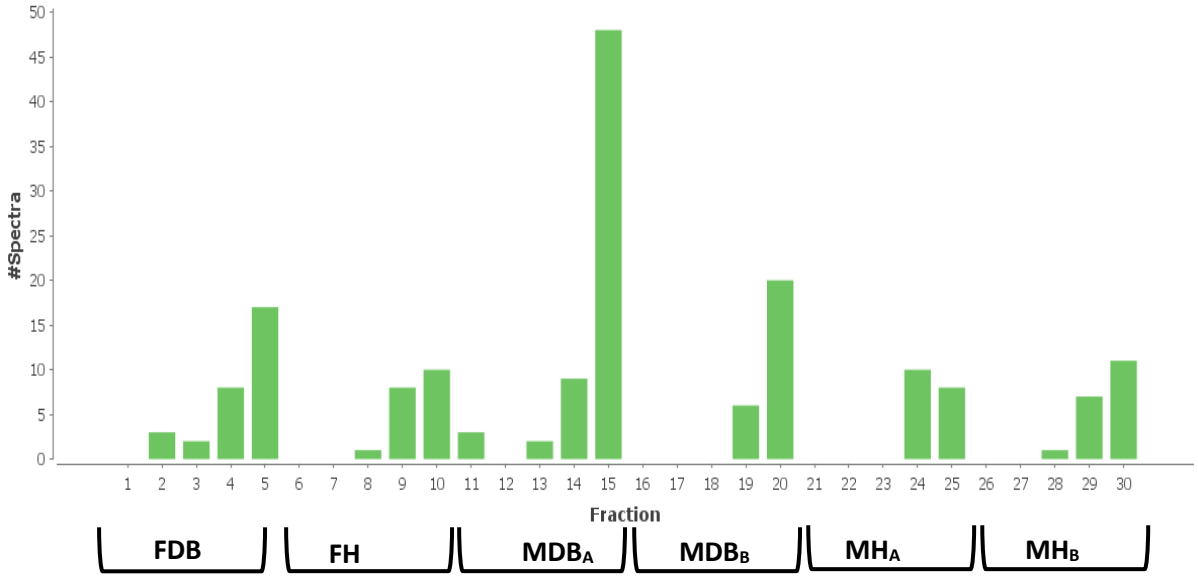


Figure 22: Number of spectral counts for the "haemoglobin subunit beta" in the five protein bands excised from the individual lanes of an SDS-PAGE gels comparing platelet proteins from diabetic and non-diabetic healthy individuals. Each of the fractions FDB (female diabetic), FH (healthy female), MDB$_A$ (male diabetic A), MDB$_B$ (male diabetic B), MH$_A$ (healthy male A) and MH$_B$ (healthy male B) had five sub-fractions (numbered) representing the five excised bands cut from each lane.

3.4.2 Data analysis of in-solution digests

For in-solution digests, three samples from non-diabetic healthy individuals and three selected samples from diabetic patients which showed protein band differences were used. Prior to LC-MS/MS analysis, the protein samples were stored at -80°C in the presence of a protease inhibitor cocktail. This was an important precautionary procedure to prevent protein degradation by proteases which are often released during isolation of protein (Ryan 2011). To ascertain that protein degradation did not occur while the samples were stored, SDS-PAGE was done to see if a pattern similar to the samples used for gel electrophoresis and in-gel digests would be obtained. The gel was stained with the Acqua stain and compared with the one shown in Figure 13. The comparison showed that there was no protein degradation in the samples stored in the protease inhibitor, which confirmed that the protease inhibitor cocktail successfully prevented proteolysis. The samples were then reduced, alkylated and cleaned-up using the HILIC MagResyn bead method which also involved tryptic digestion of these samples. Following this, LC-MS/MS analysis of the samples was performed, and the spectral data was searched using MaxQuant, which prepared the way for quantitative analysis of the data using Spectronaut™. Figure 23 is a total ion chromatogram of the

pooled samples which shows the summed intensity across the entire range of masses that were detected at every point in the analysis. This chromatogram indicates the complexity of the data that was obtained for protein analysis.

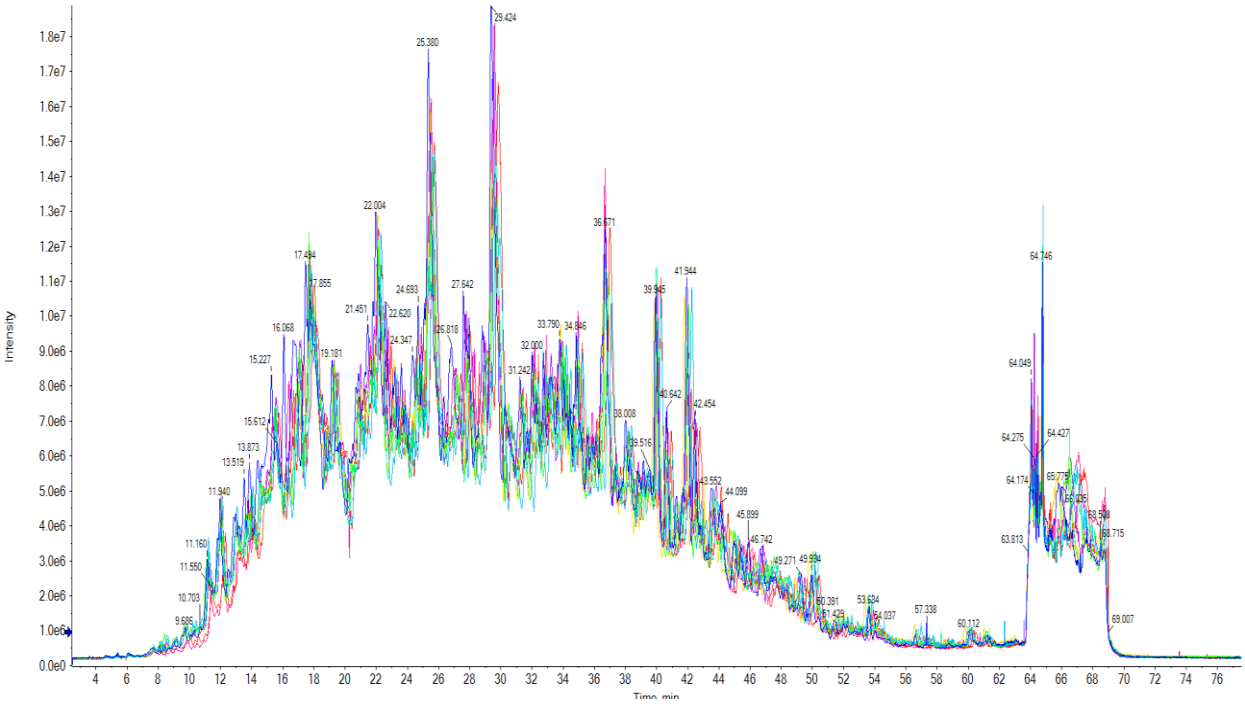


Figure 23: Total ion chromatogram (TIC) of the pooled samples from diabetic patients and non-diabetic healthy individuals. The TIC displays the summed intensity across the entire range of masses detected at every point in the analysis.

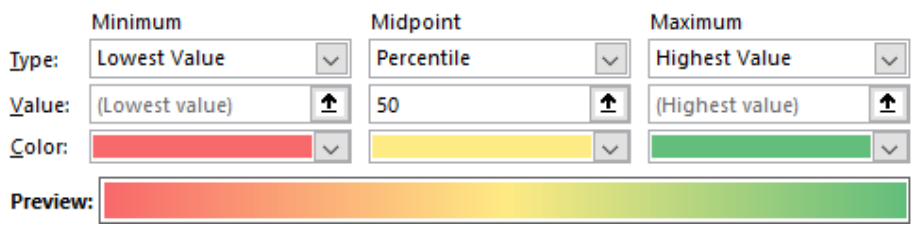
Quantitative analysis of the data to determine if there were protein differences between diabetic patients and non-diabetic healthy individuals involved calculation of fold change and comparing normalized protein intensities measured in arbitrary units, where the protein quantities were indicated using different colour intensities to show the change in quantity between the diabetic patients and non-diabetic patients. These results are shown in Tables 3 and 4.

Table 3: Data exported from Spectronaut highlighting the fold change of platelet proteins from diabetic patients and non-diabetic healthy individuals. The fold change of proteins showing differences in abundance between the two groups is highlighted in the second column.

Average log 2	Fold Change	Q-value	Protein Description	Protein name
-0.87716426	0.544436517	7.90E-22	P01024	Complement C3
-0.981876314	0.50632081	9.71E-15	P0C0L4;P0C0L4-2;P0C0L5	Complement C4-A
-0.742541185	0.597685652	5.10E-14	P07355	Annexin A2
-1.423151132	0.372896941	3.00E-10	P00738	Haptoglobin
0.381894541	1.303051895	1.22E-07	O15127	Secretory carrier-associated membrane protein 2
-3.512839644	0.087605202	3.91E-07	HBB_HUMAN;P68871	;Hemoglobin subunit beta
0.824309544	1.770687406	6.53E-07	Q13423	NAD(P) transhydrogenase, mitochondrial
-0.751988311	0.593784644	6.33E-05	P46940	Ras GTPase-activating-like protein IQGAP1
-0.826161665	0.564027862	9.01E-05	P02790	Hemopexin
-0.96604958	0.511905858	0.00011308	P04217	Alpha-1B-glycoprotein
-0.805436705	0.572188852	0.00028345	P01042-2	Isoform LMW of Kininogen-1
0.612731181	1.529151323	0.00028908	Q9Y277;Q9Y277-2	Voltage-dependent anion-selective channel protein 3
0.04683751	1.032998035	0.0003553	O75964	ATP synthase subunit g, mitochondrial
-0.804731407	0.572468649	0.00039651	P00751	Complement factor B
-0.725618752	0.604737625	0.00055153	P08603	Complement factor H
-0.605239356	0.657362309	0.00084301	P00747	Plasminogen
0.649236583	1.568338074	0.00102385	O00139;O00139-1	Kinesin-like protein KIF2A
0.018705073	1.013049783	0.00104685	Q13201	Multimerin-1
-0.636617782	0.643219129	0.00128895	P05109	Protein S100-A8
-1.800417555	0.287091485	0.0017123	P32119	Peroxiredoxin-2
-0.875745471	0.544972195	0.00270945	Q14554	Protein disulfide-isomerase A5
0.88039547	1.840879852	0.00384168	P12259	Coagulation factor V
0.781144414	1.718493523	0.00798256	P13073	Cytochrome c oxidase subunit 4 isoform 1
0.52563693	1.43956899	0.00026906	Q14644	Ras GTPase-activating protein 3

Table 4: Data exported from Spectronaut™ indicating the different protein intensities between diabetic patients and non-diabetic healthy individuals. The relative protein abundance in arbitrary units is indicated according to the colour code shown below the table. FDB = female diabetic patient; FH = female healthy individual; MDB = male diabetic patient and MH = male healthy individual.

