HbA1c as a screening tool for diabetes mellitus and its use with traditional and novel biochemical parameters to predict cardiovascular risk in a local urban community

By

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DECLARATIONS

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With regard to Chapters 3-6 on pages 47 - 135 in this dissertation, the nature and scope of my contribution were as follows:

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2. no other authors contributed to Chapters 3-6 besides those specified above, and
3. potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapters 3-6 of this dissertation.

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ABSTRACT

Introduction

The global obesity pandemic has reached Africa and the diabetes mellitus (DM) prevalence is increasing in parallel. A high prevalence of DM and risk for cardiovascular disease (CVD) has been described in the South African mixed ancestry population. Recent guidelines advocate using HbA1c as a diagnostic tool for DM and prediabetes, which is more convenient. However, various studies have challenged these cut-offs. There is a paucity of studies validating these cut-offs in Africa. As DM is considered a CVD risk equivalent, emerging markers of CVD and adiposity also need evaluation. The adipokine adiponectin has anti-diabetic, anti-atherogenic and anti-inflammatory properties and levels decrease in obesity. E-selectin, a marker of endothelial cell dysfunction, is associated with subclinical atherosclerosis and hyperglycaemia. Carotid intima-media thickness (CIMT) is a non-invasive measure of subclinical atherosclerosis. The aim of this investigation was to verify recommended HbA1c cut-offs to diagnose DM and prediabetes and to examine the usefulness of emerging markers of subclinical CVD in our population.

Methods

This investigation consists of four substudies and was performed on participants of the Bellville South Africa Study. In the first, we challenged the recommended HbA1c cut-off of 6.5% to diagnose DM in 946 participants using oral glucose tolerance test (OGTT), fasting blood glucose (FBG), and receiver operator characteristic (ROC) curves. In the second, we derived an optimal HbA1c cut-off to detect prediabetes in 667 participants and validated this in two populations, using OGTT and ROC curves. In the third, we determined high molecular weight (hmw)-adiponectin levels in 101 participants, compared these in participants with and without hyperglycaemia and investigated their relationship with two polymorphisms (rs17300539 and rs266729) reported to affect adiponectin values. In the fourth, we determined E-selectin levels in 307 participants, compared these in participants with and without hyperglycaemia and assessed their effect on CIMT.

Results

The recommended HbA1c cut-off was not sensitive enough to detect DM. Using FBG, 117 (14%) participants were diagnosed with DM and 50% had an HbA1c of ≥ 6.5%; using OGTT

147 (18%) had DM and 46% had an HbA1c of ≥ 6.5%. Comparing HbA1c to FBG and OGTT, a cut-off of 6.1% gave a better sensitivity and specificity (area under curve (AUC) 0.85 and 0.82 respectively). Also, the recommended HbA1c cut-off to detect prediabetes was not appropriate and we determined that 5.75% was best. However, the low sensitivity and specificity (64.8% and 60.4% respectively for the derivation and first validation sample and 59.6% and 69.8% for the second validation sample), confirmed that HbA1c alone would miss a significant number of prediabetics. Hmw-adiponectin levels were not affected by glycaemia (median 11.6 μg/mL in normoglycaemia vs. 10.5 μg/mL in hyperglycaemia; p=0.3060) nor by two common polymorphisms. Using robust correlations, a significant correlation was found between hmw-adiponectin and high density lipoprotein cholesterol (HDL-c) (r=0.45; 95%CI: 0.27-0.59), which was similar in both normo-and hyperglycaemia (p>0.99). This association was attenuated in robust linear regressions adjusted for gender and adiposity. E-selectin levels were significantly higher in hyperglycaemia (median 139.8 μg/L vs. 118.8 μg/L in normoglycaemia; p=0.0007) but not associated with CIMT. Significant correlations were found between E-selectin and age, markers of glycaemia and inflammation, central obesity and lipid variables. Associations remained significant only with age, hyperglycaemia and C-reactive protein (CRP) in multivariable robust linear regression models. In similar regressions models, age and gender were the main predictors of CIMT, which was not associated with E-selectin.

Conclusion

The international HbA1c cut-offs recommended to detect DM and prediabetes were not appropriate in our population. Though a cut-off of 6.5% to diagnose DM is a good diagnostic tool with high specificity, the low sensitivity limits its screening use. Similarly, recommended HbA1c values to detect prediabetes may underestimate the true numbers. This emphasizes the importance of local evidence-based values being established. Additionally, hmw-adiponectin was not affected by glycaemia or polymorphisms, but correlated significantly with HDL-c which may explain its beneficial cardiovascular effect. Though E-selectin was influenced by glycaemia, possibly reflecting early endothelial damage, it did not correlate with CIMT, which was determined by age and male gender.

OPSOMMING

Inleiding

Die globale obesiteits pandemie is ook in Afrika aanwesig, en daarmee saam is daar ‘n styging in die prevalensie van diabetes mellitus (DM). Daar is bevind dat die Suid Afrikaanse gemengde afkoms populasie ‘n hoë prevalensie van DM asook ‘n verhoogde risiko van kardiovaskulere siekte (KVS) het. Huidige riglyne beveel aan dat HbA1c gebruik word as ‘n diagnostiese toets vir DM en prediabetes, wat baie geriefliker is. Maar studies in verskillende populasiegroepe bevraagteken hierdie afsnypunte. Daar is ‘n gebrek aan validasie studies van hierdie afsnypunte vanuit Afrika. Aangesien DM beskou word as ‘n KVS risiko ekwivalent, behoort nuwe merkers van KVS en obesiteit in ons populasie ge-evalueer te word. Die adipokine adiponektien het anti-diabetiese, anti-aterogene en anti-inflammatoriese kenmerke en vlakke daal in obesiteit. E-selektien, ‘n merker van endoteelsel disfunksie, is geassosieer met subkliniese aterosklerose en hiperglisemie. Meting van die karotis intima-media dikte (KIMD) is ‘n nie-indringende metode om subkliniese aterosklerose te bepaal. Die doel van hierdie studie is om die afsnypunte van HbA1c om DM en prediabetes te diagnoseer te verifieer en om die nut van nuwer merkers van subkliniese KVS in ons populasie te evalueer.

Metodes

Hierdie ondersoek bestaan uit vier substudies en is uitgevoer op deelnemers van die Bellville-South Africa Projek. In die eerste studie, bevraagteken ons die HbA1c afsnypunt van 6.5% om DM te diagnoseer in 946 deelnemers deur middel van die orale glukose toleransie toets (OGTT) of ‘n vastende bloedglukose (VBG) te bepaal, en het die optimale HbA1c afsnypunt te bepaal met behulp van “receiver operator characteristic (ROC)” kurwes. In die tweede, het ons die optimale HbA1c afsnypunt om prediabetes te diagnoseer in 667 deelnemers bepaal, en hierdie waarde bevestig in twee daaropvolgende studiegroepe met die hulp van OGTT en ROC kurwes. In die derde, het ons hoë molekulere gewig (hmg)-adiponektien vlakke bepaal in 101 deelnemers, dit vergelyk dié met en sonder hiperglisemie en te ondersoek of hierdie vlakke beïnvloed word deur twee polimorfismes (rs17300539 en rs266729) wat beskryf is om adiponektien vlakke te beïnvloed. In die vierde het ons E-selektien vlakke op 307 deelnemers bepaal, die vlakke vergelyk in dié met en sonder hiperglisemie, en gekorreleer met KIMD.

Resultate

Die aanbevole HbA1c afsnypunt om DM te diagnoseer is nie sensitief genoeg is in ons bevolking nie. Deur net VBG te gebruik, is 117 (14%) met DM gediagnoseer en slegs 50% het ‘n HbA1c waarde van ≥ 6.5% gehad. Deur middel van OGTT is 147 (18%) met DM gediagnoseer en 46% het ‘n HbA1c waarde van ≥ 6.5% gehad. Deur HbA1c met VBG en OGTT te vergelyk, is daar bepaal dat ‘n afsnypunt van 6.1% ‘n beter sensitiwiteit en spesifisiteit gee (Area onder Kurwe (AOK) 0.85 en 0.82 respektiewelik). Ons het verder bevind dat die aanbevole HbA1c afsnypunt om prediabetes te diagnoseer nie toepaslik is nie en dat ‘n afsnypunt van 5.75% beter is. Maar die lae sensitiwiteit en spesifisiteit (64.8% en 60.4% onderskeidelik vir die afleiding en eerste bevestigings groep en 59.6% en 69.8% onderskeidelik vir die tweede bevestigingsgroep) het bewys dat HbA1c alleen ‘n beduidende hoeveelheid mense met prediabetes sou mis. Hmg-adiponektien vlakke was nie geaffekteer deur glisemie nie (mediaan 11.6 μg/mL in normoglisemie en 10.5 μg/mL in hiperglisemie; p=0.3060) en is ook nie geaffekteer deur twee algemene polimorfismes nie. Deur middel van robuuste korrelasies is ‘n beduidende korrelasie gevind tussen hmg-adiponektien en hoëdigtheidslipoproteïen cholesterol (HDL-c) (r=0.45; 95%CI: 0.27-0.59), wat soortgelyk was in die normo- en hiperglisemiese deelnemers (p>0.99). Hierdie assosiasie is betekenisvol verswak in robuuste liniêre regressive berekeninge wat gekorrigeer het vir geslag en obesiteit. E-selektien vlakke was betekenisvol hoër in hiperglisemie (mediaan 139.8 μg/L teenoor 118.8 μg/L in normoglisemie; p=0.0007) maar was nie geassosieerd met KIMD nie. Betekenisvolle korrelasies is gevind tusen E-selektien en ouderdom, merkers van glisemie en inflammasie, sentrale obesiteit en lipiedwaardes. Met meerveranderlike robuuste liniêre regressie modelle, het hierdie verhoudings betekenisvol gebly slegs met ouderdom, hiperglisemie en C-reaktiewe proteien (CRP). In soortgelyke regressie modelle, was ouderdom en geslag die hoof voorspellers van KIMD, wat nie geassossieer was met E-selektienvlakke nie.

Gevolgtrekking

Die internasionale aanbevole HbA1c afsnypunte om DM en prediabetes te diagnoseer is nie toepaslik in ons bevolking nie. Alhoewel die afsnypunt van 6.5% om DM te diagnoseer ‘n goeie diagnostiese metode is met ‘n hoë spesifisiteit, beperk die lae sensitiwiteit die siftings gebruik hiervan. Die aanbevole HbA1c afsnypunt om prediabetes te diagnoseer mag die toestand in ons bevolking onderdiagnoseer. Dit beklemtoon die belangrikheid dat

uitkomsgebaseerde afsnypunte vir Afrikabevolkings bepaal en bevestig moet word. Daarbenewens is gevind dat hmg-adiponektien nie geaffekteer is deur glisemie of polimorfismes nie, maar dat dit betekenisvol korreleer met HDL-c wat die voordelige kardiovaskulere effekte van hierdie merker mag verduidelik. Alhoewel die endoteeldisfunksie merker E-selektien beïnvloed was deur hiperglisemie, moontlik as gevolg van vroeë endoteelskade, het dit nie gekorreleer met KIMD nie. Laasgenoemde is wel beïnvloed deur ouderdom en manlike geslag.

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ORGANIZATION OF THE THESIS

This thesis is presented in the format of four articles. These have been published in peer-reviewed journals (Zemlin et al. 2011; Zemlin et al. 2015; A. E. Zemlin et al. 2016; Annalise E Zemlin et al. 2016).

Chapter 1 is a brief overview of the research topic and outline of the aims and hypotheses. Chapter 2 is the literature review which led to this project. Chapter 3 is the original research article published in 2010 examining the ideal HbA1c cut-off to diagnose diabetes in our population (Zemlin et al. 2011). Chapter 4 is the original research article where we determined and validated the ideal HbA1c cut-off to diagnose prediabetes in our population (Zemlin et al. 2015). These two studies are important, as the American Diabetes Association (ADA) in 2010 advocated the use of HbA1c as a diagnostic tool for diabetes. However it is important that these cut-offs be validated in different populations.

Chapter 5 examines high molecular weight adiponectin levels in our population and their association with cardio-metabolic traits in normo- and hyperglycaemic subjects and their correlation with two common polymorphisms known to influence adiponectin levels (Annalise E Zemlin et al. 2016). Chapter 6 examines levels of E-selectin in our population and correlating these with cardio-metabolic traits in normo- and hyperglycaemic subjects and with a known marker of atherosclerosis, the carotid intima-media thickness (CIMT) (A. E. Zemlin et al. 2016). Chapter 7 provides the conclusion derived using work presented in this thesis.

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LIST OF ABBREVIATIONS

AACC	American Association of Clinical Chemistry
ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADA	American Diabetes Association
ADAG	A1c-Derived Average Glucose
ADIPOQ	Adiponectin gene
AGE	Advanced glycation end-product
AMPK	Adenosine monophosphate kinase
ARIC	Atherosclerosis Risk in Communities
AUC	Area under curve
BAT	Brown adipose tissue
BMI	Body Mass Index
CAP	College of American Pathologists
CI	Confidence interval
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
EASD	European Association for the Study of Diabetes
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer
HbA1c	Glycated haemoglobin

HDL-c	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HPLC-MS	High performance liquid chromatography mass spectrometry
HPLC-CE	High performance liquid chromatography capillary electrophoresis
Hmw	High molecular weight
HOMA-IR	Homeostatic model assessment-insulin resistance
IDF	International Diabetes Federation
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL	Interleukin
LDL-c	Low density lipoprotein cholesterol
MS	Metabolic syndrome
MRC	Medical Research Council
NCD	Non-communicable disease
NGSP	National Glycohemoglobin Standardization Program
NF-kB	Nuclear factor-kappa B
NHI	National Health Insurance
npv	negative predictive value
NT-proBNP	N-terminal fragment of pro-brain natriuretic peptide
OGTT	Oral glucose tolerance test
PPAR-γ	Protein phosphorylation activator receptor-gamma
PHA	Paradoxically high adiponectin
ppv	positive predictive value
ROC	Receiver operator characteristic
SD	Standard deviation
SEMDSA	Society of Endocrinology, Metabolism and Diabetes of South Africa

SULF2	Sulfatase-2
TB	Tuberculosis
TNF	Tumour necrosis factor
UKPDS	United Kingdom Prospective Diabetes Study
UCP	Uncoupling protein
WAT	White adipose tissue
WHO	World Health Organization
WHR	Waist to hip ratio

CHAPTER 1

GENERAL INTRODUCTION

1.1 **INTRODUCTION**

From both a public and clinical perspective, there is an increasing need to detect people at risk for the future development of diabetes mellitus (DM), as this is a strong risk factor for cardiovascular disease (CVD), peripheral vascular disease and cerebrovascular disease. (Decode Study Group 2001; Khaw et al. 2004; Rizos & Mikhailidis 2000). Other complications of DM include retinopathy leading to loss of vision, nephropathy and eventual renal failure, neuropathy and the risk of foot ulcers and amputations (Alberti & Zimmet 1998). Until recently, health problems in sub-Saharan Africa have consisted mainly of Human Immunodeficiency Virus (HIV) infection and tuberculosis (TB). However, with Africa mirroring the global increases in obesity, the prevalence of non-communicable diseases (NCD), mainly DM and CVD are increasing (Kengne, June-Rose Mchiza, et al. 2013). In 2008, NCD, mainly CVD, contributed to 28% of deaths in Africa and this is expected to rise to 64% within the next two decades (Kengne, June-Rose Mchiza, et al. 2013). Some of the drivers of this increase in obesity and DM in Africa may be attributed to urbanization, an aging population and lifestyle habits such as sedentary lifestyle and poor diet (Kengne, Echouffo-Tcheugui, et al. 2013; Levitt 2008). As a low body mass index (BMI) amongst Africans is regarded with negativity due to its association with poverty and HIV infection, and increased weight is associated with health and affluence, this may contribute to the rising prevalence rates of obesity (Levitt 2008). According to the South African Medical Research Council (MRC), 61% of the South African population is overweight, obese or severely obese, and NCD is starting to overshadow HIV and TB as major health issues in South Africa (Baleta & Mitchell 2014). Additionally, HIV and its treatment may increase the chances of developing DM, which may in turn have increased susceptibility to TB and increase the already high prevalence of TB associated with HIV, due to its suppressive effect on the immune system (Baleta & Mitchell 2014; Peer et al. 2014).

According to the International Diabetes Federation (IDF), South Africa in 2014 had a national diabetes prevalence of 8.3% (Guariguata et al. 2014). Earlier studies may have underestimated the prevalence of DM as recent studies have shown an increasing prevalence of DM with 90% of cases being due to type 2 diabetes (Hall et al. 2011; Mbanya et al. 2014). For example, in a study published 1999, the prevalence of DM in the mixed ancestry population was reported to be 10.8 % (Levitt et al. 1999). However, this prevalence may have been underestimated since the 1985 World Health Organization (WHO) diagnostic criteria were used. Just thirteen years later, Erasmus, conducted a cross-sectional study on 642

participants and reported that according to the updated WHO criteria (Alberti & Zimmet 1998), the mixed ancestry population of Cape Town had a crude type 2 DM prevalence of 28.2% (age-adjusted 26.3%; 95% CI 22.0-30.3) (Erasmus et al. 2012). This included those previously diagnosed with type 2 DM. The prevalence of undiagnosed type 2 DM was reported as 18.1% (age-adjusted 16.8% (95% CI 13.3-20.4). This was a dramatic increase from the 10.8% described just more than a decade ago (Levitt et al. 1999). Thus early detection and community intervention with respect to behavioural lifestyle changes may have a significant impact in reversing this trend. Several studies have reported success in delaying the onset of DM by diagnosing the so-called prediabetic state which consists of either impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) and can precede the onset of type 2 DM by several years (Buysschaert et al. 2014; Buysschaert et al. 2011; Decode Study Group 2001). Prediabetes and DM are associated with CVD, with DM being a cardiovascular risk equivalent (Khaw et al. 2004; Schnell 2005; Schnell & Standl 2006). The early detection of these conditions will in turn allow for early and effective lifestyle interventions and treatment options to be implemented, leading to cost saving and decreased morbidity due to CVD. As a result, an ongoing mission exists to enhance screening tests for the early detection of DM and CVD. These include the establishment of HbA1c as an alternative marker for the diagnosis of DM and prediabetes and the investigation of inflammatory markers, CIMT and adipokines as potential early markers of CVD.

The discovery that adipose tissue is not just an inert depot but an active endocrine organ secreting adipokines has led to certain adipokines being described as risk factors for CVD and DM (Adamczak & Wiecek 2013; de Oliveira Leal & Mafra 2013; McGown et al. 2014). New potential biochemical markers of CVD include the adipokine, adiponectin, which has anti-inflammatory, anti-diabetic and anti-atherogenic properties and whose levels are decreased in obesity (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014; Rabin et al. 2005; Yadav et al. 2013). The underlying pathophysiology of CVD is atherosclerosis with associated endothelial dysfunction. Another potential CVD marker is E-selectin, which reflects endothelial dysfunction and has been shown to correlate with hyperglycaemia and cardiovascular risk including carotid-intima media thickness (CIMT), an early indicator of atherosclerosis (Beckman et al. 2002; Constans & Conri 2006; Hope & Meredith 2003a; Hope & Meredith 2003b; Miller & Cappuccio 2006).

1.2 STATEMENT OF THE PROBLEM

This rise in NCD will inevitably challenge our healthcare systems, already buckling under the burden of HIV and TB epidemics. For this reason, prevention and early diagnosis on NCD is essential. This has been prioritised and 93% of sub-Saharan African countries have a dedicated unit in the National Ministry of Health to tackle this issue (Kengne, June-Rose Mchiza, et al. 2013).The South African Government has recently launched a Strategic Plan for the Prevention and Control of Non-communicable Diseases 2013-2017 whose main aims are to decrease the risk factors and implement early detection and timely treatment (Baleta & Mitchell 2014). Additionally, the much anticipated National Health Insurance (NHI) will focus on NCD.

There are challenges with the detection of DM in Africa – specialised tests are often needed, with the need for skilled laboratory personnel. This is often not possible in an already understaffed and resource limited health care setting (Peer et al. 2014). Additionally, the costs of these tests may be prohibitive. However, studies still need to be performed to provide evidence as to whether new emerging markers are effective in our population as well. Even though the costs may be high, evidence is still lacking on our population. Therefore the aim of this study and other studies involving the Bellville South mixed ancestry cohort is to determine risk factors, early screening methods and potential preventative strategies for NCD caused by hyperglycaemia in an at risk population.

1.3 AIMS OF THE STUDY

Each aim of this study was written up as a manuscript submitted for peer-review publication. The first two aims are to assess the recommended HbA1c cut-offs to diagnose DM and prediabetes in our population, and the second two aims are to examine new novel markers of adiposity and endothelial dysfunction, even though the prohibitive costs of these tests may prevent their introduction into routine clinical use at the moment.

I. To determine the optimal HbA1c cut-off to diagnose DM in our population as compared to the cut-off recommended by the American Diabetes Association (ADA)

II. To determine the optimal HbA1c cut-off to diagnose prediabetes in our population as compared to the cut-off recommended by the American Diabetes Association (ADA)

III. To determine levels of high molecular weight (hmw) adiponectin in subjects with normo- and hyperglycaemia in our population and to correlate these levels with two common adiponectin polymorphisms known to affect adiponectin levels

IV. To determine E-selectin levels in subjects with normo-and hyperglycaemia in our population and to correlate these with carotid intima-media thickness and cardio-metabolic traits

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Until recently, the burden of disease in sub-Saharan Africa consisted mainly of infectious diseases such as Human Immunodeficiency Virus (HIV) infection and tuberculosis (TB). However, due to the global obesity pandemic reaching Africa with the adoption of unhealthy lifestyles and overeating, the prevalence of non -communicable diseases (NCD) is increasing. Diabetes Mellitus (DM) in particular and cardiovascular disease (CVD) are increasing at an alarming rate (Kengne et al. 2013). The prevalence of DM has been found to be high in the mixed ancestry population with a large number of people living with undiagnosed DM (Erasmus et al. 2012). This population group also has a high CVD risk profile according to the Framingham 30-year risk calculator (Matsha et al. 2012).

Recognition of prediabetes which is either impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (Buysschaert et al. 2011) is important, as not only does this precede type 2 DM, but both are associated with an increased risk of CVD (Decode Study Group 2001; Buysschaert et al. 2014). Until recently, HbA1c was only used for the glycaemic control and follow-up of diabetics and the diagnosis of DM relied on either an oral glucose tolerance test (OGTT) or fasting blood glucose. Recently the American Diabetes Association (ADA) advocated the use of HbA1c as a diagnostic tool for the detection of DM and prediabetes (American Diabetes Association 2010) which later was also adapted by the World Health Organization (World Health Organization 2011). However, as part of evidence-based practice, these advocated HbA1c cut-offs may not apply to all population groups and should be validated before being applied to clinical practice to ensure that no misdiagnosis is being made.

As DM is a CVD risk equivalent (Lorenzo et al. 2007) and poor glycaemic control is known to be associated with an increased risk of CVD (Khaw et al. 2004; Schnell & Standl 2006; Schnell 2005), the early detection of CVD is important in subjects with hyperglycaemia. Both DM and atherosclerosis, the underlying pathophysiological defect of CVD, are known to be associated with inflammation with an increase in inflammatory cytokines (Beckman et al. 2002; Constans & Conri 2006; Hope & Meredith 2003a; Lontchi-Yimagou et al. 2013; Leinonen et al. 2004; Porta et al. 2008; Wellen & Hotamisligil 2005; Monteiro & Azevedo 2010)

A surrogate marker for atherosclerosis is the measurement of the carotid intima-media thickness (CIMT) (De Groot et al. 2008). Additionally, in atherosclerosis, endothelial

activation stimulates the expression of adhesion molecules such as E-selectin which facilitate the tethering and rolling of leucocytes leading to their ultimate entry into the sub-endothelial space (Constans & Conri 2006; Hope & Meredith 2003a; Telen 2014). These leucocytes then engulf oxidised low density lipoprotein cholesterol (LDL-c) and lead to the formation of an atherosclerotic plaque. E-selectin levels have been found to increase in CVD and DM and correlate with severity of atherosclerosis (Baldassarre et al. 2009; Boulbou et al. 2004; Leinonen et al. 2003; Porta et al. 2008; Vaidya et al. 2011).

Adipose tissue is no longer considered to be just an inert storage area of excess triglycerides. Adipocytes, especially those in the visceral area, express numerous “adipokines” (de Oliveira Leal & Mafra 2013; McGown et al. 2014). The most abundant of these adipokines is adiponectin, whose secretion is suppressed in obesity by inflammatory cytokines and has anti-atherogenic, anti-inflammatory and anti-diabetic properties (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014; Yadav et al. 2013). Adiponectin levels have been found to correlate with insulin resistance and its gene is situated close to the diabetes susceptibility gene.

2.2 Diabetes mellitus

Diabetes mellitus (DM) is classified as either type 1 or type 2 (American Diabetes Association 2010). Type 1 DM was originally known as insulin dependent diabetes and is caused by pancreatic β-cell destruction which leads to an absolute insulin deficiency. Type 2 DM, previously known as non-insulin dependent diabetes is caused by progressive defective insulin secretion due to insulin resistance. A third type, gestational diabetes is defined as diabetes in pregnancy.

For the purpose of this study, we will be referring to type 2 DM.

