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Carotid intima media thickness versus plaque score: associations with stroke

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CAROTID INTIMA MEDIA THICKNESS VERSUS PLAQUE SCORE: ASSOCIATIONS WITH STROKE

Pitchou Zonga Gazwa

A dissertation submitted to the Faculty of Medicine, University of the Witwatersrand, for the degree of Master in Sciences (Medical).

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Abstract

Ischemic stroke caused by large artery occlusion accounts for most cases of stroke. The majority of cases of ischemic stroke occur as a consequence of thrombus formation, following rupture, erosion or calcification of atherosclerotic plaque. Conventional cardiovascular risk factors such as hypertension, diabetes mellitus, smoking, and dyslipidemia account for the large burden of stroke in South Africa. However, the extent to which measures of carotid atherosclerosis (intima-media thickness or the presence of plaque) add to conventional risk factors in identifying those at risk of stroke in communities of African ancestry where at least 50% of stroke occurs in patients younger than 50 years of age, is uncertain. In the present study, carotid IMT and the presence of atherosclerotic plaque (B mode ultrasound) were determined in 137 patients of African descent with ischemic stroke admitted to a neurology unit and 195 age and sex-matched healthy participants selected from a community study (random selection). Patients with stroke had an average age of 49.4±14.5 years. With adjustments for conventional risk factors related to stroke (hypertension and high density lipoprotein concentrations), carotid IMT (mm) was increased in patients with stroke (0.740±0.010) as compared to the control group (0.679±0.009, $p<0.0001$). The multivariate adjusted odds of having a carotid IMT $\geq$0.9 (mm) was also independently associated with stroke (Odds ratio=1.44, confidence intervals=1.13-1.86, $p<0.005$). However, only 25.6% of patients with stroke had carotid plaque as compared to 17.4% of controls ($p=0.073$). Whilst carotid IMT showed a significant performance for stroke detection (area under the receiver operator characteristic curve, [AUC] =0.623±0.031, $p<0.0005$), plaque failed to show significant performance for stroke detection (AUC=0.541±0.023, $p=0.074$). In conclusion, the current study suggests that in a group of African ancestry where the average age of stroke reflects at least half of all patients being under 50 years of age, that carotid IMT may add to

conventional risk factors in identifying the risk of stroke. However, the presence of carotid plaque may add little to conventional risk factors in identifying the risk of stroke.

Declaration

I declare that this dissertation is my own work. It is being submitted for the degree of Master in Sciences in the Faculty of Medicine, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this university, or part of it, for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references. Data in this dissertation have been presented in the form of a poster at the Stroke and Hypertension Conference held in Johannesburg, August 2016. The title of the presentation was: "*Does IMT Add to the Ability of Conventional Risk Factors to Predict Stroke in South Africa?*" (Authors: **Pitchou Z. Gazwa**, Olebogeng HL Majane, Grace Tade, Eitzaz Sadiq, Gavin R Norton, Angela J Woodiwiss, Girish Modi).

I certify that studies in this thesis have the approval of the Committee for Research in Human Subjects of the University of the Witwatersrand, Johannesburg. The ethics approval number is: **M140429**

Dr.Pitchou Zonga Gazwa

.....day of 01/12/....., 2017

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Dedication

To my wife Charmante Zonga Asumani Malkya for being so patient during the time of my study, you are wonderful and I am very proud of you. To my parents Vicky Kanalina and Raphael Zonga, and to my brothers and sisters, who have always been there for me, thank you so much for your love and support. To my grandmother Cecile Mayeteleka who inspired me to always try harder, make no excuses, and never give up. My only regret is that you, who from the start of this journey, when, at the age of five I first attended school, could not be here to share in the celebration of the current achievement. Here, I am trying to fight against stroke, which caused your death. I miss you grandmother.

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Statement of contribution to work

Carotid imaging was performed by myself. The diagnosis of stroke of ischaemic origin was made by clinical neurologists at the Charlotte Maxeke-Johannesburg Academic Hospital. The blood samples were sent to an accredited laboratory (National Health Laboratory Services) for analysis. To ensure appropriate measurements, the ultrasound images of the carotid artery were assessed by an experienced sonographer (Belinda Stevens). The anthropometric data was collected with the assistance of a qualified nurse. I analysed all the data by myself in consultation with my supervisors.