Protein ID	Accesion No.	Intensity in arbitrary units					
		FDB	FH	MDB	MDB	MH	MH
Hemoglobin subunit beta	P68871	9788	1427	34401	3633	1107	1693
Kinesin-like protein KIF2A	O00139	29.72	51.13	24.66	28.02	45.18	31.70
Secretory carrier-associated membrane protein 2	O15127	317.9	1697	28.84	351.6	584.6	935.6
ATP synthase subunit g, mitochondrial	O75964	127.5	116.1	53.50	60.25	122.8	152.1
Haptoglobin	P00738	5596	2487	3107	3533	958.4	1160
Plasminogen	P00747	125.5	77.77	87.74	75.56	30.84	57.03
Complement factor B	P00751	169.1	101.1	109.9	105.2	58.09	61.43
Complement C3	P01024	1073	556.3	804.7	750.4	365.0	499.2
Isoform LMW of Kininogen-1	P01042-2	201.4	96.88	189.4	153.5	92.18	123.3
Immunoglobulin heavy constant alpha 1	P01876	1487	681.4	1417	901.8	783.73	858.9
Hemopexin	P02790	1535	858.8	1027	1033	510.4	669.2
Alpha-1B-glycoprotein	P04217	321.6	89.70	204.3	257.4	149.4	133.4
Protein S100-A8	P05109	1502	1555	2336	1448	1048	794.6
Annexin A2	P07355	317.6	212.6	360.2	240.0	227.9	110.7
Complement factor H	P08603	83.80	47.30	71.58	63.70	47.23	38.44
Complement C4-A;Isoform 2 of Complement C4-A;(	P0C0L4	380.0	234.4	281.1	280.0	120.1	123.3
Coagulation factor V	P12259	63.27	113.71	56.97	68.66	162.8	73.19
Cytochrome c oxidase subunit 4 isoform 1,	P13073	123.3	179.80	57.49	91.94	143.5	147.4
Peroxiredoxin-2	P32119	376.0	212.0	1087	315.8	129.0	171.7
Ras GTPase-activating-like protein IQGAP1	P46940	54.96	42.20	76.56	42.69	39.30	22.68
Multimerin-1	Q13201	548.9	974.4	246.9	569.9	586.8	615.9
NAD(P) transhydrogenase, mitochondrial	Q13423	45.00	82.01	51.85	63.65	104.2	99.82
Protein disulfide-isomerase A5	Q14554	35.62	45.43	64.36	53.55	64.06	130.2
Ras GTPase-activating protein 3	Q14644	54.03	91.68	55.42	77.06	98.35	90.36
Voltage-dependent anion-selective channel protein 3	Q9Y277	122.2	150.8	100.1	103.5	164.9	184.1



The proteins had to be 1.5-fold up or down-regulated for the protein differences to be considered significant and the Log2 calculation was useful in indicating whether the protein was up- or down-regulated in the diabetic patients. A negative sign before the Log2 value for fold change indicated that the protein level was high in the diabetic patients.

The data derived from the in-solution digests indicated that the total protein recovery was lower than that of the in-gel digests. The in-solution digest method failed to identify several proteins that were identified from the in-gel digests, which was an observation conflicting to the results from the study conducted by Yang *et al*. (2009) where the number of identified proteins from the two digestion methods was comparable (Yang *et al.* 2009). This discrepancy between in-gel and in-solution digests could have been caused by the increased concentration of detergent used in this method. Due to this increased concentration, the detergent might not have been completely cleaned out from the sample leading to peptide loss, as it has been observed that the presence of detergents in the peptide samples severely suppress ionization in MS analysis (Yeung and Stanley 2010). Another possible explanation for low protein recovery is that the peptides could have been lost due to the many wash steps involved in the HILIC clean-up method or adsorbance of the proteins to the MagResyn beads. Additionally, analysis of samples in this approach was done using the SWATH MS analysis and the number of proteins identified in this kind of analysis is largely limited by the composition of the spectral library (Krasny *et al.* 2018). Therefore, if the spectral library consisted of only a fraction of the reported proteins, a number of proteins would not be identified with this approach. Impaired protein identification might have also been caused by the protease inhibitor added to the sample to prevent protein degradation, which would inhibit trypsin and block digestion of the sample if still present in high enough concentrations during digestion (Clifton *et al.* 2011). Regardless of the low protein recovery in this analysis, a few protein differences which were not identified by the in-gel digest approach were identified between the diabetic patients and the non-diabetic healthy individuals. This indicated the importance of performing a global proteomic analysis of the samples to identify possible protein differences between diabetic patients and non-diabetic healthy individuals which might not have been detected by the in-gel approach. Five proteins were found to be in low abundance in the diabetic patients and these were nicotinamide adenine dinucleotide (phosphate)

transhydrogenase (NAD(P) transhydrogenase), voltage-dependent anion-selective channel protein 3 (VDAC3), kinesin-like protein, platelet coagulation factor V (PFV) and cytochrome C oxidase subunit 4 isoform 1. All of these proteins were down-regulated by a fold change over 1.5 and also exhibited greater intensities in the non-diabetic healthy individuals as shown in Figure 4, which showed that there was a correlation between the two methods that were used to quantify the proteins. The functions of these proteins were also deduced from KEGG and GO, and the observed differences correlated to the wound healing process to assess whether they can potentially influence the process.

NAD(P) transhydrogenase catalyses the chemical reaction: NADPH + NAD^+ ↔ $NADP^+$ + NADH which is crucial in the control of reactive oxygen species (ROS) (Arkblad *et al.* 2005; Rydström 2006). High levels of ROS are proinflammatory (Mitra and Abraham 2006) therefore low levels of NAD(P) transhydrogenase would mean ROS is not optimally regulated resulting in continued inflammation of DFUs. This observation agrees with the results obtained in a previous study where a defective gene for NAD(P) transhydrogenase was seen to be linked to Type 2 diabetes (Toye *et al.* 2005).

The down-regulation of VDAC3 in diabetes patients that was observed in this study was also noted in a previous study (Springer *et al.* 2009), which suggests that diabetes could be playing a role in lowering the expression of the protein. VDAC3 is a protein highly expressed in platelets and is located on the outer mitochondrial membrane. Its function is to regulate the exchange of metabolites between the cytosol and mitochondrial membrane of the platelet (Wright *et al.* 2016). In addition to this, VDAC3 is a key regulator of the intrinsic apoptosis pathway and thus contributes to the platelet lifespan (Shoshan-Barmatz *et al.* 2010). Therefore, based on these functions, a decreased expression of VDAC3 would lead to increased apoptosis of the platelets, an occurrence more common in diabetes patients as reported in a previous study (Zharikov and Shiva 2013). This would in turn lead to a decrease in the number of platelets in the circulation and affect downstream secretion of growth factors and other mediators involved in the wound healing process.

The kinesin-like protein (KIF2A) is one of the three types of motor proteins involved in cargo transport, cell motility and cell division, as well as cytoskeletal reorganization for platelet activation (Sadoul 2015). KIF2A works in antagonistic equilibrium with dynein, another form of motor proteins to maintain the flat, discoid shape of circulating quiescent platelets (Miki *et al.* 2001). Once KIF2A is inhibited, dynein slides microtubules apart leading to extension and weakening of the platelet marginal band, which in turn causes the disc-to-sphere transition of platelets that activates them (Sadoul 2015). Therefore, the implication of having down-regulation of KIF2A as observed in this study, would be continuous activation of platelets which also means increased secretion of pro-inflammatory mediators from the platelets. This could contribute to the constant inflammatory state of diabetic wounds which hampers healing.

PFV plays a central role in the haemostatic process, where it acts as a cofactor in the blood coagulation cascade following its activation (Bos and Camire 2010). The pro-coagulant molecule interacts with other clotting proteins such as platelet factor X to increase the production of thrombin which is a key haemostatic enzyme that converts soluble fibrinogen to fibrin, thus aiding in clot formation (Perrotta 2008). The formation of the clot by fibrin is a crucial step in the wound healing process as it provides a matrix scaffold for the recruitment of tissue cells including endothelial cells and fibroblasts to the wounded site, thus promoting healing (Bryant and Nix 2015). Therefore, if there are low levels of PFV, the clot formation process might be hampered which could contribute to delayed healing of DFUs. The low levels of PFV observed in diabetic patients in this study also supported the results obtained in the study conducted by Springer *et al.* (2009).

Cytochrome C oxidase subunit 4, which also known as complex IV is one of the components forming the oxidative phosphorylation system. In platelets, oxidative phosphorylation occurs in the inner mitochondrial membrane where substrates generated from glycolysis and β-oxidation are converted to energy in the form of ATP. This is done through the electron transport chain where cytochrome C oxidase subunit 4 is a terminal component and catalyses transfer of electrons from cytochrome C to oxygen (Diaz 2010). The process of oxidative phosphorylation is important for platelet activation and aggregation which are critical processes for wound healing (Ravi *et al.*

2015). A down-regulation of this enzyme could therefore hinder platelet activation and aggregation and possibly impair wound healing in diabetic patients.

To confirm whether haemoglobin which showed up as a distinct band at 15 kDa on the SDS-PAGE gels could have been a possible contaminant in diabetic patient samples, the fold change of haemoglobin subunit beta was also analysed. The fold change was below the threshold of 1.5 therefore it could not be concluded that this protein was more abundant in the diabetic patients. However, the intensity level of the protein in one of the representative diabetic patients in Figure 4 was higher than the other two diabetic samples. This occurrence occurred in one out of three patients, which implies that in a sample size of 30 patients, it is possible that ten patients or more would have this high plasma haemoglobin which would support the notion that the distinct band seen at about 15 kDa could have been caused by contamination of the samples from diabetic patients by haemoglobin, due to haemolysis.

Therefore, this method was partially successful in identifying platelet proteins as well as their differences between the diabetic patients and non-diabetic healthy individuals. The functions of the proteins showing differences are linked to the wound healing process which means that a defect in these proteins could well be contributing to delayed wound healing in diabetic patients. An increase in the sample size as well as further development of the HILIC clean-up method to improve protein recovery would be important as the method shows great potential to identify platelet protein differences, which would help to develop targeted therapies for DFUs.

Chapter 4: Conclusions and considerations

4.1 Conclusions

Diabetic foot ulcers negatively affect the quality of life of diabetic patients and are a costly complication to the health sector, hence it is of utmost importance that effective therapies are developed to circumvent the catastrophic effects of these foot ulcers. Since the current treatments have been met with high rates of failure and recurrence, the aim of the study was to identify possible proteomic and glycoproteomic differences between platelets from diabetic patients and healthy individuals to further understand the role of platelets in impaired wound healing of DFUs in diabetic patients. This was done in a bid to form a basis in development of more effective targeted systemic therapies for DFUs.

To achieve the aims of this study, a set of defined objectives were followed, the first being screening of patients using the HbA1c test. Non-diabetic healthy individuals had to have an HbA1c level below 5.7% (39 mmol/mol) and the diabetic patients an HbA1c level above 6.5% (48 mmol/mol). Based on these HbA1c levels, participants were successfully recruited for the study. This was particularly important to confirm the long-term hyperglycaemic status of diabetic patients and the normal glucose levels for the non-diabetic healthy volunteers that participated in the study, to make sure each participant was eligible for the study.

Following this, blood was drawn from each of the participants and separated using differential centrifugation. Platelet rich plasma was collected from each of the separated blood samples and platelets were successfully isolated from the PRP. To confirm the activation status of these platelets from all the study participants, flow cytometry analysis using known general platelet and activated platelet markers was done. It was important to check for the activation status as the goal was to harvest platelets that were not activated or had minimal activation. Activated platelets usually degranulate to release proteins such as growth factors, therefore use of non-activated proteins was preferred as there would be no protein loss. The results obtained from flow cytometry analysis showed that none of the platelet samples from non-diabetic healthy individuals were activated and twenty-four of the platelet samples from diabetic patients showed slight activation of about 3% on average. This activation observed in the diabetic patient platelet samples was not surprising as it has been previously reported in literature that circulating platelets from diabetic patients exhibit a degree of

activation. Therefore, platelet isolation was successfully achieved and the protocol that was used for isolation showed no or minimal activation of the platelets.

