CAROTID ARTERY INTIMA-MEDIA THICKNESS MEASUREMENT IN SUBJECTS WITH TYPE 2 DIABETES IN CAPE TOWN, SOUTH AFRICA

BY

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF CAPE TOWN IN PART FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF PHILOSOPHY IN ENDOCRINOLOGY AND METABOLISM

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Declaration

I declare that this is an original copy of my work, under the supervision of Prof NS Levitt, Head of Division of Endocrinology, Metabolism and Diabetic Medicine, Groote Schuur Hospital, University of Cape Town and Prof AD Marais, Head of the Lipidology Division of the department of Internal Medicine, at the University of Cape Town (UCT) Health Science Faculty.

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DEDICATION

This work is dedicated to my dear husband, David Taiwo Isiavwe, and our four children Ufuoma, Ogaga, Fiona and Runo, for their support and encouragement throughout the duration of this study; My parents; Chief and Chief (Mrs.) F.U.S. JARIKRE, and my siblings too numerous to mention; for their encouragement and prayers, and above all to God Almighty, for His grace and unfailing love, and for creating this unique opportunity for me to study in South Africa.
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ABBREVIATIONS

ACC – American College of Cardiology
ACEI – Angiotensin Converting Enzyme Inhibitor
ADA – American Diabetes Association
AGEs – Advanced Glycation End products
AHA – American Heart Association
AIDS – Acquired Immuno Deficiency Syndrome
apo A – Apolipoprotein A
apo B – Apolipoprotein B
B – Black Subjects
BMI – Body Mass Index
BP – Blood Pressure
CAD – Coronary Artery Disease
CCA – Common Carotid Artery
CET – Cholesteryl Ester Transfer
CHC – Community Health Centres
CHD – Coronary Heart Disease
CIMT – Carotid Intima Media Thickness
CM – Chylomicron
CRP – C Reactive Protein
CVD – Cardiovascular Disease
DCCT – Diabetes Control and Complications Trial
DM – Diabetes Mellitus
EDIC – Epidemiology of Diabetes Intervention and Complications
EDTA – Ethylenediamine tetraacetic acid
FFA – Free Fatty Acid
FMD – Flow Mediated Dilatation

GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase

GFAT – Glutamine Fructose 6 Phosphate Amidotransferase

GFR – Glomerular Filtration Rate

GGE – Gradient Gel Electrophoresis

GlcNAc – N-acetylGlucosamine

GSH – Groote Schuur Hospital

HbA1c – Glycosylated Haemoglobin

HDL – High Density Lipoprotein

HDLc - High Density Lipoprotein Cholesterol

HIV – Human Immunodeficiency Virus

Hs-CRP – Highly sensitive C-Reactive Protein

HT - Hypertension

ICA – Internal Carotid Artery

IDF – International Diabetes Federation

IGFBP-1 – Insulin-like Growth Factor Binding Protein-1

IHD – Ischaemic Heart Disease

IMT – Intima Media Thickness

LDL – Low Density Lipoprotein

LDLc – Low Density Lipoprotein cholesterol

LP - Lipoprotein

LpA – Lipoprotein A

LpB – Lipoprotein B

NADPH – Reduced Nicotinamide Adenine Dinucleotide Phosphate

NB – Non-black subjects

NHANES – National Health and Nutrition Examination Survey
NHLS – National Health Laboratory Service
PCSK9 – Proprotein Convertase Subtilisin/Kexin type 9 Serine protease gene
PKC – Protein Kinase C
PLTP – Plasma Phospholipids Transfer Protein
PVD – Peripheral Vascular Disease
SMC – Smooth Muscle Cell
SSA- Sub Saharan Africa
T2DM – Type 2 Diabetes
TC – Total Cholesterol
Tg – Triglyceride
TNG - Nitroglycerine
UCT – University of Cape Town
UDP - GlcNAc – Uridine Diphosphate-N-Acetyl Glucosamine
UK – United Kingdom
UKPDS – United Kingdom Prospective Diabetes Study
US – United States of America
VLDL – Very Low Density Lipoprotein
WHO – World Health Organisation
WHR – Waist Hip Ratio
Summary

The current prevalence of ischaemic heart disease (IHD) is still low in black Africans in Sub Saharan Africa (SSA) compared with the Western world, largely owing to at least in part owing to a less atherogenic lipid profile. However, there appears to be an upward trend in prevalence among the more educated and wealthier sector of society in SSA, who have adopted a western diet and lifestyle. African Americans who have had extended exposure to risk factors have higher Cardiovascular Disease (CVD) rates than whites in the United States of America. This suggests that the black SSA population may be at an early stage of the epidemiological transition of the CVD epidemic.

Diabetes mellitus too, once considered a rare medical condition in Africans, is now on the increase. Approximately 13.6 million people have diabetes in Africa and it is estimated that approximately 7 million people have diabetes in SSA. CVD is the major cause of mortality in people with diabetes in industrialized societies and is likely to become increasingly important in SSA.

The aim of this study is to test the hypothesis that, for similar duration of diagnosed diabetes (DM); black South Africans have less atherosclerosis as measured by Carotid Intima Media Thickness (CIMT) than non-black South Africans.

METHODS

This is a cross sectional study involving consenting adult subjects with type 2 diabetes. A total of 47 subjects were recruited from both the Groote Schuur Hospital (GSH) diabetes clinic and the Community Health Centres (CHC). They were grouped into black (B) and non-black (NB) subjects: there were 23 B and 24 NB subjects. After consent and completing a questionnaire, the subjects had fasting blood samples collected for measurement of HbA1c, lipogram, LDL particle size, and urine for urinary microalbumin-creatinine ratio. Each subject had anthropometry, blood pressure, CIMT and Flow Mediated Dilatation (FMD) measured.
Results

The black subjects were younger than the non-black subjects but the duration of DM was similar. The mean age for B was 49.4 (SD ± 11.7) years; and 57.8 (SD±8.1) years for NB, \( P = <0.008 \). Mean Duration of DM was 10.9 years in B and 10.3 years in NB. History of macrovascular disease and cardiovascular risk factors were significantly more in the NB cohort: 13 NB had a history of IHD compared to 1 B subject \( (P = 0.003) \). There was an increased trend towards peripheral vascular disease in the NB cohort \( (p = 0.072) \). Cigarette smoking as assessed by pack years, was significantly higher in the NB \( (P = 0.0001) \). Total cholesterol was significantly higher in the NB \( (P = 0.05) \), despite this group’s significantly increased use of lipid modifying agents \( (p \text{ value } 0.02) \). The CIMT and FMD were similar in both groups.

Conclusion

This small study showed no difference in IMT between B and NB subjects, despite increased CVS risk factors, and significantly older age in the non-black subjects. There was, however, a significantly higher prevalence of atherosclerosis complications that may relate to smoking as well as other differences in risk factors in the NB subjects. This data requires confirmation in a larger study population as does the comparison with the respective non-diabetic controls. Additionally, more detailed investigations are required to determine factors that predispose to the complications of atherosclerosis between the two groups.
Chapter 1.

Review of Diabetes and Vascular Disease with Special Reference to the African

1.1 Introduction

Diabetes mellitus (DM) is a silent killer. It cuts across all races, sexes, and age groups. Indeed no part of the world is spared. It is currently estimated that about 246 million people throughout the world are living with diabetes. The number is expected to rise to 333 million by 2025\(^1\), i.e. about 6.3% of the world's population will be living with diabetes, with the majority of the new cases occurring in the developing world, where health systems are ill-equipped to handle the double burden of infectious diseases and chronic diseases. It is estimated that in developed countries, diabetes accounts for 8% of a nation's health budget (World Health Organisation – W.H.O)\(^2\).

Diabetes can be complicated by both microvascular (neuropathy, retinopathy and nephropathy) and macrovascular diseases (peripheral vascular disease (PVD), coronary artery disease (CAD) and cerebrovascular disease). The World Health Organization (WHO) estimates that, annually, about 3.2 million deaths are due to diabetes, i.e. 6 deaths every minute\(^3\). People with type 2 diabetes (T2DM) are at increased risk of fatal and non-fatal macrovascular events. The life expectancy for a 40 year old newly diagnosed T2DM patient is about 8 years shorter than for the general population\(^4\) largely because of the macrovascular events complicating diabetes. Traditional risk factors do not fully explain this increased risk for cardiovascular events. Moreover, coronary events are often silent in diabetic patients. Research has lately focused on improving risk assessment with new tools in an effort to identify individuals at increased risk of cardiovascular events and thus facilitate early intervention.

"Conventional" risk factors for atherosclerosis in diabetes include lipid-rich diet, obesity coupled to energy excess and physical inactivity, and moderate dyslipidaemia (small dense lipoprotein (LDL), hypertriglyceridaemia, and reduced high density lipoprotein (HDL)). Other factors are smoking, hypertension, age, sex and duration of diabetes. These factors individually convey an increased risk for CAD, and the occurrence of multiple risk factors in the same individual further increases the risk by several fold\(^5\). There is increasing recognition of emerging "non-traditional" risk factors for cardiovascular disease (CVD) in
people with diabetes. These include endothelial dysfunction (enhanced vasoconstriction, defective vasodilatation, prothrombotic factors, proinflammatory factors, oxidative stress, and raised homocysteine levels), and sub-clinical atherosclerosis.

The extent of atherosclerosis is difficult to assess for the individual. Several imaging techniques exist for assessing cardiovascular risk. The measurement of carotid intima media thickness (IMT) using high resolution B-mode (bright mode) ultrasonography is a noninvasive method for indirectly determining the anatomic extent of atherosclerosis. Different investigators have demonstrated the reproducibility of this technique, and a strong correlation exists between IMT and classic risk factors like diabetes, hypertension, hypercholesterolaemia, smoking, male sex, and obesity. Several randomized and epidemiological studies demonstrated the value of carotid IMT as a marker of increased risk for cardiovascular events, and some established that in T2DM carotid IMT provides a similar predictive value for coronary events as the Framingham score, and suggest a combination of these two indexes significantly improves risk prediction for these patients.

In the past, despite a high prevalence of coronary heart disease (CHD) risk factors like hypertension, obesity, and smoking in westernized black Africans, CHD remained a rarity. It was even initially thought that black Africans were immune to developing acute myocardial infarction (AMI). The current prevalence of ischaemic heart disease (IHD) is still low in black Africans in Sub Saharan Africa (SSA). They have a less atherogenic lipid profile compared to other ethnic groups. African Americans who have had extended exposure to risk factors have higher CVD rates than whites in the United States of America. There also appears to be an upward trend in prevalence among the more educated and wealthier sector of society in SSA, who have adopted a western diet and lifestyle, leading to suggestions that the black African population in SSA may be at an early stage of the CVD epidemiological transition. A study by Vermaak et al. interestingly suggested that black Africans are biochemically less responsive to an atherogenic diet than whites and that these differences are already present at birth. It is possible that both genetic and environmental (lifestyle) factors could protect B subjects from atherosclerosis and/or its complications. There is little information on clinical and other relevant parameters in atherosclerosis for B and NB subjects in SSA.
1.2 Objectives of this study

This study aims to test the hypothesis that, for a similar duration of diagnosed diabetes; black South Africans have less atherosclerosis as measured by carotid IMT than non-black South Africans.

1. To compare the carotid IMT of diabetic patients of black African ancestry with those of diabetic patients of European and mixed ancestry.

2. To compare the lipid profile and LDL particle size in black diabetic South Africans versus non black diabetic South Africans.

3. To determine the relationship between carotid IMT and several cardiovascular risk factors: FMD (flow mediated dilatation), blood pressure (BP), triglycerides, microalbuminuria, total-to-HDL cholesterol ratio, HDL cholesterol, obesity as assessed by waist to hip ratio, waist circumference, and BMI (body mass index) in diabetic patients of black African and European ancestries.

1.3 Literature Review

1.31 Epidemiology of Diabetes Mellitus in the Developing World

Based on data from the IDF atlas, the prevalence of type 2 diabetes mellitus (T2DM) is on the increase. It is estimated that by 2025 the majority of new cases of T2DM will be in the developing world and this is of major concern for Africa. The increase in T2DM can be attributed to changing lifestyles in developing countries due to adoption of an unhealthy eating pattern, rising rates of physical inactivity, obesity and urbanization. At the heels of the rise in prevalence of diabetes are its complications; both micro- and macrovascular. The great health and socio-economic impact of this rise in prevalence of diabetes mellitus combined with the burden of infectious diseases like HIV/AIDS must be taken seriously. The importance of primary prevention of T2DM and early diagnosis and management of its complications cannot be over emphasized.

1.32 Diabetes Mellitus and Cardiovascular Disease in Africans

Diabetes mellitus, once considered a rare medical condition in Africans, is now on the increase. The estimated prevalence of diabetes mellitus varies from about 1% in rural areas to between 5% and 7% in urban sub-Saharan Africa, and between 8% and 13% in more
developed areas such as South Africa\textsuperscript{13}. The risk factors for diabetes in Africans are not markedly different from those reported in other populations\textsuperscript{14}. The majority of patients (70\% to 90\%) present with typical type 2 DM, while up to 25\% of all diabetics are considered to have type 1 DM. Of those considered to be type 1, it is currently estimated that approximately 15\% may represent atypical presentations of diabetes, especially type 1b or ketosis-prone atypical diabetes (see appendix 7 for definition), and tropical diabetes (see appendix 7 for definition) \textsuperscript{13}. Irrespective of the cause, diabetes results in several complications; amongst which macrovascular and microvascular complications feature prominently.

Macrovascular complications include PVD, cerebrovascular disease and CAD. It is difficult to compare the prevalence of the different macrovascular complications because the reports have not used standard methodologies. Information on several categories will thus be provided.

PVD is on the increase in the region. Literature from Nigeria by Akanji et al showed evidence of PVD in 54\% of 50 Nigerian patients with diabetic foot lesions\textsuperscript{15}. While in Tanzania, Abbas et al showed rates of PVD of 21\% among diabetes patients with foot ulcers\textsuperscript{16}. Other published data on PVD show rates of 4\% to 28\% \textsuperscript{13} and 2.9 to 78.7\% \textsuperscript{17}. The considerable increase in PVD may be in part explained by the use of more sophisticated methods of detection.

As reviewed by Kengne AP et al\textsuperscript{13} although data on cerebrovascular disease in diabetes in SSA was scarce, almost 15\% of patients with stroke had diabetes, while about 5\% of patients with diabetes presented with cerebrovascular accident (CVA) at diagnosis. This scarceness of data was attributed to the associated mortality and lack of proper records of cause of death in Africa. However, data from Tanzania, where a morbidity and mortality surveillance system was set up, showed 3 to 6 fold the prevalence of England and Wales, with 4.4\% of type 2 DM presenting with stroke at diagnosis. About 7.3\% of stroke patients in Burkina Faso, and 8\% of stroke patients in Mauritania had documented diabetes. The prevalence of cerebrovascular disease in a cross-sectional study in the Sudanese diabetic population was 5.5\%, and stroke associated deaths occurred in 7.6\% of a Nigerian cohort of patients with type 2 DM. In a South African study, diabetics were 3 to 4 times more likely to present with a stroke than nondiabetic subjects. Furthermore, subjects with diabetes were at a greater risk of permanent cerebral ischemia without prior warning of a transient event.
than their non-diabetic counterparts. This parallels the fact that diabetics are also prone to having silent myocardial infarction. Cardiovascular disease (CVD) is the major cause of mortality and morbidity in industrialised societies. Although infectious diseases are the commonest cause of death in the developing world, they are set to be overtaken by CVD in the near future. The reason for this is the increasing prevalence of risk factors, including diabetes. It is estimated that two of every three diabetic patients will die as a result of cardiovascular complications, and approximately 30% of patients treated in cardiovascular intensive care units have diabetes. In Africa, history of diabetes mellitus also has a strong relationship to AMI. A study by Ntyintyane LM et al found half of the subjects in their cohort of 40 urbanised black South African subjects with established CAD had previously undiagnosed abnormal glucose regulation; comprising impaired glucose tolerance in 30% and DM in 20% of the subjects. Cardiovascular disease in subjects with diabetes is also associated with severe and extensive coronary artery atherosclerotic lesions and an excess cardiac death.