List of abbreviations

AF	Atrial fibrillation
APOGH	African Programme on Genes in Hypertension
AUC	Area under the curve
BP	Blood pressure
CAD	Coronary heart disease
CCA	Common carotid artery
CHD	Cardiac heart disease
CSF	Cerebrospinal fluid
CV	Cardiovascular
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
ICA	Internal carotid artery
ICH	Intra-cerebral haemorrhage
IDL	Intermediate-density lipoprotein
IL-6	Interleukin 6
IMT	Intima-media thickness
LDL	Low-density lipoprotein
MI	Myocardial infarction
NCDs	Non-communicable diseases
NHSL	National Health Systems Laboratories
OR	Odds ratio

ROC	Receivers operating curve
SAH	Subarachnoid haemorrhage
SAS	Statistical analyses software
SD	Standard deviation
SEM	Standard error of the mean
SSA	Sub-Saharan Africa
TOAST	Trial of Org 10172 in Acute Stroke
VLDL	Very low-density lipoprotein
WC	Waist circumference
WHO	World Health Organization
YR	Year

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Preface

In sub-Saharan Africa (SSA) increasing urbanization is predicted to enhance the prevalence of risk factors for vascular disease and hence increase the incidence of stroke. In this regard, stroke in SSA often occurs at a much younger age than in other countries, with many patients experiencing a stroke when they are less than 50 years of age, an age which is often considered to be the most productive years of life. The impact on the ability of those suffering a stroke to care for families; earn a living; and enjoy life is often devastating, frequently driving families below the poverty line and the socio-economic consequences of job and skills losses as a consequence of stroke are inestimable. Despite the high public health burden of stroke in South Africa, there is considerable uncertainty as to how best to identify at risk patients in groups of African descent and particularly in younger individuals. In this regard, improving risk prediction using measures (biomarkers) of the pathophysiological changes that account for stroke may be an important approach to prevent strokes.

Pathological arterial intimal thickening, which represents the earliest manifestation of progressive atherosclerosis, results in an increase in intima-media thickness (IMT), a change which can be detected non-invasively using ultrasound in several vessels, but easily measured in the carotid artery. Moreover, with advanced atheroma formation, obvious arterial plaque may occur which is often noted in the area of the carotid bifurcation. Whether carotid IMT or plaque assessment are useful predictors of stroke in black African communities, especially when stroke occurs at a young age, is an important question to answer. In order to attempt to improve on risk prediction in groups of African ancestry in Africa, in the present dissertation I therefore aimed to determine the extent to which carotid IMT or plaque add to risk

prediction for stroke in a group of patients with stroke where at least half were younger than 50 years of age.

In chapter 1 of the present dissertation, I first review the scientific literature that relates to cardiovascular risk factors and other risk factors associated with stroke, with a specific focus on what is known about stroke and indices of atheroma in groups of African descent. In this chapter I discuss the different types of stroke and the variety of mechanisms through which risk factors could lead to the progression of atherosclerosis. In the process of reviewing the scientific evidence related to these topics I highlight the missing evidence in groups of African descent whom often develop stroke at a young age. In doing so I thus argue in favour of conducting the present study. In chapter 2 of the present dissertation, I then describe the methods employed in the present study and in chapter 3, I present the results of my study. In chapter 4 of the present dissertation I finally discuss my data in the context of previously published evidence and suggest the possible relevance of my findings,