SDS-PAGE coupled to different staining procedures was performed to separate and compare platelet proteins from both groups. This was done in a bid to identify possible differences between non-diabetic healthy individuals and diabetic patients. After running a few pilot gels, it was determined that 10 µg of protein per well was sufficient to generate high resolution protein mass fingerprints for comparative analysis. Separation of the proteins from all 60 study participants was carried out successfully and similarities in the general pattern, with subtle band differences between the diabetic patients and non-diabetic healthy individuals were observed. The major difference between the two groups was seen at about 15 kDa were the protein bands were more distinct in the diabetic patients. This was thought to be haemoglobin contamination due to haemolysis of red blood cells which commonly occurs in diabetic patients. Another possible protein appearing at that molecular mass would be the platelet basic protein which is highly expressed in diabetic patients and cleaved into peptides that act as chemo-attractants and activators of neutrophils. This would have been a possibility since 90% of the study participants had diabetic foot ulcers, and these foot ulcers appear to be locked in the inflammatory phase of the wound healing process where neutrophils play a major role. The comparison of the same samples visualised by different stains also highlighted the difference in sensitivity between these imaging techniques used for band visualization. Stain-free™ imaging and Acqua staining which is one of the many forms of colloidal Coomassie stains, showed sensitivity of a similar order, though a few extra bands were observed on the gels stained with the Acqua stain and this is attributed to the Acqua stain targeting more protein moieties than Stain-free™ imaging. The silver and Oriole ™ stains showed greater sensitivity as more bands were detected when these two stains were used. Overall, more protein bands were detected when the gels were visualized using an imaging technique with greater sensitivity. This confirmed that the imaging techniques were functional and useful in the comparative analysis of protein between samples. Since SDS-PAGE is more of a qualitative analysis technique and the differences between the two groups highlighted the technical variance, it was difficult to report significant platelet protein differences between the non-diabetic healthy individuals and diabetic patients. Owing to that, LC-MS/MS analysis was important for identification of possible protein differences.

The next objective was to identify possible non-enzymatic protein glycosylation on the platelet proteins of diabetic patients due to hyperglycaemia. This was initially assessed by performing SDS-PAGE and staining the gels with the PAS stain which targets glycosylated proteins. Despite the required protein-load as indicated on the kit's protocol was loaded onto the gels, only the positive control could be detected. The detection of the positive control indicated that the stain was working, but by implication, no glycosylated proteins were present in the samples. This led to the proposition that glycosylated proteins in the diabetic patients' samples could have been further processed to advanced glycated end products which would not be detected by the PAS stain. Therefore, to test this proposition, western blot and HPLC analysis were performed. Although the two techniques were successfully carried out, the data produced in this study suggested that no difference exists in platelet-protein glycation when comparing diabetic patients and non-diabetic healthy individuals which can possibly eliminate glycation of critical platelet derived proteins as a potential cause of delayed healing of DFUs. The expectation was to observe more AGE formation in the diabetic patients due to hyperglycaemia and reports made in previous studies that proteins from diabetic patients are heavily glycated in comparison to non-diabetic healthy individuals. Consequently, it is imperative to use more sensitive techniques such as enrichment of glycated protein followed by mass spectrometry analysis to validate the results since the methods used and numbers of participants recruited present limitations that might have avoided detection of glycated protein.

The last objective was to perform proteomic analysis to determine if there were any significant protein differences between diabetic patients and non-diabetic healthy individuals. Two approaches were used to achieve this objective. In the first approach, a selection of bands exhibiting differences between diabetic patients and non-diabetic healthy individuals were excised from SDS-PAGE gels, digested with trypsin and analysed and sequenced using LC-MS/MS. The spectral data obtained from LC-MS/MS analysis was analysed using three search engines in open source SearchGUI and further analysed using Peptide Shaker to visualize the identified proteins. The major differences observed between diabetic patients and non-diabetic healthy individuals were in the protein abundance of seven platelet specific proteins. The functionalities of these platelet proteins were deduced from KEGG and GO and the observed protein differences in this study indicated that the wound healing process

can be influenced directly or indirectly, which in turn contributes to the delayed wound healing observed in diabetes patients.

The second proteomic approach was characterised by a global analysis of platelet proteins from selected participants to assess differences between diabetic patients and non-diabetic healthy individuals. In this approach, platelet protein samples from the two groups were also digested with trypsin, but the difference from the first approach was that the proteins were digested in-solution. Sample clean-up of the samples was done using the HILIC MagReSyn bead method and the spectral data searched using MaxQuant. Following this, quantitative analysis using Spectronaut™ was done where a fold change ≥ 1.5 and arbitrary units for protein intensity were used to determine protein differences between the diabetic and non-diabetic healthy individuals. The method failed to identify several proteins identified from the in-gel digests which could have been a result of differences in peptide intensities from the different sample preparation methods or different detergent concentrations that inhibited trypsinization to different extent or data loss from DIA MS analysis. However, the data also showed differences in five other platelet specific proteins whose functionalities may be altered leading to delayed wound healing in diabetes patients.

Overall, the large number of SDS-PAGE results obtained from the different staining techniques showed very strong similarities in the protein mass fingerprint between the diabetic patients and the non-diabetic healthy individuals, yet further analysis of these same samples by LC-MS/MS analysis identified and showed differences in abundance of several proteins, that could be important in platelet function, between the two groups. This study highlighted the superiority of mass spectrometric analysis over SDS-PAGE in that SDS-PAGE cannot identify any proteins and proved that the assumption that a single stained band on a gel does not represent a single protein. LC-MS/MS that has the ability, when coupled to bioinformatics, to positively identify and quantify proteomic differences in disease states for diagnostic purposes easily outperformed the electrophoretic methodology and shows strong potential to identify targets for subsequent targeted therapy.

The proteins that were observed to have differences in abundance between the two groups, particularly talin-1 and alpha-synuclein are crucial key players in the wound healing process. Therefore, from these observations, it can be proposed that

differences in the abundance of these proteins might be contributing to delayed wound healing of DFUs. Consequently, the use of autologous PRP from diabetic patients to promote healing of DFUs would result in little treatment enhancement, as these platelets show proteomic differences that could provide undesirable proteins at the wound site.

Further research, with the knowledge gained from this study would help to make more substantiated conclusions as to whether the differences in protein abundance have a significant effect on the wound healing process. If the wound healing process is significantly affected by these differences, then therapies targeting these proteins can be developed to improve healing of DFUs.

4.2 Limitations and considerations

The use of mass spectrometry-based methods to assess protein glycation may have provided a better comparison between the two groups. Since PTMs such as glycation are generally present at much lower levels than those of unmodified proteins, and there is a possibility to miss them during standard approaches used to prepare samples for LC-MS/MS analysis, removal of high abundance proteins using relatively expensive separation methods can facilitate analytical work towards detection of the low abundance proteins (Clifton *et al.* 2011). An example of a technique that could directly target glycated proteins is the development of a HILIC-phase based sample preparation method for enrichment of the glycated peptides derived from glycated proteins during digestion. This technique relies on a stationary phase with high affinity for glycopeptides, resulting in selective and effective extraction of the glycated peptides from the more abundant non-glycated peptides from the same protein within sample. The hydrophilicity of glycopeptides makes them ideal candidates for separation by HILIC as suggested by Boersema *et al*. (Boersema, Mohammed and Heck 2008). Following the enrichment process, the glycated samples would need to be deglycosylated prior to MS analysis, since N-glycation occurs on the lysine and AGE at the arginine residues which are also targeted by trypsin. Therefore, this would be a necessary step to prevent missed trypsin cleavages and failure to identify the proteins that are altered by glycation (Lapolla *et al.* 2004; Rabbani, Ashour and Thornalley 2016).

LC-MS/MS analysis during this study was also limited to 3 subjects from each group due to high costs associated with the analysis, which meant that the observed changes could not be reported as definite differences between the two groups. Therefore, increasing the number of samples analysed to have at least the complete analysis of all the study participants would help to validate the findings.

It was also observed that less proteins were recovered from the in-solution digests, which was a surprising a result when comparing to previous studies where the general trend showed more proteins are recovered from in-solution digests than from in-gel digests. One of the propositions made was that there was possible loss of protein on the MagResyn beads used for the HILIC sample clean-up and digestion process. To prevent this loss, the method would need to be optimised by adding selected buffering salts to the mobile phase which can decrease the retention of peptides on the stationary phase after the enzymatic digestion, which were the MagResyn beads. A number of other potential limiting parameters such as the buffer concentration, counter ions present, pH, detergent concentration and time per cycle would need to be investigated

If these limitations are considered and improvements to avoid these limiting parameters implemented in future studies, more differences could possibly be identified between the platelet proteins of diabetic and non-diabetic healthy individuals, which can aid in development of effective targeted therapies for DFUs.

Overall this study could only define some minor differences in the platelet proteome between the diabetic and non-diabetic health individuals but did indicate that changing the techniques used and by optimising these techniques, it would be possible that significant markers could be identified that could be used as targets to enhance wound healing in diabetic patients.

References

ADEGHATE, J., NURULAIN, S., TEKES, K., FEHER, E., KALASZ, H. & ADEGHATE, E. 2017. Novel biological therapies for the treatment of diabetic foot ulcers. *Expert Opin Biol Ther,* 17**,** 979-987.

AHMED, M. U., THORPE, S. R. & BAYNES, J. W. 1986. Identification of N epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem,* 261**,** 4889-94.

AHMED, N. 2005. Advanced glycation endproducts--role in pathology of diabetic complications. *Diabetes Res Clin Pract,* 67**,** 3-21.

AMABLE, P. R., CARIAS, R. B., TEIXEIRA, M. V., DA CRUZ PACHECO, I., CORREA DO AMARAL, R. J., GRANJEIRO, J. M. & BOROJEVIC, R. 2013. Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. *Stem Cell Res Ther,* 4**,** 67.

AMERICAN DIABETES ASSOCIATION 2004. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care,* 27 Suppl 1**,** S5-s10.

AMERICAN DIABETES ASSOCIATION 2014. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care,* 37**,** S81-S90.

ANFOSSI, G., RUSSO, I., DORONZO, G., POMERO, A. & TROVATI, M. 2010. Adipocytokines in atherothrombosis: focus on platelets and vascular smooth muscle cells. *Mediators of inflammation,* 2010**,** 174341.

ARASTEH, A., FARAHI, S., HABIBI-REZAEI, M. & MOOSAVI-MOVAHEDI, A. A. 2014. Glycated albumin: an overview of the In Vitro models of an In Vivo potential disease marker. *Journal of Diabetes & Metabolic Disorders,* 13**,** 49.

ARBESU, I., BUCSAIOVA, M., FISCHER, M. B. & MANNHALTER, C. 2016. Platelet-borne complement proteins and their role in platelet–bacteria interactions. *Journal of Thrombosis and Haemostasis,* 14**,** 2241-2252.

ARKBLAD, E. L., TUCK, S., PESTOV, N. B., DMITRIEV, R. I., KOSTINA, M. B., STENVALL, J., TRANBERG, M. & RYDSTROM, J. 2005. A Caenorhabditis elegans mutant lacking functional nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative stress. *Free Radic Biol Med,* 38**,** 1518-25.

ASHRAF, J. M., AHMAD, S., CHOI, I., AHMAD, N., FARHAN, M., TATYANA, G. & SHAHAB, U. 2015. Recent advances in detection of AGEs: Immunochemical, bioanalytical and biochemical approaches. *IUBMB Life,* 67**,** 897-913.

AVILA, C., HUANG, R., STEVENS, M., APONTE, A., TRIPODI, D., KIM, K. & SACK, M. 2012. Platelet mitochondrial dysfunction is evident in type 2 diabetes in association with modifications of mitochondrial anti-oxidant stress proteins. *Exp Clin Endocrinol Diabetes,* 120**,** 248-51.

BAKKER, K., APELQVIST, J., SCHAPER, N. C. & ON BEHALF OF THE INTERNATIONAL WORKING GROUP ON THE DIABETIC FOOT EDITORIAL, B. 2012. Practical guidelines on the management and prevention of the diabetic foot 2011. *Diabetes/Metabolism Research and Reviews,* 28**,** 225-231.