Cardiovascular disease was once considered rare in sub-Saharan Africa; but recent studies are showing an increasing burden in the region with diabetes mellitus as a major contributor. The prevalence of CHD reaches 5 to 8% in subjects with diabetes, and up to 50% have cardiomyopathy. The INTERHEART Africa study data contradicted the theory that black African people, with exposure to cardiovascular disease risk factors, were immune to developing acute myocardial infarction (AMI). It suggested that black Africans were in the early stages of the cardiovascular epidemic; Risk factors for AMI among Africans were found to be similar to those in other parts of the world. There is a need for treating cardiovascular risk factors in diabetic patients as aggressively as in nondiabetic patients with a prior myocardial infarction. This has been strongly supported by amongst others, the observation by Haffner MS et al who showed that diabetic patients without previous myocardial infarction had as high a risk of myocardial infarction as nondiabetic patients with a previous myocardial infarction. In a study by Kalk WJ and Joffe BJ, risk factors for CHD in 744 subjects with type 2 diabetes subjects of African and European ancestry were evaluated. CHD was thought to be less common in diabetic Africans (4% versus 23% in whites) because of their more favorable lipid profile and lesser degree of insulin resistance. In this study traditional risk factors for CVS disease were significantly less in the black subjects than in the white subjects. These lower levels of traditional risk factors, however, were insufficient to explain the 4-7 fold differences in CVD rates among
the African and White diabetic patients. Among the two groups, subjects with established CHD had a similar risk factor profile, suggesting that diabetic subjects of African ancestry are also susceptible to these CVD risk factors.

Prior to the 1970s, among African American women and men, protective factors such as higher levels of high density lipoprotein cholesterol may have reduced the relative incidence of CHD and AMI, thus resulting in much lower incidence of CHD and cardiovascular related deaths, when compared to non-African American people. By the 1990s, however, with adoption of unhealthy lifestyles, this protection from cardiovascular disease appears to have been almost completely lost in women, and partially lost in men. Elevated systolic blood pressures, higher serum cholesterol levels, history of diabetes, and cigarette smoking have been found to be independent risk factors for CHD in African-American women and men. Current reviews have put to rest many erroneous beliefs or outdated clinical concepts concerning cardiovascular disease among African Americans; for example, that "coronary heart disease is rare or uncommon in African American persons". In fact, Gillum et al. in the NHANES I Epidemiologic follow up study of coronary heart disease in African Americans found that the total incidence of CHD was higher in African-American women aged 25 to 54 years than in white women of the same age and was lower in African American men aged 25 to 74 years than in white men of the same ages; high rates of early death from competing non-coronary causes in African-American men and older African-American women could play a role in the lower rates of coronary death in these groups. In a 20-year follow up of more than 1000 African American women free of CHD, a clear and consistent relationship of medical history of diabetes and CHD incidence and CHD death was established. The population risk attributable to CHD incidence associated with a medical history of diabetes was 8.7% in African American women and 6.1% in European women. Medical history of diabetes was a significant predictor of coronary heart disease incidence and mortality in African American women and explained some of the excess coronary incidence in younger African American compared to European American women. Despite this reduced incidence or protection from cardiovascular disease in African Americans, the Diabetes Heart Study showed that with access to adequate healthcare, African Americans had significantly lower rates of clinical coronary artery disease than whites. It also showed markedly lower levels of coronary and carotid artery calcified plaque among African American men than white men, despite increased carotid artery IMT and conventional risk factors; suggesting that
susceptibility to subclinical cardiovascular disease differs markedly according to ethnicity and sex. Their data also suggested that African Americans may be less susceptible than white to hyperglycaemia-induced macrovascular disease.

1.33 Chronic Complications of Diabetes Mellitus

Diabetic macrovascular complications include PVD, cerebrovascular disease and CAD. Microvascular complications include retinopathy, neuropathy and nephropathy. Hyperglycaemia is believed to be the initiating cause of diabetic tissue damage, with genetic make up, and co-existing medical conditions like hypertension, hyperlipidaemia (figure 1) modifying this process$^{29,30}$.

![Figure 1. General features of hyperglycaemia-induced diabetic tissue damage. From Brownlee M. The pathobiology of diabetic complications A unifying mechanism. Diabetes. 2005; 54:1615-1625.](image)

1.34 Hypotheses for Pathogenesis of Diabetes Complications

Different hypotheses exist for the pathogenesis of diabetic complications. The most important hypotheses are hyperglycaemia, dyslipidaemia with hyperoxidative stress and insulin resistance.
1.341 Hyperglycaemia Hypothesis:

Chronic hyperglycaemia is thought to be the initiating cause of diabetic tissue damage\textsuperscript{29,30}. It is proposed that it involves increased flux through the Polyol pathway, hexosamine pathway, activation of protein kinase C (PKC) and formation of advanced glycation end products (AGEs) (Fig 2). This hypothesis is supported by two landmark studies. The DCCT (Diabetes Control and Complications Trial)\textsuperscript{31} showed that intensive glycaemic control with insulin in subjects with type 1 diabetes reduced the development and progression of diabetes complications by about 50\%. The UKPDS (United Kingdom Prospective Diabetes Study)\textsuperscript{32} showed a reduction in microvascular complications by good glycaemic control in subjects with type 2 diabetes. Hyperglycaemia, however, does not explain all the complications. Good glycaemic control has more impact on microvascular complications than macrovascular complications\textsuperscript{33}.

1.342 Dyslipoproteinaemia And Hyperoxidative Stress Hypothesis:

This theory suggests that “the development of endothelial dysfunction in T2DM is a consequence of the effect of dyslipoproteinaemia, in particular increased circulatory concentrations of modified small dense LDL and of hyperoxidative stress on the formation, action and disposal of nitric oxide, by diverse molecular mechanisms”\textsuperscript{34}. Additionally, hyperglycaemia induces mitochondrial superoxide overproduction and activates the 4 major damaging pathways by inhibiting activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the key glycolytic enzyme (figure 2).

Evidence for this hypothesis includes the fact that reactive oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) are generated during glycoxidation of glucose, and accelerate the production of AGEs. Increased flux through the polyol pathway depletes NADPH (reduced nicotinamide adenine dinucleotide phosphate) leading to a reduction in glutathione-redox cycling. Glutathione redox cycling is thought to be an important mechanism for scavenging free radicals. In addition the use of antioxidants have been shown to reduce many adverse effects of hyperglycaemia on endothelial function e.g reduced endothelial dependent relaxation and delayed cell replication\textsuperscript{35}.

1.343 Insulin Resistance Theory:

It has been suggested that hyperglycaemia and CHD may both have the same origin of hyperinsulinaemia or insulin resistance. Evidence for this hypothesis includes the fact that T2DM and CHD appear to have many similar risk factors such as age, hypertension, dyslipidaemia, and abdominal obesity\textsuperscript{36}. Macrovascular complications are thought to be due to insulin resistance\textsuperscript{29, 30}, leading to increased free fatty acid (FFA) flux from adipocytes
into arterial endothelial cells, and increased FFA oxidation by the mitochondria, with resultant overproduction of reactive oxygen species (ROS), activating the same damaging pathway mechanisms as hyperglycaemia. Increased levels of the glycolytic metabolite, fructose-6-phosphate, leads to increased flux through the hexosamine pathway, with subsequent conversion of Fructose-6-P to Uridine diphosphate-N-acetylglicosamine (UDP-GlcNAc) by the enzyme GFAT (Glutamine Fructose-6-phosphate AmidoTransferase) See Figures 2 and 3.


For microvascular complications, hyperglycaemia is believed to be the major determinant.
1.35 Screening for Cardiovascular Complications in Type 2 Diabetes

The landmark study by Haffner et al.\textsuperscript{21} highlighted the increased risk of CAD in subjects with type 2 DM. Screening for cardiovascular complications in type 2 DM is important to detect disease that may be silent and for guidance on appropriate therapy. This is an important step towards reducing the burden of cardiovascular morbidity and mortality in type 2 DM patients. Various surrogate markers for enhancing prediction of cardiovascular risks in type 2 DM exist; including both traditional and non-traditional risk factors. Several guidelines exist for screening for CHD: the American Diabetic Association (ADA), American College of Cardiology (ACC), American Heart Association (AHA).

The Framingham risk equation\textsuperscript{37} is one of the most widely used models for predicting CAD risk. It utilizes traditional risk factors like age, sex, smoking status, blood pressure, diabetes, and cholesterol. It has a positive predictive value of 75-80\% for 10 year CAD risk. It is limited by the fact that it was based on an almost entirely white population and may not be as accurate when applied to other populations. Furthermore, it has now been surpassed by placing the diabetic into high risk as a coronary risk equation.

The ADA and other associations have recommended treatment goals for traditional modifiable risk factors in patients with type 2 DM; these include goals for blood pressure, LDL cholesterol, non-HDL cholesterol, HDL cholesterol, triglycerides, and smoking status. In addition to the above, the ADA recommends\textsuperscript{38} testing for the following to assess presence of microvascular and macrovascular complications; haemoglobin A\textsubscript{1c}, urine albumin, serum creatinine, dilated fundoscopic examination, neurological examination, foot inspection, evaluation of autonomic neuropathy, pedal pulses, and ankle brachial index (ABI).

Nontraditional cardiovascular disease risk factors not currently recommended for routine screening for cardiovascular disease include haemoglobin A\textsubscript{1c}, urine albumin, serum creatinine, glomerular filtration rate (GFR), Cystatin-C, High-sensitivity C-reactive protein (hs-CRP), Lipoprotein-associated phospholipase A2, Carotid Intima-media thickness, and adiponectin\textsuperscript{39}. Cystatin-C is a cysteine protease inhibitor with potential superiority over serum creatinine for estimation of GFR and also appears to correlate with cardiovascular mortality and events. Hs-CRP is an inflammatory marker and acute phase reactant. Lipoprotein-associated lipase A\textsubscript{2} is a proinflammatory enzyme, secreted by macrophages,
that degrades phospholipids and circulates primarily bound to low-density lipoprotein. Adiponectin is a peptide hormone secreted exclusively by adipocytes with insulin-sensitizing and anti-inflammatory effects.

Several imaging techniques exist for assessing cardiovascular risk. Traditionally, methods are based on detecting obstructive luminal disease; which are however often absent outside the acute coronary syndrome. The need thus arises for imaging techniques to visualize atherosclerosis in the presymptomatic stages in the arterial wall. This has received mounting attention in recent years.

Endothelial dysfunction is regarded as an early functional marker and IMT as an early morphological marker of atherosclerosis. In a study of diabetic subjects with inadequate glycaemic control and without clinical evidence of atherosclerosis, endothelial function assessed by flow mediated dilatation (FMD) was significantly impaired and IMT was significantly greater compared to nondiabetic healthy subjects. It was recommended that large clinical trials should evaluate if, in clinical practice, FMD and IMT are useful in identification of high-risk subjects. Traditional imaging modalities for obstructive CAD include nuclear and echocardiographic stress testing, and atherosclerosis plaque imaging with carotid IMT and coronary artery calcium for risk stratification of diabetic patients.

1.36 Carotid Intima Media Thickness in DM

IMT is the distance between the lumen-intima and media adventitia interfaces as seen on the image generated by ultrasound examination. (Fig 4)
Increased IMT is a structural marker of atherosclerosis which also correlates with vascular risk factors, and relates to the extent of coronary artery disease. It may also predict the likelihood of cardiovascular events. In the elderly [>65 years] as well as younger age groups, increased IMT, even in the absence of obstructive luminal disease of the carotid arteries, has been associated with risk of myocardial infarction and stroke. An increase in 0.03 mm per year in IMT has been associated with a two-fold increase in relative risk of myocardial infarction and cardiac death during follow-up.

In diabetic as well as non-diabetic subjects, IMT bears a modest relationship with obstructive CAD, indicating that this surrogate is a new marker of generalized atherosclerosis burden rather than an index of the severity of CAD and the presence of diabetes in an individual has been recognized to cause a two to three fold increase in risk of CAD. Increased IMT in diabetes has been associated with risk factors for atherosclerosis such as high serum triglycerides levels and high total to HDL cholesterol ratio, age, BMI, lipoprotein (a), microalbuminuria, endothelial dysfunction and low-grade inflammation. Serum high sensitivity C-reactive protein (CRP) is correlated with carotid IMT and the risk factors of cardiovascular diseases, and may be useful to predict accelerated atherosclerotic process in type 2 diabetic subjects. LDL particle size is...
independently associated with Carotid IMT in patients with T2DM, and this is regardless of antidiabetic and lipid lowering medication\textsuperscript{47}. Postprandial hypertriglyceridaemia was associated with increased carotid IMT in a cohort of type 2 diabetics of Asian ancestry\textsuperscript{48}. Plasma cholesteryl ester transfer protein (CETP) is a positive determinant of IMT in type 2 diabetic and non-diabetic subjects; with plasma cholesteryl ester transfer protein mass being a determinant of CET with an increasing effect at higher triglycerides\textsuperscript{49}. Elevated plasma phospholipids transfer protein (PLTP) activity is a determinant of carotid IMT in type 2 diabetes; suggesting that high PLTP activity is involved in accelerated atherosclerosis in this disease\textsuperscript{50}.

Diabetes is an independent predictor of IMT progression, and IMT appears to progress faster in diabetes than other conditions. This accelerated progression appears partly related to glycaemic control, baseline age, carotid wall thickness, systolic hypertension, and nephropathy\textsuperscript{7}. Recent data\textsuperscript{51} show that the increased IMT in diabetes is largely associated with lower plasma adiponectin, to which IMT is related independently of conventional cardiovascular risk factors, insulin resistance and CRP; and that lower adiponectin concentrations in men may largely account for the gender difference of IMT. The Hoorn study\textsuperscript{52} found that individuals with T2DM and prediabetes had significantly increased IMT and carotid artery stiffness compared to normal individuals. The Hoorn study also indicated that the changes in arterial wall stiffness occurred before onset of diabetes. A study by Brohail et al\textsuperscript{53} also showed T2DM to be associated with increased carotid artery IMT, which was 13% larger in the diabetes group than in the control groups; and the difference corresponded to a 10-year increase in age compared with age matched controls, and was associated with a nearly 40% increase in cardiovascular risks. Patients with IGT had an increase in IMT about one third of that observed in diabetic patients. An inverse relationship is said to exist between IMT and insulin sensitivity, and it is currently speculated that the mechanisms leading to accelerated atherosclerosis in subjects with diabetes may involve C-peptide –induced proliferation of intimal smooth muscle cells (SMC) in the setting of insulin resistance and hyperinsulinaemia\textsuperscript{54}.

Although IMT is an independent predictor of cardiovascular events in the general population, such evidence is of modest strength in diabetes, and little is known of the prognostic significance of IMT progression in both diabetic and nondiabetic patients\textsuperscript{7}. Intensive diabetes therapy during the Diabetes Control and Complications Trial (DCCT) resulted in decreased progression of IMT six years after the end of the trial (Epidemiology
of Diabetes Interventions and Complications research Group-EDIC). Although the differences in IMT between the intensive treatment and the conventional treatment group could be attributable to the less atherogenic lipid profile and decreased level of microalbuminuria seen with intensive therapy during DCCT, the decreased progression of IMT remained even after adjusting for these variables\textsuperscript{55}. Some studies have shown insulin resistance, an important feature of T2DM, to correlate with IMT\textsuperscript{56,57}, while others have not\textsuperscript{58}. Reduced insulin-like growth factor binding protein 1 (IGFBP-1) has been found to have an inverse relationship with IMT in subjects with type 2 diabetes\textsuperscript{59}. As diabetic retinopathy has been found to be associated with IMT and arterial stiffness, this suggests that common pathogenic mechanisms might predispose to diabetic micro- and macroangiopathy\textsuperscript{60}.

Genetic contribution to IMT is modest, with increased IMT being observed in non-diabetic subjects with a positive family history of DM\textsuperscript{61,62}, supporting the hypothesis that atherosclerosis and T2DM share common genetic risk factors\textsuperscript{61}. Ethnic differences of IMT also exist. Studies in the US and UK suggest there is increased carotid IMT in US and UK citizens of African descent compared to non-Africans\textsuperscript{63,64}.

1.37 B-Mode Ultrasonography

This is a relatively inexpensive, safe, and noninvasive, method of directly assessing atherosclerotic plaque burden and arterial stiffness. IMT is not (yet) recommended by AHA and ACC as a routine modality for detection of generalized atherosclerosis, but it is rapidly becoming an accepted modality for this purpose\textsuperscript{39}.