CHAPTER ONE

INTRODUCTION

1.1. Introduction.

Non-communicable diseases (NCDs), which include cardiovascular disease (CVD), were formally recognized at the United Nations General Assembly as an important missing element in the Millennium Development Goals (Temu et al. 2014). Despite considerable progress made in recent years in the management of known risk factors for CVD, this group of disorders remains the leading cause of death worldwide (World Health Organization, 2015). For several decades, ischaemic heart disease and stroke have been the most common causes of death and disability in all regions of the world except sub-Saharan Africa (SSA) where communicable disease, trauma, infant mortality, and human immunodeficiency virus (HIV) infections have been the dominant causes of death (Ntsekhe et al. 2013). An epidemiological transition is now occurring in the developing world where the major causes of death are changing from infections to non-communicable diseases (diseases of lifestyle), with an increasing prevalence of CVD (Kapiga et al. 2011). Sub-Saharan Africa is currently experiencing one of the most rapid epidemiological transitions characterized by increasing urbanization and changing lifestyle (Fezeu et al. 2006), which in turn has raised the incidence of NCDs, especially CVD (Kadiri et al. 1997). The overall burden of CVD is predicted to rise by approximately 150% in the developing world within the next 20 years. In Africa alone, it is predicted that it will affect 1.3 million people per annum during this period (Sliwa, 2008). In South Africa, CVD is the second leading cause of death after HIV accounting for up to 40% of deaths among adults (Peer et al. 2008).

1.1.1. Burden of stroke in the world and in Africa

Cardiovascular disease includes several disorders of the heart and vessels, many of which share similar risk factors. A major CVD in most countries is stroke. Stroke is the second leading cause of death and the number-one cause of physical disability worldwide (Di Carlo et al. 2009, Lopez et al. 2006). Of the approximately 56 million deaths that occur annually worldwide, one-quarter of these deaths are attributable to vascular disease and of these ischemic heart disease (7.2 million deaths annually) and stroke (5.5 million deaths annually) represent the top two causes of mortality globally. Stroke therefore accounts for approximately 9.7% of all deaths worldwide (Strong et al. 2007). There is a rising epidemic of stroke in SSA directly resulting from the epidemiologic transition (Owolabi et al. 2015). Estimates suggest that 8% of all first-ever strokes occur in Africa and that 5% of the 30 million stroke survivors worldwide live in Africa (World Health Organization, 2010). The subtypes of stroke differ remarkably in Africa as compared to developed countries with relatively recent epidemiological data indicating that the proportions of ischaemic and haemorrhagic stroke in Africa are 66% and 34% respectively, as compared to 91% of ischaemic stroke and 9% of haemorrhagic stroke in high income countries (O'Donnell et al. 2010).

1.1.2. Relevance of stroke to South Africa.

Stroke is among the top 4 causes of death in South Africa (Mudzi et al. 2012). In South Africa, stroke may contribute to 6% of deaths and is the leading cause of mortality in those older than 65 years of age (Kahn et al. 2013). Over the period 1997 to 2004, an increase in stroke-related deaths was noted in the 20-35 year old age group (Statistics South Africa

2005 , Anderson et al. 2010). These figures are in-line with World Health Organisation predictions that by 2020, 69% of diseases in developing countries such as South Africa will be NCD, of which the largest burden will be derived from cardiovascular disease (Boutayeb et al. 2005). Stroke is the forerunner of vascular disease in the epidemiologic or health transition, and all evidence suggests that South Africa is well into the transition. (Connor et al. 2005).

1.2. Types of stroke.

Stroke is defined as 'rapidly developing clinical signs of focal disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin'. A stroke (cerebrovascular accident [CVA]), occurs when the blood flow to the brain is interrupted. This could occur either because a blood vessel to the brain ruptures, causing bleeding, or because a blood vessel occludes as a consequence of a thrombus. The brain requires about 50 ml/100g/min blood flow with an oxygen metabolic rate of 3.5 cc/100g/min. If the blood flow drops below 10 ml/100g/min, brain cell functions are severely affected, while neurons are unable to survive at levels below 5 ml/100g/min (Caplan, 2000). Stroke is divided into two main categories i.e. ischaemic and haemorrhagic stroke, with the latter including intracerebral haemorrhage and subarachnoid haemorrhage.

1.2.1. Haemorrhagic stroke.

Haemorrhagic stroke differs from ischaemic stroke in that it is caused by a ruptured blood vessel as opposed to a thrombus. The ruptured vessel interrupts blood flow and delivery of essential nutrients and oxygen to the affected area of the brain. Approximately 10-15% of all strokes are haemorrhagic and these have an estimated 40%-50% mortality rate (Woo et al. 2004, Go et al. 2010). Intra-cerebral haemorrhage (ICH) refers to bleeding directly into the brain parenchyma. The most common causes of ICH are hypertension, trauma, bleeding diatheses, amyloid angiopathy, illicit drug use (mostly amphetamines and cocaine), and vascular malformations. Subarachnoid haemorrhage (SAH) refers to bleeding into the cerebrospinal fluid within the subarachnoid space that surrounds the brain. The two major causes of SAH are rupture of arterial aneurysms and bleeding from vascular malformations.