BEANES, S., DANG, C., SOO, C. & TING, K. 2003. Skin repair and scar formation: the central role of TGF-[beta]. *Expert reviews in molecular medicine,* 5**,** 1-22.

BEAULIEU, L., LIN, E., MICK, E., KOUPENOVA, M., WEINBERG, E., KRAMER, C., GENCO, C., TANRIVERDI, K., LARSON, M. & BENJAMIN, E. 2014. Interleukin 1 receptor 1 and interleukin 1β regulate megakaryocyte maturation, platelet activation, and transcript profile during inflammation in mice and humans. *Arteriosclerosis, thrombosis, and vascular biology,* 34**,** 552-564.

BECK, F., GEIGER, J., GAMBARYAN, S., SOLARI, F. A., DELL'AICA, M., LOROCH, S., MATTHEIJ, N. J., MINDUKSHEV, I., PÖTZ, O., JURK, K., BURKHART, J. M., FUFEZAN, C., HEEMSKERK, J. W. M., WALTER, U., ZAHEDI, R. P. & SICKMANN, A. 2017. Temporal quantitative phosphoproteomics of ADP stimulation reveals novel central nodes in platelet activation and inhibition. *Blood,* 129**,** e1-e12.

BENDER, M., ECKLY, A., HARTWIG, J. H., ELVERS, M., PLEINES, I., GUPTA, S., KROHNE, G., JEANCLOS, E., GOHLA, A., GURNIAK, C., GACHET, C., WITKE, W. & NIESWANDT, B. 2010. ADF/n-cofilin-dependent actin turnover determines platelet formation and sizing. *Blood,* 116**,** 1767-75.

BHATTACHARJEE, S., DAS, A. K. & MANDAL, S. K. 2004. *Isolation and characterization of alkaline phosphatase of Saccharopolyspora erythraea from fermentation broth of erythromycin production*.

BLAKYTNY, R. & HARDING, J. 1992. Glycation (non-enzymic glycosylation) inactivates glutathione reductase. *Biochemical Journal,* 288**,** 303-307.

BLAKYTNY, R. & JUDE, E. 2006. The molecular biology of chronic wounds and delayed healing in diabetes. *Diabet Med,* 23**,** 594-608.

BLUMBERG, S. N., BERGER, A., HWANG, L., PASTAR, I., WARREN, S. M. & CHEN, W. 2012. The role of stem cells in the treatment of diabetic foot ulcers. *Diabetes research and clinical practice,* 96**,** 1-9.

BOERSEMA, P. J., MOHAMMED, S. & HECK, A. J. R. 2008. Hydrophilic interaction liquid chromatography (HILIC) in proteomics. *Analytical and Bioanalytical Chemistry,* 391**,** 151-159.

BOS, M. H. A. & CAMIRE, R. M. 2010. Blood coagulation factors V and VIII: Molecular Mechanisms of Procofactor Activation. *Journal of coagulation disorders,* 2**,** 19-27.

BOURNE, R. R., STEVENS, G. A., WHITE, R. A., SMITH, J. L., FLAXMAN, S. R., PRICE, H., JONAS, J. B., KEEFFE, J., LEASHER, J., NAIDOO, K., PESUDOVS, K., RESNIKOFF, S. & TAYLOR, H. R. 2013. Causes of vision loss worldwide, 1990-2010: a systematic analysis. *Lancet Glob Health,* 1**,** e339-49.

BREM, H., SHEEHAN, P. & BOULTON, A. J. 2004. Protocol for treatment of diabetic foot ulcers. *The American journal of surgery,* 187**,** S1-S10.

BRUHN-OLSZEWSKA, B., KORZON-BURAKOWSKA, A., GABIG-CIMINSKA, M., OLSZEWSKI, P., WEGRZYN, A. & JAKOBKIEWICZ-BANECKA, J. 2012. Molecular factors involved in the development of diabetic foot syndrome. *Acta Biochim Pol,* 59**,** 507-13.

BRUNELLE, J. & GREEN, R. 2014. One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). *Methods Enzymol,* 541**,** 151-9.

BRYANT, R. & NIX, D. 2015. *Acute and chronic wounds: current management concepts*, Elsevier Health Sciences.

BULLDOG BIO INC. 2017. *AcquaStain: Protein Gel Stain* [Online]. Available: http://www.bulldog-bio.com/acquastain.html [Accessed 12 December 2017].

BURKHART, J. M., VAUDEL, M., GAMBARYAN, S., RADAU, S., WALTER, U., MARTENS, L., GEIGER, J., SICKMANN, A. & ZAHEDI, R. P. 2012. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood,* 120**,** e73-82.

BUTTAR, H. S., LI, T. & RAVI, N. 2005. Prevention of cardiovascular diseases: Role of exercise, dietary interventions, obesity and smoking cessation. *Experimental & Clinical Cardiology,* 10**,** 229-249.

CAVANAGH, P., LIPSKY, B., BRADBURY, A. & BOTEK, G. 2005a. Treatment for diabetic foot ulcers. *The Lancet,* 366**,** 1725-1735.

CAVANAGH, P. R., LIPSKY, B. A., BRADBURY, A. W. & BOTEK, G. 2005b. Treatment for diabetic foot ulcers. *The Lancet,* 366**,** 1725-1735.

CERAMI, C., FOUNDS, H., NICHOLL, I., MITSUHASHI, T., GIORDANO, D., VANPATTEN, S., LEE, A., AL-ABED, Y., VLASSARA, H., BUCALA, R. & CERAMI, A. 1997. Tobacco smoke is a source of toxic reactive glycation products. *Proc Natl Acad Sci U S A,* 94**,** 13915-20.

CHADWICK, P., EDMONDS, M., MCCARDLE, J. & ARMSTRONG, D. 2013. Best practice guidelines: Wound management in diabetic foot ulcers. [Accessed 13-04-2015].

CHEVALLET, M., LUCHE, S. & RABILLOUD, T. 2006. Silver staining of proteins in polyacrylamide gels. *Nature Protocols,* 1**,** 1852-1858.

CHIASSON, J.-L., ARIS-JILWAN, N., BÉLANGER, R., BERTRAND, S., BEAUREGARD, H., ÉKOÉ, J.-M., FOURNIER, H. & HAVRANKOVA, J. 2003. Diagnosis and treatment of diabetic ketoacidosis and the hyperglycemic hyperosmolar state. *Canadian Medical Association Journal,* 168**,** 859-866.

CIANFARANI, F., TOIETTA, G., DI ROCCO, G., CESAREO, E., ZAMBRUNO, G. & ODORISIO, T. 2013. Diabetes impairs adipose tissue-derived stem cell function and efficiency in promoting wound healing. *Wound Repair and Regeneration,* 21**,** 545-553.

CLIFTON, J., HUANG, F., RUCEVIC, M., CAO, L., HIXSON, D. & JOSIC, D. 2011. Protease inhibitors as possible pitfalls in proteomic analyses of complex biological samples. *Journal of proteomics,* 74**,** 935-941.

CROMPTON, M., RIXON, K. C. & HARDING, J. J. 1985. Aspirin prevents carbamylation of soluble lens proteins and prevents cyanate-induced phase separation opacities in vitro: a possible mechanism by which aspirin could prevent cataract. *Exp Eye Res,* 40**,** 297-311.

CUBEDO, J., PADRÓ, T., GARCÍA-ARGUINZONIS, M., VILAHUR, G., MIÑAMBRES, I., POU, J. M., YBARRA, J. & BADIMON, L. 2015. A novel truncated form of apolipoprotein A-I transported by dense LDL is increased in diabetic patients. *Journal of Lipid Research,* 56**,** 1762-1773.

DAI, M., YU, C., FANG, T., FU, L., WANG, J., ZHANG, J., REN, J., XU, J., ZHANG, X. & CHEN, W. 2015. Identification and functional characterization of glycosylation of recombinant human platelet-derived growth factor-BB in pichia pastoris. *PLoS ONE,* 10.

DANAEI, G., LAWES, C. M., VANDER HOORN, S., MURRAY, C. J. & EZZATI, M. 2006. Global and regional mortality from ischaemic heart disease and stroke attributable to higher-than-optimum blood glucose concentration: comparative risk assessment. *Lancet,* 368**,** 1651-9.

DEMIDOVA-RICE, T., HAMBLIN, M. & HERMAN, I. 2012. Acute and Impaired Wound Healing: Pathophysiology and Current Methods for Drug Delivery, Part 1: Normal and Chronic Wounds: Biology, Causes, and Approaches to Care. *Adv Skin Wound Care,* 25**,** 304-314.

DEVANGELIO, E., SANTILLI, F., FORMOSO, G., FERRONI, P., BUCCIARELLI, L., MICHETTI, N., CLISSA, C., CIABATTONI, G., CONSOLI, A. & DAVÌ, G. 2007. Soluble RAGE in type 2 diabetes: association with oxidative stress. *Free Radical Biology and Medicine,* 43**,** 511-518.

DHURAT, R. & SUKESH, M. S. 2014. Principles and Methods of Preparation of Platelet-Rich Plasma: A Review and Author's Perspective. *Journal of Cutaneous and Aesthetic Surgery,* 7**,** 189-197.

DIAZ, F. 2010. Cytochrome c oxidase deficiency: Patients and animal models. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1802**,** 100-110.

DUNN, M. J. & BRADD, S. J. 1993. Separation and Analysis of Membrane Proteins by SDS-Polyacrylamide Gel Electrophoresis. *In:* GRAHAM, J. M. & HIGGINS, J. A. (eds.) *Biomembrane Protocols: I. Isolation and Analysis.* Totowa, NJ: Humana Press.

DZANDU, J. K., DEH, M. E., BARRATT, D. L. & WISE, G. E. 1984. Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double-staining technique. *Proceedings of the National Academy of Sciences,* 81**,** 1733-1737.

EDMONDS, M. 2006. Diabetic Foot Ulcers. *Drugs,* 66**,** 913-929.

ELDOR, R., RAZ, I., BEN YEHUDA, A. & BOULTON, A. 2004. New and experimental approaches to treatment of diabetic foot ulcers: a comprehensive review of emerging treatment strategies. *Diabetic Medicine,* 21**,** 1161-1173.

ESCHENBRUCH, M. & BÜRK, R. R. 1982. Experimentally improved reliability of ultrasensitive silver staining of protein in polyacrylamide gels. *Analytical Biochemistry,* 125**,** 96-99.

FALANGA, V. 2005. Wound healing and its impairment in the diabetic foot. *The Lancet,* 366**,** 1736-1743.

FALET, H. 2017. Anatomy of the Platelet Cytoskeleton. *In:* GRESELE, P., KLEIMAN, N. S., LOPEZ, J. A. & PAGE, C. P. (eds.) *Platelets in Thrombotic and Non-Thrombotic Disorders: Pathophysiology, Pharmacology and Therapeutics: an Update.* Cham: Springer International Publishing.

FLEMING, T. H., HUMPERT, P. M., NAWROTH, P. P. & BIERHAUS, A. 2011. Reactive metabolites and AGE/RAGE-mediated cellular dysfunction affect the aging process: a mini-review. *Gerontology,* 57**,** 435-43.

FORD, T. C., GRAHAM, J. & RICKWOOD, D. 1990. A new, rapid, one-step method for the isolation of platelets from human blood. *Clinica Chimica Acta,* 192**,** 115-119.

FUENTES, E., ROJAS, A. & PALOMO, I. 2014. Role of multiligand/RAGE axis in platelet activation. *Thromb Res,* 133**,** 308-14.

GARUD, M. & KULKARNI, Y. 2014. Hyperglycemia to nephropathy via transforming growth factor beta. *Curr Diabetes Rev,* 10**,** 182-189.