A potential problem with this procedure in subjects with diabetes is the presence of calcification on the arterial wall which causes shadowing and loss of visualization of IMT in the calcified segment. Secondly, due to obesity the neck blood vessels may lie quite deeply, making it impossible to visualize those segments. These two potential problems are commoner with diabetic subjects. The presence of tortuous blood vessels (this is not peculiar to subjects with diabetes) sometimes renders optimal visualization of segments impossible.
1.38 Flow Mediated Dilatation in Type 2 Diabetes

Flow-mediated dilatation (FMD) is a phenomenon whereby increased flow in an artery results in vasodilatation mediated by changes in shear stress, detected by endothelial cells. FMD of the brachial artery assessed by high-resolution ultrasound can be used to measure endothelial function (see figure 5).

Figure 5. Flow-mediated endothelium-dependent relaxation can be assessed non-invasively in vivo by continuously measuring the brachial artery diameter change in response to occlusion and reflow. (Shimokawa 2000) Reference Clinical Assessment of Endothelial Function by Shimokawa H. Keynote Lecture. Japanese Circulation Society.

Damage to or removal of the endothelium abolishes this phenomenon 65. The initial step in atherosclerosis is thought to be endothelial dysfunction. Studies support the concept that endothelial dysfunction has a significant relationship to atherogenesis 66, 67. A significant negative correlation between the IMT of the carotid artery and percent FMD exists 67. Impaired endothelial function and increased IMT are associated with cardiovascular risk factors, and the presence of carotid and femoral IMT is significantly correlated with endothelial dysfunction 68.
Vascular studies in T2DM \(^{69}\) have shown impaired muscarinic, agonist stimulated, endothelium dependent response and impaired endothelium independent response. Other factors like degree of acute hyperglycaemia, chronicity of hyperglycaemia, accumulation of advanced glycosylated end products, insulin concentrations, and diabetic complications such as autonomic neuropathy and microalbuminuria may modulate endothelial function in T2DM \(^{69}\). Morphological abnormalities of diabetic vasculature include enhanced endothelial adhesion of leucocytes, platelets and fibrin-like material, increased endothelial cell proliferation, increased numbers of multinucleated endothelial cells, increased basement membrane thickness, increased endothelial cell surface area, increased fluidity of endothelial cell membrane, and increased capillary diffusion capacity \(^{66}\). It has also been suggested that T2DM may be preceded by endothelial dysfunction \(^{66},^{69}\). Diabetic patients have been shown to have decreased FMD and increased arterial stiffness compared with age-and sex-matched nondiabetic subjects; these functional changes correlate well with the structural changes of the arteries measured by IMT \(^{70}\).
Chapter 2.

Study Design, Subjects and Methods

2.1 Design

This was a cross sectional study involving adult diabetic subjects who consented to provide information and to undergo venesection, clinical examination, ultrasound and FMD investigations.

2.2 Subjects

Two groups of subjects were studied;

(1) Black diabetic subjects attending Groote Schuur Hospital (GSH) or Community Health Centres. These were Xhosa speaking.

(11) Non-black diabetic subjects attending GSH, including subjects with mixed ancestry and whites.

Inclusion Criteria

2. Age range 30 to 80 years.
3. Both male and female subjects were recruited; but later only female subjects were recruited because fewer male volunteers could be found attending the clinics.

Black subjects were to be matched by duration of diagnosis of diabetes, gender, with non-black diabetic South Africans of mixed or European ancestry.

Exclusion Criteria

1. Type 1 diabetes, Gestational diabetes and diabetes classified as “other causes”
2. Non-consenting subjects with type 2 diabetes
3. Age less than 30 years and greater than 80 years.
4. Intercurrent illnesses interfering with evaluation.

2.3 Setting

The study was carried out at the clinical research facility of the Lipidology Division of the department of Internal Medicine, at the University of Cape Town (UCT) Health Science Faculty. Patients were recruited both from the diabetes clinic at Groote Schuur Hospital (GSH) and the Community Health Centres at Cross Roads in the Cape Metropole.

2.4 Methods

A patient information sheet (see appendix 3) was given to the patients prior to the study. Patients were instructed to refrain from smoking and the consumption of caffeine-containing drinks for 24 hours prior to the study and to have fasted overnight before the study. On the morning of the study, after obtaining written consent from the patient, a questionnaire was administered (see appendix 2 for biodata form) and the following measurements taken:

2.41 Sampling/Selection Method

Subjects were identified for invitation to participate in this study by direct contact at Groote Schuur Hospital and Cross Roads Clinics to enable adequate numbers of non-black and black participants respectively. No particular process was followed to ensure that representativity of subjects was ideal as there were no databases from which to randomly select the small numbers that entered this study. It has to be assumed that the selection was thus reasonably random as attendance at the clinics was not selective.

2.4a. Blood Pressure

Blood Pressure (BP) measurements were done using an automated Omron BP monitor. Blood pressures were measured using the participant’s left arm while seated and three minutes after standing. The appropriate cuff size was used with a large cuff for subjects...
with an arm circumference ≥ 33 cm. Subjects were also asked to refrain from smoking and caffeine consumption for 30 minutes prior to measurement.

2.4b Anthropometry Measurements:
Mass, height, waist and hip measurements were taken:
Mass was measured to the nearest 0.1 kg with the subject wearing light clothing and standing barefoot on a portable scale that was regularly calibrated.
Height was measured to the nearest 0.1 cm with the participant standing barefoot using a stadiometer.
The waist measurement was taken as the smallest circumference between the xiphi-sternum and the umbilicus on expiration. Measurements were to the nearest 0.1 cm after normal expiration while in an upright position. Waist circumference was assessed for abdominal obesity. The latest IDF cut points for waist circumference were used, that is, ≥ 80 cm for women and ≥ 94 cm for men (European cut off points are also recommended for Africans until appropriate ethnic cut-off is established).

Hip measurements were taken to the nearest 0.1 cm at the maximum posterior protuberance of the buttocks with the participant standing upright with feet together.

Waist Hip Ratio (WHR) was then calculated.

Body mass index (BMI) was calculated as the individual’s mass in kilograms divided by the height in meters squared (kg/m²). Overweight was defined as BMI 25 – 29.9 kg/m² and obesity as BMI ≥ 30 kg/m². Underweight was defined as a BMI < 18.5 kg/m² (Bray, 2003).

2.4c. Laboratory Methods
i. Biochemical measurements
Patients’ fasting blood and urine samples were taken for biochemical tests. Routine Lipid profile, LDL particle size, Glycated haemoglobin, and urine for microalbumin, creatinine and proteinuria were assessed. Lipids were analyzed using enzymatic methods. The LDL cholesterol was calculated by the Friedewald formula: \( \text{LDLC} = \text{TC} - \text{TG}/2.8 - \text{HDLC} \). Glycated haemoglobin was measured by ion exchange high performance liquid
chromatography. All investigations were done at the NHLS laboratory; except the gradient gel electrophoresis for LDL particle size, which was done at the Lipid Laboratory. NHLS Laboratory reference ranges were:

\[ \text{Glycated haemoglobin:} \quad < 6\% \text{ normal non-diabetes,} \quad <7\% \text{ target range,} \quad >8\% \text{ additional action suggested.} \]

Microalbumin/creatinine ratio – 0.0 -2.2 mg/mmol

Microalbuminuria was defined as microalbumin/creatinine ratio of 3.4 – 33.9 mg/mmol on 2 of 3 random samples.

**CVS risk stratification and Cholesterol target (S.A guidelines)**

<table>
<thead>
<tr>
<th>Fasting</th>
<th>Category 1 Risk</th>
<th>Category 2 Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/l)</td>
<td>&lt;4.5</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>LDLC (mmol/l)</td>
<td>&lt;2.5</td>
<td>&lt;3.0</td>
</tr>
<tr>
<td>HDLC (mmol/l)</td>
<td>(Males)</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td></td>
<td>(Females)</td>
<td>&gt;1.2</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>&lt;1.7</td>
<td>&lt;1.7</td>
</tr>
</tbody>
</table>

**Category 1 risk**

Established atherosclerosis: coronary heart disease, cerebrovascular atherosclerotic disease, peripheral vascular disease.

Type 2 diabetes

Type 1 diabetes plus microalbuminuria or proteinuria

Genetic dyslipidaemia e.g familial hyperlipidaemia, familial hypercholesterolaemia

**Category 2 risk**
Non-denaturing gradient gel electrophoresis (GGE) for lipoprotein (LP)

The technique of non-denaturing gradient acrylamide gel electrophoresis allows separation of lipoproteins (LP) by size. The LP may be demonstrated by staining lipid or protein. Pre-staining the sample with a lipid stain allows specific visualisation of lipoproteins in the original glass sandwich while protein staining has to be done after removing the gel and is only specific for large, apolipoprotein B-containing lipoproteins owing to plasma proteins occurring in the size range of HDL. Separation of chylomicrons (CM) and very low density lipoprotein (VLDL) is difficult because of the size of these LP and these triglyceride-rich lipoproteins are seen as broad size ranges. The intermediate density lipoprotein (IDL) range is also not a distinct band whereas LDL is. Gels may be selected to demonstrate apo-B containing (LDL) or apo-AI-containing (HDL) lipoproteins. A 2-16% gradient is usually recommended for LDL and for HDL, 4-30% but this lab uses a mini-gel of 2-8% acrylamide gradient for LDL.

ii. Assessment of carotid atherosclerosis and vascular function by carotid IMT and flow mediated dilation

All the measurements were done by the same experienced ultrasonographer who had undergone certification by an international collaborative study. The CV for repeated scans of the six segments is 6.6% with a range of 5.4 to 8.2% for the lowest and highest of the different segments. The subject was placed in the supine position in a quiet room. The procedure for performing the IMT scans was done using the ACUSON 128 and SONY DKR-700 machines. Carotid IMT measurement was performed according to a protocol standardized (see appendix 5), which involves measuring the carotid arterial segments. Scanning of the common carotid artery, the carotid bulb and the internal carotid arteries was done using this protocol. The images were saved and subsequently analysed by commercially available software (Mat lab) that integrated the IMT over a distance that could clearly resolve the IMT.
iii. Flow-mediated dilation (FMD) for vascular reactivity

This was carried out according to a protocol in use (appendix 6) in the Lipidology Division of the department of Internal Medicine, at the University of Cape Town (UCT) Health Science Faculty. The room temperature was recorded. The same certified ultrasonographer also performed the FMD.

1.) The subject rests for 15 minutes, during which BP is measured twice. A baseline Scan is then done: Ideally there is a video recording for 2mins. During this time a 2D caliper diameter Measurement is carried out, and all calculations are worked on 2D diameter and mean.

2.) The BP cuff on the forearm is then pumped up to 50mm Hg above systolic (upper arm band). The arm remains ischemic for 5 minutes; the BP cuff is then released during which time measurement of the brachial artery occurs over a further 4 minutes.

3.) After a rest period of about 10 minutes, the other hand is placed in an ice bucket for about 30 seconds, and more measurements of the arm’s blood vessel are taken over a period of 2 minutes.

4.) After a rest period of 5 minutes, two sprays of Nitroglycerine are administered sublingually, except in subjects in whom contraindications existed (hypertrophic obstructive cardiomyopathy, constrictive pericarditis, and low blood pressure). Side effects of this spray are uncommon but include headache, transient hot flushing and palpitations, nausea, vomiting, low blood pressure, dizziness and syncopy. The brachial artery is visualized over a further 4 minutes after the spray.

2.5 Statistical analysis

Data obtained was captured using the Paradox® soft ware and expressed as mean ± SD or median (range). QuattroPro (Corel) was used as a spread sheet and Prism (graph pad) was used for statistical analysis. Tables, graphs and charts were used to display summarized data. The statistical significance of differences was estimated using the student T-Test and Chi-test, where applicable. P-values of <0.05 was taken as significant.

A multivariate analysis was carried out. A matrix of scatter plots between all the variables considered for regression analysis was done. Multiple regression models of associations
between cardiovascular risk factors and carotid IMT in the 47 subjects was done to determine their correlations.

2.6 Ethical approval

The study was commenced after ethical approval was obtained from the Research Ethics Committee of the University of Cape Town.

2.7 Timing

The study took one year for completion.

2.8 Funding

The study was supported by the funds raised through contract research in Lipidology as well as from the Endocrine unit.
Chapter 3.

Results

A total of 60 subjects were recruited from the diabetes clinic at Groote Schuur Hospital, and Cross Roads CHC, and 47 subjects participated in the study. Of the remainder two were admitted for intercurrent problems, six could not be studied because of a general strike, and five couldn’t participate because the study date was not suitable for them. There were five men and 42 women. The gender bias reflects attendance at the diabetes clinic, and greater cooperation from women. There were 23 subjects of indigenous African descent and 24 of non-indigenous African lineage (of white and mixed ancestries). The subjects of Black African descent are subsequently referred to as black and those of non-African descent as non-black in this study. As relatively few black patients attended Groote Schuur diabetes clinic, they were largely recruited from the Cross Roads CHC which, is a feeder community health center for Groote Schuur hospital.

3.1 Socio-Demographic Characteristics

The black subjects were urbanized; most had lived in Cape Town for over 20 years, and a number of the younger participants were born and reared in Cape Town. Despite attempts to match for age, the B group was significantly younger than the NB group. The two groups had similar socioeconomic status assessed by housing density and levels of education, and the majority were unemployed. The duration of T2DM as per design was also similar. The socio-demographic characteristics are summarized below in table 1.
Table 1. Socio-Demographic and Selected Diabetes-Related Details of Subjects

<table>
<thead>
<tr>
<th></th>
<th>Black N=23</th>
<th>Non-black N=24</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-years (S.D)</td>
<td>49.4 (11.7)</td>
<td>57.6 (8.1)</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>Gender (M: F)</td>
<td>3:20</td>
<td>2:22</td>
<td></td>
</tr>
<tr>
<td>Duration of DM</td>
<td>10.9</td>
<td>10.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Housing Density</td>
<td>2.7</td>
<td>3.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Level of Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;Grade 7 (%)</td>
<td>14 (60.9)</td>
<td>12 (50)</td>
<td>0.6</td>
</tr>
<tr>
<td>Grade 8-12 (%)</td>
<td>1 (4.3)</td>
<td>9 (37.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Secondary (%)</td>
<td>6 (26.1)</td>
<td>3 (12.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Tertiary (%)</td>
<td>2 (8.7)</td>
<td>0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

3.2 Clinical Characteristics of the Subjects

The CVD risk profile was strikingly different in the two groups. Although hypertension, history of CVA and current smoking rates were similar in the two groups, history of IHD was strikingly higher in the NB than the B group (p=0.0003) Table 2 and figure 6.
Table 2. History of Macrovascular Disease and Cardiovascular risk factors

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>Non-black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=23</td>
<td>N=24</td>
<td></td>
</tr>
<tr>
<td>IHD (%)</td>
<td>1 (4.3)</td>
<td>13 (54.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>PVD</td>
<td>2</td>
<td>8</td>
<td>0.072</td>
</tr>
<tr>
<td>CVA</td>
<td>1</td>
<td>3</td>
<td>0.61</td>
</tr>
<tr>
<td>Hypertension</td>
<td>22</td>
<td>21</td>
<td>0.60</td>
</tr>
<tr>
<td>Current Smoking</td>
<td>0</td>
<td>4</td>
<td>0.11</td>
</tr>
<tr>
<td>Pack years Smoked</td>
<td>0.1</td>
<td>13.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>Family history of</td>
<td>17 (73.9)</td>
<td>23 (95.8)</td>
<td>0.0479</td>
</tr>
<tr>
<td>DM (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are number (percentage) of subjects

Figure 6. Subject Distribution Based on History of Ischaemic Heart Disease (IHD)

![Bar chart showing subject distribution based on history of IHD](image-url)
PVD tended to be more common in the former (P=0.075) as well. Pack year of smoking was greater in the NB than B group (P-value <0.0001) and family history of diabetes was also more common in NB subjects (P=0.04). There was no difference in the use of medications for diabetes or hypertension between groups (Table 3).