1.2.2. Ischaemic stroke.

A significant proportion of strokes are ischaemic in nature, and one of the leading causes is internal carotid artery (ICA) atherosclerosis. It is estimated that 20-25% of all strokes can be attributed directly to atherosclerosis of the carotid bifurcation (Shah et al. 2008). The consequence of atheroma formation is local thrombosis and this results in *in situ* obstruction of an artery. Atheroma may however lead to obstruction either directly through disease of the arterial wall (thickening of the wall) and luminal obstruction or indirectly through a superimposed thrombus overlying an atherosclerotic lesion. Ischaemic stroke may also be caused by embolic events where particles of debris originating elsewhere obstruct arterial access to a particular brain region. These particles often originate from the heart

(cardioembolic stroke). Ischaemic stroke may also be produced by systemic hypoperfusion, where a more general circulatory problem manifests itself in the brain and perhaps other organs.

The classification of ischaemic stroke is of clinical and pathophysiological importance. Two major approaches to the classifications of ischaemic stroke are currently being used. The most widely used system is the Trial of Org 10172 in acute stroke treatment (TOAST) classification. With the advances in modern diagnostic technology, new stroke subclassification systems, such as the causative classification system (CCS) and the Chinese ischaemic stroke subclassification (CISS) system, have been developed to enhance the accuracy of TOAST. The A-S-C-O (**A**therosclerosis, **S**mall vessel disease, **C**ardiac source, **other** cause) phenotypic classification system makes an effort to identify the most likely aetiology, but without neglecting the possibility of other potential multiple causes (Pei-Hao et al. 2012). The following describes the TOAST classification systems in more detail.

1.2.2.1. TOAST Subtype classification system

The TOAST classification system is straightforward and follows a logical progression. It is clinically relevant and can supplement other independent assessments, including assessments of the severity of the neurological deficits. While the TOAST classification notes the presence or absence of risk factors, the onset and course of the stroke, and the nature of the neurological findings, diagnoses are strongly influenced by the results of diagnostic tests. The TOAST classification system includes five categories:

1.2.2.2 Large-artery atherosclerosis.

Large artery atherosclerosis is identified as a possible cause when patients have clinical and brain imaging findings of either significant (>50%) stenosis or occlusion of a major brain artery or branch cortical artery, presumably due to atherosclerosis. Cortical or cerebellar lesions and brain stem or subcortical hemispheric infarcts greater than 1.5 cm in diameter on CT or MRI are considered to be of potential large-artery atherosclerotic origin. Supportive evidence by duplex imaging or arteriography of a stenosis of greater than 50% of an appropriate intracranial or extracranial artery is needed. Diagnostic studies should exclude potential sources of cardiogenic embolism. The diagnosis of stroke secondary to large artery atherosclerosis cannot be made if duplex or arteriographic studies are normal or show only minimal changes (Adams et al.1993, Pei-Hao et al. 2012)

1.2.2.3. Cardioembolism.

This category includes patients with arterial occlusions presumably due to an embolus arising from the heart. Cardiac sources are divided into high-risk and medium-risk groups based on the evidence of their relative propensities for embolism. At least one cardiac source for an embolus must be identified for a possible or probable diagnosis of cardioembolic stroke. A stroke in a patient with a medium-risk cardiac source of embolism and no other cause of stroke is classified as a possible cardioembolic stroke (Adams et al.1993, Pei-Hao et al. 2012)

1.2.2.4. Small-artery occlusion (lacune).

This category includes patients whose strokes are often labelled as lacunar infarcts in other classifications. The patient should have one of the traditional clinical lacunar syndromes and should not have evidence of cerebral cortical dysfunction. A history of diabetes mellitus or hypertension supports the clinical diagnosis. The patient should also have a normal CT/MRI examination or a relevant brain stem or subcortical hemispheric lesion with a diameter >1,5mm. Potential cardiac sources for embolism should be absent, and evaluation of the large extracranial arteries should not demonstrate a stenosis of greater than 50% in an ipsilateral artery (Adams et al.1993, Pei-Hao et al. 2012).