2.3 Cardiovascular Disease

In 1948, the Framingham study was established to study the history, risk factors and prognosis of CVD (Dawber et al. 1951). Subsequent offspring were studied and the Third Generation cohort was recently described (Splansky et al. 2007). Risk factors for CVD include modifiable risk factors such as smoking, blood pressure, serum lipids, waist circumference and body mass index, nutrition, physical activity, socioeconomic status and alcohol intake and non-modifiable risk factors such as age, gender, family history, ethnicity and mental health. Additionally, certain related conditions such as DM, kidney failure,

familial hypercholesterolaemia and atrial fibrillation may also contribute to CVD risk (Anderson et al. 1991). Several tools are available to calculate CVD risk (Anderson et al. 1991; Conroy et al. 2003) of which the Framingham risk calculator (Pencina et al. 2009) has been found to perform best. This score predicts a 30 year risk of cardiovascular disease by examining age, gender, levels of cholesterol and high density lipoprotein, presence of DM and hypertension (Pencina et al. 2009).

2.4 HbA1c

2.4.1 History

Increases in glycated haemoglobin (HbA1c) in diabetes were first described in Tehran in 1969 by Rahbar et al (Rahbar 1968). A survey on haemoglobin electrophoresis patterns in 1200 patients described an abnormal fast moving haemoglobin fraction in two patients. Both of these subjects had DM. Rahbar et al then proceeded to examine other patients known with DM and found that they too exhibited this haemoglobin fraction. HbA1c is formed by the spontaneous nonenzymatic attachment, known as glycation, of glucose to the amino terminus of the β-chain of haemoglobin (Bunn et al. 1976; Koenig et al. 1977). Glucose binds nonenzymatically to haemoglobin to form a labile Schiff base, which subsequently undergoes Amadori rearrangement to form the characteristic stable ketoamine linkage (Gillery 2013). HbA1c levels are indicative of glucose control for about approximately the last 120 days – the time period that corresponds to the average lifespan of normal red blood cells. Any abnormality of red blood cell survival or abnormal haemoglobins will obviously affect HbA1c levels and lead to false interpretation. HbA1c measurement may also be unreliable in renal failure, where the urea by-product, isocyanic acid, binds to haemoglobin to form carbamylated haemoglobin and a falsely raised value. Additionally, glycation rates may differ in individuals, the so-called “glycation gap” (Gillery 2013).

Since these early results were published, HbA1c has been widely used in DM care as a reliable marker of long-term glycaemic control. However until recently, due to poor assay performance and lack of standardization, HbA1c was not recommended as a diagnostic tool. Expected levels of between 4-6% have been proposed in non-diabetics and the ADA recommends levels of 7% or less for adequate glycaemic control in diabetics (American Diabetes Association 2010).

2.4.2 HbA1c and diabetic complications and CVD

Two landmark trials have shown a linear correlation between average HbA1c levels and the risk of diabetic microvascular complications:

The well-known Diabetes Control and Complication (DCCT) trial (DCCT 2005) was a multicentre trial conducted on 1441 type 1 diabetics from 1983 to 1989. Participants were followed up in 1993 after a mean of 6.5 years. It was found that the relative risk of microvascular complications such as retinopathy or nephropathy decreased by 39% for each 10% relative decrease in HbA1c levels. In the long-term, the risk of CVD was decreased by 41%. However, due to the young age of the participants, the effects on CVD risk were not significant. It was concluded that intensive glycaemic control with insulin in type 1 DM slowed the onset and progression of microvascular complications with hypoglycaemia being a potential side-effect.

The United Kingdom Prospective Diabetes Study (UKPDS) (UK Prospective diabetes study (UKPDS) group 1998) was commenced in 1977 and the results were reported in 1998. The investigators demonstrated that intensive glycaemic control with either insulin or sulphonylureas in newly diagnosed type 2 diabetics with a median age of 54 years, as measured by lower HbA1c levels, reduced clinical outcomes. The aim of intensive treatment was to maintain fasting blood glucose levels of less than 6 mmol/L. It was found that a relatively small change in HbA1c level had a significant impact on the rate of microvascular complications. They also described an increased incidence of hypoglycaemic episodes in the intensive treatment group with no significant decrease in macrovascular complications.

A smaller study from Japan, the 8 year prospective Kumamoto Trial (Shichiri et al. 2000) examined whether intensive glycaemic control in diabetics could decrease the frequency of microvascular complications. A total of 110 type 2 diabetics (half without and half with retinopathy) were assigned to receive either conventional or intensive treatment with insulin. Intensive control with HbA1c levels of less than 6.5% decreased the occurrence of both retinopathy and nephropathy.

Numerous other trials have reported an increased risk of CVD with poor glycaemic control. The Action to control Cardiovascular Risk in Diabetes (ACCORD) (ACCORD 2007) was a large randomized, double-blind study involving 10251 participants with type 2 DM and increased risk of CVD. Patients with type 2 diabetes were found to die of CVD at rates two to

four times higher than those without. Additionally, for every 1% increase in HbA1c, the risk of CVD increased by about 18%.

The European Prospective Investigation into Cancer (EPIC)-Norfolk study was a large prospective population study in Norfolk, United Kingdom to investigate the relationship between HbA1c and CVD (Khaw et al. 2004). In this study, a 1% increase in HbA1c was associated with a 21% increase in CVD risk. Higher HbA1c levels were also associated with increased all-cause mortality. Additionally, HbA1c levels were found to be elevated well in advance of the clinical development of type 2 DM.

Since then numerous publications have highlighted HbA1c's association with CVD and mortality. O'Sullivan studied an older group of patients undergoing vascular surgery with and without DM. They described that suboptimal HbA1c had significant prognostic implications irrespective of whether the patient was diabetic or not (O'Sullivan et al. 2006). A meta-analysis by Selvin et al studying the relationship between HbA1c and CVD in DM found that chronic hyperglycaemia is associated with an increased risk for CVD in persons with DM (Selvin et al. 2004). This association has been extended to nondiabetic patients, as the relationship of CVD with glycaemia is believed to be a continuum without a threshold effect (Khaw et al. 2004; Selvin et al. 2004). A study on the original cohort of the Framingham Heart Study suggested that HbA1c adequately reflects the glucose status even in nondiabetics (Meigs et al. 1996).

2.4.3 HbA1c standardization

The landmark DCCT trail used a high performance liquid chromatography (HPLC) method in one central laboratory to determine HbA1c. However, when the trial was published in 1993 (DCCT 2005), the state of HbA1c assays was in disarray. There were multiple methods available, there was no standardization of the assay, or efficient quality control programs such that results were not comparable between laboratories and methods. Additionally, many methods were nonspecific. As a result of the important study findings linking HbA1c to outcomes, separate standardization programs for HbA1c were implemented in Japan, Sweden, USA (Hoelzel et al. 2004). The National Glycohemoglobin Standardization Program (NGSP) was founded in 1996 to implement the protocol developed by the American Association of Clinical Chemistry (AACC) subcommittee (www.ngsp.org.). Their goal was to harmonize glycated haemoglobin results to the values reported by the DCCT and UKPDS trials (DCCT 2005; UK Prospective diabetes study (UKPDS) group 1998). The NGSP

introduced a proficiency program and a College of American Pathologists (CAP) survey in 2007 found that 99% of laboratories were using NGSP-certified methods to determine HbA1c (Little & Rohlfing 2013). This harmonization to the DCCT trial method reduced interlaboratory variation in HbA1c results from >12% to <5% (Figure 2.1).

However, the method was not truly standardized, as a true reference method had not been established and no true reference material had been isolated. In 1994, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established a working group with the aim of standardizing HbA1c measurement (Finke et al. 1998). Their primary aim was to have one worldwide reference system and to develop an Internal Reference Method and purified HbA1c standards and calibrators. Two reference methods namely high performance liquid chromatography mass spectrometry (HPLC-MS) or high performance liquid chromatography capillary electrophoresis (HPLC-CE) were developed (Jeppsson et al. 2002). Using these methods, HbA1c was defined and purified calibrators prepared.

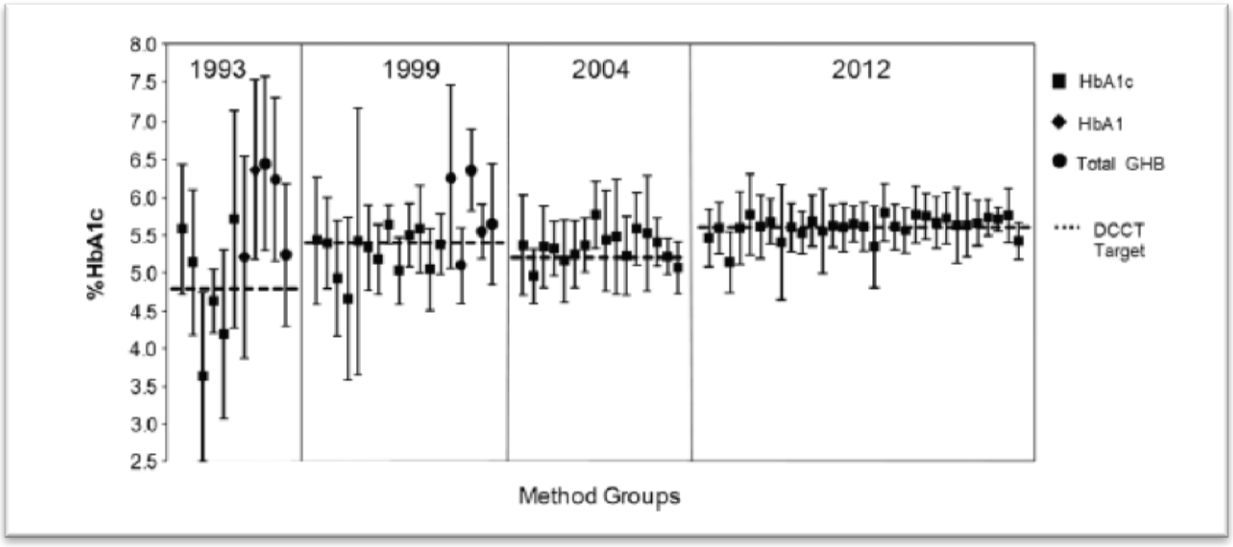

Figure 2.1: Improvement in coefficient of variation (CV) for HbA1c method over the years following DCCT harmonization and standardization (with permission Little & Rohlfing 2013)

A meeting was held in 2004 between the International Diabetes Federation (IDF), European Association for the Study of Diabetes (EASD) and ADA with the IFCC working group and it was recommended that the new IFCC reference method be implemented (ADA Report 2004). However, as HbA1c levels measured by the standardized IFCC method were 1.5-2% lower than those measured by NGSP harmonized methods, there was the potential to confuse

clinicians and patients, which could have a detrimental effect on glycaemic control. In 2007 a consensus statement was issued recommending that results be reported in SI units (mmol HbA1c/mol total Hb) (Mosca et al. 2007). Results of the A1c-Derived Average Glucose (ADAG) study in 2008 reported on the feasibility of expressing HbA1c as mean blood glucose (Nathan et al. 2008) and concluded that HbA1c can be expressed as estimated blood glucose for most patients with type 1 or type 2 diabetes. However, some authors have argued against this stating that it is not possible to have a single equation to convert HbA1c to estimated glucose (eBG) levels as the rate of haemoglobin glycation is influenced by environmental and genetic parameters as well as red blood cell factors. Additionally, the ADAG study was a relatively small study performed on mainly Caucasians (Young IS 2010). Despite these conflicting reports, some countries such as the United States report eBG values when sending out HbA1c results.

The decision to choose the format of reporting has been left to each country and in South Africa both the National Health Laboratory Service (NHLS) and private pathology laboratories in South Africa report HbA1c in both units and eBG results to clinicians.

2.4.4 HbA1c as a diagnostic tool

Until recently, the diagnosis of DM relied on either a fasting blood glucose level ≥ 7 mmol/L with symptoms of DM or results indicative of DM following a standard 75g OGTT. However, for both these tests the patient is required to fast and the OGTT is also cumbersome and uncomfortable for the patient.

As mentioned earlier, numerous studies have found an association between microvascular complications such as retinopathy a common complication of DM and HbA1c. A study by Cheng et al published in 2009 found that HbA1c was a better predictor of retinopathy than fasting blood glucose and that HbA1c levels ≥ 6.5% caused a steep increase in retinopathy prevalence (Cheng et al. 2009).

As HbA1c methods were previously not well harmonized with large variability between methods and laboratories, HbA1c could not be considered as a diagnostic tool. However, improvements in the standardization and methods for HbA1c determination led to the ADA in 2009, reporting a strong correlation between HbA1c and retinopathy (International Expert Committee 2009). They recommended that an HbA1c cut-off of 6.5% could be used to

diagnose DM. This is the cut-off to detect the least moderate retinopathy. A level of 6.0-6.4% was recommended to detect prediabetes.

This was positively welcomed, as it could potentially eliminate the need for patients to be fasting or have timed blood samples taken to diagnose DM. Another advantage was that HbA1c assays were now standardized and aligned to DCCT/UKPDS while the glucose assay was less well standardised. Additionally, HbA1c gives a better index of overall glycaemic exposure and is relatively unaffected by acute events. The blood sample for HbA1c does not have the same pre-analytical instability that glucose samples have. Also, the biological variability of HbA1c is less than that of glucose (International Expert Committee 2009)

However there are also some disadvantages that need to be considered (David B Sacks et al. 2011). The HbA1c test is more expensive than a glucose measurement but not more expensive than performing an OGTT (ZAR27 for a glucose test and ZAR54 for OGTT versus ZAR77 for HbA1c). Haemoglobinopathies and states of altered red blood cell turnover, such as haemolytic anaemia, transfusions, malaria and iron deficiency anaemia may affect HbA1c levels. This is especially important in Africa where these conditions are more common. Some studies found higher HbA1c levels in African Americans (racial differences) and some individuals have different rates of glycation – the so-called slow or rapid glycators (David B. Sacks et al. 2011). HbA1c measurement may also be unreliable in renal failure, where the carbamylated haemoglobin causes a falsely raised value. Additionally, glycation rates may differ in individuals, the so-called “glycation gap” (Gillery 2013). Besides the glucose concentration, other unknown factors are thought to play a role in the glycation of haemoglobin. Fructosamine 3-kinase is a deglycation enzyme and genetic variations of this enzyme may play a role and are being investigated. Although Delpierre et al (Delpierre et al. 2006) found that fructosamine 3-kinase levels do not affect HbA1c levels, this was a small study on 57 subjects and further larger studies on other populations have been suggested.

As a result of ethnic and other differences in HbA1c levels (Herman et al. 2009; Herman et al. 2007; Mosca et al. 2013; Tsugawa et al. 2012; Ziemer et al. 2010), it is recommended that these cut-offs should be tested in various population groups. As Africa has a high incidence of malaria and iron deficiency which affect red blood cell turnover, this is especially important in our setting. Additionally, certain areas of Africa have a higher incidence of haemoglobinopathies (Mbanya et al. 2014). Recent Society of Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA) guidelines have adopted the use of HbA1c ≥ 6.5%

to diagnose DM, but not to detect prediabetes (SEMDSA Guidelines 2012). To our knowledge, the ADA recommended HbA1c cut-offs to detect DM and prediabetes have not yet been assessed in our mixed ancestry population.

2.5 Diabetes mellitus and 30 year CVD risk in our population

Non-communicable diseases (NCDs) are increasing in South Africa and will soon be more of a health burden than infectious diseases such as HIV and TB. Early detection with preventative interventions will thus be important to prevent future morbidity and thereby curb healthcare costs.

Studies on our cohort have established that the mixed ancestry population of South Africa is a high risk population. Erasmus et al studied 642 subjects from Bellville South study and found an exceptionally high crude type 2 DM prevalence of 28.2% (age-adjusted 26.3%; 95% CI 22.0-30.3). However this included those previously diagnosed with type 2 DM. The prevalence of undiagnosed type 2 DM was reported as 18.1% (age-adjusted 16.8% (95% CI 13.3-20.4). The prevalence of prediabetes was 4.4% (age-adjusted 3.2%, 95% CI 1.6-4.9) for impaired fasting glucose (IFG) and 15.3% (age-adjusted 15.0%, 95% CI 11.4-18.6) for impaired glucose tolerance (IGT) (Erasmus et al. 2012). This was a dramatic increase from the prevalence for type 2 DM of 10.8% reported a decade ago by Levitt et al on a similar population in Mamre, Western Cape (Levitt et al. 1999). However, the following may have contributed to the different prevalences found: Different diagnostic cut-offs were used for these studies, namely the updated WHO criteria for Erasmus' study, which are stricter and may have led to this increased diabetes prevalence. Also, the subjects in Levitt's study were much younger – they started at 15 years and most were less than 45 years old. Erasmus' study was mainly on older participants with a mean age of 50.9 years. Lastly, even though Mamre was described as an urban population, until recently it was described as a rural population. As the prevalence of diabetes increases with urbanization, this may also have contributed to the higher prevalence in Erasmus' study. A further study on the Bellville South cohort by Matsha et al established that using the 30-year Framingham risk calculator for CVD in 583 subjects, there was a higher risk in hyperglycaemic subjects, with younger and normoglycaemic subjects also being at an increased risk (Matsha et al. 2012).

These studies have public health implications, as our mixed ancestry population is at a high risk for NCD which will impact negatively on the disease burden of the Western Cape. Therapeutic lifestyle interventions and effective primary care screening should be

implemented to attempt to curb these conditions and to ease the burden on our already strained, resource-limited healthcare system.

2.6 Obesity and adipose tissue

According to the South African Medical Research Council (MRC), 61% of the South African population is overweight, obese or severely obese (Baleta & Mitchell 2014) and this has been attributed for the increase in NCDs in South Africa. A sedentary lifestyle and intake of calorie-dense food has led to a drastic increase in obesity globally. With this increase in obesity, there has been a concomitant increase in obesity-related diseases, namely type 2 DM, CVD, insulin resistance, dyslipidaemia, hypertension, non-alcoholic fatty liver disease, chronic kidney disease and certain cancers (Harms & Seale 2013). Obesity influences metabolic and hormonal responses and visceral adipose tissue has been described to be a functional endocrine organ secreting many “adipokines” (McGown et al. 2014). Adipose tissue is no longer considered just a storage depot for excess fatty acids, but is now known to have distinct endocrine, paracrine and autocrine functions (Adamczak & Wiecek 2013) as shown in Table 2.1. Visceral adipose tissue is more highly active metabolically and is known to be an independent predictor of insulin sensitivity, IGT, high blood pressure and dyslipidaemia and it also plays a central role in the metabolic syndrome (Monteiro & Azevedo 2010).

Table 2.1: Physiological functions affected by adipose tissue - adapted by author from (Adamczak & Wiecek 2013)

Physiological functions affected by adipose tissue
Lipid metabolism and energy storage
Energy homeostasis and metabolism
Bone metabolism
Steroid hormone conversion
Coagulation and fibrinolysis
Vasoconstriction and vasorelaxation
Sexual maturation
Kidney function
Angiogenesis
Modulation of immune system
Haematopoiesis

There are two types of adipose tissue, namely brown adipose tissue (BAT) and white adipose tissue (WAT) (Harms & Seale 2013). The former is metabolically active, is a key site of heat production or thermogenesis, has numerous mitochondria and is associated with non-shivering thermogenesis (Harms & Seale 2013). Higher levels of BAT are found in infants and small animals which hibernate in winter months. Thermogenesis is possible due to an increased amount of mitochondria in these cells which contain uncoupling protein-1 (UCP-1). WAT lacks the energy-burning ability of brown adipose tissue, stores lipids and produces adipokines. Recently a third type of adipocyte, the so-called “beige” or “brite” adipose tissue has been described – these develop in WAT due to certain activators and may be a potential target for future weight-loss therapies (Harms & Seale 2013). Due to an increased amount of mitochondria, these cells are capable of thermogenesis; albeit to a lesser extent than BAT.

Obesity is associated with chronic inflammation and adipose tissue contains other cell types such as macrophages, monocytes and fibroblasts and releases pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6 (McGown et al. 2014). These inflammatory cytokines influence the expression of cytokines in obese states and contribute to the inflammation associated with obesity (de Oliveira Leal & Mafra 2013).

Leptin was the first adipokine to be described as being released from WAT in 1994 (Zhang et al. 1994), followed by adiponectin in 1995 (Scherer et al. 1995). Following these discoveries, WAT was recognised to be an active endocrine organ secreting a large number of active adipokines. Since then numerous more adipokines are being continually discovered which contribute to the metabolic effects of obesity (de Oliveira Leal & Mafra 2013).

2.7 Adiponectin

2.7.1 Introduction

Adiponectin was described in 1995 and is a 244 amino acid protein secreted by adipocytes, is the most abundant adipokine and levels are decreased in obesity due to the inhibitory effect of increased TNF-α and IL-6 secreted in obesity (Lee & Kwak 2014; Gable et al. 2006; Litvinova et al. 2014; Rabin et al. 2005). Adiponectin mRNA is less in visceral than in subcutaneous WAT (de Oliveira Leal & Mafra 2013). Therefore increased visceral adiposity (central obesity) is associated with decreased adiponectin levels (Monteiro & Azevedo 2010). Adiponectin is the so-called "good adipokine" - high levels are advantageous, due to its anti-inflammatory, anti-atherogenic and ant-diabetic effects (Balsan et al. 2015; Yadav et al. 2013). It contributes to the overall relationship between obesity, insulin resistance and CVD (Packard & Libby 2008) and levels are inversely associated with obesity, DM and cardiovascular risk (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014).

2.7.2 Actions of adiponectin

Adiponectin levels correlate with waist circumference and are decreased by visceral adiposity (Litvinova et al. 2014; Matsuzawa 2006; Moriyama et al. 2014; Suriyaprom et al. 2014). Higher levels have been described in premenopausal females (Gable et al. 2006), and levels also tend to increase with age (Kruger et al. 2011).

Adiponectin activates AMP protein kinase (AMPK) in the liver and skeletal muscle and thereby increases insulin sensitivity (de Oliveira Leal & Mafra 2013). Adiponectin also decreases the expression of adhesion molecules and the transformation of macrophages into foam cells, thereby decreasing atherosclerosis (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014). Its ant-inflammatory effects have been found to be due to its ability to inhibit the TNFα-induced nuclear factor-kappa B (NF-κB) activation (de Oliveira Leal & Mafra 2013). Adiponectin also activates protein phosphorylation activator receptor gamma (PPAR-

γ) leading to decreased triglyceride levels and increased insulin sensitivity (Gable et al. 2006).

Additionally, adiponectin also has been found to have an influence on high density lipoprotein cholesterol (HDL-c) levels through its effect on the reverse cholesterol transport system (Suriyaprom et al. 2014; Van Linthout et al. 2010; Yadav et al. 2013). Decreased adiponectin levels are associated with decreased HDL-c levels due to inhibition of aopA1 synthesis (Oku et al. 2007). Similarly, a study by von Linthout et al found that mice where HDL-c synthesis was stimulated with apoA1 transfer also demonstrated increased adiponectin synthesis (Van Linthout et al. 2010). Adiponectin has been found to account for 10% of the variation in HDL-c levels (Ferris et al. 2005). This association between adiponectin and HDL-c has been described to be due to hmw-adiponectin (Christou et al. 2012; Moriyama et al. 2014) (see next paragraph).

Another beneficial effect may be due to changes in the metabolism of postprandial lipoprotein remnants. In 1979, Zilversmit hypothesized that postprandial chylomicron remnants as well as oxidized LDL-c played a role in atherogenesis (Zilversmit 1979). However, recently proof of a causal role of postprandial lipids has been questioned (Katan 2004). Hyperglycaemic states such as DM have been found to have a “persistence” of postprandial apo-B lipoprotein remnants in their plasma after meals (Ramasamy 2016). Work by Chen et al found that type 2 DM impaired the clearance of these remnants by inducing the expression of sulfatase-2 (SULF2), a factor which suppresses the uptake of remnant lipoproteins (Chen et al. 2010). This increase in SULF2 can be mediated by advanced glycation end-products (AGEs). Adiponectin may have a beneficial effect on this postprandial remnant path by inhibiting the expression of SUF2 and thereby facilitating the clearance of postprandial remnants (Chen et al. 2010).

Table 2.2: The effects of adiponectin - adapted by author from (Adamczak & Wiecek 2013)

Insulin-sensitizing effects	Anti-atherogenic effects	Anti-inflammatory effects
Stimulation of glucose utilization in skeletal muscle and liver Stimulation of fatty acid oxidation in skeletal muscle and liver Enhanced insulin signalling in skeletal muscle Increased glucose uptake in skeletal muscle Suppression of gluconeogenesis in liver	Decreased adhesion molecules Increased nitric oxide production Decreased transformation of macrophages into foam cells Down-regulation of scavenger LDL-c receptors Stabilization of atherosclerotic plaque Attenuation of thrombus formation and platelet aggregation	Suppression of TNFα and IL-6 production by macrophages Production of anti-inflammatory cytokine IL-10 production by macrophages Suppression of superoxide production

2.7.3 Molecular forms of adiponectin

Adiponectin is secreted in low (trimer), medium (hexamer) and high molecular weight (hmw) (oligomer) forms (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014). The hmw form has been described to be the active form, with increased binding to AMP-kinase. It also has increased affinity to collagen and is thus able to bind exposed collagen in damaged vasculature, decrease apoptosis of endothelial cells and increase reverse cholesterol transport due to its effect on HDL-c amongst some of its functions (Suriyaprom et al. 2014; Yadav et al. 2013). Higher levels of low molecular weight adiponectin have been described in males (Rabin et al. 2005). Yet few studies have examined this fraction due to the fact that until recently there was no reliable assay available to determine this fraction (Ebinuma et al. 2006).