Table 3. Distribution of Subjects according to Medication Used

<table>
<thead>
<tr>
<th>Medication</th>
<th>Black</th>
<th>Non-black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>16</td>
<td>20</td>
<td>0.48</td>
</tr>
<tr>
<td>Sulphonylureas</td>
<td>9</td>
<td>11</td>
<td>0.16</td>
</tr>
<tr>
<td>Insulin</td>
<td>12</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>21</td>
<td>21</td>
<td>1.00</td>
</tr>
<tr>
<td>Beta blocker</td>
<td>6</td>
<td>8</td>
<td>0.75</td>
</tr>
<tr>
<td>ACEI</td>
<td>19</td>
<td>19</td>
<td>0.70</td>
</tr>
</tbody>
</table>

There was however a difference in the use of lipid modifying agents, these were seldom prescribed in the black group p=0.02, see figure 7 below.

Figure 7. Distribution of Subjects According to Use of Lipid Modifying Agents.

![Graph showing the distribution of subjects by lipid modifying agents use.](image-url)
The groups were similarly obese and had similarly increased abdominal obesity as assessed by waist circumference and waist-hip-ratio. Only 4.3% of the black and 4.2% of the non-black group had a normal BMI. Mean systolic blood pressure was similar in both group, but diastolic blood pressure was higher in the black subjects, \( p = 0.04 \) (Table 4).

Table 4. Anthropometric Measurements, Blood Pressure Measurements and Heart Rates of Subjects

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>Non-black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=23</td>
<td>N=24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.6 (8.2)</td>
<td>33.5 (7.2)</td>
<td>0.62</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>105.5 (13.3)</td>
<td>105.6 (18.7)</td>
<td>0.75</td>
</tr>
<tr>
<td>WHR</td>
<td>0.9 (0.07)</td>
<td>0.9 (0.06)</td>
<td>0.46</td>
</tr>
<tr>
<td>Heart Rate bpm</td>
<td>87.5 (16.1)</td>
<td>74.4 (11.9)</td>
<td>0.027</td>
</tr>
<tr>
<td>Systolic BP mmHg</td>
<td>126.7 (20.8)</td>
<td>120.8 (13.7)</td>
<td>0.29</td>
</tr>
<tr>
<td>Diastolic BP mmHg</td>
<td>74.5 (17.3)</td>
<td>66.5 (7.9)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values are mean±SD

However heart rate was significantly higher \( (p=0.03) \) in the black subjects (table 4) even after excluding subjects on beta-blockers, figure 8.

Figure 8. Heartrate in non-Beta blocker users
3.3 Biochemical Parameters

Glycaemic control was similarly poor in both groups, with a mean HbA1c of 9.4% in the black and 8.7% in the non-black group (see table 5);

Table 5. Biochemical Measurements in the Participants

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>Non-black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>N=23</td>
<td>N=24</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.4 (2.44)</td>
<td>8.7 (2.11)</td>
<td>0.292</td>
</tr>
<tr>
<td>U-albumin/creatinine (mg/mmol)</td>
<td>21.3 (55.59)</td>
<td>3.4 (4.71)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/l)</td>
<td>4.7 (1.39)</td>
<td>5.3 (1.20)</td>
<td>0.0515</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.7 (1.21)</td>
<td>1.9 (1.06)</td>
<td>0.25</td>
</tr>
<tr>
<td>HDLC (mmol/l)</td>
<td>1.4 (0.61)</td>
<td>1.3 (0.31)</td>
<td>0.52</td>
</tr>
<tr>
<td>LDLC (mmol/l)</td>
<td>2.4 (0.98)</td>
<td>3.1 (1.04)</td>
<td>0.024</td>
</tr>
<tr>
<td>TC/HDLC (mmol/l)</td>
<td>3.6 (1.16)</td>
<td>4.2 (1.14)</td>
<td>0.0276</td>
</tr>
<tr>
<td>TG/HDL (mmol/l)</td>
<td>1.3 (1.06)</td>
<td>1.6 (1.12)</td>
<td>0.287</td>
</tr>
</tbody>
</table>

Values are mean±SD.

All the lipid profiles were included in this analysis. Untreated lipid profiles were not traceable for separate analysis.

The black subjects had a more favourable lipid profile than the non-black subjects, with significantly lower total cholesterol (TC), LDLC and TC/HDLC ratio, p-values 0.05, 0.024 and 0.028 respectively (table 5). HDL, triglyceride, and TG/HDL ratio were similar in both groups. There was no difference in the distribution of small dense LDL particle size in the two groups.

Only 17.4% of B and 29.2% NB subjects had HbA1c of ≤ 7% (see table 6), thus meeting the ADA 2007 guidelines criteria.
Table 6. Proportion of Subjects (%) Achieving Treatment Target

<table>
<thead>
<tr>
<th></th>
<th>Black N=23</th>
<th>Non-black N=24</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>17.4</td>
<td>29.2</td>
<td>0.49</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td>56.5</td>
<td>16.7</td>
<td>0.0065</td>
</tr>
<tr>
<td>HDLC</td>
<td>73.9</td>
<td>66.7</td>
<td>0.75</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>65.2</td>
<td>54.2</td>
<td>0.56</td>
</tr>
<tr>
<td>LDLC</td>
<td>47.8</td>
<td>20.8</td>
<td>0.068</td>
</tr>
<tr>
<td>Microalbumin-creatinine ratio</td>
<td>4.3</td>
<td>54.2</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

The microalbumin-creatinine ratio was significantly higher in the B group with 4.3% of B and 54.2% of NB subjects not having microalbuminuria. See table 6. Only 17.4% of B and 29.2% of NB subjects achieved HbA1c target of ≤7.0%. Total cholesterol target of <4.5 was present in 56.5% of B subjects and only in 16.7% of NB subjects. Over half of both groups had target HDL levels; 73.9% in B subjects and 66.7% in NB subjects. Likewise more B subjects had target serum triglycerides and LDLC levels compared to NB subjects. This is a striking finding, given that many NB subjects were on treatment with lipid modifying drugs. Microalbuminuria was commonly present in the B group, with only 4.3% having normal microalbumin-creatinine ratio compared to 54.2% in the NB subjects.

3.4 Carotid IMT of Patients

No carotid IMT measurements could be done in areas with calcification, or where the neck structure made it difficult for visualization of the IMT; hence the varying sample sizes for the IMT analyses. See table 7.
Table 7. Carotid IMT of Subjects

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>Non-black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>CCA</td>
<td>23</td>
<td>24</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>0.85 (0.28)</td>
<td>0.79 (0.15)</td>
<td></td>
</tr>
<tr>
<td>ICA</td>
<td>15</td>
<td>22</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>0.69 (0.23)</td>
<td>0.63 (0.14)</td>
<td></td>
</tr>
<tr>
<td>Bulb</td>
<td>18</td>
<td>22</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.86 (0.16)</td>
<td>0.78 (0.17)</td>
<td></td>
</tr>
<tr>
<td>Carotid Mean</td>
<td>19</td>
<td>23</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.81 (0.20)</td>
<td>0.75 (0.11)</td>
<td></td>
</tr>
<tr>
<td>CCA/BULB</td>
<td>18</td>
<td>22</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>0.96 (0.21)</td>
<td>1.01 (0.11)</td>
<td></td>
</tr>
<tr>
<td>CCA/ICA</td>
<td>15</td>
<td>22</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>0.78 (0.18)</td>
<td>0.83 (0.25)</td>
<td></td>
</tr>
<tr>
<td>ICA/BULB</td>
<td>15</td>
<td>22</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>1.23 (0.44)</td>
<td>1.32 (0.44)</td>
<td></td>
</tr>
</tbody>
</table>

The numbers of subjects for each measurement are given in bold above the respective results which are in mm and the data supplied are mean ± standard deviation.

There were no intergroup differences in any of the individual carotid IMT measurements. The median common carotid IMT was 0.78 mm in B (range 0.54 to 1.76) and 0.78 mm in NB subjects (range 0.58 to 1.26).

There were however noticeable anatomical differences in the carotid arteries of both groups of patients (table 8).
Table 8. Anatomical Differences in IMT

<table>
<thead>
<tr>
<th>Anatomical features</th>
<th>B - YES</th>
<th>B - NO</th>
<th>NB - YES</th>
<th>NB - NO</th>
<th>P value (Fishers exact)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep location of vessels</td>
<td>5</td>
<td>18</td>
<td>0</td>
<td>24</td>
<td>0.02</td>
</tr>
<tr>
<td>Calcification of vessels</td>
<td>5</td>
<td>18</td>
<td>1</td>
<td>23</td>
<td>0.09</td>
</tr>
<tr>
<td>Normal IMT</td>
<td>8</td>
<td>15</td>
<td>19</td>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td>High risk IMT</td>
<td>7</td>
<td>16</td>
<td>3</td>
<td>21</td>
<td>0.17</td>
</tr>
</tbody>
</table>

The B subjects had significantly deeper localization of blood vessels compared to NB, (p = 0.02). There was a trend towards the greater presence of calcifications in the carotid artery in the B subjects, p = 0.09, while significantly more NB subjects had a normal IMT, p = 0.003. There was, however, no difference in the number of subjects with high risk IMT (>1.0 mm in diameter).

3.5 Flow Mediated Dilatation

Similar to the carotid IMT, there were no intergroup differences in any of the FMD measurements.

All the patients were able to tolerate the period of occlusion. One patient in the non-black group often had severe headaches from nitoglycerine, therefore it was not administered to her. See table 9 below.
Table 9. FMD of Subjects

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>Non-Black</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=23</td>
<td>N=24</td>
<td></td>
</tr>
<tr>
<td>Baseline Velocity</td>
<td>0.80 (0.20)</td>
<td>0.79 (1.15)</td>
<td>0.88</td>
</tr>
<tr>
<td>Baseline Diameter</td>
<td>4.13 (0.82)</td>
<td>4.11 (0.68)</td>
<td>0.97</td>
</tr>
<tr>
<td>% FMD change (ischaemia)</td>
<td>6.41 (2.40)</td>
<td>5.44 (1.58)</td>
<td>0.35</td>
</tr>
<tr>
<td>Before TNG</td>
<td>4.14 (0.75)</td>
<td>4.16 (0.68)</td>
<td>0.87</td>
</tr>
<tr>
<td>After TNG</td>
<td>4.48 (0.70)</td>
<td>4.49 (0.69)</td>
<td>0.85</td>
</tr>
<tr>
<td>%FMD change</td>
<td>8.67 (4.10)</td>
<td>7.85 (4.14)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

3.6 Correlations Analysis

There were no significant associations with carotid IMT. Microalbumin-creatinine ratio had the highest correlation (table 10), but this was caused entirely by two outliers. Other risk factors like age, waist circumference, blood pressure, total cholesterol, and HbA1c did not have a high correlation with IMT.

Table 10. Multiple Regression Model of Associations Between Cardiovascular Risk Factors and Carotid IMT in the 47 Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microalbumin/creatinine</td>
<td>0.002</td>
<td>(0.001 to 0.003)</td>
<td>0.001</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.038</td>
<td>(-0.004 to 0.08)</td>
<td>0.078</td>
</tr>
</tbody>
</table>
3.6a LDL species correlations

When analysed in tertiles, LDL species correlated significantly with levels of serum triglycerides. The greater the triglyceride concentration, the more likely the presence of small dense LDL particles (I, IB, B). P-value 0.0009. There was however no correlation of LDL size with HbA1c, WHR, BMI and waist circumference.
Chapter 4.

Discussion

The chief findings in this study of black and non-black subjects with a similar duration of diabetes, were that the carotid IMT was similarly thickened despite the slightly younger age, lower exposure to smoking, milder dyslipidaemia and less prominent history of atherosclerotic complications, but higher rates of early nephropathy in the former group. The educational status, and agents used for hypertension and glycaemic control were similar in the two groups, but the black group received less lipid lowering medication and had a lesser smoking history. This study also reveals reason for concern about glycaemic and dyslipidaemia control.

4.1 Diabetes

Although the duration of diagnosed diabetes was similar in both groups (~10 years) as determined from the clinic records and subject history, there is no certainty that the period of metabolic derangement prior to diagnosis was also similar. However, the two groups of patients did not differ with regard to the degree of glycaemic control or range of antidiabetic medications received ie; sulphonylureas, metformin and insulin. None was on the thiazolidinediones, as these were not available to the public sector. The only microvascular complication to be actively assessed at the time of this study, was nephropathy and it was notable that the black group had more early nephropathy than the non black group. In addition, the former, had significantly higher resting heart rates. This may relate to sympathetic over activity due to cardiac autonomic dysfunction, although this cannot be confirmed in the absence of autonomic function testing, which was not undertaken, or less likely, higher levels of anxiety in the black subjects, as most of them were recruited from the day hospital, and as such were in an unfamiliar environment in the Lipids Laboratory. The reasons for the higher rate of microvascular complications in the black group are unknown. They may have had a longer phase of undiagnosed hyperglycaemia prior to diagnosis of diabetes or alternatively, poorer long term glycaemic control. The fact that the recent glycated haemoglobin levels did not differ between the groups at the time of the study, may not reflect the long term situation.
4.2 Cardiovascular disease

In diabetic as well as non diabetic subjects carotid IMT bears a modest relationship with obstructive CAD, indicating that this surrogate is a new marker of atherosclerosis burden rather than an index of the severity of CAD. A review of co-morbid medical conditions and medications used showed a significantly greater number of non-black subjects had IHD p=0.003; 54% in non-black compared with 4% in black subjects. Consequently more non-black subjects were on lipid modifying medications; 70% in non-black versus 30% in black subjects. This difference reflects the reluctance of the Provincial Government of the Western Cape’s to provide statins for diabetes in general and may also reflect in part, the notion that vascular disease is less common in black Africans. The non-black subjects had a significantly higher pack year of smoking with a mean cigarette pack year of 13.8 compared to 0.1 in the black subjects p<0.0001. Although history of CVA was similar in both groups, there was a trend towards increased PVD in non-blacks p=0.07, in keeping with the increased prevalence of IHD in this group. Despite the increased CVS risk factor profile and significantly older age in the non-black subjects, carotid IMT was surprisingly similar in both groups. There are a number of possible explanations for the lower rate of IHD and trend to lower rate of PVD in the black subjects. These include their younger age, lower pack year history of smoking, lower LDLC, or innate protection against complications associated with thicker IMT. In the light of literature from the US and UK where their populations of African descent have been shown to have a higher carotid IMT, it appears the same may be true in our region. Thus complications of atherosclerosis may in future be expected to increase dramatically especially if dietary and smoking patterns change. Larger studies and prospective studies will be needed to confirm this. It is of note that the LDLC average for black subjects was on target without treatment, and that for non-black subjects was above target despite a significantly higher proportion of non-black subjects taking lipid modifying medications. Other cardiovascular disease risk factors like history of hypertension and current smoking history were similar in the two groups, but the diastolic blood pressure control was worse in the black subjects. Black subjects have been shown to have a higher risk for elevated blood pressure.
4.3 Vascular Status by ultrasound

The carotid IMT was similar in dimensions in both groups of subjects. The mean carotid IMT in blacks was 0.81mm (range 0.56mm – 1.28mm) versus 0.74mm (range 0.57mm – 0.97mm) in non-blacks, p=0.25. This was a surprising finding, considering the fact that the black subjects were significantly younger and had a better CVS risk profile. A review of the anatomical structures however showed some differences. A significant number of black subjects had their carotid arteries deeply sited. This made IMT measurements technically difficult to perform, but those that could be done, were reliable. There was also a trend towards increased calcification in the black subjects. Significantly more non-blacks had a normal IMT structure and the number of subjects with high risk IMT (>1.0 mm in diameter) was similar in both groups. Putting all these findings together, IMT may actually be worse in this cohort in black subjects.

Several factors have been shown to affect carotid IMT. Some of them include presence of diabetes, effects of glycaemic control, presence of microalbuminuria, ethnicity, gender, and gene polymorphisms. The level of glycaemia is independently related to carotid IMT. The presence of diabetes has been associated with accelerated carotid atherosclerosis, and greater IMT. In subjects with diabetes, the postprandial period is associated with abnormalities of fatty acid and triglyceride metabolism. This is suspected to play a role in the development of atherosclerosis. The Niemann-Pick C1-like 1 (NPC1L1) regulates intestinal absorption of cholesterol. NPC1L1 deficient mice have been shown to have a reduced intestinal absorption of cholesterol. Diabetic patients have also been demonstrated to have more NPC1L1 mRNA than control subjects. Microalbuminuria is associated with increased CCA IMT. It has been suggested that inheritance may play a role in IMT in type 2 DM. Ethnicity has also been shown to affect carotid IMT. Freedman et al observed African American men to have higher IMT in a bi-racial cohort from families enriched for members with T2DM. Likewise in UK African Carribbeans, carotid IMT has been shown to be increased compared to UK whites. Several factors like gene polymorphism, environmental factors, inflammation and chronic infection have been suggested to play a role in these ethnicity variations in IMT. In S. Africa however, as at the time of this study there are no studies comparing IMT in the different ethnic groups. In our cohort IMT was not obviously correlated with any parameters except for albumin-creatinine ratio and possibly triglycerides. The small number of subjects in the study could have contributed to this finding.
Likewise the FMD was similar in both groups, which may mean this test is not useful for discriminating IHD. Future studies are required to determine the utility of carotid IMT for predicting coronary artery disease in the presence or absence of diabetes.