1.2.2.5. Acute stroke of other determined aetiology.

This category includes patients with rare causes of stroke, such as nonatherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders. Patients in this group should have clinical and CT or MRI findings of an acute ischaemic stroke, regardless of the size or location. Diagnostic studies such as blood tests or arteriography should reveal one of these unusual causes of stroke. Cardiac sources of embolism and large-artery atherosclerosis should be excluded by other studies(Adams et al.1993, Pei-Hao et al. 2012).

1.2.2.6. Stroke of undetermined etiology.

In several instances, the cause of a stroke cannot be determined with any degree of confidence. Some patients will have no likely aetiology determined despite an extensive evaluation. In others, no cause is found but the evaluation was cursory. This category also includes patients with two or more potential causes of stroke so that the physician is unable to make a final diagnosis (Adams et al.1993, Pei-Hao et al. 2012)

1.3. Risk factors for stroke.

The risks factor for stroke can be divided into modifiable and non-modifiable types and include hyperlipidaemia, hypertension, cigarette habituation, diabetes mellitus, age, sex, physical inactivity, heart diseases (including atrial fibrillation [AF]) and obesity. Several other risk factors of stroke have been proposed but are less well documented. Some of these risk factors include oral contraceptive use, inflammatory processes, migraine, sleep apnea syndrome, prothrombin activator inhibitor complex deficiency, hypotension, high C-reactive protein concentrations, neurocysticercosis, Chlamydia pneumoniae, Helicobacter pylori, Legionella pneumophilia, chronic bronchitis, periodontal disease, ethnicity, and hyperuricemia (Lopez et al. 2006, Palm et al. 2009, Nagel et al. 2014).

1.3.1. Non-modifiable risk factors.

1.3.1.1. Age and sex.

Age is the strongest determinant of stroke and most individuals who have a stroke are over 65 years of age. The chance of having a stroke approximately doubles for each decade of life after age 55 years, and while stroke is more common among the elderly, an increasing number of people under 65 are also having strokes (Asplund et al. 2009). Controversies regarding differences in stroke epidemiology between men and women continue. Petrea et al (2009) noted that stroke incidence increased with each decade of life in both women and men with no significant sex difference in stroke subtypes, severity or case fatality rates. Data from the Framingham Study further suggested that 1 in 5 women and 1 in 6 men reaching the age of 55 years free of stroke, will develop a stroke event during their remaining lifetime (Seshadri et al. 2006). However, Barker-Collo et al (2015) noted that the global burden of stroke has been increasing for both men and women but that these increases have been greater among men (Suzanne et al. 2015).

1.3.1.2. Ethnicity.

At all ages, blacks are at a considerably higher risk of incident stroke compared with whites, although the effect is most marked in younger age groups (Huxley et al. 2014). Indeed, in the United States of America (USA) prevalence by ethnicity is 1.8% for Asians, 2.7% for Caucasians, 3.6% for Afro-Americans, and 3.9% for American Indians and residents of Alaska (Go et al. 2010). The higher risk of stroke in groups of African ancestry as compared to Asians and Caucasians is most likely due to blacks having a greater burden of

stroke risk factors rather than because there is any substantial race differences in the associations between risk factors and stroke. (Huxley et al. 2014).

1.3.1.3. Heart diseases

Through embolic events (cardio-embolic stroke), there is an approximately five-fold greater risk of stroke with atrial fibrillation and this is independent of alternative risk factors and occurs at any age (Fuster et al. 2011). As a result, heart disease is responsible for approximately 15% of all strokes and for 20% of all ischaemic strokes (World Health Organisation 2005). Arrhythmias of the heart, valvular disease, previous myocardial infarction, and congenital heart defects are noted as significantly increasing the risk of stroke or transient ischaemic attack (Goldstein et al. 2001). These effects are produced by embolic phenomena (emboli originating from a thrombus on a region of the heart where stasis occurs).