GAWLOWSKI, T., STRATMANN, B., RUETTER, R., BUENTING, C., MENART, B., WEISS, J., VLASSARA, H., KOSCHINSKY, T. & TSCHOEPE, D. 2009. Advanced glycation end products strongly activate platelets. *European journal of nutrition,* 48**,** 475-481.

GHADERIAN, S. B., HAYATI, F., SHAYANPOUR, S. & BELADI MOUSAVI, S. S. 2015. Diabetes and end-stage renal disease; a review article on new concepts. *Journal of Renal Injury Prevention,* 4**,** 28-33.

GKOGKOLOU, P. & BÖHM, M. 2012. Advanced glycation end products: Key players in skin aging? *Dermato-endocrinology,* 4**,** 259-270.

GOLDSTEIN, D., LITTLE, R., WIEDMEYER, H., ENGLAND, J. & MCKENZIE, E. 1986. Glycated hemoglobin: methodologies and clinical applications. *Clin Chem,* 32**,** B64-70.

GUELL, C. & UNWIN, N. 2015. Barriers to diabetic foot care in a developing country with a high incidence of diabetes related amputations: an exploratory qualitative interview study. *BMC Health Services Research,* 15**,** 377.

GUEMES, M., RAHMAN, S. A. & HUSSAIN, K. 2016. What is a normal blood glucose? *Arch Dis Child,* 101**,** 569-74.

GUEST, G. M. 1949. Diabetic coma. *The American Journal of Medicine,* 7**,** 630-646.

GUNDRY, R. L., WHITE, M. Y., MURRAY, C. I., KANE, L. A., FU, Q., STANLEY, B. A. & VAN EYK, J. E. 2009. Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow. *Curr Protoc Mol Biol,* Chapter 10**,** Unit10.25.

GUO, S. & DIPIETRO, L. 2010a. Factors Affecting Wound Healing. *J Dent Res.,* 89**,** 219-229.

GUO, S. & DIPIETRO, L. A. 2010b. Factors Affecting Wound Healing. *Journal of Dental Research,* 89**,** 219-229.

HALING, J. R., MONKLEY, S. J., CRITCHLEY, D. R. & PETRICH, B. G. 2011. Talin-dependent integrin activation is required for fibrin clot retraction by platelets. *Blood,* 117**,** 1719-1722.

HARVEY, R., CLARK, M., FINKEL, R., REY, J. & WHALEN, K. 2012. *Pharmacology*, Lippincott Williams & Wilkins.

HO, D. H., BADELLINO, K., BAGLIA, F. A., SUN, M.-F., ZHAO, M.-M., GAILANI, D. & WALSH, P. N. 2000. The Role of High Molecular Weight Kininogen and Prothrombin as Cofactors in the Binding of Factor XI A3 Domain to the Platelet Surface. *Journal of Biological Chemistry,* 275**,** 25139-25145.

INTERNATIONAL DIABETES FEDERATION 2015. *Diabetes Atlas*.

INTERNATIONAL DIABETES FEDERATION 2017. IDF Diabetes Atlas. 8th ed. Brussels,Belgium.

ITO, R. & SANGHERA, G. 2001. *Measurement of glycated hemoglobin*. US patent application US 95710497 A. 2001/01/16.

JAFARI, M., PRIMO, V., SMEJKAL, G. B., MOSKOVETS, E. V., KUO, W. P. & IVANOV, A. R. 2012. Comparison of in-gel protein separation techniques commonly used for fractionation in mass spectrometry-based proteomic profiling. *Electrophoresis,* 33**,** 2516-26.

JAISSON, S., SOUCHON, P., DESMONS, A., SALMON, A., DELEMER, B. & GILLERY, P. 2016. Early formation of serum advanced glycation end-products in children with type 1 diabetes mellitus: Relationship with glycemic control. *Journal of Pediatrics,* 172**,** 56-62.

JUSTER-SWITLYK, K. & SMITH, A. G. 2016. Updates in diabetic peripheral neuropathy. *F1000Research,* 5**,** F1000 Faculty Rev-738.

KALOUSOVA, M., ZIMA, T., MALBOHAN, I. M. & STIPEK, S. 2002. [Determination of advanced glycation end products]. *Sb Lek,* 103**,** 427-34.

KHARROUBI, A. T. & DARWISH, H. M. 2015. Diabetes mellitus: The epidemic of the century. *World J Diabetes,* 6**,** 850-67.

KIEFFER, N., GUICHARD, J., FARCET, J. P., VAINCHENKER, W. & BRETON-GORIUS, J. 1987. Biosynthesis of major platelet proteins in human blood platelets. *European Journal of Biochemistry,* 164**,** 189-195.

KINTER, M. & SHERMAN, N. E. 2005a. Basic Polyacrylamide Gel Electrophoresis. *Protein Sequencing and Identification Using Tandem Mass Spectrometry.* John Wiley & Sons, Inc.

KINTER, M. & SHERMAN, N. E. 2005b. An Introduction to Protein Sequencing Using Tandem Mass Spectrometry. *Protein Sequencing and Identification Using Tandem Mass Spectrometry.* John Wiley & Sons, Inc.

KRASNY, L., BLAND, P., KOGATA, N., WAI, P., HOWARD, B. A., NATRAJAN, R. C. & HUANG, P. H. 2018. SWATH mass spectrometry as a tool for quantitative profiling of the matrisome. *Journal of Proteomics*.

KURIEN, B. T. & SCOFIELD, R. H. 2006. Western blotting. *Methods,* 38**,** 283-293.

KUTTI, J., WADENVIK, H., HENESTAM, B. & STENSTRÖM, G. 1986. Evaluation of Platelet Reactivity in Diabetes Mellitus. *Acta Medica Scandinavica,* 219**,** 195-199.

LADNER, C. L., EDWARDS, R. A., SCHRIEMER, D. C. & TURNER, R. J. 2006. Identification of trichloroethanol visualized proteins from two-dimensional polyacrylamide gels by mass spectrometry. *Anal Chem,* 78**,** 2388-96.

LAEMMLI, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature,* 227**,** 680.

LANGHAUSER, F., GOB, E., KRAFT, P., GEIS, C., SCHMITT, J., BREDE, M., GOBEL, K., HELLUY, X., PHAM, M., BENDSZUS, M., JAKOB, P., STOLL, G., MEUTH, S. G., NIESWANDT, B., MCCRAE, K. R. & KLEINSCHNITZ, C. 2012. Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation. *Blood,* 120**,** 4082-92.

LAPOLLA, A., FEDELE, D., REITANO, R., ARICÒ, N. C., SERAGLIA, R., TRALDI, P., MAROTTA, E. & TONANI, R. 2004. Enzymatic digestion and mass spectrometry in the study of advanced glycation end products/peptides. *J Am Soc Mass Spectrom,* 15.

LIPPI, G., MERCADANTI, M., ALOE, R. & TARGHER, G. 2012. Erythrocyte mechanical fragility is increased in patients with type 2 diabetes. *European Journal of Internal Medicine,* 23**,** 150-153.

LÖNDAHL, M., KATZMAN, P., NILSSON, A. & HAMMARLUND, C. 2010. Hyperbaric oxygen therapy facilitates healing of chronic foot ulcers in patients with diabetes. *Diabetes care,* 33**,** 998-1003.

MAHMOOD, T. & YANG, P.-C. 2012. Western Blot: Technique, Theory, and Trouble Shooting. *North American Journal of Medical Sciences,* 4**,** 429-434.

MAILLARD, L. 1912. Reaction of amino acids on sugars: melanoidin formation through a methodologic way. *C R Acad Sci,* 156**,** 148-149.

MAJEK, P., REICHELTOVA, Z., STIKAROVA, J., SUTTNAR, J., SOBOTKOVA, A. & DYR, J. 2010a. Proteome changes in platelets activated by arachidonic acid, collagen, and thrombin. *Proteome Sci.,* 8**,** 56.

MAJEK, P., REICHELTOVA, Z., STIKAROVA, J., SUTTNAR, J., SOBOTKOVA, A. & DYR, J. E. 2010b. Proteome changes in platelets activated by arachidonic acid, collagen, and thrombin. *Proteome Sci,* 8**,** 56.

MANGARAJ, M., NANDA, R. & PANDA, S. 2016. Apolipoprotein A-I: A Molecule of Diverse Function. *Indian Journal of Clinical Biochemistry,* 31**,** 253-259.

MAYNARD, D. M., HEIJNEN, H. F., HORNE, M. K., WHITE, J. G. & GAHL, W. A. 2007. Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost,* 5**,** 1945-55.

MERRIL, C. R., GOLDMAN, D., SEDMAN, S. A. & EBERT, M. H. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science,* 211**,** 1437-8.

MICHELSON, A. D. 1996. Flow cytometry: a clinical test of platelet function. *Blood,* 87**,** 4925-36.
MICHELSON, A. D., BARNARD, M. R., KRUEGER, L. A., VALERI, C. R. & FURMAN, M. I. 2001. Circulating Monocyte-Platelet Aggregates Are a More Sensitive Marker of In Vivo Platelet Activation Than Platelet Surface P-Selectin. *Studies in Baboons, Human Coronary Intervention, and Human Acute Myocardial Infarction,* 104**,** 1533-1537.
MIKI, H., SETOU, M., KANESHIRO, K. & HIROKAWA, N. 2001. All kinesin superfamily protein, KIF, genes in mouse and human. *Proceedings of the National Academy of Sciences,* 98**,** 7004-7011.
MITRA, S. & ABRAHAM, E. 2006. Participation of superoxide in neutrophil activation and cytokine production. *Biochim Biophys Acta,* 1762**,** 732-41.
MUNCH, G., KEIS, R., WESSELS, A., RIEDERER, P., BAHNER, U., HEIDLAND, A., NIWA, T., LEMKE, H. D. & SCHINZEL, R. 1997. Determination of advanced glycation end products in serum by fluorescence spectroscopy and competitive ELISA. *Eur J Clin Chem Clin Biochem,* 35**,** 669-77.
NAGAI, R., SHIRAKAWA, J.-I., FUJIWARA, Y., OHNO, R.-I., MOROISHI, N., SAKATA, N. & NAGAI, M. 2014. Detection of AGEs as markers for carbohydrate metabolism and protein denaturation. *Journal of clinical biochemistry and nutrition,* 55**,** 1.
NAKAYAMA, M., IZUMI, G., NEMOTO, Y., SHIBATA, K., HASEGAWA, T., NUMATA, M., WANG, K., KAWAGUCHI, Y. & HOSOYA, T. 1999. Suppression of N (epsilon)-(carboxymethyl) lysine generation by the antioxidant N-acetylcysteine. *Peritoneal dialysis international,* 19**,** 207-210.
NATIONAL DIABETES INFORMATION CLEARINGHOUSE. 2014. *Your Guide to Diabetes: Type 1 and Type 2* [Online]. National Institute of diabetes and digestive kidney diseases. Available: http://diabetes.niddk.nih.gov/dm/pubs/type1and2/specialtimes.aspx [Accessed 11-04 2015].
NCD RISK FACTOR COLLABORATION 2016. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants. *The Lancet,* 387**,** 1513-1530.
NURDEN, A., NURDEN, P., SANCHEZ, M., ANDIA, I. & ANITUA, E. 2008a. Platelets and wound healing. *Front Biosci,* 13**,** 3532-48.
NURDEN, A. T., NURDEN, P., SANCHEZ, M., ANDIA, I. & ANITUA, E. 2008b. Platelets and wound healing. *Front Biosci,* 13**,** 3532-48.
OGURTSOVA, K., DA ROCHA FERNANDES, J. D., HUANG, Y., LINNENKAMP, U., GUARIGUATA, L., CHO, N. H., CAVAN, D., SHAW, J. E. & MAKAROFF, L. E. 2017. IDF Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Research and Clinical Practice,* 128**,** 40-50.
OLD, W. M., MEYER-ARENDT, K., AVELINE-WOLF, L., PIERCE, K. G., MENDOZA, A., SEVINSKY, J. R., RESING, K. A. & AHN, N. G. 2005. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. *Mol Cell Proteomics,* 4**,** 1487-502.
OLIVEIRA, M. I. A., SOUZA, E. M. D., PEDROSA, F. D. O., RÉA, R. R., ALVES, A. D. S. C., PICHETH, G. & REGO, F. G. D. M. 2013. RAGE receptor and its soluble isoforms in diabetes mellitus complications. *Jornal Brasileiro de Patologia e Medicina Laboratorial,* 49**,** 97-108.
ORNELAS, A., ZACHARIAS-MILLWARD, N., MENTER, D. G., DAVIS, J. S., LICHTENBERGER, L., HAWKE, D., HAWK, E., VILAR, E., BHATTACHARYA, P. & MILLWARD, S. 2017. Beyond COX-1: the effects of aspirin on platelet biology and potential mechanisms of chemoprevention. *Cancer Metastasis Reviews,* 36**,** 289-303.
PANICCIA, R., PRIORA, R., ALESSANDRELLO LIOTTA, A. & ABBATE, R. 2015. Platelet function tests: a comparative review. *Vascular Health and Risk Management,* 11**,** 133-148.
PARK, S. M., JUNG, H. Y., KIM, H. O., RHIM, H., PAIK, S. R., CHUNG, K. C., PARK, J. H. & KIM, J. 2002. Evidence that alpha-synuclein functions as a negative regulator of Ca(++)-dependent alpha-granule release from human platelets. *Blood,* 100**,** 2506-14.
PAUL, R. G. & BAILEY, A. J. 1996. Glycation of collagen: the basis of its central role in the late complications of ageing and diabetes. *Int J Biochem Cell Biol,* 28**,** 1297-310.
PEMAYUN, T. G., NAIBAHO, R. M., NOVITASARI, D., AMIN, N. & MINULJO, T. T. 2015. Risk factors for lower extremity amputation in patients with diabetic foot ulcers: a hospital-based case-control study. *Diabet Foot Ankle,* 6**,** 29629.