4.4 Lipids

There were major differences in the lipid profiles in the black and non-black subjects in this study. The black subjects had a significantly lower total cholesterol and LDL cholesterol, but the mean triglyceride, and HDLC were similar in both groups. The diet was not analysed although the black urban population tend to have a westernized diet in SA and our cohort of black subjects was urbanized. Possibly different childhood exposure could also explain these as traditional diets are under change. Lower LDL may also be due to diet or genes such as PCSK9, whose mutation is associated with a reduced mean LDL and coronary heart disease risk. TC/HDLC ratio was better in the black subjects, and may reflect reduced apo B/ apo A1 ratio as described in black control subjects in the Interheart Africa study. The LDL particle size was similar in both groups and although not all had small dense LDL particles, the proportion was higher, as is expected with diabetes. The relationship of small dense LDL to TG was upheld but there was no other obvious relationship with BMI, and waist/hip ratio. Atherogenesis did not correlate to LDL species but TG did. Lp (a) was not measured, but is generally higher in blacks but figures are not available for local non-blacks. Poor renal function causes elevated triglycerides, whether the poorer renal function in the black subjects contributed to their having a similar serum triglyceride level with the non-black subjects, is unclear.

Using the IDF definition of metabolic syndrome, 69.6% of black and 66.7% of non-black subjects had the metabolic syndrome. This is concerning as the African region already burdened by infectious diseases cannot afford to take on the extra burden of non-communicable diseases like diabetes and its sequelae. There is therefore a need for emphasis of adoption of healthier lifestyles and diet in our region as the energy content of the local diet is adequate to support the metabolic syndrome. This study did not analyse the dietary intakes of cholesterol and fatty acids; nor did it analyse the fatty acid distribution that may have a bearing on atherosclerosis complications.
4.5 Treatment

Using the ADA 2007 guideline, Glycaemic control as measured by HbA1c was similarly below target in the two groups of patients, as only 17.4% of black and 29.2% of non-black subjects achieved HbA1c target of ≤ 7.0%. Total cholesterol target of <4.5mmol/l was attained in significantly more black subjects (56.5%) and only 16.7% of non-black subjects (p = 0.013). Over half of both groups had target HDLC levels; 73.9% in blacks and 66.7% in non-blacks (p = 0.75). Likewise more black subjects had target serum triglycerides (p = 0.55) and LDLc levels (p = 0.07) compared to non-black subjects, but this was not statistically significant. Only 4.3% of black subjects compared with 54.2% of non-black subjects achieved target for microalbumin-creatinine ratio (p = 0.0003) see table 6.

Diabetes control in our cohort of subjects was generally poor as previously reported from other clinics in the world. DM is regarded as secondary prevention equivalent risk, and guidelines recommend LDLc <2.5mmol. The Western Cape Provincial government accepted statins for diabetes in 2005, but it appears that these guidelines may not be applicable to black cohorts who have a better profile. Target values for Black Subjects with T2DM need to be established by detailed studies. Currently no interventional studies are available for black but should be in view of anticipated increase of IHD in the developing world. In this cohort non-black subjects may have been favoured for statin treatment owing to higher prevalence of IHD and higher lipid levels, but still were not on target. HbA1c was also not near target for either group. Measures to improve control like exercise, diet, home glucose monitoring and earlier introduction of insulin, should be considered in these subjects. Majority of the subjects in each group had blood pressure controlled to target; with 61% of the black subjects, and 75% of the non-black subjects having systolic blood pressure controlled to target. Likewise diastolic blood pressure control was good in the majority of subjects; 87% of black subjects and 92% of non-black subjects had diastolic blood pressure controlled to target.

4.6 General

Risk estimation for primary prevention by using UKPDS calculation (www.dtu.ox.ac.uk/riskengine), though not studied in these populations, would be interesting to calculate and compare risk for CVS events, especially if adjusted for cholesterol, and susceptibility to thick IMT versus susceptibility to complications. The black cohort seemed more susceptible to thick IMT but less to complications associated with this IMT compared to the non-black cohort. This could be that other risk factors have
not operated to increase IMT: higher LDL, more smoking, and age; yet, this is unlikely given the higher rate of atherosclerotic complications. It could also be that the coagulation and/or shear stress handling of the arterial wall is different. This could be due to nutritional or genetic differences. Higher LDL receptor activity due to low fat diet and/or cholesterol-lowering PCSK9 mutations could decrease postprandial remnants and their contributions to complications. Also higher prevalence of CD36 deficiency in blacks could influence concentration of NEFA (not measured). It is also possible that different levels of inflammation may play a role in the extent of IMT in the groups studied as the inflammatory process is thought to be important in the development of atherosclerosis\textsuperscript{82}. The review of literatures show conflicting results of association of Helicobacter pylori infection, with some studies associating increased prevalence of Helicobacter pylori seropositivity in subjects with CVD\textsuperscript{83}. Increased carotid IMT has been associated with increased inflammation as assessed by hs-CRP\textsuperscript{45}. The measurement of hs-CRP and helicobacter pylori serology were however beyond the scope of this study. It should be noted that hs-CRP has been found to be higher in Black subjects suggesting that comparisons with normal black subjects may be required.

Reference values for IMT need to be determined and compared to establish the value of IMT for risk within black and non-black cohorts. Prospective vascular studies would be of interest to determine rate of progression. Future studies will also be indicated to record and determine risk of vascular complications in black subjects, especially value of IMT.

STATISTICS. At the outset of this study, it was difficult to do a proper evaluation to assess the numbers of subjects needed for the findings about differences in IMT to be conclusive, as there was no valid information for these populations on which to base the calculation. As far as can be told from the literature, there had been no survey of carotid IMT in subjects of mixed ancestry or in black subjects in Southern Africa. The methods of performing IMT analysis are also not directly transferable from study to study though the equipment delivers reproducible measurements. Using a single operator improved the reproducibility. The data obtained in this study for a given segment of the carotid can be used to determine the sample size that would correctly resolve the difference between the two means with similar standard deviations in the two groups. For the carotid bulb the dimensions were 0.86 and 0.78 mm for black and non-black subjects respectively, the difference being 0.08 mm and
the standard deviations were similar (0.16 and 0.17 respectively). The number suggested to be able to determine this difference is calculated as $N = \frac{8}{(SD/\text{precision})(SD/\text{precision})}$ where SD is the (larger) standard deviation and precision is 0.06mm and reflects the half-width of the of the 95%CI of the difference. This derives a value of 64. Calculating the sample size of each group needed to discriminate the difference detected (0.08mm) between the two means, using an alpha of 0.05 and beta of 0.1 in the power index $\frac{(za-zb)}{(za-zb)}$, the formula applied is: $N = 2 \cdot SD \cdot SD \cdot \text{power index}/(0.08 \times 0.08)$. The power index is 8.6. Hence $N = 2 \cdot 0.17 \cdot 0.17 \cdot 8.6 / (0.0064) = 77.7$ or 78 people.

### 4.7 Limitations

Limitations of this study include the small sample size, absence of carotid IMT and brachial FMD values for comparisons with non-diabetic control populations. Studies have a continuation or graduation from small/pilot to large and definitive, with varying power to resolve the issue being examined. This is not only due to numbers but because assumptions for calculations may not be well-founded. Even the very SD obtained in this study may be in error and factors such as treatment may modify it and thus could make the calculations unreliable. (An example is the ENHANCE study with IMT recently).

A significant proportion of the patients were also obese. This is problematic because in subjects with increased BMI, neck blood vessels lie quite deeply making it impossible to visualize those segments. Other limitations include presence of calcification on the arterial wall, which causes shadowing and loss of visualization of the IMT segment. These latter two (i.e. increased BMI and calcification) are common with diabetic subjects. This study did not have HIV positive subjects in whom the co-existence of chronic inflammation and HIV treatment could promote atherosclerosis.
Chapter 5

Conclusion and recommendation

This study in a small cohort of Black and non-Black type 2 DM subjects in Cape Town has revealed interesting information that makes future investigations essential to discern whether there are significant differences in atherosclerosis development and outcome between these two groups. These studies include information not only about diet and lifestyle factors, but additionally carotid IMT and vascular function studies in black and non-black subjects with and without diabetes. Long term prospective studies are required to establish whether IMT is similarly associated with atherosclerosis in black and non-black subjects with diabetes. The atherosclerosis risk in Black diabetic subjects should be established through a large cross sectional study to establish whether the small above target LDLC requires statins in this population for whom resources are limited.

Much attention needs to be paid to the management of T2DM in the public sector of the region. Public education about the risks factors for T2DM and aggravating the rise in IHD, PVD and CVAs is essential. Smoking is a particularly important issue. Monitoring of risk factors and diabetes control at primary care level is essential. This includes meticulous attention not only to glycaemic control, but also to blood pressure and lipids in order to reduce risk in T2DM.

I would not recommend that carotid IMT measurement be used for routine clinical management until more information is available from this region on its clinical utility. There is also a need to establish the reference range of carotid IMT of normal subjects from the different racial groups in S. Africa to determine if black S. Africans also have increased carotid IMT as found in other black Africans in the UK and USA.
Bibliography


Appendix 1

INFORMED CONSENT FORM

RESEARCH STUDY: Carotid Intima Media Thickness (CMIT) measurement in Type 2 Diabetics
I hereby consents to participate in the study CIMT measurement in type 2 diabetes.

Dr. has explained to me the nature of the study with its benefits and risks to me. I understand that the study is to be carried out solely for the purpose of medical research and I am willing to act as a volunteer for that purpose. I recognize that the result of the study may be of significant benefit to mankind.

Date (Signed) (Witness to patient’s signature)

I confirm that the purpose and nature of the study and the risks involved including the fact that any refusal to participate will not in any way affect your normal care by me or any other members of this institution. All information obtained in this study is strictly confidential. Your responses will be identified by a study code number. Your name will NOT be used in this study. If any information is published, there will not be any information which would identify you as a participant. I know the consequences of any false declaration on this or any other form.

Date (Signed)

Appendix 2

DIABETES IMT STUDY

Study Date
Reference number
Surname Contact Phone numbers:
Date of Birth Age 1. (<20) 2. (20-39) 3. (40-59) 4. (≥ 50)
SEX 1. male □ 2. Female □
Ethnicity:
1. Black African-B □
2. Mixed ancestry-M □
3. European ancestry-W □
What grade did you finish at school? Grade ≤ 7 □ Grade 8-12 □ post school training □
University □
DM Duration □ Hypertension-Yes □ No □ Hypertension Duration □
↑Cholesterol-Yes □ No □ IHD-Yes □ No □ CVA-Yes □ No □ PVD Yes □ No □
Current Smoking Yes □ No □ Previous smoker Yes □ No □ Pack years □
+ve DM Family hx Yes □ No □ +ve HPT family hx Yes □ No □
Housing Density
Number of rooms in your house other than kitchen and bathroom □
Number of people in each room □
Height □ Weight □ BMI □ Waist Circumference □ Hip Circumference □ WHR □
Blood pressure: Erect □ Supine □ BP control- Good □ Poor □ Postural drop-Yes □ No □
Medications ACEI, TZD, Metformin
5. Aspirin □ 6. Others
Fundoscopy- Normal □ Abnormal □
BIOCHEMISTRY
Trigs
T.Chol
HDL
LDL
HbA1c
GGE
Microalbuminuria Yes □ No □ Microalbumin/creatinine ratio □
Resting Tachycardia-Yes □ No □
CIMT □ FMD
Appendix 3

Information regarding the study:

Purpose:
This study examines whether people with diabetes who are of African, white or mixed ancestry have similar changes in the big blood vessels of the neck. People with diabetes have an increased risk of diseases of the blood vessels compared to people without the disease. Previous studies have suggested that people of African ancestry may be protected from having diseases of the blood vessels, but more recent studies show a change from traditional diet and lifestyle may remove this protection. This study wants to find out if people of African ancestry with diabetes have a lower risk of diseases of the blood vessels compared to South Africans of European and mixed ancestries with diabetes. We also want to find out the effect of the presence of diabetes complications of the nerves on the tone of the blood vessels.

Who can participate?
People with diabetes within the age of 25 – 75 will take part in the study.

Benefits:
You will be informed about your body fat, diabetic control, and if you have other conditions that can occur in diabetes like high blood pressure and high cholesterol. You will be referred to your doctor or clinic if this is necessary. The findings of this research can be used to help manage heart and stroke diseases in South African people.

Procedures and potential risks:
On the morning of the examination, you will not take any medications or food, and should not have eaten 8-12 hours before the study; as you would do on a normal diabetic clinic day. You must not exercise within 4-6 hours of the study. No caffeine, high fat foods, vitamin C should be taken within 4-6 hours of the study. No smoking including passive smoking (that means you should not be in the same room with someone smoking) should occur within 4-6 hours of the study. You should not take any alcoholic beverages within 24 hours of the study. You should bring along your breakfast and medications, which you will take after your blood samples have been withdrawn.
**Ultrasound scanning**
When you arrive at the venue of the study, you will be taken to a quiet room, where you will lie down on the examination couch, in a comfortable position; the aim is for you to be properly relaxed. The ultrasonographer will then scan the large blood vessels of your hand; and your blood pressure will be measured. This will involve the blood pressure cuff on your arm being kept inflated for a period of about 4 or 5 minutes and you may experience some discomfort. After this the blood vessel will be scanned, following which your other hand will be placed in an ice bucket for about 30 seconds, and more measurements of your arm’s big blood vessel will be taken. You may also experience some discomfort during this process. The large blood vessels of your neck will also be measured using a probe (if you have had an ultrasound scan previously, maybe when pregnant; the method is similar; some gel will be applied on your neck and the probe will be used to scan your neck). A nitroglycerine spray (which is relatively safe, but not free of side effects and contraindications to its use) may be administered if you do not have any contraindication to its use. You will not receive it if you have any of these medical conditions; heart muscle diseases - hypertrophic obstructive cardiomyopathy, constrictive pericarditis, low blood pressure. The side effects of this spray include headache, transient hot flushing and palpitations, nausea, vomiting, low blood pressure, dizziness or fainting.

**Blood tests**
About 2 table spoons full of blood (10 ml) will be withdrawn from your blood vessel, you may experience some bruising during this process. After this, you will take your breakfast and medications like you usually do.

**Questionnaires**
You will then be asked some questions which will be recorded on a form.

**Clinical examination**
Your height, weight and waist and hip measurements will be recorded. Your eyes will be examined, your blood pressure will be measured from a sitting and standing position, and your ECG will be recorded.

The whole examinations, interview and investigation process will last an average of two hours.
Assurances:
Participation in this research study is absolutely voluntary. You do not have to take part if you do not want to. You may withdraw at any time without stating a reason and without prejudice. The doctor or researcher can also withdraw you from the study. You will be provided with all your own results.

Your own results will only be given to you. All records will be kept in a locked room and in a secure computer database in the research unit. Your name will not be used in any publication of the results.

The University of Cape Town and its team of researchers, who are working under the mandate of the University, will be responsible for treating any adverse or untoward events arising from participation in this research study.

If medical problems are identified during this study you will be referred to your usual doctor for further help.

Thank you for your participation. Please contact me if you would like to ask any questions or you experience any problems during or after the tests.
Appendix 4

Non-denaturing gradient gel electrophoresis (GGE) FOR LP

The technique of non-denaturing gradient acrylamide gel electrophoresis allows separation of lipoproteins (LP) by size. The LP may be demonstrated by staining lipid or protein. Pre-staining the sample with a lipid stain allows specific visualisation of lipoproteins in the original glass sandwich while protein staining has to be done after removing the gel and is only specific for large, apolipoprotein B-containing lipoproteins. Separation of CM and VLDL is difficult because of the size of these LP and these triglyceride-rich lipoproteins are seen as broad size ranges. The IDL range is also not so distinctly separated. Gels may be selected to demonstrate LpB series (LDL) and LpA series (HDL). A 2-16% gradient is usually recommended for LDL and for HDL, 4-30% [1] but our lab uses a mini-gel of 2-8% acrylamide gradient for LpB, and a 4-18% for LpA.