2.7.4 Genetics of adiponectin

The adiponectin gene (ADIPOQ) is located on chromosome 3q27, a susceptibility locus for type 2 DM and the metabolic syndrome and its components (Gable et al. 2006; Li et al. 2014; Yadav et al. 2013). It is believed that between 30-70% of the variability in adiponectin levels

may be influenced by genetic factors (Comuzzie et al. 2001; Rabin et al. 2005; Suriyaprom et al. 2014). Numerous functional single nucleotide polymorphisms (SNPs) and missense mutations have been identified in various population groups which may affect adiponectin levels and influence risk of DM and CVD (Vasseur et al. 2006). Two that may be associated with risk of DM are rs17300539 (11391 G>A) and rs266729 (11377 C>G) (Han et al. 2011). Both these SNPs are in the promoter region of the adiponectin gene and affect adiponectin levels (Karmelić et al. 2012). The polymorphism rs266729 decreases adiponectin levels and is associated with an increased risk of type 2 DM and the rs17300539 increases adiponectin levels. Ethnic differences in adiponectin levels have been described with Blacks having lower adiponectin levels and increased insulin resistance (Ferris et al. 2005). A possible mechanism by which mutations affect adiponectin action may be by impairing the assembly of adiponectin multimers. Waki et al describes how certain genetic mutations impair the formation of adiponectin multimers and therefore the metabolically active hmw form (Waki et al. 2003).

However, these findings vary in different studies and may be due to differences in age, genetic and ethnic backgrounds of the various study populations. A large mendelian randomization study by Yaghootkar et al indicated no causal association of alleles related to hypoadiponectinaemia with diabetic or related states (Yaghootkar et al. 2013).

2.7.5 Studies of adiponectin in DM and CVD

Several studies have described an association between low adiponectin levels and DM. A meta-analysis of thirteen studies by Li et al found that high adiponectin levels were associated with a decreased risk of type 2 DM in various races. This association was found irrespective of the assay used to determine adiponectin (Li et al. 2009). A study on the KORA (Cooperative Health Research in Region of Augsburg) Study and Framingham Offspring cohorts described the association between decreased adiponectin levels and increased risk of type 2 DM (Hivert et al. 2011). Several studies have specifically examined this risk with the hmw-adiponectin fraction and found that decreases in this fraction increased the risk of insulin resistance and type 2 DM (Aso et al. 2006; Hara et al. 2006; Nakashima et al. 2006; Seino et al. 2007). Heideman described how this association between hmw-adiponectin and glycaemia was even present in nondiabetics (Heidemann et al. 2008).

Several studies have also described a higher incidence of CVD associated with decreased adiponectin levels. Decreased adiponectin levels have been found to be associated with traits

of the metabolic syndrome (Ding et al. 2015; Hata et al. 2015; Ryo et al. 2004). This association between decreased adiponectin levels and CVD has been described to be stronger in younger females (Pischon et al. 2011). However, Sattar et al found no association between hmw-adiponectin and CVD in older women (Sattar et al. 2008).

Paradoxically, some studies have also described a higher mortality with higher adiponectin levels (Alehagen et al. 2015; Choi et al. 2015; Hascoet et al. 2013; Kizer 2014; Lee et al. 2013; Wannamethee et al. 2007; Witberg et al. 2016; Wu et al. 2014). Kruger et al found that adiponectin levels increased in age in White and African males from South Africa. Although this age-related increase in adiponectin with age had previously been attributed to decreasing renal function and hence clearance of adiponectin, this study found that this may not be the case in African males. (Kruger et al. 2011). Ferris et al studied three population in South Africa (Whites, Africans and Indians) and found that the relationship between adiponectin and insulin resistance varied across ethnic groups (Ferris et al. 2005).

Although the beneficial effects of adiponectin were well-described, some studies found an increased mortality with higher adiponectin levels, especially in the elderly and those with pre-existing CVD (Alehagen et al. 2015; Choi et al. 2015; Hascoet et al. 2013; Kizer 2014; Lee et al. 2013; Wannamethee et al. 2007; Witberg et al. 2016; Wu et al. 2014). This led to the speculation that adiponectin may be protective in low risk subjects but is actually leads to adverse effects in a high risk population (Wannamethee 2008). A prospective study on 4046 elderly men aged 60-79 years by Wannamethee et al found that a higher adiponectin level was associated with an increased incidence of CVD and all-cause mortality (Wannamethee et al. 2007). In a prospective study on 2879 elderly men in 2010, Wannamethee described that increased levels of N-terminal fragment of pro-brain natriuretic peptide (NT-proBNP) may explain this increased adiponectin found in CVD (Wannamethee et al. 2011). In 2013, they found that adiponectin was not associated with an increased risk of stroke in this same population (Wannamethee et al. 2013). The reason for this paradoxical effect of adiponectin is still unknown, but adiponectin resistance at receptor level, increased adiponectin levels in response to raised NT-proBNP, and an increase in pro-inflammatory cytokines have been postulated (Choi et al. 2015; Kizer 2014; Lee et al. 2013; Wannamethee et al. 2011; Witberg et al. 2016). It has also suggested that this increase may be due to renal impairment.

Doumetry described the concept of paradoxical hyperadiponectinaemia associated with the so-called metabolically healthy obese phenotype in African Americans (Ayo P Doumatey et

al. 2012). They described how obesity is not always associated with a decreased level of adiponectin.

The inconsistencies in these studies may be due to different assays used, renal status of subjects, molecular forms of adiponectin studied and the influence of polymorphisms of the adiponectin gene (A P Doumatey et al. 2012; Ferris et al. 2005; Sobngwi et al. 2007; Obot et al. 2013). Due to the beneficial effect of adiponectin found in the majority of studies, adiponectin has been identified as a potential therapeutic target to lower the risk of type 2 DM and CVD. There is a paucity of studies on adiponectin, specifically hmw-adiponectin from South Africa (Dessein et al. 2013; Omar et al. 2014). However, to our knowledge adiponectin levels in the mixed ancestry population has not yet been studied.

2.8 Atherosclerosis, Inflammation and CVD and DM

2.8.1. Inflammation in DM and CVD

The recent worldwide obesity epidemic has seen a dramatic increase in the global prevalence of DM which is expected to increase over the next two decades (Mena et al. 2014). Obesity and type 2 DM are both inflammatory disorders (Wellen & Hotamisligil 2005; Hotamisligil 2006; Monteiro & Azevedo 2010; Lontchi-Yimagou et al. 2013). DM is associated with an increased risk of CVD even with intensive glycaemic control and the common thread may be the underlying inflammation (Lontchi-Yimagou et al. 2013; Hadi & Suwaidi Al 2007; Wellen & Hotamisligil 2005), as atherosclerosis is also an inflammatory condition. The inflammation associated with obesity and DM has been reported to cause endothelial cell dysfunction via numerous pathways, including increased oxidative stress, decreased synthesis of nitric oxide, endoplasmic reticulum stress, and increased synthesis of pro-inflammatory cytokines (Beckman et al. 2002; Hadi & Suwaidi Al 2007; Monteiro & Azevedo 2010; Wellen & Hotamisligil 2005). Numerous factors associated with type 2 DM may lead to increased inflammation (Lontchi-Yimagou et al. 2013; Monteiro & Azevedo 2010) as shown in table 2.3.

Table 2.3: Factors associated with type 2 DM and lead to inflammation (Lontchi-Yimagou et al. 2013)

Potential triggers of chronic inflammation in DM
Obesity especially central
High fat diet
Periodontal disease
Gut microflora imbalance
Air and other pollutants
Vitamin D deficiency

2.8.2. DM and endothelial dysfunction

The endothelium was previously thought to be merely a barrier between circulating blood and the underlying tissues, but is now recognised as an important modulator of vascular function with numerous paracrine and autocrine functions (Hadi & Suwaidi Al 2007; Potenza et al. 2009). The endothelial cells produce numerous mediators of vascular function such as nitric oxide, inflammatory responses such as prostacyclins, coagulation factors, growth factors and selectins and other adhesion molecules. Insulin has certain effects on the endothelium and can stimulate the production of endothelin-1, E-selectin and nitric oxide (Potenza et al. 2009).

Longstanding hyperglycaemia leads to the nonenzymatic binding of glucose to proteins leading to the formation of AGES. This leads to structural and functional changes in these proteins and has been described as the basis for diabetic complications (Saleh 2015). This glycation reaction can occur between reducing sugars and proteins, lipids and nucleic acids and contributes to the aging of macromolecules and diabetic complications (Yamagishi & Matsui 2016). Additionally, AGEs can bind receptors on endothelial cells leading to increased synthesis of adhesion molecules including E-selectin (Hadi & Suwaidi Al 2007; Hope & Meredith 2003b). The intake of dietary AGES from food browned at high heat contributes to this AGES pool , induces oxidative stress and endothelial dysfunction and can reduce adiponectin levels in type 2 diabetics (Yamagishi & Matsui 2016). Advanced glycation of the lipid component of LDL-c may also occur which may lead to atherosclerosis by decreased clearance of LDL-c and deposition of this modified LDL-c in the vessel wall (Vlassara & Palace 2002). Diabetics also often present with comorbidities such as

atherogenic dyslipidaemia, hypertension and abnormal coagulation which increase their CVD risk (Beckman et al. 2002). DM may also contribute to atherogenesis and endothelial dysfunction as hyperglycaemia decreases levels of endothelial nitric oxide sythetase with subsequent decreased levels of nitric oxide, and also activates NF-κB which leads to increased synthesis of adhesion molecules (Beckman et al. 2002). DM is now considered a CVD risk equivalent (Lorenzo et al. 2007) and most diabetics die from CVD.

2.8.3 Atherosclerosis

Arteriosclerosis is derived from the Greek meaning "hardening of the arteries" and is classified into three lesions: atherosclerosis, Mönckeberg medial calcific sclerosis and arteriosclerosis (Fishbein & Fishbein 2009). Although the terms atherosclerosis and arteriosclerosis are often used interchangeably, strictly speaking atherosclerosis affects elastic and large muscular arteries and the atheroma is the underlying lesion, whereas arteriosclerosis affects smaller arterioles and is associated with hypertension and DM (Fishbein & Fishbein 2009). Atherosclerotic plaques are formed due to the accumulation of modified lipids, extracellular matrix, monocyte-derived macrophages and activated smooth muscle cells in the arterial wall (Hermus et al. 2010). The rupture of an unstable plaque leads to thrombus formation and the symptoms of CVD. A vulnerable plaque is characterised by inflammation, lipid accumulation, apoptosis, proteolysis, thrombosis and angiogenesis (Hermus et al. 2010).

Although originally thought to simply be the cell layer lining blood vessels, the endothelium has been identified to have various important paracrine, endocrine and autocrine functions and is involved in the pathogenesis of various diseases such as CVD (Bonetti et al. 2003; Rajendran et al. 2013). These functions affect haemostatic balance, platelet and leucocyte interaction, the regulation of vascular tone and growth and cell proliferation and angiogenesis. Endothelial dysfunction or activation is defined as an alteration in any of these endothelial functions due to oxidative stress or vascular inflammation with reduced vasodilatation, increased thrombosis and a local proinflammatory state and the ensuing proinflammatory, proliferative, and procoagulatory milieu favours the progression of atherogenesis (Bonetti et al. 2003; Rajendran et al. 2013).

Endothelial dysfunction is considered the first step in atherosclerosis and occurs when the endothelium loses its physiological properties and instead enters a vasoconstrictive, pro-thrombotic and pro-inflammatory state (Potenza et al. 2009). DM contributes to endothelial

dysfunction by means of hyperglycaemia, hyperinsulinaemia and insulin resistance and this dysfunction is the basis of micro- and macrovascular diabetic complications (Potenza et al. 2009). Insulin plays a critical role in the maintenance of the endothelium, as endothelial cells express the insulin receptor. In health these effects are not important, but impairment in insulin resistance leads to vasoconstriction by interfering with the action of nitric oxide and can affect blood flow (Rajendran et al. 2013).

Endothelial dysfunction leads to increased formation of adhesion molecules such as E-selectin (Beckman et al. 2002; Constans & Conri 2006; Potenza et al. 2009). These adhesion molecules facilitate for the tethering and rolling of leucocytes along the endothelial surface and their entry into the subendothelial space. There they engulf oxidised LDL and lead to the formation of a fatty streak and ultimately an atherosclerotic plaque (Potenza et al. 2009; Ross 1993).

It is difficult to assess endothelial function in the peripheral and coronary circulations. Non-invasive tests such as Doppler echocardiography are available, but gold-standard tests are invasive (Hadi & Suwaidi Al 2007). The measurement of carotid intima-media thickness (CIMT) has been described as a surrogate marker of atherosclerosis burden and is used for CVD risk assessment (De Groot et al. 2008). A CIMT > 0.8 mm in an adult is considered thickened and is associated with an increased CVD risk. The CIMT is measured by an ultrasound scanner and the average of three readings is taken. As E-selectin is produced exclusively by the endothelial cells in response to endothelial dysfunction, its measurement has also been suggested as a potential marker of endothelial dysfunction.

2.9 E-selectin

2.9.1. Selectins

E-selectin is one of the three selectins which form part of the groups of cellular adhesion molecules which are expressed on cell surfaces during periods of inflammation or stress. E-selectin is produced specifically by activated endothelial cells, whereas P-selectin is found on platelets and L-selectin on leucocytes (Hope & Meredith 2003a).

The extracellular binding domain binds ligands on other cells such as leucocytes or interacts with other adhesion molecules. The intracytoplasmic component is important for interactions with the cell cytoskeleton and for intracellular signalling pathways (Hope & Meredith

2003a). When adhesion molecules are shed from the cell surface and found in the circulation, they are referred to as “soluble” adhesion molecules (Hope & Meredith 2003a).

All selectins are involved in the earliest stages of leucocyte adhesion in inflammatory lesions and they also have important roles in embryogenesis, the immune response, embryogenesis and malignant metastases (Hope & Meredith 2003a). E-selectin is an adhesion molecule specific for the endothelium and its synthesis is increased in endothelial dysfunction associated with DM and CVD (Constans & Conri 2006). E-selectin synthesis is stimulated within 6 hours by pro-inflammatory cytokines, TNFα and IL1β (Hope & Meredith 2003a; Constans & Conri 2006; Telen 2014) and levels are detectable for 24 hours. Figure 2.2 shows the general structures of the three selectins. These selectins have varying number of consensus repeats (2 in L-selectin, 6 in E-selectin and 9 in P-selectin) and share 60% homology (Telen 2014).

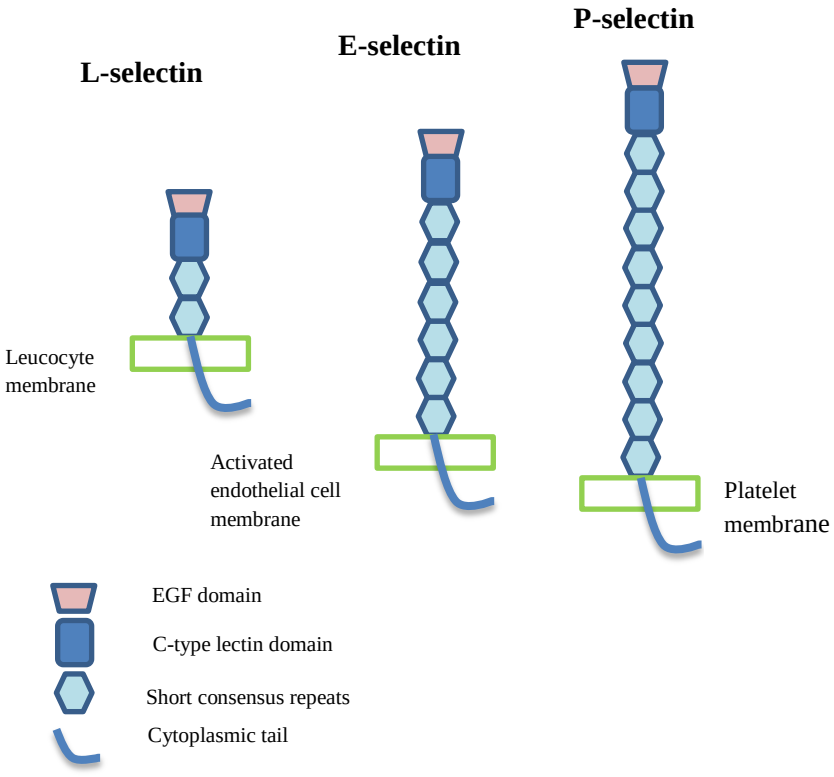

Figure 2.2: Structure of the various selectins – adapted by author (Telen 2014)

2.9.2. Actions of E-selectin

E-selectin is poorly expressed by resting endothelial cells, but its production is stimulated by inflammatory cytokines such as TNFα and IL-1 (Hope & Meredith 2003a; Telen 2014). E-selectin binds ligands on passing leucocytes, facilitates their tethering to the endothelium and slows them down by gentle rolling along the cell surface (Jubeli et al. 2012; Hope & Meredith 2003a; Telen 2014; Constans & Conri 2006). The process is shown in Figure 2.3. Other adhesion molecules such as vascular adhesion molecules then capture the cells and they are able to migrate into the subendothelial space.

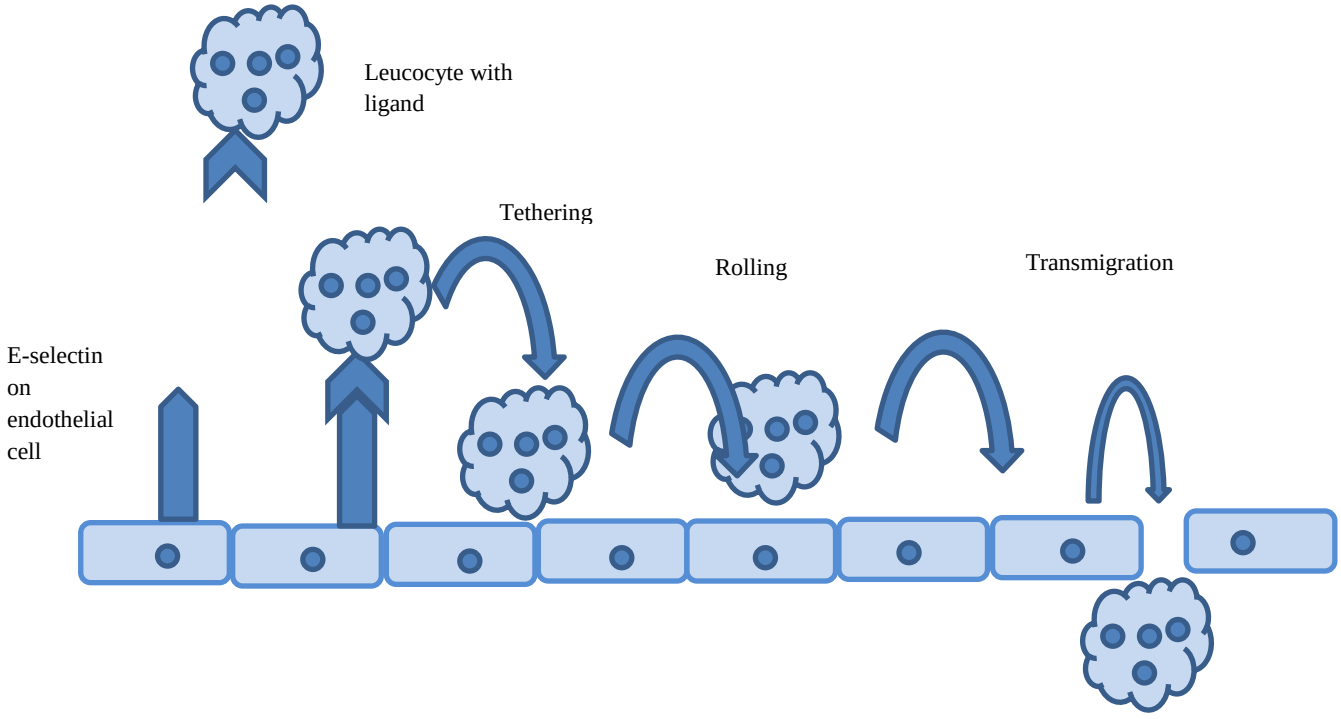

Figure 2.3: Tethering and rolling of leucocytes as facilitated by selectins - adapted by author (Telen 2014)

2.9.3. Studies of E-selectin in CVD and DM

Since the results of the Atherosclerosis Risk in Communities (ARIC) study were published in 1997 and reported that plasma levels of adhesion molecules including E-selectin, may be markers of atherosclerosis and future CVD risk, there have been numerous studies examining the use of soluble adhesion molecules to predict CVD risk (Chambless et al. 1997). High levels of E-selectin have also been described in high CVD risk disorders, such as smoking, DM, obesity, hypertension and hypercholesterolaemic subjects (Hope & Meredith 2003b; Constans & Conri 2006; Jubeli et al. 2012).

The increased synthesis of E-selectin in obesity is most likely due to increased synthesis of TNFα which is associated with visceral obesity (Zanni et al. 2010). Hyperglycaemia and AGEs bind receptors on the endothelial cells and increase the synthesis of adhesion molecules such as E-selectin leading to vascular inflammation, the recruitment of leucocytes and atherosclerosis (Hope & Meredith 2003b). Intensive glycaemic control has been shown to decrease E-selectin levels. In a study on 81 diabetics and 41 controls, Ryysy and Yki-Järvinen described a 71% increase in E-selectin levels in type 2 DM and found that there was a sustained decrease in their concentrations with intensive glycaemic control (Ryysy & Yki-Järvinen 2001). Leinonen et al found that E-selectin correlated with HbA1c levels, BMI, markers of inflammation and correlated inversely with HDL-c levels in type 2 diabetics (Leinonen et al. 2003). In a small study on diabetics with and without peripheral arterial disease, Boulbou described how E-selectin levels were increased in those with peripheral arterial disease and correlated with duration of DM (Boulbou et al. 2004). Another small study with only 29 type 2 diabetics and 12 controls described increased levels in E-selectin in type 2 DM (Elhadd et al. 2004).

A study by Lu et al found that E-selectin levels increased in CVD and that this was due to the underlying inflammatory process of atherosclerosis. With increasing severity of CVD, the levels of E-selectin increased (Lu et al. 2010). A cross-sectional study on 505 Japanese men found that both E-selectin and C-reactive protein were associated with CIMT and could be used as biomarkers for atherosclerosis (Sakurai et al. 2009). The MIAMI study is a prospective multicentre clinical study to investigate the relationship between changes in CIMT and markers of inflammation, thrombosis and endothelial dysfunction, and they described that E-selectin levels correlated with CIMT and were indicative of atherosclerotic burden (Porta et al. 2008). However, a further study by Leinonen found that both E-selectin

and CIMT were increased in diabetics, but that E-selectin was not associated with CIMT (Leinonen et al. 2004). Moreover, Malik et al found that soluble adhesion molecules including E-selectin were not strongly associated with CVD and were unlikely to add any value to follow-up (Malik et al. 2001).

Taken together, the associations between novel markers such as E-selectin and adiponectin and their degree of CVD risk are not consistent and need to be investigated in local populations to establish them to be CVD risk markers. To our knowledge no such studies on emerging markers of CVD have yet been performed on the mixed ancestry population of South Africa.

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CHAPTER 3

HbA1c and the diagnosis of Diabetes Mellitus

Zemlin et al.

HbA1c of 6.5% to diagnose diabetes mellitus – does it work for us? The Bellville South Africa study.

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HbA1c of 6.5% to Diagnose Diabetes Mellitus – does it work for us? - The Bellville South Africa Study.

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Abstract

Background: HbA1c has been the gold standard for glycaemic control follow-up for decades. In 2009, a level of 6.5% (48 mmol/mol) was proposed as diagnostic for diabetes. We test this cut-off in our community.

Methods: Participants (946) from a community-based study were screened for diabetes using either a fasting blood glucose or oral glucose tolerance test (OFTT). The HbA1c cut-off of 6.5% was tested for each group. A receiver operator characteristic (ROC) curve for both groups was generated to establish an optimal cut-off.

Results: Our study included 224 (23.7%) males and 722 (76.3%) females. Using fasting blood glucose alone, 117 (14%) were diagnosed with diabetes - 50% had an HbA1c value of ≥ 6.5% (48 mmol/mol). Using an OGTT, 147 (18%) were diagnosed with diabetes - 46% had an HbA1c value of ≥ 6.5% (48 mmol/mol). ROC curves found a level of 6.1% (43 mmol/mol) to be optimal in both groups (AUC 0.85 and 0.82 respectively). The sensitivities were 80% and 75% and the specificities 77% and 78% respectively.

Conclusions: A cut off of 6.5% (48 mmol/mol) is a good diagnostic tool with its high specificity; however the low sensitivity limits its use. We found a level of 6.1% (43 mmol/mol) to be optimal. This emphasizes the need for evidenced based values to be established in various population groups.