PEREZ, A. G., LANA, J. F., RODRIGUES, A. A., LUZO, A. C., BELANGERO, W. D. & SANTANA, M. H. 2014. Relevant aspects of centrifugation step in the preparation of platelet-rich plasma. *ISRN Hematol,* 2014**,** 176060.

PERROTTA, P. L. 2008. Proteomics: moving from a discovery to a quality assurance tool. *Transfusion,* 48**,** 408-10.

PETRICH, B. G., MARCHESE, P., RUGGERI, Z. M., SPIESS, S., WEICHERT, R. A. M., YE, F., TIEDT, R., SKODA, R. C., MONKLEY, S. J., CRITCHLEY, D. R. & GINSBERG, M. H. 2007. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. *The Journal of Experimental Medicine,* 204**,** 3103-3111.

PULCINELLI, F. M., PIGNATELLI, P., CELESTINI, A., RIONDINO, S., GAZZANIGA, P. P. & VIOLI, F. 2004. Inhibition of platelet aggregation by aspirin progressively decreases in long-term treated patients. *Journal of the American College of Cardiology,* 43**,** 979-984.

RABBANI, N., ASHOUR, A. & THORNALLEY, P. J. 2016. Mass spectrometric determination of early and advanced glycation in biology. *Glycoconjugate Journal,* 33**,** 553-568.

RABBANI, N. & THORNALLEY, P. 2012. Glycation research in amino acids: a place to call home. *Amino acids,* 42**,** 1087-1096.

RAHBAR, S. & FIGAROLA, J. L. 2003. Novel inhibitors of advanced glycation endproducts. *Arch Biochem Biophys,* 419**,** 63-79.

RAHBAR, S., NATARAJAN, R., YERNENI, K., SCOTT, S., GONZALES, N. & NADLER, J. L. 2000. Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation. *Clin Chim Acta,* 301**,** 65-77.

RAO, G. N., LARDIS, M. P. & COTLIER, E. 1985. Acetylation of lens crystallins: a possible mechanism by which aspirin could prevent cataract formation. *Biochem Biophys Res Commun,* 128**,** 1125-32.

RAPALA, K. 1996. The effect of tumor necrosis factor-alpha on wound healing. An experimental study. *Ann Chir Gynaecol Suppl,* 211**,** 1-53.

RAVI, S., CHACKO, B., KRAMER, P. A., SAWADA, H., JOHNSON, M. S., ZHI, D., MARQUES, M. B. & DARLEY-USMAR, V. M. 2015. Defining the effects of storage on platelet bioenergetics; the role of increased proton leak. *Biochimica et biophysica acta,* 1852**,** 2525-2534.

REDDY, S., BICHLER, J., WELLS-KNECHT, K. J., THORPE, S. R. & BAYNES, J. W. 1995. N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry,* 34**,** 10872-8.

RENNE, T. 2012. The procoagulant and proinflammatory plasma contact system. *Semin Immunopathol,* 34**,** 31-41.

RITSU, M., KAWAKAMI, K., KANNO, E., TANNO, H., ISHII, K., IMAI, Y., MARUYAMA, R. & TACHI, M. 2017. Critical role of tumor necrosis factor-α in the early process of wound healing in skin. *Journal of Dermatology & Dermatologic Surgery,* 21**,** 14-19.

RIVERO-GUTIERREZ, B., ANZOLA, A., MARTINEZ-AUGUSTIN, O. & DE MEDINA, F. S. 2014. Stain-free detection as loading control alternative to Ponceau and housekeeping protein immunodetection in Western blotting. *Anal Biochem,* 467**,** 1-3.

ROBLES, D. T. & BERG, D. 2007. Abnormal wound healing: keloids. *Clin Dermatol,* 25**,** 26-32.

ROOHK, H. & ZAIDI, A. 2008. A review of glycated albumin as an intermediate glycation index for controlling diabetes. *J Diabetes Sci Technol,* 2**,** 1114-1121.

ROUTLEDGE, P. A. 1986. The plasma protein binding of basic drugs. *British Journal of Clinical Pharmacology,* 22**,** 499-506.

ROY, S. & KUMAR, V. 2014. *A Practical Approach on SDS PAGE for Separation of Protein*.

RYAN, B. J. 2011. Avoiding proteolysis during protein chromatography. *Methods Mol Biol,* 681**,** 61-71.

RYDSTRÖM, J. 2006. Mitochondrial NADPH, transhydrogenase and disease. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1757**,** 721-726.

SAAD SETTA, H., ELSHAHAT, A., ELSHERBINY, K., MASSOUD, K. & SAFE, I. 2011. Platelet-rich plasma versus platelet-poor plasma in the management of chronic diabetic foot ulcers: a comparative study. *International Wound Journal,* 8**,** 307-312.

SADOUL, K. 2015. New explanations for old observations: marginal band coiling during platelet activation. *Journal of Thrombosis and Haemostasis,* 13**,** 333-346.

SANTILLI, F., SIMEONE, P., LIANI, R. & DAVÌ, G. 2015. Platelets and diabetes mellitus. *Prostaglandins & Other Lipid Mediators,* 120**,** 28-39.

SARWAR, N., GAO, P., SESHASAI, S. R., GOBIN, R., KAPTOGE, S., DI ANGELANTONIO, E., INGELSSON, E., LAWLOR, D. A., SELVIN, E., STAMPFER, M., STEHOUWER, C. D., LEWINGTON, S., PENNELLS, L., THOMPSON, A., SATTAR, N., WHITE, I. R., RAY, K. K. & DANESH, J. 2010. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet,* 375**,** 2215-22.

SCHLEICHER, E. & WIELAND, O. 1986. Kinetic analysis of glycation as a tool for assessing the half-life of proteins. *Biochim Biophys Acta,* 884**,** 199-205.

SCHMAIER, A. H., ZUCKERBERG, A., SILVERMAN, C., KUCHIBHOTLA, J., TUSZYNSKI, G. P. & COLMAN, R. W. 1983. High-molecular weight kininogen. A secreted platelet protein. *Journal of Clinical Investigation,* 71**,** 1477-1489.

SCHROR, K. 1997. Aspirin and platelets: the antiplatelet action of aspirin and its role in thrombosis treatment and prophylaxis. *Semin Thromb Hemost,* 23**,** 349-56.

SELVIN, E., STEFFES, M., ZHU, H., MATSUSHITA, K., WAGENKNECHT, L., PANKOW, J., CORESH, J. & BRANCATI, F. 2010. Glycated hemoglobin, diabetes, and cardiovascular risk in nondiabetic adults. *New England Journal of Medicine,* 362**,** 800-811.

SHOSHAN-BARMATZ, V., DE PINTO, V., ZWECKSTETTER, M., RAVIV, Z., KEINAN, N. & ARBEL, N. 2010. VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Molecular Aspects of Medicine,* 31**,** 227-285.

SINGH, G. M., DANAEI, G., FARZADFAR, F., STEVENS, G. A., WOODWARD, M., WORMSER, D., KAPTOGE, S., WHITLOCK, G., QIAO, Q., LEWINGTON, S., DI ANGELANTONIO, E., VANDER HOORN, S., LAWES, C. M. M., ALI, M. K., MOZAFFARIAN, D., EZZATI, M., GLOBAL BURDEN OF METABOLIC RISK FACTORS OF CHRONIC DISEASES COLLABORATING, G., ASIA-PACIFIC COHORT STUDIES, C., DIABETES EPIDEMIOLOGY: COLLABORATIVE ANALYSIS OF DIAGNOSTIC CRITERIA IN, E., EMERGING RISK FACTOR, C. & PROSPECTIVE STUDIES, C. 2013. The Age-Specific Quantitative Effects of Metabolic Risk Factors on Cardiovascular Diseases and Diabetes: A Pooled Analysis. *PLOS ONE,* 8**,** e65174.

SINGH, V. P., BALI, A., SINGH, N. & JAGGI, A. S. 2014. Advanced Glycation End Products and Diabetic Complications. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology,* 18**,** 1-14.

SONG, B. J., AIELLO, L. P. & PASQUALE, L. R. 2016. Presence and Risk Factors for Glaucoma in Patients with Diabetes. *Curr Diab Rep,* 16**,** 124.

SPEAR, M. 2013. Acute or chronic? What is the difference? *Plastic Surgical Nursing,* 33**,** 98-100.

SPRINGER, D., MILLER, J., SPINELLI, S., PASA-TOLIC, L., PURVINE, S., DALY, D., ZANGAR, R., JIN, S., BLUMBERG, N., FRANCIS, C., TAUBMAN, M., CASEY, A., WITTLIN, S. & PHIPPS, R. 2009. Platelet Proteome Changes Associated with Diabetes and during Platelet Storage for Transfusion. *Journal of Proteome Research,* 8**,** 2261-2272.

STEINBERG, T. H. 2009. Protein gel staining methods: an introduction and overview. *Methods Enzymol,* 463**,** 541-63.

STOYCHEV, S., GERBER, I., JORDAAN, J., NAICKER, P., KHUMALO, F., MAMPUTHA, S. & VAN DER WESTHUIZEN, C. 2017. *Universal unbiased sample-clean up pre-mass spectrometry using MagResyn HILIC for SPE* [Online]. ReSyn Biosciences Pty Ltd [Accessed 14 December 2017 2017].

SUSLOVA, T., SITOZHEVSKII, A., OGURKOVA, O., KRAVCHENKO, E., KOLOGRIVOVA, I., ANFINOGENOVA, Y. & KARPOV, R. 2015. Platelet hemostasis in patients with metabolic syndrome and type 2 diabetes mellitus: cGMP- and NO-dependent mechanisms in the insulin-mediated platelet aggregation. *Frontiers in Physiology,* 6.