Qualitative differences in LDL may be important. The first relation between smaller denser LDL and heart disease was shown by Melissa Austin [4], who recognised two types of LDL. Dormans [5] suggested that 3 LDL subtypes could be identified by either ultracentrifugation or GGE, although the latter method could on occasion identify 5 bands. Tashiro [6] found a "midband", which is probably Lp(a), that predicts heart disease in FH subjects. Similarly, HDL3 and HDL2 subtypes may influence atherosclerosis. The GGE and HDL ultracentrifugation subtypes have not been compared yet in our lab. In this system, small dense LDL can be demonstrated in the lowest quintiles of triglyceride, waist/hip ratio, body mass index and fasting glucose concentration. However, small dense LDL is seen usually at a triglyceride concentration of >1.7mmol/L, almost always at TG >2.5mmol/L. Similarly, a waist/hip ratio of >1, BMI >30 or glucose >7mmol/L is highly associated with small dense LDL. It has been shown that a TG/HDLc ratio, in molar terms, of >1.33 is also strongly predictive of small dense LDL [15]. The system is also good for diagnosing dysbetalipoproteinaemia [14].

Samples may be plasma or serum or isolated lipoproteins. Samples should immediately be placed on ice and processed as soon as possible, but have been satisfactory for LDL characterisation up to 1 week when stored at 4 celsius, either stained immediately or after a delay. The samples can be frozen at -20celsius for a few weeks, and for several (>12) weeks at -80. There is a factor in the LPDS that can increase the size of LP on incubation. It is heat labile and dialysable but is neither CETP nor PLTP.
Samples from animals with cloven hooves may carry foot and mouth disease virus. Samples can be sterilised by heating to 56°C for ≥30 mins. This has been tested and shown not to affect human samples. After processing the same samples, should be placed at pH <6 or >9.

**MATERIALS**

SUDAN BLACK STAIN. 1% sudan black is added to ethylene glycol. Filter the solution. It keeps for several weeks.

SATURATED SUCROSE with a spatula tip of bromophenol blue.

SOLUTIONS FOR SDS-PAGE as declared in the laboratory methods but omit SDS from all. Fresh acrylamide solution is better for clarity of separation. Depending on the brand and batch of acrylamide slight adjustments may need to be made for optimal performance, adjusting the denser solution to 7 to 8%.

NILE RED STAIN. Dissolve 1mg in 100ul of dimethylsulphoxide as a stock solution (50X), then dilute by adding 2ul to 98ul of DMSO for use on GGE. Add 10 ul to 50ul of plasma. This stain with similar sensitivity to sudan black but requires UV light exposure for visualising the lipoproteins.

Minigel apparatus and powerpack in cold room or refrigerator.

**PROCEDURE LDL FOR (LpB) GEL**

Cast a 2-8% polyacrylamide gel and a 3% stacking gel with a minimal but definite layer of gel between the bottom of the wells and the separation gel. Mostly 2 gels are run in the system, with 15 lanes per gel. Label the glass plates. (The stacking gel may be coloured by a small amount of phenol red to make loading easier.)

To 100ul of plasma add 50ul of lipid stain, mix and incubate for a minimum of 1 hour at 4 celsius. Spin for 20min at 10000G. Mix an equal volume of supernatent with saturated sucrose and load approximately 12ul per well.

Prefocus the gel at 20V for 30mins. This is ideal but not essential. Run the gel at 130V for 18-24 hours. The progress is visible! Remove the glass sandwich from the stand, dry with a paper tissue.

Recording of the gel. Write the gel identification on the dry glass plate. Mark the point at which the separation and stacking gels meet by placing a dot next to the spacer. The gel is
now placed face-down on a photocopy machine and covered with white paper before a photocopy is taken. Afterwards, photograph the gel if necessary. The gel can also be scanned in the Hoefer densitometric scanner while in the glass plates, or it may be recorded on the Biorad Geldoc videocamera. Hereafter it is dried on filter paper or cellophane.

**PROCEDURE HDL (LpA) GEL**

Cast a 4-18% gradient gel and a 3% stacking gel. Prepare the plasma sample with sudan black in ethylene glycol as for LDLGEL but load directly, 16ul/lane.

Prefocus the gel at 20V for 30mins, run at 130V for 4 hours. The progress is visible! It is analysed in the same way as the LDLGEL.

**INTERPRETATION OF LpB GGE**

**REPORTING.**

The gel is inspected without knowledge of clinical or biochemical detail. Comment should be made about material in the stacking gel as occasionally there may be significant precipitate in the stacking gel, either from granules of sudan black that were not removed by the spin, or from lipoprotein aggregates. The origin (top or least dense part) of the separation gel reflects the largest particles. The minigel is about 60mm long. In our system the stain reflecting lipoproteins in the LDL density range, is from approximately the junction of the top 2/3 with the bottom 1/3. The chief LDL-related bands are in the bottom 1/6 of the stained area.

**TERMINOLOGY.**

The gel is described by simple terminology avoiding terms inferring separation by density. Since the gel was developed to look at "LDL", the zone between the origin and the LDL being referred to as "mid" and staining in this region was consequently referred to as M, which was later modified according to an earlier or later observation to M-early, or M-late to describe particles that were respectively larger or smaller. Subsequently it became clear that chylomicron-like particles tended to remain at the origin of the separation gel, and hence were termed O. At the "LDL" range, the largest particle is designated "A" while some intermediate "I" bands may be discerned or the "B" band as the smallest (densest) LDL which has been associated with a higher risk of ischaemic heart disease in FCH and with hypertriglyceridaemia. These common species of A and B appear to agree with the
prevalences of A and B reported in most studies although they have not been specifically prepared. Subsequently it became clear that even further size distinctions can be made, now abbreviated to A-early, A/I, I/B and B-past. Sometimes a band between A and M-late could be discerned, and referred to as pre-A. This band specifically disappears on adding a reducing agent, proving it is Lp(a). A small letter is used to designate that the band stains faintly, and a capital letter is used to demonstrate dominant bands. A sharply focussed band in the M-early range is seen in cholestatic jaundice, and is referred to as M-focussed.

Describing particles from large to small on this gel would thus be: O, M-early (Me), M-focus (Mf, in Me range usually), M-late (Ml), pre-A, A-early (Ae), A, A/I, I, I/B, B, B-past (Bp). Chylomicrons correspond to O, VLDL1 (Sf 60-400) to Me, LpX to Mf, VLDL2 (Sf 20-60) to Ml, pre-A is Lp(a), Ae to Bp are all species of LDL.

**RELATION TO DYSLIPIDAEMIAS AND FREDRICKSON TYPES.**

All hypertriglyceridaemias give rise to staining in the M region, but LpX which represents a hypercholesterolaemia, will also have staining in the M region. In type I and IV and V there is intense staining of the M region, often with something visible near the origin and the LDL bands are often only slightly visible, if not absent. In type III dyslipoproteinaemia the band peaks in the M range, with minimal LDL banding and there may be some staining at the origin.

Hypercholesterolaemias will stain mainly the LDL range with some entry into the M region if IDL is present. The LDL-A pattern is seen in most normal persons and hypercholesterolaemias, including FH, in which there is no hypertriglyceridaemia. Thus in normal and Fredrickson IIA one expects large species of LDL. In type IIB it would appear that almost always there is the in mid-portions, particularly Me, indicating an excess of VLDL-like LP. The LDL particle size in X-linked ichthyosis is large, so that agarose gel electrophoresis to demonstrate fast-migrating beta-lipoprotein is still preferable to prove this diagnosis.

The situation of Lp(a) is preA. LpX is seen in the M zone, usually as a sharp band and the distinct impression is that it is detected more sensitively than in agarose electrophoresis. LpX often gives the sudan black a greenish tinge.

The colour of the lipoproteins can vary considerably from brown to blue, and the sharpness of the bands is much better with fresh acrylamide. Lipid staining of isolated lipoproteins is also more intense than the staining in whole plasma.
CALIBRATION OF LpB GGE.

Currently there is no standardisation for assays of small or dense LDL. Small, dense LDL can be demonstrated by density (ultracentrifugation), or size (non-denaturing polyacrylamide gradient gel electrophoresis (GGE) or size exclusion chromatography such as FPLC). The reported size of LDL varies, with a slightly broader range reported [11] between 21 and 29 nm and the acrylamide gradients can vary [10]. Nuclear magnetic resonance can detect lipoproteins by unique emission signals according to their mass, allowing the discrimination of up to 15 lipoproteins [11] with estimates for LDL at 19 to 22 nm. Ultracentrifugation uses a few ml of fresh plasma and permits compositional analysis but takes hours to days and gravitational forces may cause the loss of some apoproteins. Typically, three fractions are separated [12]: LDL-I at 1.025-1.034 g/ml, LDL-II at 1.034-1.044 g/ml, and LDL-III 1.044 - 1.060 g/ml. The concentration can be reported as lipoprotein mass or protein or cholesterol.

It has not been possible to calibrate the system accurately so that the diameter of the particles can be properly calculated. Currently we readily identify A and B patterns amongst the 15 lanes and use one of each LDL type to carry across from gel to gel to bring some standardisation about. Lanes 7 and 8 contain an A and B species that are repeated used from gel to gel for a few weeks, but mixing them is not a good idea as some changes in size can occur. The observation is also that small LDL can enlarge with time. Of course gels that are prepared individually for each run are not exactly reproducible. However, it has been found that the classification is extremely reproducible on repeat samples.

Latex beads tended to clump and cause "ladders" and were thus not useful. We have generally accepted that LDL particles will have the typically published diameters and that there is a broad size range for all other particles. There is even disagreement in diameters for LDL from different laboratories using size markers, and also with other methods of determining lipoprotein particle size. This makes accurate diameter determinations less useful. The gel is primarily used in our laboratory to describe patterns of major dyslipoproteinaemias involving apolipoprotein B-containing lipoproteins with particular reference to dysbetalipoproteinaemia and lipoprotein X, and to determine LDL particle size categories and whether there are significant changes in these as a result of interventions.

Retardation factors. The system has been standardised against ultracentrifugally prepared VLDL1 (Sf 60-400), VLDL2 (Sf 20-60), IDL (Sf 12-20) and LDL (Sf 0-12) . Taking the B
particle as Rs=1.0, the following Rs values are typically found for V1: 0.2-0.45. For V2 the
Rs is 0.45-0.7; for IDL it is 0.7-0.85 and for LDL A-B it will be 0.85-1.0 and about 1.05 for
B-post.

**DIAGNOSTIC ASPECTS OF LDL GGE.**

Hypertriglyceridaemias have M bands, and usually B or Bposts. In chylomicronaemia
there is usually no LDL bands. Mixed hyperlipidaemias also have M bands. Dysbetalipoproteinaemia has a preponderance of Ml band with very little or no LDL bands, although in some instances the pattern may change to only a LDL band in this condition
upon successful therapy. If there is a lapse of dietary and drug compliance, the lipoproteins
may increase in size. Most hypercholesterolaemias have A bands, sometimes I bands and
occasionally there is a B band; the B usually being associated with a M or m. This pattern is
almost the norm when the fasting triglyceride concentration is >1.8mM/L, or waist hip ratio
is more than 0.95. It is also prevalent in diabetics, but more so in men. PreA is seen
variably and generally correlates with an apo(a) concentration of >50mg/dl, and some size
variation can be seen. There is a distinct impression that LDL particles distribution
distribute more strongly to a single species compared with ultracentrifugally derived
subfractions. In a population survey, it was found that about 2% of adults have no visible
LDL bands, and about 2 % have 2 distinct bands, sometimes of equal intensity.
Occasionally there may be more than 2 bands visible in the LDL zone. Interventions that
modulate triglyceride concentration, will usually alter LDL size at between 2 and 3 weeks.
An interesting pattern of smearing through the preA region into the LDL region has not
been explored.

**INTERPRETATION OF THE LpA GGE**

**REPORTING.**

This is done blinded and after selecting from the many lanes, likely equivalents of HDL 2
and 3 species. The bands tend to be poorly staining and broad, and only occasionally can
speciation be distinguished in the two regions. The LDL bands at the top may be
classifiable as A or B but are often inadequately separated for comment.
TERMINOLOGY OF "HDL GEL".

Particles relating to HDL2b and HDL2a as well as HDL3a, HDL3b, HDL3c are described in the review by Silverman [7]. In our system we find mostly 2 peaks, one with Mr of 135kD and another at about 165kD. The former appears to be HDL3 and the latter HDL2. In some patients there is a smaller size lipid-staining peak, at about 115kD. In some instances larger particles are seen in hyperalphalipoproteinaemia; in one patient discrete bands at 209, 229, 269, 300 and 365kD.

Current practice is to assume that the common species of smaller size is HDL3, the larger is assumed to be HDL2. The description is thus of the intensities being dominant in either one of the two bands or equivalent. This agrees remarkably well with the area under the curve and the peak intensities on our gel scanner. Occasionally there may be small species of lipoproteins, to which the label HDL4 has been given, or larger species to which the label HDL1 has been given. These can range to a size close to the LDL band. Typically the Me,B pattern of the Lp GGE has only a 3 band on the LpA GGE.

STANDARDISATION OF PARTICLE SIZE.

This has not been satisfactorily performed yet. Usually the pattern can be described by comparison with the other 14 lanes on the same run. Latex beads of defined diameter do not provide a single neat band on the LDL or HDL system, while on the HDL system the rainbow markers do not give neat bands when undenatured. Protein staining on our system would be confusing especially in the HDL range as there are many proteins at these sizes.

The utility of using haemoglobin is under investigation. Hb has a molecular mass of 64 458 and binds to haptoglobin9 (Hp) which has 2 binding sites for Hb. Hp is present in most individuals but rarely the Hp 0-0 status or anhaptoglobinaemia is found. The molecule is a tetrad of two a and b units. The a units can be a1 or a2 and slow and fast migrating forms of a1 are known. Several phenotypes of Hp are known: Hp 1-1 (a1a1b2) with mass of 80kd, Hp1-2 (a1a2b2) with a mass of 120kd and (a2a2b2) of 160kd. The Hp 1-2 and 2-2 phenotypes can dimerise to 200 and 400kd forms. Assuming one Hb to be bound per molecule, red bands should be seen on the GGE at 65, 145, 185, 225, 265 and 465kd. It is difficult to prepare suitable plasma samples to act as distinct markers, but sometimes haemolysis leads to these bands being visible in the HDL gels.

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An unusual ladder of proteins has been observed between HDL and LDL on the HDL gels from some subjects in the coronary care unit. The calculated molecular mass seems to escalate approximately 300kd intervals up to 2.5 million molecular mass.

APPLICATIONS

FLUORESCENT STAINING. The preliminary observations suggest that pre-staining the plasma lipoproteins with Nile red is as sensitive as Sudan Black staining for the LpB and LpA gels, but some optimisation is still required. There is intense staining at about 70kd on the LpA system which is not seen with a gel containing only albumin.

COOMASSIE STAINING. After the gel is run, it can be removed and placed in Coomassie staining solution according to lab protocols. If the plasma had not been pre-stained with Sudan Black, the LDL band is faint and possibly the preA band is more visible. Prestaining with Sudan Black enhances the Coomassie staining. Protein bands are visible below the LDL zone, at the edge of the gel and represent macroglobulins.

SILVER STAINING. This method becomes very sensitive for detecting protein and a decent band of LDL is seen with about 4ul of plasma in the lane. There is little protein visible in the lipoprotein range. Sudan Black enhances silver staining in a similar way that it enhances Coomassie staining and makes the entire range of lipoproteins from LDL to IDL stain very darkly. Acetone exposure of the gel for 30 minutes sensitises the unpresetained LDL to silver.

WESTERN BLOT. This allows transfer to nitrocellulose and probing for apoproteins. The transfer of apoB is slow owing to its molecular mass and can be enhanced by adding SDS if lipids are not desired in further studies.