3.1. Introduction

Diabetes is a disease fuelled by the increasing worldwide obesity epidemic with significant morbidity and mortality, and the World Health Organization (WHO) estimates that it will affect 366 million individuals worldwide by 2030 (Wild et al. 2004). Its diagnosis was previously made either according to the WHO criteria which were updated in 2006 (World Health Organization) using a fasting blood glucose sample and subsequent 75g oral glucose tolerance test (OGTT) with blood taken for glucose determination again 2 hours after an oral glucose challenge, or according to the American Diabetes Association (ADA) criteria which were updated in 2005 (American Diabetes Association 2005), using only a fasting blood glucose level. Using ADA criteria only has been found to underestimate the prevalence of diabetes and misses those individuals with impaired glucose tolerance (IGT), a pre-diabetic state (Melchionda et al. 2002). The disadvantage of both these diagnostic approaches is that they require the patient to fast and if need confirming, would require a second fasting sample. Glucose also has a large biological and diurnal variation and depends on recent carbohydrate intake and the OGTT is fairly invasive (Sacks 2009). In 2010, the ADA updated their diagnostic criteria to include an OGTT as well (American Diabetes Association 2010).

On the other hand, HbA1c, which is formed by the attachment of glucose to various amino groups of haemoglobin and has been used since 1977 for the long-term (2-3 month) glycaemic control follow up of diabetes, has recently been advocated by the ADA as a diagnostic tool. In 2009, the International Expert Committee of the ADA issued a statement proposing an HbA1c value of 6.5% (48 mmol/mol) as a diagnostic level for the diagnosis of diabetes. This value was chosen, as it was found to be the value after which the incidence of retinopathy, a common complication that often is present before the actual diagnosis of diabetes is made, is increased (International Expert Committe 2009). This test would be advantageous, as it does not require a fasting sample and has much less intraindividual variation.

The worldwide diabetes “epidemic” is expected to affect developing countries more than developed ones. Studies to determine the usefulness of HbA1c as a diagnostic tool in these populations are needed, as there is a paucity of data from these communities compared to Western countries. As there appear to be racial differences in HbA1c levels (Cohen 2007; Young IS 2010), the purpose of this study was to assess the utility of an HbA1c value of

6.5% as a diagnostic tool for diabetes in our local Coloured (mixed ancestry) population, and to establish an optimum cut-off for this population.

3.2. Methods

3.2.1. Ethical Considerations

The study was approved by the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences Ethics committee and the University of Stellenbosch Ethics committee (N09/03/090). The study was conducted according to the Declaration of Helsinki. All participants signed written informed consent after all the procedures had been fully explained in the language of their choice. In addition, permission was also sought from other relevant authorities such as the city and community authorities. These authorities granted permission to operate in the community and also to make use of designated places such as community halls or nearby schools for data and samples collection. Patient confidentiality was maintained at all times.

3.2.2. Research Setting

Bellville-South is located within the Northern suburbs of Cape Town, South Africa and is a traditionally a Coloured township formed in the late 1950s. In the South African context, the term township usually refers to the often underdeveloped urban living areas that, under the Apartheid regime, were reserved for non-whites. According to the 2011 population census, its population stands at approximately 24 642 with 88% consisting of the Coloured (mixed race) (Cape Town Census 2011). The predominant language in this community is Afrikaans and most of the residents of this community have lived there for over five years while others have been there for their entire lives.

3.2.3. Research Design and Study Population

This was a cross-sectional quantitative study aimed at establishing a cohort that can be followed up in randomly selected manner. The data was collected mid-January 2008 to March 2009. Using a map of Bellville South, multistage stratified random sampling was approached as follows: From a list of streets from each stratum, the streets were then classified as short, medium and long streets based on the number of houses. Streets with houses ≤ 22 were classified as short, medium; houses 23 – 40 and long streets were > 40 houses. A total of 16 short streets representing approximately 190 houses, 15 medium streets

representing approximately 410 houses and 12 long streets representing approximately 400 houses were randomly selected across the different strata. From the selected streets, all household members meeting the selection criteria were invited to participate in the study.

3.2.4. Recruitment Strategy

Information regarding the project was disseminated to the local residents through the local radio station, community newspaper, brochures and fliers; the latter bearing information about the project and distributed through school children and taxis to the local residents by the recruitment team. This team consisted of unemployed matriculants and was managed by a qualified retired nurse from the community. Recruited subjects were visited by the recruitment team the evening before participation and reminded of all the survey instructions. These included overnight fasting, abstinence from drinking alcohol or consumption of any fluids in the morning of participation. Furthermore, participants were encouraged to bring along their medical/clinic cards and/or medication they were currently using.

3.2.5. Pre-participation counselling

All participants except the self-reported diabetic subjects, confirmed by either medical card record or drugs in use, had blood taken for fasting blood glucose and underwent a 75g oral glucose tolerance test (OGTT) as prescribed by the WHO. DM was diagnosed both according to previous ADA criteria using only a fasting blood glucose (American Diabetes Association 2005) and according to the WHO 2006 criteria (World Health Organization) using a 75 g OGTT.

3.2.6. Anthropometric measurements and counselling

All consenting participants received a standardized interview and physical examination during which blood pressure was measured according to WHO guidelines (Chalmers et al. 1998) using a semi-automated digital blood pressure monitor (Rossmax PA, USA). Other clinical measurements included the body weight, height, waist and hip circumferences.

3.2.7. Laboratory measurements

Blood samples were transported daily in an ice-pack box for processing at an accredited laboratory. Plasma glucose was measured by enzymatic hexokinase method (Cobas 6000, Roche Diagnostics). HbA1c was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics). This method is National Glycohemoglobin Standardization

Programme (NGSP) certified. The assay has a within-run CV of 1.4% and between run CV of 2.8%.

3.2.8. Statistical Analysis

Statistical analysis of the data was performed using STATISTICA (STATISTICA 9, StatSoft Inc 1984 – 2009). The continuous variables are presented as median (confidence interval) or means ± standard deviation (SD), and categorical variable are expressed in percentage. For data where the normality assumptions were suspect, the Mann Whitney U test was used. Diabetes was diagnosed according to both WHO and ADA criteria; the ADA diagnostic cut-off of 6.5% for HbA1c was tested separately for each group. A Receiver operator characteristic (ROC) curve for both ADA and WHO diabetes criteria were generated and the area under the curve (AUC) calculated. Test sensitivity, specificity positive predictive value (ppv) and negative predictive value (npv) was calculated.

3.3. Results

A total of 946 subjects participated, comprising 642 random subjects between the ages 35 -65 years and 304 voluntary subjects, age range 16 – 95. One hundred and twenty two subjects with known diabetes and 5 that did not consent for blood collection were excluded. Therefore, for this study, 819 subjects with the median (confidence interval) age of 52 (52, 54) were eligible.

Table 3.1 shows the general characteristics of the participants for this study. Although there were more females (722) than males (224) in the cohort, this is not reflective of the female to male ratio of the population, but of the willingness to participate. The females had significantly higher BMI and subsequently a higher prevalence of diabetes mellitus. No significant differences were observed between male and female HbA1c values; these ranged from 4.4% (25 mmol/mol) to 13.3% (122 mmol/mol) (median 5.8% (40 mmol/mol) and 5.9% (41 mmol/mol) respectively).

Table 3.1: Characteristics of all participants (946), stratified by gender

Characteristics	Male (N = 224)	Female (N = 722)	p
Age (years)	57 (44, 68)	53 (43, 64)	0.023
BMI (kg/m^2)	25.1 (21.9, 29.2)	30.3 (25.8, 34.8)	< 0.0001
FBG (mmol/L)	5.5 (5.0, 6.5)	5.6 (5.0, 6.4)	0.567
PostBG (mmol/L)	6.4 (5.3, 8.4)	6.9 (5.7, 8.9)	0.013
HbA1c (%)	5.9 (5.5, 6.3)	5.8 (5.5, 6.3)	0.856

FBG, fasting blood glucose; PostBG, post 2-hour blood glucose;

One hundred and seventeen subjects were newly diagnosed with diabetes using fasting blood glucose and 147 when using an OGTT. Using ROC curves, the optimal cut-off for HbA1c for diabetes as diagnosed according to either fasting blood glucose or OGTT gave a value of 6.1% (43 mmol/mol), AUC 0.85 and 0.82 respectively (Figure 3.1). The sensitivities at this cut-off were 80% and 75% respectively and the specificities 77% and 78% respectively (Figure 1). The ROC's were repeated with age categories of < 60 and ≥ 60 years and the optimal cut-off for HbA1c as a screening tool for diabetes remained at 6.1% (43 mmol/mol) for both age categories.

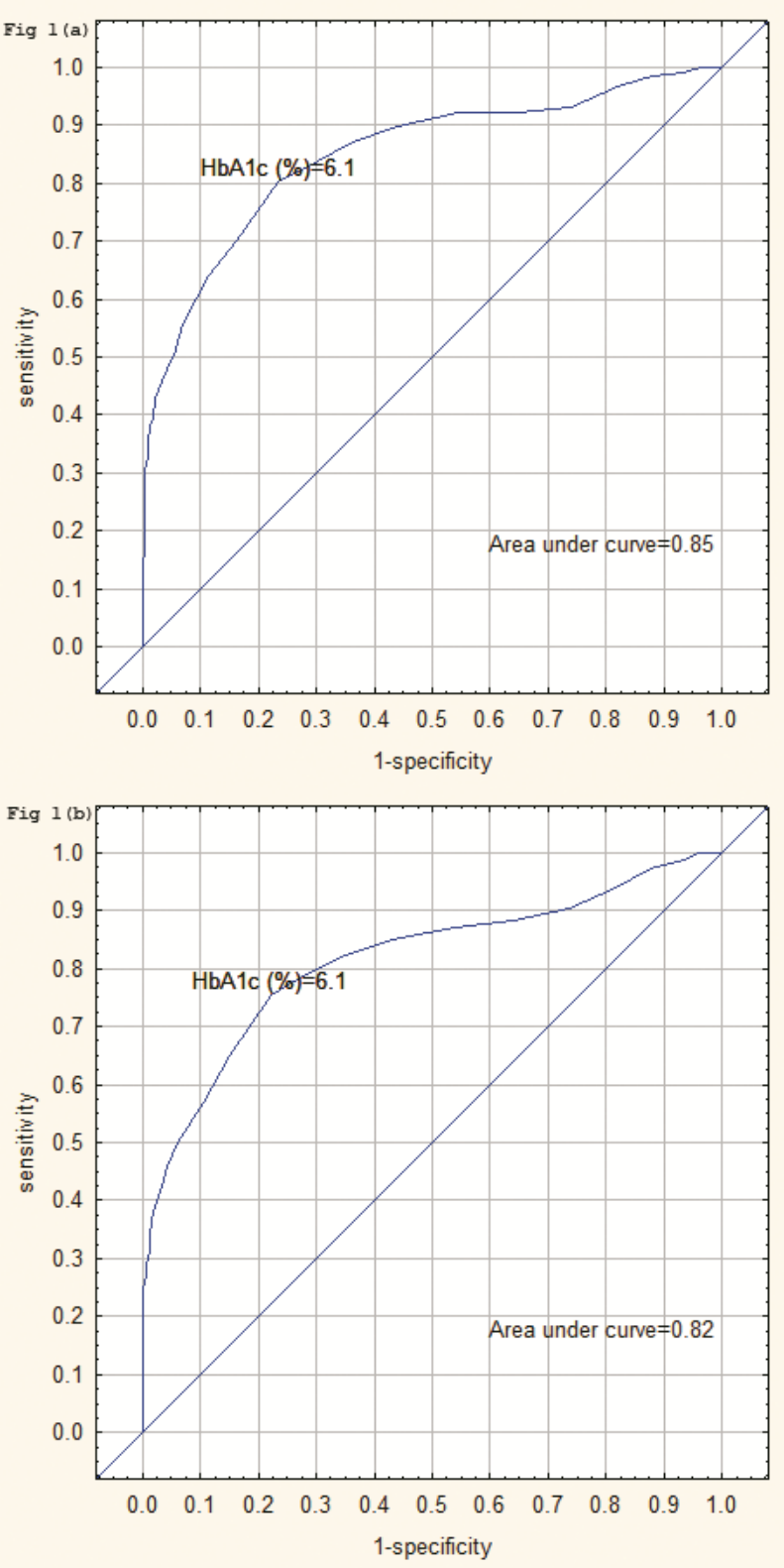

Figure 3.1: ROC curves depicting an HbA1c cut-off value of 6.1% as optimal for the diagnosis of DM according to fasting blood glucose and the OGTT. Figure 3.1 (a) area under curve = 0.85, sensitivity = 70%, and specificity = 77%. Figure 3.1 (b) area under curve = 0.82, sensitivity = 75%, and specificity = 78%.

Table 3.2 summarises the stratification of diabetic and normal subjects according to HbA1c cut-off of 6.5% (48 mmol/mol) and 6.1% (43 mmol/mol). When using a cut-off of 6.5% as recommended by the ADA, high specificities were obtained, but sensitivity was sacrificed. An HbA1c value of 6.5% (48 mmol/mol) gave a sensitivity of 50% and specificity of 95% using fasting blood glucose for diagnosis and a sensitivity of 46% and specificity of 96% when using an OGTT.

Table 3.2: Stratification of diabetic and normal subjects according to HbA1c cut-off of 6.5% and 6.1%

	HbA1c < 6.5%	HbA1c <6.1%	HbA1c ≥ 6.5%	HbA1c ≥6.1%
Diagnosis using FBG				
Normal (%)	97.1	83.3	2.9	16.7
DM (%)	49.6	19.7	50.4	80.3
Diagnosis using OGTT				
Normal (%)	97.5	80.5	2.5	19.5
DM (%)	54.1	24.7	45.9	75.3

3.4. Discussion

Type 2 diabetes is increasing in developing countries due to growth and aging of the population, and urbanisation with its introduction of an unhealthy diet and increasing obesity and sedentary lifestyles. In a recent article Bradshaw et al estimated that 5.5% of all South Africans ≥ 30 years had diabetes (Bradshaw et al. 2007). Levitt et al studied the prevalence of diabetes in 974 residents from the mixed ancestry (coloured) community of Mamre, a rural town near Cape Town in the Western Cape. The age standardized prevalence of type 2 diabetes in the age group 30-65 years, South African mixed ancestry population group was 10.8 % and that of impaired glucose tolerance (IGT) 10.2 % (Levitt et al. 1999). Age, physical inactivity, family history of diabetes and waist circumference was all identified as independent risk factors (Levitt et al. 1999). Another study that specifically examined the prevalence of diabetes in elderly coloured subjects found a prevalence of 28% for men and 29% for women (Charlton et al. 1997; Steyn et al. 2006). A population of Indian origin studied in sub-Saharan Africa found prevalences of diabetes of between 12 and 13%. This increasing incidence of diabetes with its subsequent complications in Sub-Saharan African countries such as South Africa places an even greater burden on health care systems already buckling under the challenges of diseases such as malaria, tuberculosis and HIV (Beran & Yudkin 2006). The incidence of diabetes in this study was more than 20% using either fasting blood glucose or an OGTT for diagnosis. For this reason, a quick and simple diagnostic test such as HbA1c would be advantageous.

HbA1c is formed by the attachment of glucose to various amino groups. The Diabetes Control and Complications Trial (DCCT), which determined HbA1c using a precise HPLC method, showed that a reduction of HbA1c led to a reduction in diabetic complications (DCCT 2005). This opened the door for HbA1c standardization, as there are numerous analytical methods available for its determination (Goodall 2005). In 2009, the International Expert Committee of the ADA issued a statement proposing an HbA1c value of 6.5% (48 mmol/mol) as a diagnostic level for the diagnosis of diabetes. This value was chosen, as it was found to be the value after which the incidence of retinopathy, a common complication that often is present before the actual diagnosis of diabetes is made, is increased (International Expert Committe 2009).

Previous studies proposed using HbA1c as a screening tool for the detection of diabetes, but their cut-off values differed (Bennett et al. 2007; Kumar et al. 2010; Rowley et al. 2005;

Saudek et al. 2008). In the present study, we have shown a cut-off of 6.1% (43 mmol/mol) as optimal for all mixed ancestry ages groups from South Africa. Our findings are similar to those reported by Bennett et al who performed a meta-analysis of nine studies and found that most recommended an HbA1c cut off of 6.1% (43 mmol/mol) as optimal (Bennett et al. 2007). A recent study by Kumar et al assessed the validity of HbA1c as a screening and diagnostic test for diabetes. They found a value of 6.1% (43 mmol/mol) had optimal sensitivity and could thus be used for screening and 6.5% to have optimal specificity and could thus be used as a diagnostic test (Kumar et al. 2010). In 2008, Saudek et al found an HbA1c cut off of 6.0% (42 mmol/mol) to be optimal for screening and 6.5% (48 mmol/mol) for diagnosis of diabetes (Saudek et al. 2008). Rowley et al found that a value of 7% (53 mmol/mol) had the best specificity and could therefore be used as a diagnostic test. However, this study was published in 2005 (Rowley et al. 2005).

Our study has the following strengths: (1) the number of participants is large and from the same population group and (2) the diagnosis of diabetes was made using either fasting blood glucose or an OGTT and compared.

However, our study also has some limitations: (1) the haemoglobin and iron status was not determined simultaneously, and these can affect the red blood cell survival and thus render HbA1c levels unreliable; (2) the presence of renal impairment can also affect HbA1c levels and was not investigated; (3) HbA1c levels are affected by various haemoglobinopathies and thalassaemias and these were not determined; (4) the extent to which haemoglobin glycation occurs also varies between individuals and may be affected by environmental parameters, such as lipid peroxidation and hereditary factors, and (5) medication use, specifically those such as antiretrovirals which may affect glucose metabolism, was not examined Although we did not look for haemoglobinopathies and thalassaemias, their incidence is fortunately low in this population. Another possible shortcoming, but not necessary limitation, of this study was that HbA1c was not determined on an HPLC-based method as used in the DCCT and may thus be more prone to interferences. However, the assay is NGSP- certified and had acceptable CV's.

In conclusion, we recommend an HbA1c value of 6.1% (43 mmol/mol) as an optimal cut off to screen for diabetes in our local population. A cut off of 6.5% (48 mmol/mol) would be a good diagnostic tool with its high specificity (95% using fasting blood glucose and 96%

using OGTT), however the low sensitivity limits the use of this value (50% using fasting blood glucose and 46% using OGTT).

Our study emphasizes the need for evidence based values to be established in various population groups.

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CHAPTER 4

HbA1c and the diagnosis of prediabetes

Zemlin et al.

Derivation and validation of an HbA1c optimal cutoff for diagnosing prediabetes in a South African mixed ancestry population.

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Derivation and validation of an HbA1c optimal cut-off for diagnosing prediabetes in a South African Mixed Ancestry population

Running title: HbA1c and prediabetes

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Keywords: prediabetes, HbA1c, optimal cut-off, ROC curve

Abstract

Background: Prediabetes compromises impaired fasting glucose and impaired glucose tolerance and is a high risk for future diabetes mellitus and cardiovascular disease. Traditional diagnostic methods involve a fasting sample or oral glucose tolerance test, which are cumbersome, time-consuming and inconvenient. An HbA1c-based approach has been incorporated into new guidelines, but cut-offs may vary and have not been defined for all population groups. We derived and validated HbA1c cut-offs to diagnose prediabetes in Mixed Ancestry South Africans.

Methods: Participants were 667 (derivation sample), 234 (validation sample 1) and 674 (validation sample 2) diabetes-free individuals. They underwent standard 2-hour OGTT with HbA1c test. Receiver-operator characteristic curves were used to determine optimal HbA1c cut-off to predict prediabetes.

Results: A total of 27.7% participants in the derivation sample had prediabetes versus 17.5% (validation sample 1) and 15.4% (validation sample 2). The optimal cut-off was 5.75% in all three cohorts with sensitivity and specificity of 64.8% and 60.4% in combined derivation and validation sample 1, and 59.6% and 69.8% in validation sample 2.

Conclusions: The discriminatory capacity of HbA1c for predicting prediabetes in this population is modest at the derived cut-off. The use of HbA1c alone in this setting may result in an inaccurate diagnosis.

4.1. Introduction

Globally, type 2 diabetes mellitus is rising at an alarming rate and has become a health crisis that now threatens economies of all nations. In developing countries, a 69% increase is expected in the next two decades among adults compared to a 20% increase in developed countries (Shaw et al. 2010). Most people with diabetes go through a prediabetic phase for several years during which there is an opportunity to identify them and institute preventative measures (Buysschaert et al. 2011; Pour & Dagogo-Jack 2011). The concept of an intermediate state between normoglycaemia and diabetes was first introduced in 1979 by the National Diabetes Data Group. The Expert Committee on the Diagnosis and Classification of Diabetes extended this in 1997 by including subjects with impaired fasting glucose (IFG) in addition to subjects with impaired glucose tolerance (IGT) (National Diabetes Data Group 1979). Both categories, referred to as prediabetes, are risk factors for the progression to the full stage of type 2diabetes. The underlying pathology is different in individuals with IFG or IGT with the former having hepatic insulin resistance and the latter being characterised by muscular insulin resistance (Abdul-Ghani MA, Tripathy D 2006) demonstrating that the two metabolic states have different pathophysiological mechanisms (Ferrannini et al. 2011). Although individuals with prediabetes may regress to normoglycaemia or progress to overt diabetes, this stage is also characterized by an increased risk of diabetic microvascular complications and cardiovascular disease (Buysschaert et al. 2011).

HbA1c is good marker of chronic hyperglycaemia that characterizes diabetes mellitus and has been traditionally used to monitor glycaemic control. However, in 2010, the American Diabetes Association (ADA) incorporated it into clinical practice guidelines to be used for the diagnosis of DM (≥ 6.5%) and prediabetes (5.7% - 6.4%) (American Diabetes Association 2010). However there is considerable debate about these cut off points. Age, ethnicity, genetic make-up, life span of erythrocytes and degree of glycosylation can all affect these cut off points. Racial differences, particularly in black populations, in the relationship between blood glucose and HbA1c have been observed (Herman et al. 2007; Herman et al. 2009; Tsugawa et al. 2012; Ziemer et al. 2010).

According to the revised ADA guidelines of 2010 (American Diabetes Association 2010), prediabetes is classified as:

- Impaired glucose tolerance (IGT): 2-hour glucose 7.8 – 11.1 mmol/L post 75 g oral glucose tolerance test (OGTT) OR

- Impaired fasting glucose (IFG): fasting blood glucose ≥ 5.6 – 7 mmol/L OR
- HbA1c level 5.7% - 6.4%

The mixed ancestry population of South Africa has one of the highest prevalence rates of diabetes mellitus in Africa and high progression rates from the pre-diabetic stage to diabetes (Matsha et al. 2012). HbA1c as a screening test that requires no special patient preparation and has good reproducibility might be an ideal test to screen for prediabetes in this population group as both IGT and IFG are traditionally diagnosed during OGTT which is cumbersome, time consuming and impractical. However, the cut-offs for defining pre-diabetes have not been defined for all population groups and may not necessarily agree with the recommended values suggested by ADA. In this study we examined the distribution of HbA1c and explored the optimal cut off points for identifying subjects with prediabetes in mixed ancestry South Africans.

4.2. Methods

4.2.1 Ethical consideration

The derivation and the validation studies were approved by the Cape Peninsula University of Technology Faculty of Health and Wellness Sciences ethics committee (Reference Number: CPUT/HW-REC 2008/002, CPUT/HW-REC 2010, NHREC: REC - 230 408 – 014 and N14/01/003) as well as the Health Research Ethics Council of the University of Stellenbosch (N09/03/090). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent after all the procedures had been fully explained in the language of their choice.

4.2.2 Research setting

This section was covered in chapter 3.

4.2.3. Research design and Study population

This section was covered in chapter 3.

Participants were 667 (derivation sample), 234 (validation sample 1) and 674 (validation sample 2) diabetes free individuals examined respectively in 2008, 2011, and 2014 in the Bellville South Community in Cape Town.

4.2.4. Recruitment strategy

This section was covered in chapter 3.

4.2.5. Pre-participation counselling

This section was covered in chapter 3.

4.2.6. Anthropometric measurements and counselling

This section was covered in chapter 3.

4.2.7. Laboratory measurements

Blood samples were transported daily in an ice-pack box for processing at an accredited laboratory. Plasma glucose was measured by enzymatic hexokinase method (Cobas 6000, Roche Diagnostics). HbA1c was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics). This method is National Glycohemoglobin Standardization Programme (NGSP) certified. The assay has a within-run CV of 1.4% and between run CV of 2.8%. The follow-up examination in 2011(3 years from baseline) and the new cohort in 2014 were conducted using similar procedures.

4.2.8. Statistical methods

General characteristics of the study groups are summarized as count and percentage for qualitative variable, mean and standard deviation (SD) or median and 25^{th}-75^{th} percentiles for quantitative variables. Group comparisons used chi square tests and equivalents for qualitative variables, and Student's t-test and non-parametric equivalents for quantitative variables, with adjustments where relevant through logistic and linear regression models. The *pROC* package (Robin et al. 2011) of the R statistical software version 2.13.0 [13-04-2011], (The R Foundation for Statistical Computing, Vienna, Austria) was used for receiver operating characteristics (ROC) analyses. The area under the curve (AUC) was then used to assess the ability of HbA1c to predict the presence prediabetes (DeLong et al. 1988). The optimal HbA1c was determined by applying both the Youden's index approach (Youden 1950) and the closest top left point approach (Perkins & Schisterman 2006). Main analyses including the determination of optimal cut-offs were conducted in a sample of participants recruited during the initial survey (derivation sample). The derived cut-offs were then tested

in a sample of participants from the same population, recruited during the second evaluation (validation sample 1) and third evaluation (validation sample 2).