TECILAZICH, F., DINH, T. L. & VEVES, A. 2013. Emerging drugs for the treatment of diabetic ulcers. *Expert Opin Emerg Drugs,* 18**,** 207-17.

TIAN, X., YI, L.-J., MA, L., ZHANG, L., SONG, G.-M. & WANG, Y. 2014. Effects of honey dressing for the treatment of DFUs: A systematic review. *International Journal of Nursing Sciences,* 1**,** 224-231.

TOYE, A. A., LIPPIAT, J. D., PROKS, P., SHIMOMURA, K., BENTLEY, L., HUGILL, A., MIJAT, V., GOLDSWORTHY, M., MOIR, L., HAYNES, A., QUARTERMAN, J., FREEMAN, H. C., ASHCROFT, F. M. & COX, R. D. 2005. A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. *Diabetologia,* 48**,** 675-86.

TRUGILHO, M. R. O., HOTTZ, E. D., BRUNORO, G. V. F., TEIXEIRA-FERREIRA, A., CARVALHO, P. C., SALAZAR, G. A., ZIMMERMAN, G. A., BOZZA, F. A., BOZZA, P. T. & PERALES, J. 2017. Platelet proteome reveals novel pathways of platelet activation and platelet-mediated immunoregulation in dengue. *PLoS Pathog,* 13**,** e1006385.

TUOMI, T. 2005. Type 1 and type 2 diabetes: what do they have in common? *Diabetes,* 54 Suppl 2**,** S40-5.

UNITED STATES RENAL DATA SYSTEM 2014. Internation Comparisons In United States Renal Data System. *United States Renal Data System, 2014 annual data report.* Bethesda (MD), United States: National Institute of Diabetes and Digestive and Kidney Diseases.

URIOS, P., GRIGOROVA-BORSOS, A. M. & STERNBERG, M. 2007. Aspirin inhibits the formation of pentosidine, a cross-linking advanced glycation end product, in collagen. *Diabetes Res Clin Pract,* 77**,** 337-40.

VARO, N., LIBBY, P., NUZZO, R., ITALIANO, J., DORIA, A. & SCHÖNBECK, U. 2005. Elevated release of sCD40L from platelets of diabetic patients by thrombin, glucose and advanced glycation end products. *Diab Vasc Dis Res,* 2**,** 81-87.

VAUDEL, M., BARSNES, H., BERVEN, F. S., SICKMANN, A. & MARTENS, L. 2011. SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. *Proteomics,* 11**,** 996-9.

VELNAR, T., BAILEY, T. & SMRKOLJ, V. 2009. The wound healing process: an overview of the cellular and molecular mechanisms. *J Int Med Res,* 37**,** 1528-42.

VIGNESHWARAN, N., BIJUKUMAR, G., KARMAKAR, N., ANAND, S. & MISRA, A. 2005. Autofluorescence characterization of advanced glycation end products of hemoglobin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 61**,** 163-170.

VINIK, A., ERBAS, T., PARK, T., NOLAN, R. & PITTENGER, G. 2001. Platelet dysfunction in type 2 diabetes. *Diabetes care,* 24**,** 1476-1485.

WANG, X., LI, X. & LI, Y. 2007. A modified Coomassie Brilliant Blue staining method at nanogram sensitivity compatible with proteomic analysis. *Biotechnology Letters,* 29**,** 1599-1603.

WANG, Z., JIANG, Y., LIU, N., REN, L., ZHU, Y., AN, Y. & CHEN, D. 2012. Advanced glycation end-product Nepsilon-carboxymethyl-Lysine accelerates progression of atherosclerotic calcification in diabetes. *Atherosclerosis,* 221**,** 387-96.

WATALA, C., PLUTA, J., GOLANSKI, J., ROZALSKI, M., CZYZ, M., TROJANOWSKI, Z. & DRZEWOSKI, J. 2005. Increased protein glycation in diabetes mellitus is associated with decreased aspirin-mediated protein acetylation and reduced sensitivity of blood platelets to aspirin. *Journal of Molecular Medicine,* 83**,** 148-158.

WHO 2016. Global Report on Diabetes. *WHO*.

WHO 2017. Diabetes fact sheet. *WHO*.

WRIGHT, J. R., AMISTEN, S., GOODALL, A. H. & MAHAUT-SMITH, M. P. 2016. Transcriptomic Analysis of the Ion Channelome of Human Platelets and Megakaryocytic Cell Lines. *Thrombosis and Haemostasis,* 116**,** 272-284.

WU, S. C., JENSEN, J. L., WEBER, A. K., ROBINSON, D. E. & ARMSTRONG, D. G. 2008. Use of Pressure Offloading Devices in Diabetic Foot Ulcers: Do we practice what we preach? *Diabetes Care,* 31**,** 2118-2119.

YANG, X., CLIFTON, J., HUANG, F., KOVAC, S., HIXSON, D. C. & JOSIC, D. 2009. Proteomic analysis for process development and control of therapeutic protein separation from human plasma. *Electrophoresis,* 30**,** 1185-1193.

YARDUMIAN, D. A., MACKIE, I. J. & MACHIN, S. J. 1986. Laboratory investigation of platelet function: a review of methodology. *Journal of Clinical Pathology,* 39**,** 701-712.

YATSCOFF, R. W., MEHTA, A., GERRARD, J. M. & THLIVERIS, J. 1987. Glycation of platelet protein in diabetes mellitus: lack of correlation with platelet function. *Clin Biochem,* 20**,** 359-63.

YAU, J. W., ROGERS, S. L., KAWASAKI, R., LAMOUREUX, E. L., KOWALSKI, J. W., BEK, T., CHEN, S. J., DEKKER, J. M., FLETCHER, A., GRAUSLUND, J., HAFFNER, S., HAMMAN, R. F., IKRAM, M. K., KAYAMA, T., KLEIN, B. E., KLEIN, R., KRISHNAIAH, S., MAYURASAKORN, K., O'HARE, J. P., ORCHARD, T. J., PORTA, M., REMA, M., ROY, M. S., SHARMA, T., SHAW, J., TAYLOR, H., TIELSCH, J. M., VARMA, R., WANG, J. J., WANG, N., WEST, S., XU, L., YASUDA, M., ZHANG, X., MITCHELL, P. & WONG, T. Y. 2012. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care,* 35**,** 556-64.

YEUNG, Y.-G. & STANLEY, E. R. 2010. Rapid Detergent Removal From Peptide Samples With Ethyl Acetate For Mass Spectrometry Analysis. *Current protocols in protein science / editorial board, John E. Coligan ... [et al.],* CHAPTER**,** Unit-16.12.

YU, Y., LENG, T., YUN, D., LIU, N., YAO, J., DAI, Y., YANG, P. & CHEN, X. 2010. Global analysis of the rat and human platelet proteome – the molecular blueprint for illustrating multi-functional platelets and cross-species function evolution. *PROTEOMICS,* 10**,** 2444-2457.

ZARINA, S., ZHAO, H. R. & ABRAHAM, E. 2000. Advanced glycation end products in human senile and diabetic cataractous lenses. *Mol Cell Biochem,* 210.

ZEILER, M., MOSER, M. & MANN, M. 2014. Copy number analysis of the murine platelet proteome spanning the complete abundance range. *Molecular & Cellular Proteomics,* 13**,** 3435-3445.

ZHANG, B., VERBERKMOES, N. C., LANGSTON, M. A., UBERBACHER, E., HETTICH, R. L. & SAMATOVA, N. F. 2006. Detecting differential and correlated protein expression in label-free shotgun proteomics. *J Proteome Res,* 5**,** 2909-18.

ZHAO, Z., LIU, J., SHI, B., HE, S., YAO, X. & WILLCOX, M. D. 2010. Advanced glycation end product (AGE) modified proteins in tears of diabetic patients. *Mol Vis,* 16**,** 1576-84.

ZHARIKOV, S. & SHIVA, S. 2013. Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease. *Biochem Soc Trans,* 41**,** 118-23.

ZHOU, X., HONG, G.-Y., HUANG, B.-B., DUAN, Y.-M., SHEN, J.-Y., NI, M.-W., CONG, W.-T. & JIN, L.-T. 2014. Improved conditions for periodate/Schiff's base-based fluorescent staining of glycoproteins with dansylhydrazine in SDS-PAGE. *ELECTROPHORESIS,* 35**,** 1439-1447.

ZHU, R., SONG, E., HUSSEIN, A., KOBEISSY, F. H. & MECHREF, Y. 2017. Glycoproteins Enrichment and LC-MS/MS Glycoproteomics in Central Nervous System Applications. *In:* KOBEISSY, F. H. & STEVENS, J. S. M. (eds.) *Neuroproteomics: Methods and Protocols.* New York, NY: Springer New York.

ZHU, W., SMITH, J. W. & HUANG, C.-M. 2010. Mass Spectrometry-Based Label-Free Quantitative Proteomics. *Journal of Biomedicine and Biotechnology,* 2010**,** 6.

ZIMMET, P. Z., MAGLIANO, D. J., HERMAN, W. H. & SHAW, J. E. 2014. Diabetes: a 21st century challenge. *The Lancet Diabetes & Endocrinology,* 2**,** 56-64.

Appendix

Ethical Approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences Research Ethics Committee

20/10/2016

Approval Certificate
New Application

Ethics Reference No.: 358/2016

Title: A comparative proteomic and glycoproteomic study of platelets from patients with diabetes and non-diabetic healthy individuals

Dear Miss Shamiso Mlambo

The **New Application** as supported by documents specified in your cover letter dated 11/10/2016 for your research received on the 12/10/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 19/10/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**358/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

Ethics Extension

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UNIVERSITY OF PRETORIA
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Faculty of Health Sciences Research Ethics Committee

29/03/2018

Miss Shamiso Mlambo
Department of Pharmacology
University of Pretoria

Dear Miss Shamiso Mlambo

RE.: 358/2016 ~ Letter dated 2 March 2018

358/2016	**Mlambo**
Protocol Title	A comparative proteomic and glycoproteomic study of platelets from diabetics and non-diabetics.
Principal Investigator	Miss Shamiso Mlambo, Tel: Email: Dept: Pharmacology

We hereby acknowledge receipt of the following document:

- Extension of study until the end of December 2018

which has been approved at 28 March 2018 meeting.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed; PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

☎ 012 356 3085 fhsethics.up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4-59, Gezina, Pretoria

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Faculty of Health Sciences Research Ethics Committee

29/03/2018

Miss Shamiso Mlambo
Department of Pharmacology
University of Pretoria

Dear Miss Shamiso Mlambo

RE.: 358/2016 ~ Letter dated 2 March 2018

358/2016 Mlambo	
Protocol Title	NEW TITLE: A comparative proteomic and glycoproteomic study of platelets from patients with diabetes and non-diabetic healthy individuals. dd 11 Oct 2016 OLD TITLE: A comparative proteomic and glycoproteomic study of platelets from diabetics and non-diabetics.
Principal Investigator	Miss Shamiso Mlambo, Tel: Email: Dept: Pharmacology

We hereby acknowledge receipt of the following document:

- Extension of study until the end of December 2018

which has been approved at 28 March 2018 meeting.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed; PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Information leaflet and informed consent form for blood donations from patients with diabetes and non-diabetic volunteers for research purposes.