ANALYSIS ON GELDOC

The Biorad Geldoc apparatus allows convenient graphic recording of gels stained with visible dyes or of light emitted from fluorescent stains upon UV illumination. The system includes a personal computer, a videocamera mounted in a photographic black chamber with illumination facility, and a dedicated printer for images. The graphics files are saved in C: under directories created for the staff, and in file names that can trace the gel efficiently. The graphics file stored can be used to print images and to analyse selected portions of images directly on the screen or by transferring the data to other software as x,y coordinates. This latter process affords better analysis and interpretation, especially through
Graphpad prism. The University of Cape Town Information Technology has been unable to link the Windows NT system to the network and the files have to be manually transferred by floppy disk from the Geldoc to other personal computers. This also poses problems for back-up.

**CAPTURING THE GEL.**

Switch on the computer, the video camera and the printer. The computer will prompt signing on as the administrator and the password is MONALISA (case-sensitive).

Select Multi-analyst Shortcut from the programme icons by a double click. The display will have toolbars available but no image will be seen until one is created or recalled from stored files. To create a new image, go to File and select New. The videocamera view will now be on the screen.

Dry the gel with a tissue and make certain there are no avoidable marks on the gel. It is a good idea to place a dot against the left and right spacers so that the junction of separation and stacking gels can be marked for reference during analysis.

Go to Set-up. Enter the appropriate light source (white light for GGE with Sudan Black). Prepare the photographic chamber. Note that a white transforming plate is placed over the UV source for analysing gels stained with dyes absorbing in visible wavelengths. Place the gels appropriately on the white plate or on the glass for UV analysis. Close the chamber. Adjust the aperture, focus, and zoom of the camera.

Make certain that the background density is uniform (best on analytical setting at the switch on the bottom front of the camera box). Once set up, the image needs to be captured. Select Capture button at bottom left corner. This button may be masked by the lower edge of the screen, in which case click on the edge and drag it lower to display this button. Additionally click on View and remove the toolbar to allow the capture button to come into view. The capture process will prompt the file saving data: In the Save As window, select the “Work” folder, double click on the appropriate “Name” folder and enter the appropriate Subfolder and file name. Enter the file name and save as a *.bif file.

Switch off the video camera (both the UV source and camera). To exit, select File, exit. To switch off the computer, go to Start, select shutdown and machine will close the programme and declare when it is safe to switch off manually.
PRINTING THE IMAGE.

This can be done from the video camera while the gel is being recorded, or from a saved image. In both cases the picture will include the surrounds of the gels unless there is appropriate cropping.

With the programme activated, select the saved image: go to File, select Open, select Work, follow directory through Folders and then select the appropriate File. The image will be displayed, but should be maximised for better viewing. Now select from the toolbar the open square to perform cropping so that the gel only is displayed, by clicking in one corner and dragging the mouse to enclose the area of interest. Take the mouse’s cursor to the Image button and select Crop.

Now return to File, select Videoprint. Now press the button attached to the printer.

ANALYSING THE GEL.

The password for the Multianalyst is case sensitive: MONALISA. With the Multi-Analyst software activated, select the file from the appropriate subdirectory. The image will be displayed on the screen, best maximised. Select lanes to be analysed with care. The toolbar contains a button with a folded image for single lane selection. Click on this and then click the mouse to point in the typically at left top of the lane to be analysed, and drag the mouse to the right and bottom of the portion of interest. Select the display in profile to view the lane as a densitometric scan, with optical density on the vertical axis and cm migration on the horizontal axis. If several lanes are to be analysed, use the “Selector” button which will place a “+” on the gel after which pressing “F” on the vertical toolbar will find lanes automatically. Select sensitivity required, usually at midpoint. The bands thus selected are demarcated in red. The profile can be viewed by pressing “View Profile” on the horizontal toolbar. To include all the selected lanes, go to “View” on main toolbar and select “Show all lanes”.

With the scan profile image box on the screen the saving can proceed. From the File Menu select Export to Excel and select Active Window. Now open Excel and scroll far right across to columns until reaching the columns containing numerical values and labelled “cm” and “OD” for migration (x axis) and optical density (y axis). Select, copy and paste these 2 columns into a new Excel worksheet. This must now be saved as a text file (*.txt), onto a stiffy disk. On closing down the Multi-Analyst file, the process will request saving
the changes (analyses and manipulations). Do not save these changes as they will alter the original gel record.

Activate Graphpad Prism, go to Files and select Import. Select the *.txt file on the stiffy drive or directory and proceed to import. Prepare a profile tracing by making a graph from the data sheet, best displaying the image as a line without the approximately 250 data points. The baseline correction can be done according to a personally selected value by using the Analyze and then Transform selections, or by going to Remove Baseline. The data may also be pruned to the appropriate rows by selecting Analyze and Prune.

The lane is analysed with retardation standardised to the given B distance for the particular run from which it is derived. Retardation is standardised according to the B band, Rs(B) = 1.0. Peak particle size can be described as a Rs. The profile should be analysed for AUC with the baseline subtracted.

**ANALYSIS ON HOEFER SCANNER**

The first densitometric scanner for GGE scanned about a 1000 points as x,y coordinates at absorption of 595nm as it was originally designed for Coomassie stained SDSPAGE. It was found suitable for Sudan Black and thus was used for capturing data. DJB developed the method below for capturing the data and processing it in Graphpad Prism.

Locate the scan desired on the stiffy disks, bearing in mind that each scan is a single lane. Open the file using Notepad. Choose Edit and select all data. Select Edit again and do Copy command.

Open Prism and prepare a datasheet which will receive the y coordinates. Paste the data to the Y column. Create a serially numbered column in the X-axis starting at point 1 and using increments of 1 (column dialogue box is found under Change). This procedure will create a graph.

Manipulate the data via Analyse and Transform command to Prune Rows. Exclude the first 100 points according to judgement about the start of the separation gel. Optionally, prune the rows again, by averaging 4 consecutive points. Now copy the y-data to a new data sheet in which X starts at zero in a series.

To convert the data to retardation factors, create a data sheet that will span from 0 to 1.1. Compare the marker and other lanes in the photocopy of the gel for A and B markers and ascertain the Rf values from these lanes. The LDL_B is taken as Rf=1.0. Using the transform
function on all the x points, the function is X/K where K is the value identified to mark RF=1.0.

**FUTURE DEVELOPMENTS**

It is my belief that the LpB system is useful for studying LP phenotypes in the common hyperlipidaemias and also in normal to moderately disturbed metabolism. It is a simple way of confirming dense species of LDL and for looking for unusually large species of HDL. It should be possible to reproduce it on larger gel systems but electrophoresis time may become impractically long and may generate a lot of heat. The system should lend itself to transfer to membranes and immunodecoration to demonstrate qualitative differences in lipoproteins. In larger gels it may be possible to elute adequate amounts of material for sensitive methods to determine lipid (e.g. fluorimetric) and protein (e.g. immunoassays) contents. A combination of the two gels could be attempted as published from WV Brown’s unit [13].

The separation of HDL into preβ and α forms can be done in 2 dimensional electrophoresis in which the first dimension is in 0.1% agarose gels and the second dimension is in 2-30% non-denaturing acylamide gels [16].

**REFERENCES For GGE**


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Appendix 5

Lipid Laboratory IMT procedure

1. Procedure for performing IMT scans using the ACUSON 128 and SONY CAMERA DKR-700

*Intima - media thickness (IMT) = distance between lumen-intima and media-adventitia interfaces*

**To start:**
1. Plug Acuson into wall socket (never use an extension lead)
2. Move Acuson to desired position and put brakes on.
3. Plug the transducer in and lock.
4. Hook transducer cord over hook and place in holder. (Do NOT drive over transducer cord)
5. Make sure the SONY CAMERA is connected correctly: 7 items plugged in (I) Input: red, green, blue and white (sync), (II) Output: video out (III) footswitch (IV) power plug
6. Switch the Acuson on (switch at the bottom front of the machine)

**Check:**
1. Set depth at 40 mm using "Depth toggle" on bottom Right side of screen.
2. Set application to VASCULAR using application toggle.
3. M-MODE setting:
4. Press M-MODE button
5. Press CODE and SIZE (key H) together, then soft key on RT until 2/3 appears
6. Press CODE and SIZE again.
7. Check brightness setting of screen.
8. Make sure that the SONY CAMERA is switched on.
9. Make sure that the correct minidisk is in the SONY CAMERA.
10. Make sure that the minidisk has been initialised. NB This process erases all data on disc
    • NB Disc can be damaged if power is put off while busy indicator is lit.
    • Switch on Acuson and turn SONY CAMERA power switch on
    • Insert disc into the SONY CAMERA
    • The ALL ERASE/INIT button will blink (It will stop after +/- 5secs)
    • While the ALL ERASE/INIT button is blinking – press the button. (If the ALL ERASE button has stopped blinking before step 3 – press the button and it will blink – then press again to start initialisation)
    • The ALL ERASE button will light and initialisation starts. Initialisation takes +/- 3 min and ends when the ALL ERASE button light goes out.

**Enter patient's details:**
1. Press BEGIN and type patient initials (first name and surname) and date of birth (dd-mm-mm-yy)
2. Press TEXT button. Move block to top L corner of screen, space bar and press HOME SET. Enter segment name here.
To annotate scans:
1. Press TEXT and HOME soft key.
2. Label segments RCCA, RCBA, RICA, RMMC, LCCA, LCBA, LICA, LMMC, LFMA, RFMA
3. To erase text press CODE and DELETE together. (This will not remove pt details entered by pressing BEGIN)
4. Press HOME and enter next label.

Order of scans performed:
1. Right-CCA, R-CBA, R-ICA, R-M-mode
2. Left-CCA, L-CBA, L-ICA, L-M-Mode
3. Left-FMA
4. Right-FMA

The transducer:
1. Transducer has a groove on one side.
2. The groove corresponds to Acuson logo on screen.
3. Groove must face towards the top of the patient's head. For cross-sectional views, groove must face towards the left.

Scan technique

Carotid artery
2. Turn head sideways with chin at 225 degrees for scan of Right carotid artery and at 135 degrees for scans of Left side. Optional: place a small sandbag next to the head to maintain the position.
3. Start scanning with head turned sideways as above. It may sometimes be necessary to turn head to a neutral position and / or to lift chin to achieve the best picture.
4. Perform transverse scan to determine the orientation of the internal and external carotid artery.
5. Confirm internal and external carotid artery by using Doppler if necessary.
6. Perform a longitudinal scan of the far walls of the distal 1 cm of common carotid artery, then the carotid bulb and perform a third scan of the proximal 1 cm of the internal carotid artery. The orientation of the transducer should be “ear to ear” resulting in a scan at 90 degrees to the chin. When optimising the image of the internal carotid artery, stay as close as possible to this angle. The aim is to have a standard technique to allow for reproducibility during repeated scans of the same patient.
7. Start scan with RES mode off
   • Push RES button to expand 2D image
   • Press once and a 2cm box appears
   • Move box to position with trackball
   • Then press again to enlarge
8. Optimize image
   • Adjust transition zone (focus depth) using toggle next to depth toggle on R side of screen
   • Adjust gain
9. Press cine button
   • Cine records previous 3-5 seconds of screening
• No screening takes place when button is depressed
• Trackball can be used to move through saved images in the clip
10. When the best image is on screen, place arrows and print
• To place arrows press delete
• Small square appears on screen
• Move into position with trackball
• Press shift and upwards arrow at the same time, to place arrow
• Placement of first arrow: inferior edge of carotid bulb
• Placement of second and third arrows: superior edge of carotid bulb and flow divider
• Placement of fourth arrow: flow divider
• To delete arrow: use space bar
• Always mark area of interest clearly, without arrow obscuring area of interest
11. PRINT button: saves image on minidisk

**M-mode**
1. Perform M-mode of common carotid artery as close as feasible to the carotid bulb
2. Start scan with RES mode off, optimise image – horizontal, near and far walls visible
3. Push RES button to expand 2D image
• Press once and a 2cm box appears
• Move box to position with trackball
• Then press again to enlarge
4. Optimize image again
5. Adjust transition zone (focus depth) to middle of artery
6. Adjust gain to minimise reverberation in the lumen (gain button and / or gain slides)
7. Press M-mode button
8. 2 images on screen now – B-mode at top and M-mode at the bottom
• Concentrate on the image in the B-mode window and use the trackball to move the
  cursor to area with clear double lines on both near and far walls
• When the best B-mode image is on screen, focus on the M-mode image below
• Optimise image by adjusting M-mode gain if necessary (small gain button)
9. Wait for one clear trace of near and far wall double lines
10. Press freeze and PRINT

**Femoral artery**
1. Patient lies supine with legs straight and feet pointing upwards. Optional: head of bed
  can be raised for comfort.
2. Perform transverse scan to determine where the bifurcation of the femoral artery is into
  the superficial and deep femoral artery.
3. Perform a longitudinal scan of the far wall of the distal 1 cm of the common femoral
  artery on the left and right side.
4. Use RES box and cine as above and place an arrow to mark the bifurcation.
5. Save the images to minidisk by pressing the PRINT button on the ACUSON keyboard.

To review scans performed (Minidisk):
1. Press EXTERNAL VIDEO button to see images on disc
2. Press "PLAY" on SONY CAMERA if not highlighted
3. Use + and - buttons to scroll through images
Transfer images to computer
(See Appendix A)

Administration:
1. Complete ultrasound logbook – date and patient details
2. Write image numbers down in logbook
3. Transfer images from minidisk to computer (refer Appendix B)

2. Transferring images from SONY CAMERA to computer:

NB SONY camera can only connect to computer which runs Windows 98.
NB both computer and SONY CAMERA must be off when connecting
1. Switch SONY CAMERA off and disconnect from ACUSON
2. Attach SCSI lead to SONY CAMERA into top slot / plug
3. Plug in black power cable to back of SONY CAMERA
4. Switch on first the SONY CAMERA and then the computer
5. Computer: login onto server
6. Double-click on icon for ACDSee icon (Green eye)
7. To find study files, go to MY DOCUMENTS-Ultrasound.
8. Double-click on required file (the file that you want to transfer images into eg Practise5 or FH minidisk) and note number of last image (e.g. 69)
9. Click on Acquire (at top of screen). If connected correctly, images on minidisk appear on screen. If not - check SCSI connected correctly. If not connected - restart from no (1.) – Go to Album and maximise.
10. Click on next image number (e.g. 70) – image has a blue frame when selected.
11. Click on Acquire in box in mid screen - this moves image to the selected file (Check - if number of transferred image is NOT 70 – then something is wrong. Correct it now – images are transferred from SONY CAMERA without numbers and are given the next available number in the new file. If you delete image number 23 and are transferring the real number 70, it will be renamed as number 23. Transfer all images from minidisk in the same order that they are stored on the minidisk, even if the image contains incorrect information. Only delete images later when transferring to specific patient files.)
12. In file, click on SONY CAMERA on bottom bar to return to SONY CAMERA. Click on next image i.e. 71, click Acquire. Repeat for remaining images, until all are transferred.
13. Make a note in diary of last number transferred.
14. NB Never delete unwanted images in master file, as this will affect the future transfer of images from the minidisk.
15. Click on SONY CAMERA on bottom bar and close SONY CAMERA window (cross at top right corner). Eject minidisk!
16. Switch the power off and disconnect SCSI lead. Reconnect to Acuson.

Transferring images to specific files:

To make a new folder:
1. Remain in program ACDSee.
2. To make a new folder for patient, click on folder in which you want to place images. E.g.: FH pts/Clinic pts/FMD pts
3. Click on File - New - Folder, enter name for folder (patient's surname and Initial) in blue highlighted new folder box and press Enter.
4. Transfer images:
   - NB in MASTER file images after no 99, are sorted by the computer as follows:
     100 will be placed after 10, 12 after 12 etc. It is important to check only
     patient’s own images transferred to own file.