4.3. Results

4.3.1. Characteristics of the study populations

Tables 4.1 and 4.2 show the general characteristics of both the derivation and both validation samples. The derivation sample consisted of 667 participants of which 512 (77%) were women, and the first validation cohort consisted of 234 participants of which 163 (70%) were women. There were some men versus women differences in each cohort, but little evidence of gender*cohort interaction. A total of 185 (27.7%) participants in the derivation cohort, 41 (17.5%) of the validation sample 1 and 104 (15.4%) of the validation sample 2 had prediabetes.

Table 4.1: General characteristics of participants of derivation and 1st validation samples by gender and cohort

Variables	Derivation sample				Validation sample					
	Overall	**Men**	**women**	**p**	**Overall**	**men**	**women**	**p-value**	**P cohort**	**P gender*cohort**
n	667	155	512		234	71	163		0.031	
Age (years)	51.7 (15.2)	54.5 (16.7)	50.8 (14.6)	0.015	48.3 (13.8)	50.3 (15.3)	47.4 (13.1)	0.166	0.002	0.763
FBG (mmol/L)	5.2 (0.7)	5.1 (0.8)	5.3 (0.7)	0.012	5.1 (0.6)	5.1 (0.6)	5.1 (0.6)	0.557	0.010	0.048
Post BG (mmol/L)	6.6 91.6)	6.4 (1.6)	6.7 (1.6)	0.030	5.7 (1.7)	5.3 (1.7)	5.8 (1.6)	0.032	<0.0001	0.449
Fserum insulin (µU/mL)	6.2 [2.7-11.5]	3.9 [1.7-8.0]	7.0 [3.1-12.6]	<0.0001	10.1 [5.9-16.1]	9.9 [4.6-15.9]	10.3 [6.4-16.7]	0.108	<0.0001	0.944
Glucose insulin ratio	0.83 [0.46-1.81]	1.29 [0.64-2.81]	0.73 [0.43-1.60]	<0.0001	0.48 [0.33-0.79]	0.50 [0.35-1.20]	0.48 [0.31-0.73]	0.091	<0.0001	0.509
HbA1c (%)	5.7 (0.4)	5.7 (0.4)	5.7 (0.4)	0.683	5.8 (0.4)	5.7 (0.4)	5.8 (0.4)	0.006	0.007	0.051
Trigs (mmol/L)	1.4 (0.9)	1.4 (0.9)	1.3 (0.9)	0.445	1.4 (0.8)	1.6 (1.0)	1.3 (0.7)	0.054	0.643	0.186
LDL (mmol/L)	3.6 (1.0)	3.4 (1.0)	3.7 (1.0)	0.003	3.4 (1.0)	3.4 (1.0)	3.4 (0.9)	0.902	0.0002	0.078
HDL (mmol/L)	1.3 (0.3)	1.2 (0.3)	1.3 (0.3)	0.007	1.4 (0.4)	1.3 (0.4)	1.4 (0.4)	0.038	0.015	0.458
Total cholesterol (mmol/L)	5.6 (1.2)	5.3 (1.1)	5.6 (1.2)	0.002	5.4 (1.1)	5.4 (1.1)	5.4 (1.1)	0.968	0.035	0.082
SBP (mmHg)	121 (18)	125 (17)	120 (18)	0.0004	130 (23)	132 (22)	129 (23)	0.366	<0.0001	0.424
DBP (mmHg)	74 (12)	75 (11)	74 (12)	0.113	80 (15)	83 (19)	79 (13)	0.129	<0.0001	0.308
Height (m)	1.59 (0.09)	1.68 (0.07)	1.56 (0.07)	<0.0001	1.61 (0.09)	1.69 (0.10)	1.58 (0.06)	<0.0001	0.002	0.809
Weight (Kg)	73 (17)	70 (16)	74 (18)	0.016	78 (18)	78 (20)	78 (18)	0.749	0.001	0.141
BMI	29.1 (7.2)	24.9 (5.5)	30.4 (7.1)	<0.0001	30.1 (7.0)	27.5 (6.6)	31.2 (6.9)	0.0005	0.066	0.134
Hip (cm)	109 (14)	99 (9)	112 (14)	<0.0001	108 (13)	102 (13)	110 (12)	<0.0001	0.357	0.081
WC (cm)	95 (15)	91 (14)	96 (15)	<0.0001	94 (17)	93 (15)	95 (18)	0.554	0.768	0.147
WHR	0.87 (0.08)	0.91 (0.08)	0.86 (0.07)	<0.0001	0.88 (0.13)	0.92 (0.06)	0.86 (0.14)	<0.0001	0.635	0.813
Prediabetes	185	40	145	0.540	41	13	28	0.834	0.002	0.630

Table 4.2: Second validation dataset

Variables	Validation sample 2			
	Overall	Men	women	**P**
n	674	209	465	
Age (years)	45.1 (15.8)	42.2 (15.2)	46.4 (15.8)	0.001
FBG (mmol/L)	4.8 (0.6)	4.7 (0.7)	4.8 (1.0)	0.018
Post BG (mmol/L)	5.8 (1.8)	5.1 (1.7)	6.1 (1.7)	<0.0001
Fserum insulin (µU/mL)	5.6 [3.5-8.8]	4.3 [2.7-7.5]	6.1 [4.0-9.2]	<0.0001
Glucose insulin ratio	0.90 [0.60-1.30]	1.10 [0.70-1.70]	0.80 [0.50-1.10]	<0.0001
HbA1c (%)	5.6 (0.5)	5.5 (0.4)	5.6 (0.5)	0.0008
Trigs (mmol/L)	1.3 (0.9)	1.4 (1.2)	1.3 (0.8)	0.454
LDL (mmol/L)	3.0 (1.0)	2.7 (0.9)	3.1 (0.9)	<0.0001
HDL (mmol/L)	1.4 (0.4)	1.3 (0.4)	1.4 (0.4)	0.032
Total cholesterol (mmol/L)	5.0 (1.1)	4.7 (1.2)	5.1 (1.1)	0.0001
SBP (mmHg)	133 (26)	132 (27)	134 (26)	0.509
DBP (mmHg)	83 (15)	81 (17)	84 (14)	0.030
Height (m)	1.61 (0.09)	1.69 (0.08)	1.57 (0.07)	<0.0001
Weight (Kg)	70 (19)	67 (18)	71 (19)	0.038
BMI	27.1 (7.8)	23.5 (6.1)	28.7 (7.8)	<0.0001
Hip (cm)	97 (16)	90 (12)	101 (16)	<0.0001
WC (cm)	86 (16)	80 (13)	88 (16)	<0.0001
WHR	0.88 (0.10)	0.89 (0.08)	0.87 (0.11)	0.002
Prediabetes	104	24	80	0.057

4.3.2. Optimal HbA1c cut-offs for the diagnosis of prediabetes

Figure 4.1 shows the ROC curves for the prediction of prediabetes in the derivation and first validation sample as well as their combination. The area under the ROC curve (AUC) for the prediction of prediabetes was 0.631 in the derivation sample, 0.729 for the validation sample 1 and 0.642 for the combined sample. In the validation sample 2, the AUC was 0.712 in women, 0.501 in men and 0.665 in the overall sample (Figure 4.2). Table 4.2 shows the performance of HbA1c thresholds in the different cohorts. The optimal HbA1c cutoff for the diagnosis of prediabetes was 5.75% in the derivation sample (sensitivity 61.1%, specificity 64%); 5.85% in the validation sample 1 (sensitivity 72.5%, specificity 63.3%) and 5.75 in the combination of both (sensitivity 64.8%, specificity 60.4%) (Table 4.3). Results were mostly similar in men and women. In the validation sample 2, an HbA1c cutoff of 5.75% also emerged as the optimal cut point (Figure 4.2).

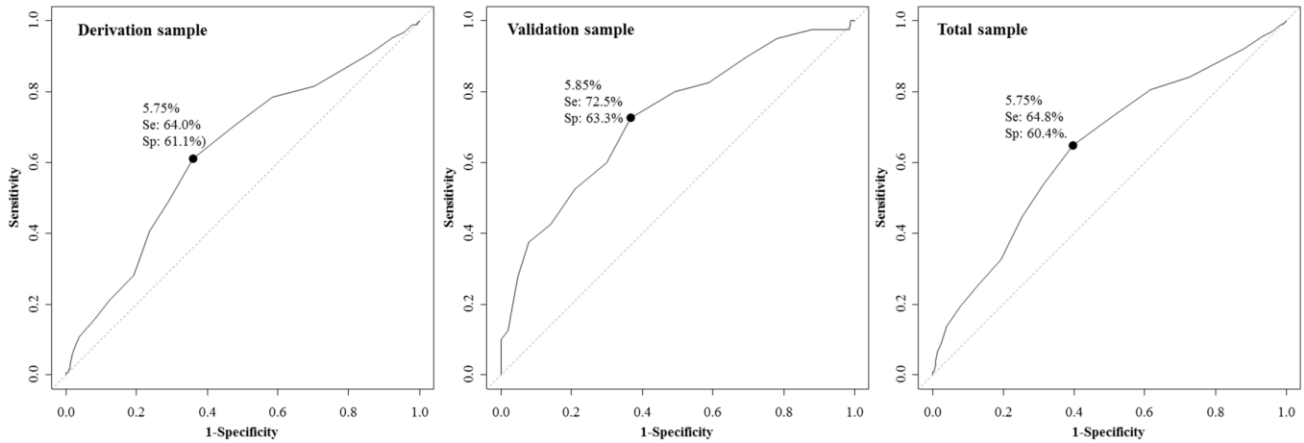

Figure 4.1: Receiver operating characteristic curves (ROC) for the prediction of the presence of prediabetes using HbA1c for the derivation and first validation samples

Definition of prediabetes is based on the IDF-WHO criteria-based presence of impaired fasting glycaemia and impaired glucose tolerance following an oral glucose tolerance test and venous blood glucose tests.

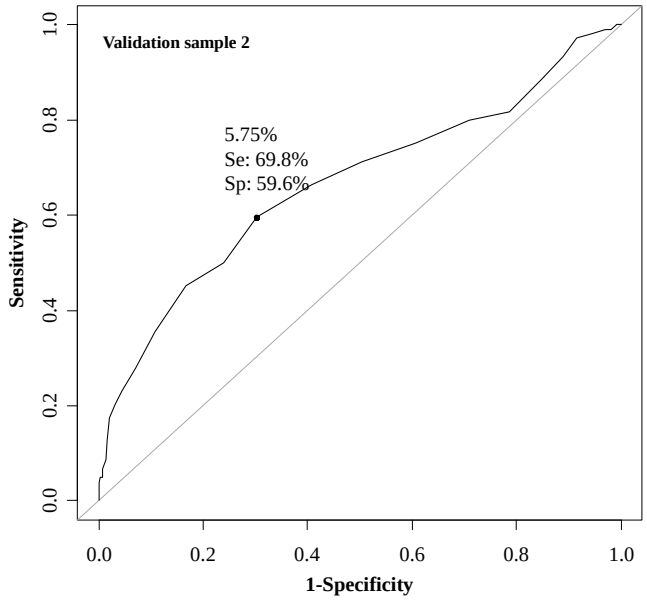

Figure 4.2: Receive operating characteristic curves (ROC) for the prediction of the presence of prediabetes using HbA1c in the second validation sample

Definition of prediabetes is based on the IDF-WHO criteria-based presence of impaired fasting glycaemia and impaired glucose tolerance following an oral glucose tolerance test and venous blood glucose tests.

Area under the ROC curve: 0.665. HbA1c optimal cut point: 5.75% (based on both the Youden index and closest-top-left methods: sensitivity (Se) 59.6%, specificity (Sp) 69.8%)).

Table 4.3: Performance of different HbA1c thresholds in different samples

Sample	HbA1c thresholds	Sensitivity	Specificity	PPV	NPV	Youden index	AUC (95% CI)
Derivation							
Overall	5.75% (CTL)	61.1%	64.0%	39.5%	81.0%	0.251	0.631 (0.583-0.678)
	5.75% (Youden)	61.1%	64.0%	39.5%	81.0%	0.251	
Men	5.75% (CTL)	55.0%	64.3%	34.9%	80.4%	0.193	0.628 (0.529-0.728)
	5.55% (Youden)	82.5%	40.9%	32.7%	87.0%	0.234	
Women	5.75% (CTL)	62.9%	63.9%	40.8%	81.2%	0.267	0.632 (0.578-0.686)
	5.75% (Youden)	62.9%	63.9%	40.8%	81.2%	0.267	
Validation sample 1							
	5.85% (CTL)	72.5%	63.3%	29.3%	91.7%	0.358	0.729 (0.639-0.818)
	5.85% (Younden)	72.5%	63.3%	29.3%	91.7%	0.358	
	5.75%	80.0%	50.8%	25.4%	92.4%	0.308	
Combined derivation and validation samples							
Overall	5.75% (CTL)	64.8%	60.3%	35.2%	83.7%	0.251	0.642 (0.600-0.684)
	5.75% (Youden)	64.8%	60.3%	35.2%	83.7%	0.251	
	5.85%	53.7%	68.7%	36.4%	81.7%	0.225	
Men	5.75% (CTL)	57.7%	64.8%	32.6%	83.8%	0.225	0.650 (0.564-0.737)
	5.85% (Younden)	51.9%	72.2%	35.5%	83.5%	0.241	
Women	5.75% (CTL)	66.9%	58.8%	36.0%	83.7%	0.257	0.640 (0.591-0.688)
	5.75% (Youden)	66.9%	58.8%	36.0%	83.7%	0.257	
	5.85%	54.3%	67.5%	36.7%	81.0%	0.218	
Validation sample 2							
Overall	5.75% (CTL)	59.6%	69.8%	26.5%	90.4%	0.294	0.665 (0.602-0.729)
	5.75% (Youden)	59.6%	69.8%	26.5%	90.4%	0.294	
Men	5.35% (CTL)	45.8%	70.3%	16.7%	90.9%	0.161	0.501 (0.354-0.650)
	5.35% (Youden)	45.8%	70.3%	16.7%	90.9%	0.161	
	5.75	62.5	28.1	10.1	85.1	-0.094	
Women	5.75% (CTL)	66.2%	68.8%	30.6%	90.7%	0.350	0.712 (0.645-0.779)
	5.75% (Youden)	66.2%	68.8%	30.6%	90.7%	0.350	

4.4. Discussion

There has been considerable interest in the use of HbA1c to identify individuals who are in the prediabetic state ever since it was recommended for the diagnosis of diabetes in 2010 (American Diabetes Association 2010). This, to the best of our knowledge, is the first study from Africa to report on the optimal HbA1c values that may be used to identify subjects with prediabetes. We found that our optimal cut-off of 5.75% had a sensitivity of 64.8% and specificity of 60.4% and observed that this value was within the range recommended by the ADA for the diagnosis of prediabetes but lower than the 6% recommended by the International Expert Committee. Furthermore, this cut-off was confirmed in a separate cohort of subjects. However, similar to observations reported in several studies, the discriminatory capacity of HbA1c for predicting prediabetes in our population was modest, with just above average sensitivity and specificity.

Other studies have confirmed this suboptimal performance of HbA1c to identify subjects with prediabetes. Bersoux et al studied 242 patients mean age 62 years in United States of America and found that reliance on HbA1c alone would miss a substantial amount of patients (Bersoux et al. 2011). A study of 5395 individuals from the NHANES study found that an HbA1c value of 5.7% to diagnose prediabetes had a low sensitivity and could not replace an OGTT (Guo et al. 2014). Ziemer et al studied 1581 Americans 18-87 years of Black and White ethnicity and found that HbA1c levels were higher in Blacks and this difference increases as glucose intolerance worsens and therefore cannot be used to screen for prediabetes (Ziemer et al. 2010).

An Italian study looking at White nondiabetics found that HbA1c alone fails to identify IGT who have significant insulin resistance. They found that the use of HbA1c alone has low sensitivity (AUC .678) (Succurro et al. 2011). A study on a cohort of 1370 Palestinian Arabs with no known diabetes aged older than 30 years old compared fasting plasma glucose and HbA1c to diagnose prediabetes. They found the area under the curve for HbA1c to diagnose prediabetes to be 63.9%; the optimal cutoff of 6.3% gave a sensitivity of 62.6% and a specificity of 94.5% (Kharroubi et al. 2014). A Chinese study examining 7641 participants older than 18 years old found that using fasting plasma glucose the prediabetes prevalence was 14.9% and using HbA1c it was 21.5% (Sun et al. 2013). Another study examined 903 Chinese individuals aged 21-79 years. Prediabetes was diagnosed according to 1999 WHO criteria post 75g OGTT (IFG and IGT) and they found a prevalence of 22.4%. An HbA1c

cut-off ≥ 6% was better with a sensitivity of 80% and specificity 89.8%. When the cutoff was lowered to ≥ 5.7% the sensitivity decreased to 59.4% and the specificity to 73.9% (Zhou et al. 2009). A more recent study by Xu et al on 98 658 Chinese adults found that using all three diagnostic criteria for prediabetes gave an overall prevalence on 50.1% amongst Chinese adults (Xu et al. 2013). A study in Korea found that HbA1c led to more subjects being diagnosed with prediabetes than fasting plasma glucose (Jeon et al. 2013). A study in Bangladesh found that an HbA1c value greater than 5.6% had a sensitivity of 68%, a specificity of 66.4% and an area under the curve of 0.714 when used to diagnose prediabetes (Bhowmik et al. 2013). A study in New Zealand examining 4721 participants older than 15 years found the prevalence of prediabetes to be 18.6% when using HbA1c as a screening tool. However, they did not compare this to an OGTT (Coppell et al. 2013).

Although the advantages of performing a single HbA1c test are unequivocal, screening with HbA1c alone will misclassify many individuals. Furthermore, HbA1c is not without limitations. It is influenced by ethnicity, age, glycation phenotype, haemoglobinpathies, anaemia and iron deficiency, the standardization is inconsistent and the cost of the test may be restrictive in our setting (Higgins et al. 2011). We found that the discriminatory capacity of HbA1c for predicting prediabetes in our population was modest and its use alone for this purpose may lead to an inaccurate diagnosis. This is in agreement with other population studies from other areas of the world. Based on our second validation of the derived cut-off, we strongly recommend an HbA1c of 5.75% for the screening of prediabetes in our population.

The study has some limitations. We did not test for iron deficiency anaemia, which may increase HbA1c results. Iron deficiency anaemia is common in our population. HbA1c may be unreliable to diagnose prediabetes and diabetes in anaemic patients. Son et al found that anaemic subjects had a higher HbA1c level and suggested that the diagnostic significance of HbA1c may be limited in them (Son et al. 2013). This higher HbA1c was more pronounced in subjects with higher glucose levels. Iron deficiency anaemia reduces erythropoiesis and increases HbA1c levels (Kim & Choi 2013). A study by Hardikar et al comparing the use of OGTT and HbA1c to diagnose prediabetes found that in iron deficiency anaemia the prevalence of prediabetes was spuriously exaggerated (23.3% (HbA1c) vs 7.8% (OGTT)) (Hardiker et al. 2012). As the Western Cape Province where this study was performed has a relatively high prevalence of iron deficiency anaemia, these values that we obtained may not be totally accurate (SANHANES). We also did not test for haemoglobinpathies, but we do

not have high prevalence of haemoglobinopathies in our population. However, its strength was that it was a large study with the cut-off values obtained validated in two separate cohorts.

4.5 Conclusion

Because there is now strong evidence that lifestyle management in those with prediabetes may reduce the rate of progression to diabetes, it is important to detect those at risk so that prevention efforts can be initiated. These may include lowering the HbA1c levels considerably to detect those at risk in our population. However, recent publications have questioned whether prediabetes may not in fact be an overdiagnosed condition. Yudkin and Montori questioned whether prediabetes is being overdiagnosed since the introduction of HbA1c into the diagnostic criteria (Yudkin & Montori 2014). Xu et al showed a recent prevalence of prediabetes of 50.1% using all three diagnostic criteria which is a dramatic increase compared to previous studies in China before the use of HbA1c (Xu et al. 2013). A meta-analysis has shown that more than half the subjects diagnosed with prediabetes due to IGT in fact do not develop diabetes within a 10 year follow up (Morris et al. 2013). As only 5 years have passed since the introduction of HbA1c into these diagnostic criteria, we do not have sufficient follow-up studies yet. This potential for overdiagnosis could potentially place further burden on a healthcare budget already overloaded by the HIV and tuberculosis pandemics in the Western Cape.

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Conflict of Interest: None

Authorship: All authors have made substantial contributions to the following: (1) the conception and design of the study, acquisition of data, analysis and interpretation of data, (2) drafting of the article and revising it critically for intellectual content, (3) final approval of submitted version.

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CHAPTER 5

High molecular weight adiponectin and polymorphisms in subjects with hyperglycaemia

Zemlin et al.

High molecular weight adiponectin levels are neither influenced by adiponectin polymorphisms nor associated with insulin resistance in mixed-ancestry hyperglycemic subjects from South Africa.

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High molecular weight adiponectin levels are neither influenced by adiponectin polymorphisms nor associated with insulin resistance in mixed-ancestry hyperglycemic subjects from South Africa

Running title: Hmw-adiponectin in hyperglycemia

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Summary

Background: High molecular weight (hmw) adiponectin has anti-atherogenic, anti-inflammatory and anti-diabetic properties and these effects have been linked to its effect on high density lipoprotein cholesterol (HDL-c). Single nucleotide polymorphisms (SNPs) in the adiponectin gene influence adiponectin levels. We examined the relationship between hmw-adiponectin levels and cardiometabolic traits in normo-and hyperglycemic mixed ancestry South Africans and correlated these levels to two common polymorphisms.

Methods: Hmw-adiponectin was determined in 101 subjects from the Cape Town Bellville-South community-based study on Mixed Ancestry population. Comparisons were made between individuals with normo- and hyperglycemia. Two common SNPs, *ADIPOQ* SNPs rs17300539 and rs266729, known to affect adiponectin levels were also tested for. Levels of hmw-adiponectin were then correlated with cardiometabolic traits in all groups.

Results: Levels of hmw-adiponectin were not significantly different in the normo-and hyperglycemic groups (median 11.6 vs. 10.5 μg/mL, p=0.3060) and in men and women (8.44 vs. 11.34 μg/mL, p=0.6834). *ADIPOQ* SNPs rs17300539 and rs266729 did not influence levels of hmw-adiponectin. Robust correlation analyses revealed a significant positive correlation between hmw-adiponectin and HDL-c (r=0.45; 95%CI: 0.27-0.59), similarly in normo- and hyperglycemic participants (p>0.99).This association was substantially attenuated in robust linear regressions adjusted for age, gender and adiposity.

Conclusions: Adiponectin levels in this population were not determined by the commonest SNPs of the adiponectin gene, were unaffected by glycemic status; but were significantly correlated with HDL-c levels, Previous studies have attributed some of the beneficial effects of adiponectin to its effect on HDL-c.

Keywords: hmw-adiponectin, hyperglycemia, cardiometabolic traits, polymorphisms

5.1. Introduction

Adiponectin is the most common adipokine secreted by adipose tissue and increased visceral fat accumulation inhibits its secretion (Litvinova et al. 2014; Gable et al. 2006; Lee & Kwak 2014). It plays an important role in regulating glucose levels and fatty acid oxidation by enhancing insulin sensitivity and decreasing free fatty acid production (Gable et al. 2006; Rabin et al. 2005). It is known to have anti-atherogenic, anti-inflammatory and anti-diabetic properties (Balsan et al. 2015; Yadav et al. 2013). Higher circulating adiponectin concentrations are associated with reduced plasma glucose and serum triglyceride levels and increased high density lipoprotein cholesterol (HDL-c) concentrations, decreased blood pressure and a lower risk of obesity and type 2 diabetes (Gable et al. 2006; Rabin et al. 2005; Yadav et al. 2013; Ryo et al. 2004). It is thought that the lower adiponectin levels observed in obesity and diabetes may be due to inhibitory effects of inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8. Additionally, adiponectin stimulates the production of nitrous oxide and inhibits the expression of adhesion molecules (Gable et al. 2006; Lee & Kwak 2014; Balsan et al. 2015; Matsuzawa 2006). As such, adiponectin levels were suggested as a therapeutic target for lowering the risk of developing metabolic syndrome, type 2 diabetes and cardiovascular disease (Fisman & Tenenbaum 2014). Subsequently, several studies have described a paradox where raised adiponectin levels are associated with increased mortality especially in subjects with pre-existing cardiovascular disease (Alehagen et al. 2015; Choi et al. 2015; Hascoet et al. 2013; Lee et al. 2013; Kizer 2014; S Goya Wannamethee et al. 2007; Witberg et al. 2016; Wu et al. 2014).