Research title:

A comparative proteomic and glycoproteomic study of platelets from patients with diabetes and non-diabetic healthy individuals

Study investigator: Shamiso Mlambo

Organization: Pharmacology Department, University of Pretoria

Sponsor of study: University of Pretoria

You are invited to participate in a study where differences in blood cells are being investigated between diabetic patients and healthy volunteers. The data will be used for a Masters degree. If you are 25 years of age or older and fall into only ONE of the following categories:

- **You are healthy and not currently taking any chronic medications**
- **You are a diabetic patient with no other medical conditions unrelated to diabetes mellitus**

You will be asked to donate blood once so that the smallest cells in the blood (known as platelets) will be studied to try and find changes that could possibly be causing poor wound healing in diabetic patients.

Your personal information and identity will be kept private and confidential when you volunteer to take part in the study. You should not feel pressured in any way to participate in this study, and any information that you may need to make the decision to take part will be provided to you.

This document is an information leaflet which includes a consent form and you will get a copy for yourself. Please sign the form and give consent **only** once you feel that you are well informed about the study and are aware of the potential risks during collection of blood samples.

Study purpose (This is only applicable if you have Diabetes Mellitus)

Diabetes mellitus is a condition where the blood glucose levels are higher than normal and it has become increasingly common in society. It is associated with a number of health complications that include poor wound healing and the increasing risk of developing diabetic foot ulcers. These foot ulcers heal slowly and in most cases the patient will need medical help to avoid the wounds becoming a major health risk that could lead to foot or leg amputation.

Platelets are the smallest cells in the blood and these cells are very important in the wound healing process as they form a clot when injury has occurred or when a wound is formed to prevent blood loss and infection. These cells also produce components known as growth factors which regulate and ensure proper functioning of the healing process. Several studies have shown that platelet-based treatment can improve the healing of several types of injuries, which has provided motivation for a study to be conducted in the Department of Pharmacology of the University of Pretoria to test platelet treatment of diabetic foot wounds in the near future. However, as a first step to back up the need for these platelet-based healing studies, we want to do a comparison of platelet samples and find if there are any differences between the platelets of diabetic patients and non-diabetics that may be responsible for slow healing of wounds in diabetic patients.

Information gathered from this study would help to develop treatment that could improve healing of diabetic foot ulcers. Participation or not in this study will not compromise any treatment regimens that you may be undergoing and will not result in any changes to the treatment of any patients.

Procedure

You will be asked to fill in a questionnaire to see whether you qualify for the study or not. If you qualify for the study, a test known as the HbA1c test will be done to assess your long-term blood sugar levels, which will be used to screen you for the study. If you are diabetic, your patient file will also be accessed to see if you qualify for the study and if you have other conditions such as kidney disease that may influence reporting of results.

The WHO guidelines on drawing blood will be followed and before donation, you will be asked if you are not anaemic, as this will qualify you to take part in this study. In the event that you do not know whether you are anaemic or not, you will be tested by having your finger pricked and testing your blood droplet density. Blood samples will be drawn from 30 diabetic patients and 30 non-diabetic individuals. Two blood collection tubes of 10 ml each (about two teaspoons full in volume) will be used to draw blood from your arm and these tubes will labelled using a code to indicate diabetic status with no further information about you.

The blood will be drawn by a person trained to draw blood such as a doctor or nurse. A restriction cuff will be placed on your upper arm, the area cleaned with a sterilising solution tissue and a needle will be inserted into your vein near the inside of your elbow. Your blood will be drawn into the two blood tubes and this will take about 5 minutes.

The blood samples will only be used to perform the HbA1c test to confirm the diabetic status and to isolate platelets for protein characterisation analysis in this study. Any leftover blood will be discarded in the biohazard containers in the lab.

Risks

The needle prick may hurt a bit. There is a small risk of bruising; a rare risk of infection and you may feel lightheaded directly after donating the sample.

Benefits

There will not be direct benefits for you, although your participation will aid in the development of potential treatments for diabetic foot ulcers.

Payments

No payments will be made to you and participation is completely voluntary.

Withdrawal from participation

You have the right not to participate or to withdraw from blood donation at any time as it is voluntary to donate the blood.

Confidentiality

Your details will be kept private and confidential and samples will be referred to by means of coding letters that will indicate your gender and diabetic status.

Sharing of results

Results will be reported in the form of a dissertation and an article which will be published in a highly recognized journal. Complete anonymity of your details will be ensured.

Has the study received ethical approval?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human studies. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Who to contact

For any questions or queries the following people can be contacted:

Study investigator: Shamiso Mlambo
012 319 2558
mlamboshamiso@gmail.com

Supervisor of Study: Prof AD Cromarty
012 3192622
duncan.cromarty@up.ac.za

Co- supervisor: Chanelle Pillay
082 2235 827
chanellepillay@up.ac.za

Consent form

Declaration by participant:

By signing this form I agree to have read the information above or had the information read to me. I have understood everything and I had the opportunity to ask questions which were answered to my satisfaction. I consent that participation in blood donation is voluntary.

Participants name printed: _____

(Please print)

Participant's signature: _____

Date _____

Declaration by study investigator:

I confirm that the participant is participating voluntarily and was given the chance to ask questions which were answered correctly and to the best of my ability. A copy of this document has been made available to the participant.

Study investigator's name printed _____

(Please print)

Study investigator's signature _____

Date _____

VERBAL PATIENT INFORMED CONSENT

I, the undersigned, Ms ……..…., have read and have explained fully to the patient, named …….. and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

I hereby certify that the patient has agreed to participate in this study.

Patient's Name _____

(Please print)

Patient's Signature _____ ***Date*** _____

Investigator's Name _____

(Please print)

Investigator's Signature _____ ***Date*** _____

Witness's Name _____

Witness's Signature _____ ***Date*** _____

(Please print)

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Code:

PATIENT OR PARTICIPANT'S QUESTIONNAIRE

Researcher: Shamiso S mlambo

Student Number: 15258442

Department of Pharmacology

University of Pretoria

Dear Patient/Participant

Thank for your willingness to participate in this research study. Your participation will help in the development of potential therapies for foot wounds in diabetic patients. The aim of this study is to compare platelets from diabetic patients to those of healthy individuals to see if there any differences that may be contributing to a delay in the healing of these wounds. This questionnaire will be used to see if you qualify for this study. You will spend 5 minutes maximum to complete this questionnaire and your details will be kept private and confidential. Samples will be referred to by means of coding letters that will identify your gender and diabetic status. Please respond by placing the letter "X" in columns to the questions with a "Yes" or "No" answer.

The Research Ethics Committee of the University of Pretoria, Faculty of Health Sciences, telephone numbers 012 356 3084 / 012 356 3085 granted written approval for this study.

Your participation in this study is voluntary. You can refuse to participate or stop at any time without giving any reason. As you do not write your name on the questionnaire, you give us the information anonymously. Once you have given the questionnaire back to us, you cannot recall your consent. We will not be able to trace your information. Therefore, you will also not be identified as a participant in any publication that comes from this study.

Note: The implication of completing the questionnaire is that informed consent has been obtained from you. Thus any information derived from your form (which will be totally anonymous) may be used for e.g. publication, by the researchers.

We sincerely appreciate your help.

Yours truly,

Shamiso Mlambo

(Principal investigator)

Part 1	**Yes**	**No**
1. Are you diabetic (Type 2 diabetes mellitus)?		
2. If you are diabetic, do you have foot wounds as a result of the disease?		
3. If you are diabetic, are you on any other chronic medication besides diabetes mellitus treatment?		
4. Are you a healthy non-diabetic individual? (healthy means you are not on any chronic medication)		
5. Have you taken any medication in the last 10 days?		
Part 2		
6. If you answered "Yes" to **Questions 3 & 5** please specify the medication you have taken or taking		
7. Gender		
8. Age		
NB: The HbA1c value and additional comments from your patient file (if you are diabetic) can only be filled in by the principal investigator of the study after making sure you qualify for the study		
9. HbA1c value		
Comments:		

Patient Information List

Non – diabetic healthy individuals						**Diabetic patients**					
Patient code	Age	Gender	HbA1c value (%)	Medication taken	Presence of DFU (Yes/No)	Patient code	Age	Gender	HbA1c value (%)	Medication Taken	Presence of DFU (Yes/No)
MH6	24	Male	5.2	Diclofenac		MD23	24	Male	11.7	Humulin	Yes
MH25	23	Male	5.0	NIL	No	MD31	24	Male	18.2	Insulin, Metformin	Yes
MH14	26	Male	4.9	Panado	No	MD7	46	Male	9.2	Insulin, Metformin, Aspirin	Yes
MH9	26	Male	5.0	NIL	No	MD28	48	Male	5.9	NIL	No
FH12	32	Female	5.3	Mefloquine (Prophylaxis)	No	FD25	52	Female	11.3	Statin, Pioglitazone, Diazepam	Yes
FH16	22	Female	5.2	Antihistamine		FD30	34	Female	15.0	Losartan, Metformin	Yes
MH20	35	Male	4.9	Panado, Puricos	No	MD18	57	Male	5.8	Gabapentin, Ibuprofen,Metformin	Yes
MH27	40	Male	5.1	NIL	No	MD29	57	Male	9.8	Metformin, Spironolactone	Yes
MH8	60	Male	5.4	Valsartan		MD15	65	Male	14.2	Metformin	Yes
MH24	57	Male	5.8	Warfarin		MD26	65	Male	10.8	Warfarin,Pioglitazone	Yes
FH18	48	Female	5.3	NIL	No	FD20	51	Female	9.2	Pioglitazone, Captopril	Yes
FH23	34	Female	5.3	NIL	No	FD21	50	Female	7.1	Simvastatin, Metformin, Aspirin	Yes
FH2	39	Female	5.4	Paracetamol	No	FD17	66	Female	7.0	Enalapril,Simvastatin, Aspirin, Isosorbide, Metformin	Yes
FH5	40	Female	5.6	Myprodol	No	FD19	64	Female	5.9	Warfarin, Metformin	Yes
FH29	54	Female	5.3	NIL	No	FD1	65	Female	9.4	Enalapril, Amlodipine,Humulin	Yes
FH30	55	Female	5.3	NIL	No	FD16	70	Female	11.0	Metformin, Rivastigmine	Yes

FH3	47	Female	5.1	Panado	No	FD4	55	Female	8.2	Allopurinol, Enalapril, Methotrexate	Yes
FH15	67	Female	5.4	NIL	No	FD8	63	Female	6.9	Simvastatin, Aspirin, Enalapril	Yes
FH17	60	Female	5.7	NIL	No	FD2	61	Female	8.4	Metformin, Verapamil	Yes
FH31	60	Female	5.5	Statin		FD3	62	Female	6.8	Insulin, Metformin	Yes
MH13	29	Male	5.0	NIL	No	MD11	66	Male	7.2	Lorsatan, Furosemide, Statin, Metformin	No
MH19	46	Male	5.1	NIL	No	MD14	72	Male	7.2	Aspirin, Valsartan, Pioglitazone	Yes
FH22	52	Female	4.8	NIL	No	FD24	71	Female	12.4	Lorsatan, Pioglitazone	Yes
FH28	32	Female	5.4	NIL	No	FD27	77	Female	6.4	Statin, Aspirin, Metformin	Yes
MH1	62	Male	5.2	NIL	No	MD6	72	Male	6.5	Insulin, Warfarin	Yes
FH4	33	Female	5.1	Fluoxetine	No	FD9	51	Female	10.9	Enalapril, Pioglitazone	Yes
MH7	64	Male	5.0	NIL	No	MD10	76	Male	7.0	Valsartan,Aspirin	Yes
MH11	35	Male	5.2	Panado	No	MD13	79	Male	8.0	Metformin, Aspirin, Furosemide	Yes
MH10	30	Male	5.3	NIL	No	MD5	70	Male	8.4	Metformin, Aspirin	Yes
MH21	33	Male	5.7	NIL	No	MD22	61	Male	7.8	Pioglitazone, Valsartan	No
			Mean = 5.25						**Mean = 9.12**		