**Transferring images to patients’ own files:**
1. Select file from which images are to be transferred e.g.: FH minidisk
2. Select 1st image for patient e.g. 70 and check last image number e.g. BloggsJ = images
   70 to 77.
3. The selected image will appear at LT bottom screen.
4. To enlarge image: double-click on image at LT bottom screen
5. Check name etc.: click on NEXT until all of patient’s images have been checked.
6. Reduce image: click on browse at top LT screen.
7. When image numbers have been determined, click on first image for patient e.g. 70
   (framed in blue when selected), move cursor to 71 and hold down Control key and click
   on 71 at the same time. Select all the required images for the patient in the same way
   (e.g. 70 to 77)
8. It is not possible to miss out an image in the sequence if it belongs to another patient or
   is not needed in the patient’s file. Transfer all images to patients own folder and delete
   once in patients file. NB Do NOT delete images in master file, as this will affect the
   future transfer of images from the minidisk.
9. Click on Edit and click copy. Now click on patient’s own folder, click on edit, then click
   on paste. (This leaves image in MASTER file with a copy in patient’s own file.)
10. Each patient will have 10 images.
11. Check to see that only the patient’s images are in the file.
12. If an incorrect or additional image has been copied to patient folder, delete it from
    patient folder only.
13. Close ACDSee (cross in top Rt corner)
14. Optional: it is possible to perform the same procedure in Windows Explorer.

3. Analysing images using Matlab (program written by Dr L John; copyright L John)

Jpeg or tiff images analysed in Matlab
1. Open Excel.
   - Minimise.
2. Open Matlab.
4. Go back to Matlab.
   - In command window-type IMT4 and enter.
   - Now minimise Matlab.
5. In IMT4 click File - Load and select image: i.e. C - Ultrasound, Clinic Patients. Select
   first image.
6. To calibrate:
   - Type 10 in block
   - Click on radio button
   - Calibrate on image
   - Crosshair on scale on left side of scan and click-repeat below.
   - If unhappy-calibrate again
7. To measure:
   • Click on measure radio button
   • Now click on intima-lumen interface and click on media-adventitia interface, in a vertical line.
   • If unhappy with markers - delete (delete yellow markers)
   • Can zoom if necessary-click on zoom radio button, then on area of interest on image
   • Always work L to R
   • Only measure what you can see
   • Use arrows as reference (segment)
8. When completed, click on Average IMT, then on Interpolate (need to have solid lines between crosses)
9. Check that row number in IMT4 should correspond to next available blank row in excel. If not, change row number in IMT4.
10. Now click on Transfer to Excel, for the first image analysed. May need to click twice (look at Excel file)
11. Save Excel table: File, Save As, use down arrow to go to
   • C, Ultrasound, NewMatlabanalysis
   • Patient name (e.g. BrownS) and SAVE
12. Go back to IMT4 and load next image
13. Measure and transfer when done
14. SAVE after each transfer
15. To measure length of segment: In IMT4, click File, Load:
   • Load image marked with 'IMT4' at the end
   • Type 10 in calibrate box and calibrate as before
   • Click on measure radio button and place crosshair on first original set of crosses
   • Then move crosshair horizontally to last set of crosses
   • Click on Average IMT, above which measurement will appear
   • Change Row number to a free row in excel and click Transfer to Excel
16. Continue till all 'IMT4' images are measured
   • SAVE
17. After one patient completed, close excel table and open a new one.
18. Load 1st image of next patient and change row number to 1 in IMT4.
19. To delete one row in Excel. Edit, Delete, Entire row.

4. IMT reports using Excel and WordPerfect

Preparing Excel report for transfer to WordPerfect:
1. Open Windows Explorer go to My Computer – Ultrasound – NewMatlabanalysis -Pts table
2. File - Save As – ExcelForReports - e.g. BrownSrep (type in ‘rep’ after name)
3. Highlight Pathname column and Delete with Delete button
4. Type in ‘Segment’ as heading
5. Type in ‘RCCA RCBA RICA’ etc (check and confirm this matches ACDC)
6. SAVE
7. Delete columns as follows:
   • Date, Data points, Mean (Edit - Delete)
   • All columns under ‘Individual-Distances’
   • SAVE
8. Highlight all the measurements and decrease decimal to 3 (on top toolbar)
   • SAVE
9. Change heading Interpolated-mean to Mean
   • SAVE
10. Highlight table minus first column
    • Copy and Paste to a free space
11. Cut LT sided measurements and paste next to RT
    • (e.g. LCCA NEXT TO RCCA)
    • SAVE
12. Cut RFMA and measurements and paste next to LFMA
    • SAVE
13. Copy headers above RT sided measurements and paste above LT sided measurements
    • SAVE
14. Highlight whole table
    • Bold
    • Format – Column - AutoFit Selection
    • Borders (block with sides and inners in bold)
    • SAVE

**To make Means/Max table**

1. Highlight whole table
   • Copy and paste a few rows down
2. Highlight columns ‘SD’ and ‘Min’ and delete
   • SAVE
3. Make a new table one column away
   • Type: Segment           Mean Max
   • Common carotid
   • Carotid bulb
   • Internal carotid
   • Carotid mean
4. Under Mean type =
   • (  
   • cursor on RCCA mean (digits)
   • +
   • cursor on LCCA mean(digit)
   • )
   • / 2 and enter
5. Copy this block and paste in two rows below
6. Repeat for Max - cursor on RCCA max etc
   • Next to carotid mean place cursor in block, select AutoSum - Average on toolbar and enter
7. Copy this block and paste in Max block
   • SAVE
8. Check that measurements have 3 decimal points and SAVE
9. Highlight this table – Bold – Format - borders-as with previous table.
   • SAVE
To make measurement distance table
1. Make a new table in a free space
2. Type Segment Distance - Segment Distance
   - RCCA 1.111 LCCA 1.111 (1.111 is example)
3. Copy values from measurements table into corresponding space.
4. Highlight table - Bold - Format - Column - AutoFit Selection - Borders as before
   - SAVE

Now ready to copy and paste into WordPerfect Report
1. Open WordPerfect
2. File – Open - C-Drive – Ultrasound - Sonographyreports2006 (pre-prepared)
3. Open pt file
4. Go back to Microsoft Excel and highlight each table, one at a time, and copy to WordPerfect
5. All 3 tables should now be in WordPerfect
6. Highlight each table in turn and use down arrow on Font Face to select Times New Roman and down arrow on Font Size to select ‘12’
   - SAVE
   - SAVE
8. Print
9. Submit for reporting
10. Original to be filed in ultrasound room.
11. Copy to be filed in lipid sleeve
   - See report template on next page
Lipid Laboratory, Internal Medicine
6th Floor Cape Heart Centre
UCT Health Science Faculty
Observatory, 7925. South Africa
Tel: 21-406 6838
Fax: 21-406 6396

Date of scan:
Sonographer:
Ultrasound machine:
Patient:
Birth date:
Gender:
Folder no:

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<td>RFMA</td>
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<th>Mean</th>
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<tbody>
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<td></td>
</tr>
<tr>
<td>Carotid bulb</td>
<td></td>
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<tr>
<td>Internal carotid</td>
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<tr>
<td>Carotid mean</td>
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<tr>
<td>RICA</td>
<td>LICA</td>
<td>RFMA</td>
<td>LFMA</td>
</tr>
</tbody>
</table>

Comments

Reported by:
Appendix F

IMT database

1. Enter data on Sonography report into Paradox database G drive – LIP – A-SONOGRAPHY - Academicscans

2. List of fields
   - Patient name
   - Date of birth
   - Date of scan
   - Hospital number
   - DNA number
   - Ultrasound Machine
   - Indication
   - Sonographer
   - Diagnosis
   - Reliability
   - RCCA mean
   - RCCA SD
   - RCCA max
   - RCCA min
   - RCBA mean
   - RCBA SD
   - RCBA max
   - RCBA min
   - RICA mean
   - RICA SD
   - RICA max
   - RICA min
   - LCCA mean
   - LCCA SD
   - LCCA max
   - LCCA min
   - LCBA mean
   - LCBA SD
   - LCBA max
   - LCBA min
   - LICA mean
   - LICA SD
   - LICA max
   - LICA min
   - LFMA mean
   - LFMA SD
   - LFMA max
   - LFMA min
   - RFMA mean
   - RFMA SD
• RFMA max
• RFMA min
• Common carotid mean
• Common carotid max
• Bulb mean
• Bulb max
• Internal mean
• Internal max
• Carotid mean of means
• Carotid mean of maxes
• Comments
• Analysis

Backup
The Paradox tables are kept on G: / drive / LIP / A- SONOGRAPHY
All other work is backed up onto T: drive
Use copy and paste in Windows Explorer to back work up.
Appendix 6

FMD Protocol for the Diabetes study

Protocol summary

- Brachial artery diameter and then FMD is measured
- Lumen diameter after ice immersion and then maximum lumen diameter after sublingual tri-nitro-glycerine (TNG) is measured
- Acquire images for carotid IMT according to Lipid Laboratory IMT Protocol

Subject preparation

- The subject should be studied in a quiet, temperature controlled room
- Withhold all vasoactive medications for at least 4 half-lives (if possible)
- No exercise within 4-6 hours of study
- No caffeine, high-fat foods, vitamin C within 4-6 hours of study
- No smoking within 4-6 hours of study
- The investigator should be cognizant of the menstrual cycle of female subjects

FMD Checklist

Equipment

- FMD worksheet
- Consent form
- Scale
- Room temperature thermometer
- Video
- Minidisk
- Ice buckets x 2
- Glass of water x 2
- Wine / grape juice or vodka (ml/kg)
- TNG spray
- BP cuff manual
- BP cuff electronic
- Pillow
- Blankets x 2
- Swivel chair
- Background music
- Fruit juice for afterwards

Mini disk recorder (SONY DKRP700 by Sony Corporation)

- Check mini disk recorder connected correctly: Acuson RGB and sync connected to video in; Acuson video in (white cable) connected to video in; little black cable from video machine connected to video out; power cable, footswitch connected
- Switch power on
- Check correct mini disk in SONY
NB It is vital that the SONY is set on SOURCE for the VCR to record what is on the ACUSON screen

**Video recorder (SONY VHS Video recorder SVO 9500 MDP by Sony Corporation)**
- Check video recorder connected correctly: Acuson video out connected to video out; little black cable from mini disk recorder connected to video in, power cable
- Switch power on
- Check video not faulty – press PLAY and RECORD and record for a few seconds
- Rewind Press EXTERNAL VIDEO on ACUSON and the PLAY on video recorder
- Rewind again and write down video start numbers on worksheet

**Acuson settings (Acuson 128 by Kardia Netherlands)**
- Vascular; depth 30 mm
- Doppler angle 60 degrees; angle L
- Doppler gate small
- Annotate screen as follows PT BEGIN XXX brachial artery, the CUFF and time, later ICE time or TNG time
- Refer to the clock on the ACUSON for time during the study

**Electronic BP apparatus (High quality electronic BP apparatus Acutorr Plus (Datascope) by Vitalcare Technologies)**
- Use electronic BP apparatus to record BP
- Use manual BP apparatus to occlude artery

**Starting the study**
- Check that the subject has fasted overnight (Cancel study if not fasted)
- Check that consent form has been signed

**Complete FMD worksheet as study progresses**
- Date
- Name
- DOB
- Sex
- Medication in past week
- Smoking
- Wt in kg
- Did the subject fast (DO NOT PROCEED IF NO)
- Video number
- Video start and stop

**Position of subject and sonographer**
- The subject is positioned supine with the arm in a comfortable position and the left brachial artery is imaged directly above the cubital fossa in the longitudinal plane
- One layer of blanket is usually required
• It is important for the sonographer to sit in a comfortable position and to support the left elbow on the bed during the scanning of the artery. Use the right hand to operate the ultrasound machine.

• Make notes during the study by annotating the screen and transcribe to the worksheet in between sections of the procedure.

• Avoid rewinding the video during the procedure as it can lead to loss of data.

Baseline protocol

Rest prior to start of study

• The subject needs to lie down and relax for 15 minutes prior to start of study.

• Record starting time on FMD worksheet.

• Record room temperature in BC at baseline and again at +30 minutes, +60 minutes and +120 minutes after drinking the wine / grape juice or vodka.

• Use the electronic BP apparatus on the right arm and do BP x 2 (± 5 minutes apart) and record on worksheet.

Image acquisition

• Subject is positioned supine with the arm in a comfortable position for imaging the brachial artery. Place paper towel under arm to protect clothing.

• Place a manual BP cuff around the upper arm. The fit should be snug. Use a pediatric cuff if necessary for very slender arms. Position the BP apparatus on the bed next to the subject.

• Arm should be straight and rotated outwards. Apply gel to the skin and find the brachial artery above the antecubital fossa. Start medially & slide laterally with transducer towards head. Use colour Doppler to confirm artery.

• Optimize image - clear anterior and posterior intimal interfaces between the lumen and vessel wall. It is seldom possible to visualize a double-line pattern on the near wall.

• Use the cubital crease as an external landmark – place the distal edge of the transducer on the cubital crease.

• Find the brachial artery in the 4 x 4 box, and then press RES.

• Start the Doppler imaging and place gate in artery (Doppler setting as specified in checklist).

• Press PLAY and RECORD simultaneously on the VCR.

• Press FREEZE, then TRACE Use trackball to place caliper on near wall and push soft key MARK Move second caliper to far wall. Press PRINT to save to MINIDISK.

• Now press CALIPER and use trackball to move crosshairs to the maximum systolic velocity.

• Press PRINT to save to MINIDISK.

FMD procedure

• Unfreeze image.

• Inflate BP cuff around forearm to 50 mmHg above systolic BP recorded at rest.

• Leave inflated for 5 minutes. Annotate screen CUFF and time cuff inflated.

• Keep recording Doppler screen for 5 minutes.

• Use TRACE and MARK to follow the diameter of the artery in the top (B-mode) window.
• Remember the baseline diameter
• Release cuff exactly 5 min after inflation
• Do not freeze image now
• Record continuously for 4 minutes after deflating the cuff
• Use TRACE and MARK to follow the diameter of the artery in the top (B-mode) window
• Memorize maximum dilatation
• Wipe ultrasound gel off arm and allow subject to bend arm
• Record diameter on worksheet

ICE procedure
• At least 10 minutes of rest is needed after reactive hyperemia
• Immersion in ice is used to calibrate minimum arterial diameter
• Place ice near operator (NOT ON CHEST OF SUBJECT YET!)
• Place second blanket (folded) on chest of subject
• Again place paper towel under elbow and apply gel to skin
• Place container with ice on chest of subject Subject can hold side of container lightly
• Subject places RIGHT hand into ice for 30 seconds Annotate screen ICE and time
• Continue recording for 2 minutes after hand out of ice
• Use TRACE and MARK to follow the diameter of the artery in the top (B-mode) window
• Memorize minimum diameter
• Stop recording
• Wipe ultrasound gel off arm and allow subject to bend arm
• Record diameter on worksheet

TNG procedure
• Sublingual nitroglycerin spray is given, to establish the maximum obtainable vasodilator response
• NB TNG can cause headache, which is worsened by sitting up suddenly Allow subject to lie down for ± 10 minutes after administering the spray TNG is short acting and wears off after about 10 minutes
• Check TNG spray and place bottle near operator
• Again place paper towel under elbow and apply gel to skin
• Find the brachial artery in the 4 x 4 box, and then press RES
• Start the Doppler imaging and place gate in artery (Doppler setting as specified in checklist)
• Press PLAY and RECORD simultaneously on the VCR
• Subject administers 2 sprays under tongue, using their right hand, during recording
• Images are recorded continuously for 4 minutes after TNG administration Dilatation starts about 2 minutes after administering the spray
• Use TRACE and MARK to follow the diameter of the artery in the top (B-mode) window
• Memorize maximum dilatation
• Wipe ultrasound gel off arm and allow subject to bend arm
• Record diameter on worksheet
• Record video stop time on worksheet
- TNG can cause headache, which is worsened by sitting up suddenly. Allow subject to lie down for ± 10 minutes after administering the spray. TNG is short acting and wears off after about 10 minutes.

**Finally**

- Rewind video and write name of subject, start time and stop time on video
- Write comments on worksheet if applicable
- Photocopy worksheet. Original saved in Sonography room.
Appendix 7

Definition of Terms

1. ketosis prone atypical diabetes: Classified by the ADA as Type 1b, or Idiopathic type 1 diabetes. Characterised by an acute initial presentation with severe hyperglycaemia and ketosis, as classical type 1 diabetes. In the subsequent clinical course after initiation of insulin therapy, prolonged remission is often possible with cessation of insulin therapy and maintenance of appropriate metabolic control. Metabolic studies show blunted insulin secretory response to glucose, partially reversible with improvement in blood glucose control.

2. Tropical diabetes: Atypical diabetes associated with chronic malnutrition and sometimes chronic pancreatitis. Characterised by early onset of non-ketosis prone diabetes in underweight patients, with very high insulin requirements.