There are conflicting reports on the association between body mass index (BMI), waist circumference, insulin resistance, duration of diabetes and adiponectin levels in Africans. The inconsistencies may be attributed to the different assays used to measure adiponectin levels, renal status, molecular forms of adiponectin and the influence of polymorphisms in the adiponectin (A P Doumatey et al. 2012; Ferris et al. 2005; Obot et al. 2013; Sobngwi et al. 2007; Vasseur et al. 2006). It is now known that adiponectin is secreted in low molecular weight (trimer), medium molecular weight (hexamer) and high molecular weight (hmw) (12-18mer) forms (Balsan et al. 2015; Bunn et al. 1976; Lee & Kwak 2014; Rabin et al. 2005; Vasseur et al. 2006; Gable et al. 2006). The hmw form has been found to be the active form and also has increased affinity to collagen and is thus able to bind exposed collagen in damaged vasculature, decrease apoptosis of endothelial cells and increase reverse cholesterol transport due to its effect on HDL-c (Suriyaprom et al. 2014b; Yadav et al. 2013). Moreover,

it is believed that between 30-70% of the variability in adiponectin levels is influenced by genetic factors (Comuzzie et al. 2001; Gable et al. 2006; Suriyaprom et al. 2014a). The adiponectin (*ADIPOQ*) gene is located on chromosome 3q27, a susceptibility locus for metabolic syndrome and its components (Gable et al. 2006; Li et al. 2014; Yadav et al. 2013). Numerous functional single nucleotide polymorphisms (SNPs) and missense mutations have been identified in various populations which may affect adiponectin levels and impact on insulin sensitivity and risk of cardiovascular disease (Vasseur et al. 2006). In this regard, two adiponectin polymorphisms, rs 17300539 (11391 G>A) and rs 266729 (11377 C>G) have been shown to be associated with risk for type 2 diabetes by modulating adiponectin levels and activity (Vasseur et al. 2006). Both these SNPs are in the promoter region of the adiponectin gene and affect adiponectin levels and thus the risk of metabolic syndrome, cardiovascular disease and type 2 diabetes (Karmelić et al. 2012). In a meta-analysis by Han et al, rs266729 was associated with lower adiponectin levels and type 2 diabetes risk in Whites, whereas rs 17300539 was associated with higher adiponectin levels and no increased risk of type 2 diabetes unless there is a family history of the disease (Han et al. 2011). However the association between the adiponectin gene polymorphisms and adiponectin levels varies across different populations and some studies have found that rs17300539 is indeed associated with increased risk of metabolic syndrome and type 2 diabetes (Karmelić et al. 2012). Kaftan et al described that both homozygous and heterozygous mutations of rs266729 are associated with and increased risk of type 2 diabetes in Iraqis, but that conflicting results have been found in other populations (Kaftan & Hussain 2015). These conflicting results of genetic studies may be due to differences in age, genetic and ethnic backgrounds of the study populations (Kaftan & Hussain 2015; Karmelić et al. 2012).

Not much is known about the effect of BMI, insulin resistance, glycemia and obesity on the hmw form of adiponectin and how these are affected by commonly reported polymorphisms in the adiponectin gene in African populations. In this study we examined the relationship between hmw-adiponectin levels and cardiometabolic traits in normo-and hyperglycemic subjects of a mixed ancestry South African population. We further investigated whether these levels were influenced by two commonly reported adiponectin polymorphisms, namely rs 17300539 and rs 266729.

5.2. Materials and Methods

5.2.1. Ethical Considerations

The study was approved by the Cape Peninsula University of Technology Faculty of Health and Wellness Sciences ethics committee (Reference Number: CPUT/HW-REC 2008/002, CPUT/HW-REC 2010, NHREC: REC - 230 408 – 014 and N14/01/003) and the University of Stellenbosch (N09/03/090). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent after all the procedures had been fully explained in the language of their choice.

5.2.2. Research Setting

This section was covered in chapter 3.

5.2.3. Research Design and Study Population

This section was covered in chapter 3.

5.2.4. Recruitment Strategy

This section was covered in chapter 3.

5.2.5. Anthropometric Measurements

All consenting participants received a standardized interview and physical examination during which blood pressure was measured according to WHO guidelines (Chalmers et al. 1998) using a semi-automated digital blood pressure monitor (Rossmax PA, USA). Other clinical measurements included the body weight, waist and hip circumferences. Participants underwent a standard two hour 75 g oral glucose tolerance test (OGTT) as prescribed by the World Health Organization (WHO), with fasting and 2-hour plasma glucose being determined. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) were diagnosed based on WHO criteria (Alberti & Zimmet 1998). Normoglycemia was defined as normal fasting glucose levels and normal response to 75g OGTT, whereas hyperglycemia was defined as known diabetes, screen detected diabetes, IGT and / or IFG.

5.2.6. Laboratory Measurements

Fasting whole blood and serum samples were collected and processed for further analysis. Separated serum was stored at -70 degrees C. Plasma glucose was measured by enzymatic hexokinase method (Cobas 6000, Roche Diagnostics). Glycated hemoglobin (HbA1c) was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics). This method is National Glycohemoglobin Standardization Programme (NGSP) certified according to Roche Diagnostics. Total cholesterol, HDL-c and triglycerides (TG) were estimated by enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics). Insulin was determined by a microparticle enzyme immunoassay (Axsym, Abbot). C-reactive protein (CRP) was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Immage® Immunochemistry System; Beckman Coulter), with a lower limit of detection of 0.2 mg/L.

5.2.7. Adiponectin ELISA

Levels of hmw-adiponectin were measured in duplicate on serum by a sandwich ELISA (DRG® Adiponectin Human (HMW) ELISA). The assay is specific for hmw-adiponectin and has no interference from medium or low molecular weight adiponectin. The limit of sensitivity of the assay is 0.5 μg/mL and the appropriate range is 1.56 – 200 μg/mL. Serum samples were stored and are stable at -70 degrees C.

5.2.8. Genotyping

Genomic DNA was extracted from whole blood samples collected in an EDTA tube. Single nucleotide polymorphism, *ADIPOQ* rs266729 and *ADIPOQ* rs17300539, were genotyped using high throughput real-time polymerase chain reaction (RT-PCR) in two independent laboratories on the ABI Prism 7900HT platform (Applied Biosystems, USA) and a BioRad Optica (BioRad, USA) using Taqman genotyping assay (Applied Biosystems, USA). Direct sequencing was used for analytical validation of high throughput genotyping against direct sequencing as the gold standard.

5.2.9. Definitions and Calculations

The BMI was calculated as weight per square meter (kg/m^2) and waist-hip-ratio (WHR) as waist/hip circumferences (cm). Diabetes was based on a history of doctor-diagnosis, fasting blood glucose concentration ≥ 7.0 mmol/L (or 126 mg/dL) and/or 2-hour post-OGTT plasma

glucose ≥ 11.1mmol/L (or 200mg/dL) (Alberti & Zimmet 1998). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: HOMA-IR= [fasting insulin concentration (mIU⁄ L) x fasting plasma glucose (mmol⁄ L]/ 22.5] (Matthews et al. 1985). Low density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al. 1972).

5.2.10. Statistical Methods

The statistical software programs, STATISTICA 12 (StatSoft, Inc) and R (version 3.0.3 [2014-03-04], The R Foundation for statistical computing, Vienna, Austria) were used for statistical analyses. The Shapiro-Wilk W test was used to determine the distribution of hmw-adiponectin. Due to the skewed distribution, nonparametric tests were used and the median and lower and upper quartiles (25Q, 75Q) were used for descriptive statistics of the general characteristics of the study group. The Mann-Whitney U test was used to compare subgroups. Robust correlations were used to investigate the continuous associations between continuous variables and adiponectin levels, and the potential effects of extraneous factors adjusted for in robust linear regression models. Single nucleotide polymorphisms were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectation via a chi square goodness of fit test. Results corresponding to p-values below 5% are described as significant. We did not adjust for multiple testing.

5.3. Results

A total of 101 mixed ancestry subjects with a median age of 58 years were analysed – 38 had normoglycemia and 63 had hyperglycemia. The demographic data of our cohort is shown in Table 5.1 with normoglycemia compared to hyperglycemia. Significant differences between the two groups were found for HOMA-IR, HbA1c, triglycerides, HDL-c, BMI and WHR. Only 14 of the subjects were males and separate analysis found no significant gender difference in hmw-adiponectin levels (median 8.44 μg/mL in males and 11.34 μg/mL in females; p = 0.67). There was no significant difference in hmw-adiponectin levels between the normoglycemic and hyperglycemic groups (median 11.6 vs. 10.5 μg/mL; p=0.306).

Table 5.1: Demographic data of study cohort according to glycemic status

	Total (N 101)	Normal (N 38)	Hyperglycemia (N 63)	
	Median (25^{th}-75^{th} percentiles)	**Median (25^{th}-75^{th} percentiles)**	**Median (25^{th}-75^{th} percentiles)**	**P-value**
Hmw-adiponectin (µg/mL)	11.1 (6.3 - 16.4)	11.6 (6.3 - 16.8)	10.5 (5.2 - 16.4)	0.31
Age (years)	58 (50 - 64)	59 (47 - 64)	57 (52 - 64)	0.77
Fasting blood glucose (mmol/L)	5.9 (5.0 - 8.0)	5.1 (4.7 - 5.3)	7.4 (6.0 - 10.0)	< **0.0001**
2-hour glucose (mmol/L)	7.6 (5.7 - 9.0)	5.7 (5.4 - 6.2)	8.9 (8.1 - 14.9)	< **0.0001**
Fasting insulin (µU/mL)	11.4 (6.1 - 17.0)	36.7 (27.8 - 61.4)	57.3 (24.9 - 129.3)	0.06
HOMA-IR	3.5 (1.6 - 5.2)	2.3 (1.2 - 3.4)	3.9 (1.8 - 7.0)	0.0021
HbA1c (%)	6.1 (5.7 - 6.8)	5.7 (5.6 - 6.0)	6.3 (6.1 - 8.1)	< **0.0001**
C-reactive protein (mg/L)	5.4 (1.9 - 10.4)	4.2 (1.2 - 10.7)	5.9 (2.3 - 10.4)	0.28
Triglycerides (mmol/L)	1.4 (1.1 - 1.9)	1.1 (0.8 - 1.5)	1.7 (1.3 - 2.1)	< **0.0001**
HDL-cholesterol (mmol/L)	1.2 (1.1 - 1.5)	1.4 (1.2 - 1.7)	1.1 (1.0 - 1.4)	**0.0006**
LDL-cholesterol (mmol/L)	3.8 (3.1 - 4.5)	3.7 (3.2 - 4.4)	3.9 (3.1 - 4.5)	0.55
Total cholesterol (mmol/L)	5.8 (5.1 - 6.6)	5.8 (5.0 - 6.5)	5.9 (5.2 - 6.7)	0.52
Height (m)	1.6(1.5- 1.6)	1.6 (1.5 - 1.6)	1.6 (1.5 - 1.6)	0.02
Weight (kg)	80.3 (72.4 - 88.0)	71.5 (59.1 - 85.0)	81.5 (75.0 - 89.5)	0.01
Body mass index (kg/m^2)	32.3 (28.2 - 36.5)	30.6 (24.6 - 34.6)	32.6 (29.1 - 38.4)	0.04
Waist circumference (cm)	100.0 (93.5 - 108.0)	93.6 (84.0 - 102.3)	102.0 (96.5 - 112.3)	**0.0007**
Hip circumference (cm)	113.0 (104.0 - 120.0)	107.7 (99.0 - 117.5)	115.0 (105.0 - 121.0)	0.06
Waist-to-hip ratio	0.88 (0.84 - 0.93)	0.87 (0.81 - 0.90)	0.89 (0.85 - 0.96)	0.01
Systolic blood pressure (mmHg)	130 (119 - 140)	126 (115 - 135)	132 (123 - 142)	0.15
Diastolic blood pressure (mmHg)	80 (71 - 86)	81 (75 - 87)	79 (69 - 86)	0.31

ADIPOQ rs17300539 was in HWE (p=0.8535), whilst *ADIPOQ* rs266729 was not (p=0.0201). The hmw-adiponectin levels were higher in individuals with heterogeneous *ADIPOQ*_2_17300539 AG when compared to homozygous GG, respectively, [median (25^{th} - 75^{th} percentile): 12.97 (9.22 - 20.12) and 11.57 (4.99 -16.40); p=0.4547], however only three individuals carried the A allele.

Table 5.2 shows correlation of hmw-adiponectin with the variables for the group as a whole and separately for the normo-and hyperglycemia groups. Using Spearman Rank Order correlations, hmw-adiponectin was found to correlate significantly with HDL-c and age and inversely with triglycerides, BMI and WHR in the total group. In the normoglycemic group, hmw-adiponectin correlated significantly with HDL-c and inversely with HOMA-IR, triglycerides, total cholesterol, BMI and WHR. In the hyperglycemic group, the only significantly correlation was with HDL-c.

Table 5.2: Correlation between hmw-adiponectin and parameters tested

	Total N101		Normal N38		Hyperglycemia N63	
	R	**P-value**	**R**	**P-value**	**R**	P-value
Age (years)	**0.1991**	**0.05**	0.2743	0.10	0.1439	0.26
Fasting blood glucose (mmol/L)	-0.0452	0.65	0.1134	0.50	-0.0591	0.65
2-hour glucose (mmol/L)	0.0492	0.67	-0.0174	0.92	0.1894	0.24
Fasting insulin (µU/mL)	-0.1581	0.12	**-0.4148**	**0.01**	0.0028	0.98
HOMA-IR	-0.1510	0.13	**-0.4212**	**0.01**	-0.0768	0.55
% HbA1c	-0.1456	0.15	-0.0906	0.59	-0.1332	0.30
C-reactive protein (mg/L)	-0.0913	0.365	-0.1845	0.27	0.0113	0.93
Triglycerides (mmol/L)	**-0.2616**	**0.01**	**-0.5130**	**<0.05**	-0.1208	0.35
HDL-cholesterol (mmol/L)	**0.3361**	**<0.05**	**0.3525**	**0.03**	**0.2758**	**0.03**
LDL-cholesterol (mmol/L)	0.1075	0.29	0.2951	0.08	0.0080	0.95
Cholesterol (mmol/L)	0.1802	0.07	0.3810	0.02	0.0777	0.55
Height (m)	-0.0730	0.47	0.2855	0.09	-0.2026	0.11
Weight (kg)	-0.1812	0.15	-0.4379	0.06	-0.0655	0.67
Body mass index (kg/m^2)	**-0.2262**	**0.02**	**-0.4072**	**0.01**	-0.0677	0.60
Waist circumference (cm)	**-0.2267**	**0.02**	**-0.4398**	**0.01**	-0.0904	0.48
Hip circumference (cm)	-0.0992	0.32	-0.3527	0.03	0.0764	0.55
Waist-to-hip ratio	**-0.2279**	**0.02**	**-0.3397**	**0.04**	-0.1607	0.21
Systolic blood pressure (mmHg)	-0.0496	0.62	0.0439	0.80	-0.0347	0.79
Diastolic blood pressure (mmHg)	-0.0640	0.53	0.0626	0.71	-0.1597	0.21

Using robust correlation statistics to eliminate the effect of outliers and account for possible non-linear correlation, we found that HDL-c was significantly correlated with hmw-adiponectin in overall, normoglycaemic and hyperglycaemic groups. BMI correlated inversely in the normal group but not in hyperglycemia. Waist circumference had inverse significant correlation in the overall sample likely driven by a negative correlation in the normoglycemic group and a non-significant correlation in the hyperglycemic group. A similar pattern was observed with WHR. The correlation with triglycerides and LDL-c was only in the normoglycemic group, with evidence that the correlation coefficients were significantly different across glycemic status for LDL-c ($p<0.001$) (Table 5.3).

Table 5.3: Robust Correlation of hmw-adiponectin (µg /mL) with biochemical and anthropometric parameters

Correlates	Overall N = 96	Normal N =36	Hyperglycemia N=60	P
Age (years)	0.20 (0.00 to 0.38)	0.22 (-12 to 0.51)	0.15 (-0.11 to 0.39)	0.76
Fasting blood glucose (mmol/L)	0.01 (-0.19 to 0.21)	0.05 (-0.28 to 0.38)	-0.01 (-0.26 to 0.25)	0.78
Fasting insulin (µU/mL)	-0.12 (-0.32 to 0.08)	-0.39 (-0.64 to 0.07)	0.06 (-0.20 to 0.31)	0.03
HOMA-IR	-0.09 (-0.28 to 0.12)	-0.35 (-0.61 to -0.03)	0.02 (-0.23 to 0.28)	0.07
% HbA1c	-0.08 (-0.28 to 0.12)	-0.14 (-0.44 to 0.20)	-0.09 (-0.34 to 0.16)	0.84
Triglycerides (mmol/L)	-0.21 (-0.14 to 0.07)	-0.46 (-0.68 to -0.15)	-0.07 (-0.32 to 0.19)	0.05
HDL-cholesterol (mmol/L)	0.45 (0.27 to 0.59)	0.42 (0.11 to 0.66)	0.42 (0.19 to 0.61)	>0.99
LDL-cholesterol (mmol/L)	0.19 (-0.01 to 0.37)	0.53 (0.24 to 0.73)	0.05 (-0.30 to 0.20)	<0.001
Body mass index (kg/m^2)	-0.17 (-0.36 to 0.03)	-0.33 (-0.59 to 0.00)	0.005 (-0.25 to 0.26)	0.11
Waist circumference (cm)	-0.23 (-0.41 to -0.03)	-0.39 (-0.64 to 0.07)	-0.04 (-0.29 to 0.22)	0.09
Hip circumference (cm)	-0.10 (-0.30 to 0.10)	-0.22 (-0.51 to 0.11)	0.04 (-0.22 to 0.29)	0.23
Waist-to-hip ratio	-0.21 (-0.40 to -0.01)	-0.39 (-0.64 to 0.07)	-0.08 (-0.33 to 0.17)	0.13
Systolic blood pressure (mmHg)	0.03 (-0.17 to 0.23)	0.16 (-0.17 to 0.47)	-0.03 (-0.28 to 0.22)	0.37
Diastolic blood pressure (mmHg)	0.01 (-0.19 to 0.21)	0.20 (-0.14 to 0.50)	0.11 (-0.35 to 0.15)	0.15

In robust linear regressions adjusted for age, gender and BMI, none of the covariates was significantly associated with adiponectin levels. For HOMA-IR, LDL-c and to lesser extent triglycerides, there was suggestion of significant interaction with status for glycemia, on their effects on adiponectin levels (Table 5.4).

Table 5.4: Robust linear regressions (age, gender, BMI adjusted) showing the effects of covariates on hmw-adiponectin levels

Variables	Overall N = 96		P interaction with glycemic status
	β (se)	**p-value**	
Age (years)	0.070 (0.098)	0.473	0.760
Sex (women)	2.031 (3.413)	0.553	0.424
Fasting blood glucose (mmol/L)	-0.179 (0.327)	0.586	0.658
Fasting insulin (uU/mL)	-0.004 (0.087)	0.961	0.156
HOMA-IR	-0.060 (0.309)	0.845	0.032
% HbA1c	-0.588 (0.391)	0.136	0.435
Triglyceride (mmol/L)	-1.243 (1.330)	0.353	0.086
HDL-cholesterol (mmol/L)	5.836 (3.520)	0.101	0.966
LDL-cholesterol (mmol/L)	0.654 (1.191)	0.584	0.058
Body mass index (kg/m^2)	-0.225 (0.167)	0.182	0.515
Waist circumference (cm)	-0.058 (0.126)	0.648	0.109
Hip circumference (cm)	0.041 (0.075)	0.584	0.357
Waist-to-hip ratio	-15.861 (10.867)	0.148	0.187
Systolic blood pressure (mmHg)	-0.001 (0.050)	0.982	0.953
Diastolic blood pressure (mmHg)	0.014 (0.090)	0.877	0.839
Normoglycemia	1.158 (1.689)	0.495	-

5.4. Discussion

In this study we investigated the relationship between two common polymorphisms reported in the adiponectin gene and hmw-adiponectin in mixed ancestry South Africans, and further examined its relationship with insulin resistance and cardiometabolic traits in normo- and hyperglycemic subjects. Most studies have established total adiponectin levels as a marker of insulin resistance. However this association has not been observed consistently in African populations. This has been attributed to variability in adiponectin's secretion due to genetic differences. Ebinuma et al developed a novel ELISA to determine hmw-adiponectin levels in 2006 which was followed by the development of ELISA assays to specifically determine this fraction of adiponectin (Ebinuma et al. 2006). A probable reason for the limited studies on hmw-adiponectin from Africa is that until recently there was no reliable assay and even when available, the cost is prohibitive.

We found no difference in hmw-adiponectin levels between the normo-and hyperglycemic groups, despite the higher degree of insulin resistance in the latter group. Epidemiological studies demonstrate an association between lower adiponectin and the prevalence and incidence of insulin resistance, and type 2 diabetes in various populations (Li et al. 2009). There are indications that this progression to diabetes associated with low adiponectin levels is modulated by insulin resistance (Hivert et al. 2011; S G Wannamethee et al. 2007). A recent meta-analysis emphasized the substantial inverse association between total plasma adiponectin levels and the incidence of type 2 diabetes, which was clearly consistent in various populations (Li et al. 2009).

A study from the United States found paradoxically high total adiponectin (PHA) levels in obese metabolically healthy Afro-Americans and also reported gender differences despite having higher BMIs and waist circumferences (Doumatey et al. 2012). We did not observe gender differences despite a higher degree of obesity in females and speculate PHA as a possible cause, though numerous other studies have described higher adiponectin levels in females (Ding et al. 2015; Gable et al. 2006; Li et al. 2014). However, Moriyama et al also found no gender difference in their study (Moriyama et al. 2014). Another possible factor that may have contributed in our study may be the extremely low number of males in our cohort (only 14 out of 101).

Recent studies have also described the so-called "adiponectin paradox" where raised adiponectin levels are associated with increased mortality, especially in older individuals with

pre-existing cardiovascular disease (Alehagen et al. 2015; Choi et al. 2015; Hascoet et al. 2013; Kizer 2014; Lee et al. 2013; S G Wannamethee et al. 2007; Witberg et al. 2016; Wu et al. 2014). The reason for this is still unknown, but adiponectin resistance at receptor level, increased adiponectin levels in response to raised N-terminal fragment of pro-brain natriuretic peptide (NT-proBNP), and an increase in proinflammatory cytokines have been postulated (Choi et al. 2015; Kizer 2014; Lee et al. 2013; Wannamethee et al. 2011; Witberg et al. 2016).

It is difficult to compare our results with these studies, as to our knowledge only two studies have reported hmw-adiponectin in South African populations. In one, levels of hmw-and total adiponectin were determined in HIV-infected women both on and off antiretroviral therapy and it was determined that levels of hmw-adiponectin were lower in the treatment group and inversely correlated with waist circumferences, insulin and HOMA-IR (Omar et al. 2014). Another study examined levels of hmw-and total adiponectin and other markers of endothelial dysfunction in Black and White South Africans with rheumatoid arthritis. They found no ethnic differences and significant associations with glucose and lipid profile (Dessein et al. 2013). Whilst Meilleur et al (Meilleur et al. 2010) and Sobngwi et al (Sobngwi et al. 2007) showed a significant association between total adiponectin levels and either insulin resistance or obesity in West Africans, Ferris et al from South Africa (Ferris et al. 2005) and a study from Nigeria (Godwill & Kasia Benedicta 2013) did not observe this relationship. Similarly Ntyintyane et al (Ntyintyane et al. 2009) from South Africa also observed no relationship. In contrast to observations made in this study, numerous Japanese studies have reported an association between hmw-adiponectin and insulin resistance (Aso et al. 2006; Goto et al. 2014; Hara et al. 2006; Moriyama et al. 2014; Nakashima et al. 2006; Seino et al. 2007) and found it to be a better predictor of insulin resistance, type 2 diabetes progression, metabolic syndrome and coronary artery disease prediction in patients with type 2 diabetes (Aso et al. 2006; Hara et al. 2006; Nakashima et al. 2006). As our study was cross-sectional, it is difficult to evaluate the prognostic value of our findings.

As variability in adiponectin levels in different ethnic groups has also been attributed to polymorphisms in the adiponectin gene, we investigated the role of two commonly reported polymorphisms and found that these did not affect hmw-adiponectin levels nor did they have an association with insulin resistance or components of the metabolic syndrome. Karmelić et al studied the same polymorphisms as in this study and found that there was a significant decrease in total adiponectin with metabolic syndrome traits and that levels correlated with

central obesity and triglyceride levels (Karmelić et al. 2012). A study by Lin et al described ethnic differences in the incidence of polymorphisms and found that rs266729 decreased adiponectin levels and increased the risk of metabolic syndrome in Chinese (Lin et al. 2012). Similarly Vasseur et al (Vasseur et al. 2006) also described an increased incidence of type 2 diabetes with the rs266729 polymorphism and an association between rs266729 and coronary artery disease was reported by Fisman and Tenenbaum (Fisman & Tenenbaum 2014). Furthermore, in a meta-analysis Han et al concluded that rs17300539 was not associated with an increased risk of type 2 diabetes, but that rs266729 decreased total adiponectin levels and increased the risk of diabetes (Han et al. 2011). In the Erasmus Rucphen study in Netherlands, rs17300539 was found to be associated with plasma insulin levels and HOMA-IR due to functional variation of the adiponectin protein which affected insulin sensitivity independently of adiponectin level (Henneman et al. 2010). Kaftan et al found that rs266729 was associated with type 2 diabetes and also had an effect on HDL-c in an Iraqi population; however they did not perform serum adiponectin levels (Kaftan & Hussain 2015). In contrast to these observations Gao et al found no association between rs266729 polymorphism and metabolic syndrome in a Chinese population which they felt was due to their small study population and additionally they did not correlate their results with serum adiponectin levels (Gao et al. 2013).

Several studies have postulated that the most important beneficial effect of adiponectin may be due to its effect on HDL-c and the reverse cholesterol transport (Suriyaprom et al. 2014b). A sub-study of The Nurses' Health Study in United States found that hmw-adiponectin was protective and that this effect was mainly due to increased HDL-c, with these effects being stronger in younger women (Pischon et al. 2011). While another study on the same Nurses' Health Study found that hmw-adiponectin was associated with an increased risk in women who developed type 2 diabetes (Heidemann et al. 2008), the British Women's Heart and Health study found no association between hmw-adiponectin and coronary heart disease, although levels were inversely associated with WHR, fasting insulin, glucose, HOMA-IR, triglycerides and HDL-c (Sattar et al. 2008). Moriyama et al determined that hmw-adiponectin was associated with the metabolic syndrome mainly due to its effect on HDL-c by influencing its size (Moriyama et al. 2014). In a South African study on total adiponectin levels, Ferris et al found that although total adiponectin did not correlate with insulin resistance after correcting for BMI and ethnicity, levels did have a role in determining HDL-c levels (Ferris et al. 2005). A previous study where adipocytes were incubated with HDL-c

found that the HDL upregulated adiponectin expression (Van Linthout et al. 2010). As both HDL and adiponectin activate AMP-kinase and decrease insulin levels, adiponectin may mediate the insulin sensitizing effects of HDL-c (Christou et al. 2012).

There are numerous limitations that require consideration. We had a small sample size with very few male participants so could not divide our adiponectin levels into quartiles or reliably determine gender differences. Because of the high prevalence of obesity in this population group the controls as well as subjects with hyperglycemia were obese which may have adversely affected the hmw-adiponectin levels in normoglycemic subjects. Our population was older and according to Pischon, adiponectin level is a better predictor in younger subjects (Pischon et al. 2011). In contrast to findings by Pischon, a meta-analysis by Wu et al found that increased adiponectin levels may in fact be associated with increased mortality in older subjects (Wu et al. 2014). The cross-sectional nature of our study is a further limitation. Despite these limitations a unique and major contribution to previous studies from Africa was the measurement of hmw-adiponectin, known to be the metabolically active form of adiponectin. Additionally, we used robust correlation and robust regressions which avoid the effect of outliers and the issues with the distribution of hmw-adiponectin and correlates.

5.5. Conclusion

In our cohort of 101 adults South Africans, we found no significant difference in hmw-adiponectin levels between normo- and hyperglycemic subjects. Furthermore, levels of adiponectin were not determined by common adiponectin polymorphisms known to influence adiponectin levels. We did find some correlations between hmw-adiponectin and traits of the metabolic syndrome. However the only correlation that remained statistically significant in the group as a whole and in both groups was with HDL-c. This is in keeping with literature which describes that one of the most important reason for adiponectin's beneficial effect may be due to its effect on HDL-c function and size and the reverse cholesterol transport system. Further follow-up studies are needed on a larger population with a more equal gender distribution with lower BMI.

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CHAPTER 6

E-selectin and carotid intima-media thickness and cardio-metabolic risk profile

Zemlin et al.

Correlation of E-selectin levels with carotid intima media thickness and cardio-metabolic profile of mixed ancestry South Africans: A cross-sectional study

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Title: **Correlation of E-selectin levels with carotid intima media thickness and cardio-metabolic profile of mixed ancestry South Africans: A cross-sectional study**

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DECLARATIONS

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Abstract

Background: E-selectin, an adhesion molecule, is a specific marker of endothelial dysfunction. High concentrations have been reported in type 2 diabetes and disorders with high risk of cardiovascular disease (CVD). Measurement of carotid intima-media thickness (CIMT) is a surrogate marker of early atherosclerosis. We examined the relationship between E-selectin levels, CIMT and cardio-metabolic traits in normo-and hyperglycaemic mixed ancestry South Africans.

Methods: E-selectin concentrations were determined in 308 subjects from the Cape Town Bellville-South Community-based study on a mixed ancestry population. Their correlation with CIMT and cardio-metabolic profile used robust correlations and linear regression models.

Results: E-selectin concentrations were significantly higher in the hyperglycaemic (median 139.8 μg/L) compared to the normoglycaemic group (median 118.8 μg/L), (p=0.0007). Significant differences between the two groups were found for markers of glycaemia and adiposity, but not for CIMT. Significant correlations were found between E-selectin and age, markers of glycaemia and inflammation, central obesity and lipid variables. Associations remained significant only with age, hyperglycaemia and C-reactive protein in robust linear regression models. In similar regressions models, age and gender were the main predictors of CIMT, which was not associated with E-selectin.

Conclusions: E-selectin concentrations in this study were associated with hyperglycaemia, possibly reflecting early endothelial damage. However, E-selectin was not useful to assess CIMT, a marker of subclinical atherosclerosis, which appeared to be determined by ageing and male gender.

Keywords: E-selectin, endothelial dysfunction, CIMT, hyperglycaemia, atherosclerosis

6.1. Introduction

Diabetics are at an increased risk of cardiovascular disease (CVD), through multiple pathophysiological pathways including endothelial cell dysfunction and damage, which are major causes of vascular complications in diabetes (Beckman et al. 2002; Hadi & Suwaidi Al 2007). Endothelial cell dysfunction is regarded as an early step in atherosclerotic plaque formation primarily due to its effect on endothelial cell activation (Hope & Meredith 2003a). The effect of hyperglycaemia on endothelial cells closely mimics that of inflammatory initiators. Advanced glycation end-products which increase due to the nonenzymatic binding of glucose to various proteins. This includes binding to receptors on the endothelial cells, leading to an increased synthesis of adhesion products such as E-selectin, a specific marker of endothelial cell dysfunction (Hadi & Suwaidi Al 2007; Hope & Meredith 2003b). This promotes vascular inflammation and the recruitment of leucocytes and atherosclerosis (Hope & Meredith 2003b). E-selectin's expression on inflamed endothelial cells is increased in response to pro-inflammatory cytokines such as tumour necrosis factor (TNF)α and interleukin (IL)-1 (Raab et al. 2002; Hope & Meredith 2003a) and has been reported to be elevated in type 2 diabetes. E-selectin recruits leukocytes into the endothelium by binding ligands on their cell surfaces. This facilitates their rolling on endothelial cells, thereby slowing down the leucocytes and allowing their eventual entry into the subendothelial space (Constans & Conri 2006; Telen 2014; Hope & Meredith 2003a). Elevated concentrations of E-selectin in obesity may be due to its increased synthesis secondary to TNFα, which is associated with the inflammatory response of visceral obesity (Zanni et al. 2010; Miller & Cappuccio 2006). Elevated concentrations of E-selectin have also been known to occur in high CVD risk disorders such as smoking, obesity, diabetes, hypertension and hypercholesterolaemia (Jubeli et al. 2012; Hope & Meredith 2003b; Constans & Conri 2006).

The obesity pandemic has fuelled a dramatic increase in prevalence of type 2 diabetes, particularly in low to middle income countries such as South Africa (Erasmus et al. 2012). Type 2 diabetes and obesity are both considered to be inflammatory conditions (Lontchi-Yimagou et al. 2013; Wellen & Hotamisligil 2005; Monteiro & Azevedo 2010; Hotamisligil 2006). The measurement of carotid intima-media thickness (CIMT) has been described as a surrogate marker of early atherosclerosis burden and is used for CVD risk assessment (De Groot et al. 2008). A CIMT > 0.8 mm is considered thickened and is associated with CVD risk.

Emerging markers of CVD risk such as soluble E-selectin concentrations have not been investigated in populations within Africa. The mixed ancestry population is a heterogeneous South African ethnic group with one of the highest reported prevalence of type 2 diabetes in Africa (Erasmus et al. 2012). This community has a high prevalence of metabolic syndrome which has been shown to be a powerful determinant of diabetes and CVD. Previous studies on this population found a high risk of diabetes and CVD and led to the establishment and continuation of the Bellville South study group. In the current study, we examined the relationship between soluble E-selectin concentrations, CIMT and cardio-metabolic traits in normo-and hyperglycaemic mixed ancestry South Africans.

6.2. Methods

6.2.1. Ethical Considerations

The study was approved by the University of Stellenbosch (N09/03/090) and the Cape Peninsula University of Technology Faculty of Health and Wellness Sciences ethics committee (Reference Number: CPUT/HW-REC 2008/002, CPUT/HW-REC 2010, NHREC: REC - 230 408 – 014 and N14/01/003). The study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent after all the procedures had been fully explained in the language of their choice.

6.2.2. Research Setting

This section was covered in chapter 3.

6.2.3. Research Design and Study Population

This section was covered in chapter 3.

6.2.4. Recruitment Strategy

This section was covered in chapter 3.

6.2.5. Anthropometric measurements

Participants were examined in the Bellville South Community in Cape Town. All consenting participants received a standardized interview and physical examination during which blood pressure was measured according to World Health Organization (WHO) guidelines

(Chalmers et al. 1998) using a semi-automated digital blood pressure monitor (Rossmax PA, USA). Other clinical measurements included the body weight, height, waist and hip circumferences. Participants underwent a standard two hour 75 g oral glucose tolerance test (OGTT) as described by the WHO, with fasting and 2-hour plasma glucose being determined. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) were diagnosed based on WHO criteria (Alberti & Zimmet 1998). According to these criteria, IFG was defined as a fasting glucose concentration of 5.6-7 mmol/L and IGT was defined as a 2-hour glucose concentration of 7.8-11.1 mmol/L. Normoglycaemia was defined as normal fasting glucose concentrations and normal response to 75g OGTT, whereas hyperglycaemia was defined as known diabetes, screen detected diabetes, IGT and / or IFG.

6.2.6. Laboratory measurements

Fasting (minimum of 8h) blood samples were collected and processed within 2 h for further analysis. Separated serum was stored at -70°C. Plasma glucose was measured by enzymatic hexokinase method on the Cobas® 6000 Roche Diagnostics (Indianapolis, IN, USA). Glycated haemoglobin (HbA1c) was assessed by turbidimetric inhibition immunoassay on the Cobas® 6000 Roche Diagnostics (Indianapolis, IN, USA). This method is National Glycohaemoglobin Standardization Programme (NGSP) certified according to Roche Diagnostics. Cholesterol, high density lipoprotein cholesterol (HDL-c) and triglycerides (TG) were estimated by enzymatic colorimetric methods on the Cobas® 6000 Roche Diagnostics (Indianapolis, IN, USA). Insulin was determined by a microparticle enzyme immunoassay on the AxSYM® Abbott (Abbott, Irving, TX, USA). C-reactive protein (CRP) was measured by a high-sensitivity CRP assay, based on the highly sensitive Near Infrared Particle Immunoassay rate methodology (Immage® Immunochemistry System, Beckman Coulter (Brea, CA, USA)), with a lower limit of detection of 0.2 mg/L. All laboratory tests were performed in an accredited laboratory where regular internal and external quality control was performed.

6.2.7 E-selectin ELISA

Serum samples for E-selectin determination were stored and are stable at -70°C according to the manufacturers' insert. Samples, controls and standards were measured in duplicate by a sandwich ELISA (abcam®, Cambridge, UK), and the concentrations were calculated from a standard curve. The limit of detection is <0.5 ng/mL and the average E-selectin concentration

for this assay as determined by the manufacturers on 80 normal subjects was found to be 51.99 ± 26.65 μg/L (range 11.78 – 160.72 μg/L). The intra-assay coefficient of variation (CV) was determined and found to be 8.9% and the inter-assay CV was 10.5%.

6.2.8. Definitions and calculations

Body mass index (BMI) was calculated as weight per square meter (kg/m^2) and waist-hip-ratio (WHR) as waist/hip circumferences (cm). Diabetes was based on a history of doctor-diagnosis, fasting blood glucose concentration ≥ 7.0 mmol/L and/or 2-hour post-OGTT plasma glucose ≥ 11.1mmol/L (Alberti & Zimmet 1998). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to the formula: HOMA-IR= [fasting insulin concentration (mIU⁄ L) x fasting plasma glucose (mmol⁄ L]/ 22.5 (Matthews et al. 1985). Low density lipoprotein cholesterol (LDL-c) was calculated using Friedewald's formula (Friedewald et al. 1972). This calculation is invalid and was not performed at TG concentrations > 4.5 mmol/L.

6.2.9. Measurement of carotid IMT

The CIMT was measured by two qualified sonographers who were blinded to the risk factor status of participants, in longitudinal section at the far wall of the distal common carotid arteries, 2 cm from the bifurcation, at 3 consecutive end-points, 5-10 mm apart. The mean of the six readings (three from each side) was taken for each participant using a portable B-mode and spectral Doppler ultrasound scanner equipped with cardiovascular imaging software. The GE LOGIQ e® (General Electric Healthcare, Germany) high-performance multipurpose colour compact ultrasound system included new imaging CrossXBeam technologies with multifrequency virtual apex on phased array cardiac transducer (3S-RS wide band phased probe 1.7-4.0 MHz) for echocardiography, and a linear wide band vascular transducer (8L-RS 4.0-12 MHz linear probe) used for improved diagnostic confidence and imaging clarity for the carotids.

6.2.10. Statistical methods

The statistical software R (version 3.2.2 [15 August 2015], The R Foundation for statistical computing, Vienna, Austria) was used to perform all the statistical analyses. The Shapiro-Wilk *W* test was used to determine the distribution of E-selectin. Due to the skewed distribution, nonparametric tests were used and the median and lower and upper quartiles (P25-P75) were used for descriptive statistics of the general characteristics of the study

group. The Mann-Whitney *U* test was used to compare categorical variables across subgroups. The association of E-selectin with baseline characteristics was assessed by comparing the distribution of those characteristics across quarters of E-selectin. We first used the Kruskal-Wallis test to test for differences in the median values of those characteristics by quarter of E-selectin, and then used the Jonckheere's trend test to investigate monotonic changes in the medians across quarters of E-selectin. Finally, we used robust correlation to investigate continuous associations between continuous E-selectin levels and continuous traits to limit the potential effects of outliers. Similar robust correlations were used to investigate the continuous association of E-selectin and other characteristics with CMIT, and the potential effects of extraneous factors adjusted for in robust linear regression models. Results corresponding to *P* values below 5% are described as significant. We did not adjust for multiple testing.

6.3. Results

The study cohort consisted of 308 participants. Sixty-six (21%) were male and 242 (79%) female. As there was no significant gender difference between E-selectin levels (median in males 129.3 μg/L versus 134.1 μg/L in females, p=0.644), the study participants were subgrouped into those with hyperglycaemia and those with normoglycaemia. The demographic data of subjects is shown in Table 6.1 overall and by glycaemic status. E-selectin levels were significantly higher in hyperglycaemic than in normoglycaemic participants (median 139.8 μg/L vs. 118.8 μg/L, p = 0.0007). Significant differences between the two glycaemic groups were found for age, HOMA-IR, fasting blood glucose, 2-h blood glucose, fasting insulin levels, HbA1c, CRP, TG, HDL-c, BMI, WHR and systolic blood pressure (SBP) (all $p < 0.05$) With no significant differences for CIMT, LDL-c and total cholesterol. BMI was raised in both groups, but much more so in the hyperglycaemic group ($p<0.0001$).

Table 6.1: Results according to glycaemic status (Mann-Whitney *U* test for non-parametrics)

	Total (N=308)	Normal (N=142)	Hyperglycaemia (N=165)	
	Median (P25, P75)	**Median (P25, P75)**	**Median (P25, P75)**	**p-value**
Age (years)	57 (47, 64)	53 (45, 63)	59 (51, 65)	**0.0004**
E-selectin (µg/L)	133.2 (100.2, 171.1)	118.8 (83.0, 156.9)	139.8 (108.5, 182.8)	**0.0007**
Total CIMT (mm)	0.88 (0.73, 1.07)	0.85 (0.72, 1.03)	0.90 (0.75, 1.08)	0.0918
HOMA-IR	2.9 (1.6, 5.1)	2.1 (1.0, 3.3)	3.9 (2.2, 7.4)	< **0.0001**
Fasting blood glucose (mmol/L)	5.6 (5.0, 7.3)	5.0 (4.6, 5.3)	7.0 (6.0, 9.4)	< **0.0001**
2-h glucose (mmol/L)	6.5 (5.4, 8.5)	5.6 (4.9, 6.3)	8.9 (8.0, 12.2)	< **0.0001**
Fasting insulin (uU/L)	11.0 (6.2, 17.5)	9.7 (5.0, 15.1)	12.3 (7.0, 19.8)	**0.0036**
HbA1c (%)	6.0 (5.7, 6.7)	5.7 (5.5, 6.0)	6.6 (6.1, 7.9)	< **0.0001**
C-reactive protein (mg/L)	5.6 (1.9, 10.3)	4.7 (1.3, 9.4)	6.2 (2.5, 10.4)	**0.0476**
TG (mmol/L)	1.4 (1.0, 1.9)	1.3 (0.9, 1. 7)	1.5 (1.2, 2.0)	< **0.0001**
HDL-c (mmol/L)	1.3 (1.1, 1.5)	1.4 (1.1, 1.7)	1.2 (1.1, 1.4)	**0.0014**
LDL-c (mmol/L)	3.4 (2.8, 4.2)	3.4 (2.7, 4.1)	3.5 (2.8, 4.3)	0.4747
Cholesterol (mmol/L)	5.6 (4.9, 6.4)	5.5 (4.9, 6.3)	5.6 (4.8, 6.4)	0.7755
Height (m)	1.6 (1.5, 1.6)	1.6 (1.5, 1.6)	1.6 (1.5, 1.6)	0.5541
Weight (kg)	78.4 (67.4, 92.1)	71.5 (61.1, 82.5)	83.1 (74.3, 95.9)	< **0.0001**
Body mass index (kg/m^2)	31.7 (27.1, 36.3)	28.5 (24.1, 33.5)	33.1 (29.3, 39.2)	< **0.0001**
Waist circumference (cm)	99.6 (89.0, 109.0)	93.3 (82.0, 103.1)	103.0 (95.8, 114.5)	< **0.0001**
Hip circumference (cm)	109.1 (101.0, 120.0)	105.1 (97.0, 113.5)	113.5 (105.0, 124.7)	< **0.0001**
Waist-to-hip ratio	0.88 (0.84 - 0.94)	0.87 (0.82 - 0.92	0.90 (0.85 - 0.96)	**0.0002**
SBP (mmHg)	132 (119, 150)	128 (116, 145)	136 (123, 152)	**0.0066**
Diastolic blood pressure (mmHg)	80 (72, 89)	79 (72, 88)	82 (71, 90)	0.4096

Bold denotes statistical significance

Across quarters of E-selectin levels, significant differences were apparent in the distribution of age, glycaemic status, fasting glucose and HbA1c, CRP, HDL and total cholesterol. A significant decreasing trend across quarters of E-selectin was observed for age, hyperglycaemia, HDL-c and total cholesterol, while an increasing trend was observed for fasting glucose and HbA1c, CRP, and waist and hip circumferences. Furthermore, there was a continuous negative correlation between E-selectin levels and age, and HDL, LDL and total cholesterol; while a significant positive correlations were observed with hyperglycaemia, fasting glucose and HbA1c, HOMA-IR and waist circumference. In robust linear regression analyses adjusted for age, gender and glycaemic status; age, hyperglycaemia, CRP and, to a lesser extent, female gender were the significant determinants of E-selectin levels (Table 6.2).

Table 6.2: Baseline characteristics across quarters of E-selectin

Characteristic	Quarters of E-selectin				P difference*	P-trend	r (95% CI)**	β (p-value)***
	Q1	Q2	Q3	Q4				
n	60	60	60	61				
E-selectin (µg/L)								
Range	1.00-105.55	105.96-136.66	136.73-174.43	174.45-426.52				
Median	76.72	120.06	152.33	211.02				
Females, n (%)	51 (85)	45 (75)	51 (85)	45 (74)	0.238	0.305	-0.10 (-0.23 to 0.02)	-15.28 (0.063)
Hyperglycaemia, n (%)	27 (45)	33 (55)	38 (63)	43 (70)	0.029	0.003	0.25 (13 to 0.37)	27.52 (<0.0001)
Age (years)	61.5 (51.0-69.2)	58.5 (51.0-66.0)	58.5 (47.7-64.0)	52.0 (47.0-58.0)	0.001	<0.0001	-0.21 (-0.33 to -0.09)	-1.44 (<0.0001)
CIMT (mm)	0.90 (0.72-1.10)	0.90 (0.73-1.10)	0.88 (0.77-1.05)	0.86 (0.70-1.03)	0.768	0.555	-0.01 (-0.14 to 0.11)	2.62 (0.836)
HOMA-IR	2.59 (1.61-4.04)	3.08 (1.43-5.12)	2.59 (1.63-4.67)	3.26 (1.85-7.48)	0.340	0.159	0.14 (0.02 to 0.27)	-0.37 (0.474)
Fasting blood glucose (mmol/L)	5.45 (4.97-6.50)	5.45 (5.00-6.62)	5.80 (5.00-6.95)	6.10 (5.40-9.30)	0.044	0.008	0.20 (0.08 to 0.32)	0.416 (0.778)
Fasting insulin (µU/L)	10.30 (6.75-14.95)	10.70 (6.27-20.05)	9.50 (5.70-17.12)	12.40 (6.90-17.90)	0.714	0.447	0.09 (-0.03 to 0.22)	-0.118 (0.484)
HbA1c (%)	6.00 (5.77-6.22)	6.00 (5.70-6.42)	6.10 (5.77-6.60)	6.40 (5.80-7.30)	0.054	0.019	0.18 (0.06 to 0.30)	1.91 (0.389)
HbA1c (mmol/mol)	42.1 (39.9-44.3)	42.1 (38.8-46.4)	43.2 (39.9-48.6)	46.4 (39.9-56.3)				
CRP (mg/L)	3.20 (1.07-6.32)	5.70 (1.87-10.10)	5.70 (2.52-10.40)	6.90 (3.80-11.40)	0.002	0.0003	0.20 (0.07 to 0.32)	0.83 (0.017)
TG (mmol/L)	1.32 (1.03-1.72)	1.33 (1.00-1.72)	1.52 (1.22-1.89)	1.39 (0.99-1.74)	0.396	0.519	0.06 (-0.06 to 0.19)	-3.09 (0.417)
HDL-c (mmol/L)	1.37 (1.17-1.64)	1.31 (1.06-1.53)	1.21 (1.05-1.52)	1.20 (1.06-1.36)	0.033	0.005	-0.20 (-0.32 to -0.08)	-4.54 (0.565)
LDL-c (mmol/L)	3.76 (2.96-4.42)	3.48 (2.89-4.08)	3.70 (2.94-4.21)	3.05 (2.63-4.10)	0.173	0.079	-0.13 (-0.25 to -0.01)	-3.66 (0.335)
Cholesterol (mmol/L)	5.90 (5.19-6.47)	5.49 (5.04-6.36)	5.76 (5.30-6.36)	5.19 (4.52-5.95)	0.026	0.018	-0.17 (-0.29 to -0.05)	-4.39 (0.187)
BMI (kg/m^2)	30.60 (26.47-34.67)	32.15 (28.95-35.75)	31.55 (27.65-38.65)	32.70 (28.20-38.90)	0.326	0.099	0.09 (-0.04 to 0.22)	0.480 (0.158)
Waist circumference (cm)	96.7 (84.4-104.1)	100.05 (93.4-108.0)	101.7 (93.2-108.9)	102.1 (92.0-114.5	0.116	0.021	0.16 (0.04 to 0.28)	0.228 (0.346)
Hip circumference (cm)	107.4 (98.1-116.0)	113.0 (102.0-120.9)	109.0 (105.1-121.2)	113.0 (103.0-122.7)	0.173	0.049	0.11 (-0.01 to 0.24)	0.350 (0.110)
WHR	0.88 (0.85-0.93)	0.87 (0.83-0.93)	0.88 (0.84-0.94)	0.89 (0.83-0.93)	0.821	0.626	0.11 (-0.02 to 0.23)	-27.74 (0.517)
SBP (mmHg)	134 (121-156)	135 (124-150)	133 (118-148)	130 (119-145)	0.526	0.161	-0.08 (-0.20 to 0.05)	-0.159 (0.196)
Diastolic blood pressure (mmHg)	80 (74-90)	81 (70-89)	79 (72-86)	80 (72-87)	0.932	0.564	-0.02 (-0.15 to 0.11)	-0.304 (0.215)

***** P-values from Kruskal-Wallis and chi square tests for the differences across quarters of E-selectin

** Robust correlation coefficients and 95% confidence intervals for the continuous associations of E-selecting with covariates

*** β-coefficients and p-values from age, gender and status for hyperglycaemia adjusted robust linear regressions for the prediction of E-selectin levels by various traits.

The correlation coefficients and 95% confidence intervals from robust correlation analyses for the relationship of CIMT with covariates in the overall as well as separately by glycaemic status are shown in Table 6.3. In the overall sample, CIMT was significantly and positively correlated with age, fasting blood glucose, HbA1c, WHR and SBP. In stratified analyses by glycaemic status, the correlations remained positive and significant in normoglycaemic and hyperglycaemic groups for age, HbA1c, and SBP, with indications that the magnitude of the effect was more important in the hyperglycaemic group for age, but not for HbA1c and SBP. The correlation with fasting glucose was significant only in the normoglycaemic group, while the correlation with AHR was significant only in the hyperglycaemic group. In both cases however, differences in the correlations across both subgroups were not significant. In stratified analyses, we further observed significant positive correlations in the hyperglycaemic group between CIMT, CRP, TG and total cholesterol. A significant correlation was observed in the normoglycaemic group between CMIT and diastolic blood pressure. With the exception of CRP where a borderline difference was apparent, all other correlations were of similar magnitude in normoglycaemic and hyperglycaemic participants. E-selectin, HOMA-IR or BMI were not correlated with CIMT.

Table 6.3: Robust Correlation of CIMT with E-selectin and other variables

Correlates	Overall (n=241)	Normoglycaemia (n=141)	Hyperglycaemia (n=100)	P-value
E-Selectin (μg/L)	-0.01 (-0.14 to 0.11)	-0.05 (-0.22 to 0.11)	-0.05 (-0.25 to 0.15)	0.98
Age (years)	0.41 (0.30 to 0.51)	0.28 (0.12 to 0.43)	0.55 (0.39 to 0.67)	0.02
Fasting blood glucose (mmol/L)	0.14 (0.01 to 0.26)	0.16 (0.00 to 0.32)	0.03 (-0.17 to 0.22)	0.29
Fasting insulin (μU/L)	0.02 (-0.11 to 0.14)	0.07 (-0.09 to 0.24)	-0.06 (-0.25 to 0.14)	0.31
HOMA-IR	0.07 (-0.05 to 0.20)	0.14 (-0.03 to 0.30)	-0.06 (-0.25 to 0.14)	0.14
HbA1c (%)	0.20 (0.08 to 0.32)	0.23 (0.06 to 0.38)	0.20 (0.00 to 0.38)	0.83
CRP (mg/L)	0.06 (-0.06 to 0.19)	-0.05 (-0.22 to 0.11)	0.19 (0.00 to 0.38)	0.06
TG (mmol/L)	0.10 (-0.02 to 0.23)	0.07 (-0.10 to 0.23)	0.15 (0.05 to 0.33)	0.54
HDL-c (mmol/L)	0.02 (-0.10 to 0.15)	0.01 (-0.16 to 0.17)	0.10 (-0.11 to 0.28)	0.88
LDL-c (mmol/L)	0.06 (-0.07 to 0.19)	-0.01 (-0.18 to 0.15)	0.16 (-0.04 to 0.35)	0.19
Cholesterol (mmol/L)	0.09 (-0.03 to 0.22)	0.01 (-0.15 to 0.18)	0.22 (0.03 to 0.40)	0.10
BMI (kg/m^2)	-0.03 (-0.15 to 0.10)	-0.10 (-0.26 to 0.06)	0.02 (-0.17 to 0.22)	0.34
Waist circumference (cm)	0.004 (-0.12 to 0.13)	-0.11 (-0.27 to 0.05)	0.07 (-0.13 to 0.26)	0.17
Hip circumference (cm)	-0.11 (-0.24 to 0.01)	-0.23 (-0.38 to 0.06)	-0.04 (-0.23 to 0.16)	0.15
WHR	0.18 (0.06 to 0.30)	0.15 (-0.01 to 0.31)	0.19 (0.00 to 0.38)	0.75
SBP (mmHg)	0.27 (0.15 to 0.38)	0.25 (0.09 to 0.40)	0.29 (0.09 to 0.46)	0.77
Diastolic blood pressure (mmHg)	0.11 (-0.02 to 0.23)	0.18 (0.01 to 0.31)	0.05 (-0.14 to 0.25)	0.34

The results of robust linear regressions are shown in Table 6.4. In a basic model containing age, gender, WHR and glycaemic status, only age and gender were significantly associated with CIMT. This basic model explained 20.2% of variations in CIMT. In the presence of variables in the basic model, E-selectin was not significantly associated with CIMT and no change was observed in the overall performance of the model. In an expanded basic model to include SBP and CRP, age, gender and SBP were the significant predictors of CIMT. The resulting model explained 21.3% of variations in CIMT. In the presence of variables in this expanded model, E-selectin was still not associated with CIMT and not change was observed in the overall performance of the model.

Table 6.4: Regression coefficients from multiple robust linear models for the prediction of CIMT by E-Selectin accounting for the potential effect of gender, age, hyperglycaemia and adiposity

	E-selectin alone		Basic covariates alone		Basic covariates and E-selectin		Expanded covariates alone		Expanded covariates and E-selectin	
	β	p	β	p	β	p	β	p	β	p
E-selectin (μg/L)	-0.0001	0.607	-	-	<-0.00001	0.992	-	-	<-0.0001	0.986
Age (years)	-	-	0.0821	<0.0001	0.0820	<0.0001	0.0075	<0.0001	0.0075	<0.0001
Gender (females)	-	-	-0.1518	0.0002	-0.1580	0.0002	-0.1537	0.0003	-0.1547	0.0003
WHR	-	-	0.0543	0.786	0.0524	0.795	0.16060	0.449	0.1567	0.463
Hyperglycaemia	-	-	0.0449	0.144	0.0449	0.158	0.0419	0.193	0.0412	0.216
SBP (mmHg)	-	-	-	-	-	-	0.0013	0.045	0.0013	0.050
CRP (mg/L)	-	-	-	-	-	-	0.0020	0.242	0.0021	0.213
R^2	0.001		0.202		0.202		0.213		0.213	

6.4. Discussion

The adhesion of circulating leukocytes to endothelial cells plays an important role in the initiation of atherosclerosis (Beckman et al. 2002; Ross 1993). Cellular adhesion molecules are poorly expressed by the resting endothelium, but are upregulated during endothelial activation associated with atherogenesis (Hope & Meredith 2003a; Jang et al. 1994). Soluble forms of cellular adhesion molecules can be found in plasma, and it has been suggested that elevated plasma levels of some cellular adhesion molecules may be an index of endothelial activation (Constans & Conri 2006) or even a molecular marker of early atherosclerosis (Ridker et al. 1998; Hope & Meredith 2003b).

The purpose of this study was to examine the relationship between E-selectin levels, CIMT and cardio-metabolic traits in normo-and hyperglycaemic mixed ancestry South Africans. Although we found that although E-selectin levels were raised in hyperglycaemic subjects, no relationship was observed between these E-selectin levels and CIMT. These results suggest that the measurement of E-selectin in this population for the purpose of CVD risk stratification is likely futile. Indeed, subclinical atherosclerosis as measured by CIMT showed significant associations only with traditional risk factors, such as age and gender and blood pressure.

Similar to our observations, Leionen et al found that E-selectin concentrations were higher in the diabetics, but not associated with CIMT (Leinonen et al. 2004). In contrast to these results several studies have reported a significant association between E-selectin concentrations and CIMT. A study by Delibasi et al found that in subjects with non-functional adrenal incidentalomas, E-selectin concentrations correlated significantly with both HOMA-IR and CIMT (Delibasi et al. 2015) while Baldassarre et al, who examined subjects with only CVD but no diabetes, found that statin therapy significantly decreased both CIMT and E-selectin concentrations indicating the treatment's ability to improve endothelial dysfunction (Baldassarre et al. 2009). In another study of 505 Japanese males, Sakurai et al reported that E-selectin concentrations were significantly associated with CIMT and suggested its usefulness as a biomarker for unstable plaques and future CVD prediction (Sakurai et al. 2009). Furthermore, Vaidy et al reported that E-selectin was associated with CIMT independent of metabolic syndrome (Vaidya et al. 2011). The MIAMI study, which examined the relationship between various circulating markers of inflammation and CIMT,

found that E-selectin was strongly correlated to atherosclerotic burden and CIMT, and inversely correlated to HDL-c (Porta et al. 2008).

Obesity, type 2 diabetes and atherosclerosis are associated with underlying inflammation (Wellen & Hotamisligil 2005; Monteiro & Azevedo 2010; Hotamisligil 2006; Lontchi-Yimagou et al. 2013). This pro-inflammatory status leads to increased secretion of TNFα and IL-6 which stimulate the synthesis of E-selectin (Hope & Meredith 2003a; Constans & Conri 2006). In the case-cohort study by the Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA)/Cooperative research, males and females with elevated concentrations of E-selectin demonstrated a significantly higher risk of type 2 diabetes after multivariate analysis (Thorand et al. 2006).

Ryysy and Yki-Järvinen measured E-selectin concentrations in type 2 diabetics before and after initiation of insulin therapy for intensive glycaemic control and described that before therapy, E-selectin levels were 71% higher in diabetics than in controls but that these levels decreased with improved glycaemic control (Ryysy & Yki-Järvinen 2001). Leinonen et al measured adhesion molecules including E-selectin in type 2 diabetics and found that concentrations correlated with HbA1c and inversely with HDL-c (Leinonen et al. 2003). A small study by Boulbou et al examined 18 type 2 diabetes with peripheral arterial disease, 18 type 2 diabetics without peripheral arterial disease and 22 controls, and found that diabetics with peripheral arterial disease had significantly increased E-selectin concentrations which correlated with duration of disease and insulin resistance (Boulbou et al. 2004). Elhadd described increased E-selectin concentrations in type 2 diabetics (Elhadd et al. 2004) and Cominacini described higher E-selectin concentrations in type 2 diabetics were dependent on glycaemic control and postulated that oxidative stress associated with poor glycaemic control may upregulate E-selectin concentrations (Cominacini et al. 1997).

HDL-c has been found to inhibit the formation of adhesion molecules (Barter et al. 2004). In support, we found a significant inverse correlation between E-selectin concentrations and HDL-c in the group as a whole but none in separate groups. Leionen et al proposed that E-selectin may influence HDL-c concentrations by influencing phospholipase A_2, which affects paraoxonase activity and thus the reverse cholesterol transport system. Additionally phospholipase A_2 decreases LDL-c size, making LDL-c more atherogenic (Leinonen et al. 2003). In cell culture studies, Nofer et al (Nofer et al. 2003) found that HDL-c inhibited the

cytokine –induced expression of E-selectin and Cockerill et al (Cockerill et al. 2001) also determined that this inhibitory effect may be due to its inhibition of IL-1.

We found an inverse correlation between E-selectin levels and age which was unexpected. However Ponthiex et al performed and age-related study of reference values for E-selectin in 2003 and found that although E-selectin concentrations decreased steadily during childhood, they were not influenced by age in adulthood (Ponthieux & Herbeth 2003). It is also important to note that although E-selectin concentrations differed significantly between the two groups, the concentrations were increased in all the participants. Our findings of higher baseline E-selectin levels may be due to participants having higher BMIs, and all had relatively increased CIMT. Additionally, Constans and Conri advocate caution when interpreting E-selectin results due to large interindividual variation of results (Constans & Conri 2006).

Our study has some limitations, namely the cross-sectional nature precluding inferences about causality. The study sample was relatively small, likely compromising our statistical power to uncover some significant association. We only determined E-selectin concentrations and did not examine other adhesion molecules. The reference intervals for E-selectin were determined by the manufacturer in normal subjects who were not described and may have differed from our population. Additionally, although the manufacturers claimed that serum samples for E-selectin determination are stable at -70°C, we did not formally verify this. Furthermore, the study participants were almost uniformly obese. Such a uniform population could narrow the spectrum of some covariates investigated in the current study and therefore mask some significant associations. However, our study also has major strength including the assessment of a wide range of cardio-metabolic risk factors, and the use of robust analytic technics to address and efficiently deal with possible effects of outliers which could distort the associations, particularly in the context of a modest sample size.

6.5. Conclusion

In conclusion, although we found higher E-selectin concentrations in hyperglycaemic subjects which may be indicative of the underlying endothelial dysfunction in hyperglycaemia, we found that levels of soluble E-selectin did not correlate with CIMT, a surrogate marker of early atherosclerosis. This is despite the fact that E-selectin is endothelium specific and is a marker of endothelial dysfunction. However, in our cohort, all participants were overweight, all had relatively high E-selectin concentrations and all had

median CIMT above the recommended cut-off of 0.8mm. This supports previous findings that this is likely a population at high risk for CVD and this may be due to underlying endothelial dysfunction. Further longitudinal studies are warranted in this population to establish the prognostic value of our findings.

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CHAPTER 7

INTEGRATED CONCLUSIONS AND SUMMARY

7.1 CONCLUSION

The aim of this project was to determine if newly-recommended ADA HbA1c cut-offs to detect DM and prediabetes were valid in our local population and to establish the usefulness of new novel biomarkers to detect endothelial dysfunction and adiposity in this same population. All four substudies were performed on participants of the Bellville South Africa study, a cross-sectional quantitative study on mixed ancestry participants from the Western Cape in South Africa.

Noncommunicable diseases (NCD) are rapidly overtaking the traditional infectious disease burden such as HIV and TB and becoming more problematic in sub-Saharan Africa (Kengne et al. 2013). Due to increasing obesity and urbanization in South Africa, DM and CVD are rapidly contributing to what is being called the "quadruple burden of disease". In South Africa, the mixed ancestry population has been found to have a particularly high prevalence of DM and 30 year CVD risk (Erasmus et al. 2012; Matsha et al. 2012). As the early detection and possible prevention of NCD is a healthcare priority, this study was undertaken to examine whether the suggested HbA1c cut-offs to detect DM and prediabetes were appropriate in our population and to establish the possible value of two novel biochemical markers of CVD as a potential early indicator of cardio-metabolic risk in subjects with hyperglycaemia. Even though the cost of the assays for these two markers is currently too high to justify their introduction into routine care, if it can be shown that they are good markers and have the possibility to prevent further morbidity and mortality, their use may be justified.

The introduction of HbA1c as a diagnostic tool for DM and prediabetes by the ADA in 2009 (American Diabetes Association 2010) was met with much enthusiasm, as this test requires minimal inconvenience to patient such as fasting and is easy to perform in most big laboratories with a good reproducibility due to recent advances in standardization of the assay. However, previous studies have described ethnic differences in HbA1c levels (Herman et al. 2007; Herman et al. 2009; Tsugawa et al. 2012; Ziemer et al. 2010) and have suggested that the cut-offs be validated in different populations before their implementation. Additionally, conditions which influence the survival of red blood cells such as iron deficiency anaemia, a common problem in developing countries, may give false results. Also, Africa is known to have a high incidence of haemoglobinopathies which may affect results.

We have demonstrated that the HbA1c cut-offs determined to be diagnostic for DM and prediabetes by the ADA cannot be reliably used in our population (Zemlin et al. 2011; Zemlin et al. 2015). This emphasizes the need for evidence-based values to be verified in various populations before they can just be accepted and introduced into routine patient care guidelines. Although the ADA recommended an HbA1c cut-off of 6.5% to diagnose DM, we performed our study on a large number (946) of subjects and found that the recommended cut-off has a low sensitivity in our population, limiting its use as a screening tool. Using this recommended cut-off, about half of subjects diagnosed as diabetics using either OGTT or fasting blood glucose would have been missed. We determined that a level of 6.1% had the best sensitivity and specificity to detect DM.

As prediabetes can precede the onset of DM by several years and is in itself associated with CVD risk (Buysschaert et al. 2011; Buysschaert et al. 2014), its detection is important to allow the implementation of therapeutic lifestyle changes to possibly prevent or delay the onset of DM. The ADA suggested HbA1c cut-offs of 5.7%-6.4% to detect prediabetes. Using 3 different samples (a derivation and two validations samples), and utilising ROC curves, we found an HbA1c cut-off of 5.75% gave the best sensitivity and specificity (Zemlin et al. 2015). Based on these observations we concluded that the use of HbA1c alone may give an inaccurate diagnosis of prediabetes. This is in agreement with previous studies (Bersoux et al. 2011; Bhowmik et al. 2013; Coppell et al. 2013; Guo et al. 2014; Jeon et al. 2013; Kharroubi et al. 2014; Succurro et al. 2011; Sun et al. 2013; Zhou et al. 2009; Ziemer et al. 2010) which found that HbA1c was not accurate to diagnose prediabetes. Indeed, Yudkin and Montori questioned whether prediabetes was not in fact being overdiagnosed since the introduction of HbA1c as a screening tool (Yudkin & Montori 2014) and a recent meta-analysis found that we may be placing unnecessary importance on prediabetes, as up to half the subjects diagnosed with prediabetes due to IGT do not develop DM within a 10 year follow up period (Morris et al. 2013). This is important as we do not want to place an extra burden on our strained healthcare environment by incorrectly over-diagnosing and following up people.

As discussed, the increase in the prevalence of DM in sub-Saharan Africa is mainly due to the increase in obesity (Baleta & Mitchell 2014). Adipose tissue is no longer considered to be just an inert reservoir for storage of excess fat, but has been found to be an active endocrine organ secreting numerous “adipokines” (Adamczak & Wiecek 2013; McGown et al. 2014). The most abundant of these, adiponectin, is decreased with obesity due to inhibition of its synthesis by inflammatory cytokines and has anti-inflammatory, anti-diabetic and anti-

atherogenic properties (Balsan et al. 2015; Gable et al. 2006; Lee & Kwak 2014; Litvinova et al. 2014; Rabin et al. 2005; Yadav et al. 2013). We determined that the active high molecular weight form of adiponectin, which reportedly is the active form, was not affected by two common polymorphisms of the adiponectin gene, and that these levels were unaffected by glycaemic status (Annalise E Zemlin et al. 2016). However, levels correlated significantly with HDL-c levels, which is in agreement with previous studies that have described a significant association between the beneficial HDL-c and adiponectin (Ferris et al. 2005; Oku et al. 2007; Suriyaprom et al. 2014; Van Linthout et al. 2010). Unfortunately, due to prohibitive costs of the hmw-adiponectin assay, this was a relatively small cross-sectional study. Further larger longitudinal studies may be required to determine the prognostic benefit of this marker.

As DM is considered a CVD risk equivalent (Lorenzo et al. 2007) and this population has previously been found to already have a high 30-year CVD risk (Matsha et al. 2012), one of our studies was to determine if the novel marker of endothelial dysfunction, E-selectin, differed in normo- versus hyperglycaemia and whether these values correlated with carotid intima-media thickness (CIMT), a non-invasive marker of subclinical atherosclerosis (De Groot et al. 2008). As expected, we found that E-selectin levels were significantly higher in hyperglycaemia, but surprisingly, these levels did not correlate with CIMT (A. E. Zemlin et al. 2016). This finding is important as it provides evidence that the introduction of this new expensive test in our population will be of little value and indeed may actually waste limited resources. Further longitudinal studies are again needed to determine if this marker has any prognostic value.

However, it must be noted that our population was generally overweight with both cases and controls having BMI in the overweight cut-off, and this may have affected the results of the last two studies. Additionally they demonstrated a central obesity pattern and visceral fat is known to influence adiponectin levels. The hmw-adiponectin levels may have been decreased in our controls as well as the cases due to this, and the controls may have had falsely increased E-selectin levels. Additionally, when we examined the CIMT, it is of interest to note that both our cases and controls had a median value of more than the recommended cut-off of 0.8mm, indicating underlying subclinical atherosclerosis even in our controls. These findings corroborate the previous studies on this population describing their high CVD risk and may have affected the results of the last two studies.

7.2 SUMMARY AND FUTURE DIRECTIONS

Based on the findings presented in this thesis, the results of our study have added knowledge and evidence to the diagnosis of DM and prediabetes in South Africa. Cut-offs published in the literature always need to be validated in local populations before their routine introduction into clinical practice. This is an important aspect of evidence-based laboratory medicine. We found that the cut-offs suggested by ADA were not ideal in our population. This is especially important where cut-offs have been established in populations different to the one being studied. Using non-validated cut-offs would lead to the incorrect diagnoses and unnecessary costs would be incurred by working up incorrectly diagnosed patients. Additionally we have shown that even though novel biomarkers are described as useful in the literature and optimistically welcomed as a potential new diagnostic marker, studies always need to be performed to verify whether the same results are found in different populations. New assays are often introduced at great cost and may not actually be beneficial to the population being served.

It was of concern that we found that this population is at a high risk of DM and CVD and almost all were overweight. The population needs to be made aware of this problem and educated, as well as the relevant healthcare providers and health authorities. The next step should be to implement educational programmes and interventions to ameliorate this obesity and DM problem in this community. This should be the aim of future studies in this community.

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APPENDICES

INFORMATION SHEET

Cape Peninsula

University of Technology

THE CARDIOVASCULAR DISEASE STUDY

INFORMATION SHEET

Invitation to participate in the study

We would like to invite members of the Bellville-South community who participated in a previous research project which determined the prevalence of diabetes to take part in this study. This phase of research is investigating cardiovascular disease (heart diseases) in Bellville South. The study will be conducted by Cape Peninsula University of Technology in conjunction with the University of Stellenbosch.

What is the study about?

The incidence of cardiovascular diseases is increasingly common in our communities. Some of the underlying risk factors for developing cardiovascular disease are hypertension (high blood pressure), diabetes, high cholesterol as well as being overweight. Following the previous research conducted by the Cape Peninsula University of Technology and University of Stellenbosch on diabetes in this community, these factors were found to be highly prevalent. Often people are not aware that they have some of these risk factors. The purpose of this research project is to provide an opportunity for early detection of people that have cardiovascular diseases or are at risk. Cardiovascular diseases are

diseases that affect the function of the heart and the blood vessels. Usually before one gets a heart attack, he/she develops signs and symptoms suggesting that in future you might get a heart attack.

Benefits of Participating in this Study

As one grows older, the chances of developing heart diseases also increase. Since you are aged 20 years or older, it would be to your benefit to have your heart examined since you would be informed of the outcome. You would also be advised by professionals on lifestyle changes that would delay the onset and/or prevention of heart disease. For those who will be found to have heart diseases, they will be referred for further examination and follow-up.

What will I need to do?

Should you be selected and agree to take part in the project:

- You will be asked to go to a central data collection site at…….. (between 7 – 9 am) of any day of the week (Monday-Friday) whichever day is convenient to you.
- At the data collection site, you will be asked questions regarding your general household information, family health history and those related to your heart health. This will take approximately ½ an hour to complete.
- A nursing sister will then take your blood pressure, weight, height, and also measure the amount of fat in your body.
- Radiologists will then check and evaluate your heart health using a special machine for which you will be informed of the outcome. This procedure will be performed in privacy and will require you to expose your chest.

Participation in this study will be voluntary and cost free, except for your time. All information will be confidential. Selection into the study will be on the basis that one participated in the diabetes survey and is also resident in Bellville South. If you don't meet the selection criteria, but would also wish to have your heart health status determined, that can be arranged with the research staff.

Do you have any questions?

If you need more information regarding the project, please feel free to contact the following people during office hours:

1. Prof. Tandi Matsha Tel. 0219596366
2. Mrs. B. George Cell Phone 0729581877
3. Mr. M.S Hassan Tel. 021 9596274
4. Prof. R.T, Erasmus Tel. 021 938 4107
5. Mr J.D. Soita Cell phone 0725558628

CONSENT FORM

THE BELLVILLE, SOUTH AFRICA CARDIOVASCULAR STUDY CONSENT FORM

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Mr MS. Hassan – 021 959 6274

Prof. RT. Erasmus – 021 938 4107

Prof. T. Matsha – 021 460 3209

Dear Participant,

You are invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate.

If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the Health and Wellness Sciences Research Ethics Committee at the Cape University of Technology and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. For research to be carried out on certain diseases it is necessary to first establish the incidence and prevalence of the disease. This project aims to determine the incidence, prevalence as well as factors which are predictive of cardiovascular disease. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Why have you been invited to participate?

The prevalence of cardiovascular disease in South Africa is not well documented and few studies have been conducted since 1994. Many subjects with cardiovascular disease are unknown to the health service, often because they are not yet diagnosed. In order to assess the magnitude of the problem, you have been approached to participate in this project to determine the incidence of cardiovascular disease amongst our adult population.

You have been selected since you previously participated in a diabetes study and that you were aged between 35-65 years at the time. You also meet other criteria used to select participants into this study.

What will your responsibilities be?

The participant will be requested to provide information about his/her medical history with specific emphasis on cardiovascular disease. You will also be asked to complete a questionnaire which will take no longer than 10 minutes. A painless procedure known as electrocardiography (ECG) will then be performed to check the health of your heart. No pharmaceutical agents (medication) will be tested in the study.

Will you benefit from taking part in this research?

You will be notified of your cardiovascular status by the medical nurse or doctor. Thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment if necessary.

In the unlikely event that the research may lead to the development of commercial applications, the participant or the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to me/the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc.

Are there any risks involved in my taking part in this research?

There are no risks involved when you take part in this study since it will not involve any invasive procedure.

Who will have access to your medical records?

The participant's identity will be kept confidential throughout. Information will not be associated with the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant.

Some insurance companies may mistakenly assume that taking part in research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

Will you or your child be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, but your transport, if required will be covered for each study visit. There will be no costs involved for you if you take part in the project.

Is there anything else that you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study.

You can contact ***Prof Erasmus*** **at Tel 938 4107 or rte@sun.ac.za** if you have any further queries or encounter any problems.

You can also contact the chairperson of Health and Wellness Sciences Research Ethics Committee of the Cape University of Technology at **021 442 6162or** engelhillssp@cput.ac.za if you have any concerns or complaints that have not been adequately addressed by the research staff.

You will receive a copy of this information and consent form for your own records if it is requested

DECLARATION BY PARTICIPANT:

I declare that:

I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.

I have had a chance to ask questions and all my questions have been adequately answered.

I understand that taking part in this study is voluntary and I have not been pressurized to take part.

I may choose to withdraw from the study at any time and will not be penalized or prejudiced in any way.

I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.

I also consent that my information may be:

- **Used and kept for future research studies** ☐
- **Used and discarded** ☐

Signed at (*place*) ..... on (*date*) 20

Signature of participant---------- **Signature of witness----------**

DECLARATION BY THE INVESTIGATOR

I *(name)*…… declare that:

I explained the information in this document to (*Names of*

Participant)……

I encouraged him/her to ask questions and took adequate time to answer them.

I am satisfied that he/she adequately understands all aspects of the research, as discussed above

Signed at (*place*) ..... on (*date*) …….. 2010.

Signature of investigator---------- **Signature of witness**----------