There are three high-risk cardiac origins of emboli for cardioembolic strokes and these include the left atrial wall, left-sided cardiac valves and the left ventricular wall. Atrial fibrillation (AF), atrial flutter, sick sinus syndrome, left atrial thrombus, left atrial appendage thrombus and left atrial myxoma are the most frequent atrial causes of emboli that produce ischaemic stroke. Mitral stenosis, prosthetic valves and infective endocarditis are the most frequent valvular causes of emboli that result in ischaemic stroke. Left ventricular thrombus, left ventricular myxoma, recent anterior myocardial infarct and dilated cardiomyopathy are the most frequent ventricular causes of emboli (Mei-Shu L et al. 2005).

1.3.2. Modifiable risk factors.

1.3.2.1. Hypertension.

Independent of other risk factors, the risk of stroke is increased 2- to 4-fold in those with hypertension (Nguyen et al. 2010). Observational, population-based studies demonstrate that although a higher blood pressure (BP) is strongly linked to an increased cardiovascular risk and cardiovascular and all-cause mortality, there is a particularly strong association between a higher BP and the risk of stroke (Peter et al. 2015). This increased risk is associated with morphologic alterations of the arterial intima and functional alterations of the endothelium that are similar to the changes observed in hypercholesterolemia and established atherosclerosis (Brian et al. 2011). Moreover, there is substantial evidence that the treatment of hypertension is associated with a markedly reduced risk of developing carotid stenosis and stroke (Paladino et al 2014).

1.3.2.2. Diabetes mellitus.

Diabetes mellitus is an important risk factor for ischaemic stroke. Individuals with diabetes have a 2- to 3-fold increased risk of stroke and other cardiovascular diseases compared with individuals without diabetes (Goldberg, 2000). In a meta-analysis of prospective studies (including 530 083 participants), the hazard ratio for ischaemic stroke was 2.3 (95% CI 2.0–2.7) in people with versus those without diabetes (Sarwar et al. 2010). Observational analyses of data from the United Kingdom Prospective Diabetes Study (UKPDS) indicate that glucose control as indexed by glycated haemoglobin levels (HbA1c)

is positively associated with risk of stroke (Stratton et al. 2000). However, the relation between disturbed glucose metabolism and ischaemic stroke is bidirectional. On the one hand, people with diabetes have more than double the risk of ischaemic stroke after correction for other risk factors, compared with people without diabetes (Sarwar et al. 2010). On the other hand, acute stroke can give rise to abnormalities in glucose metabolism, which in turn could affect outcome (Capes et al. 2001). Diabetes causes atherosclerotic changes in the heart and the cerebrospinal arteries and is associated with different subtypes of ischaemic stroke, including lacunar, large artery occlusive, and thromboembolic strokes (Cui et al. 2011, Jackson et al. 2005, Ohira et al. 2006). Moreover, the risk of atrial fibrillation, a major cause of thromboembolic stroke is increased by 40% in individuals with diabetes (Huxley et al. 2011). Strict control of post-prandial glucose levels in diabetic patients has been associated with a reduction of carotid intimal media thickness and may also help to reduce the incidence of stroke from carotid stenosis (Fazel et al. 2008).

1.3.2.3. Smoking

Smoking is known to raise triglyceride concentrations in the blood, damage endothelial cells, cause thickening and narrowing of blood vessels and result in the formation of thrombus (U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, 2010). In a study by Fazel et al (2008) smoking was strongly associated with development of carotid atherosclerotic disease. In this regard, as compared to age-matched non-smokers where the prevalence of clinically significant carotid stenosis (>50%) was 4.4%, in former smokers and current smokers the prevalence was 7.3% and 9.5% ($p<0.0001$), respectively. In addition, Lemogoum et al (2005) reported that the population attributable risk of stroke due to smoking is about 15% with a six-fold greater risk compared with non-

smokers. Importantly the Global Youth Tobacco Survey indicates that greater than 10% of 13- to 15-year-olds use tobacco in many Sub-Saharan African countries, and this prevalence can be as high as 33% in South Africa (Asma et al. 2002).

1.3.2.4. Dyslipidaemia.

The effect of cholesterol on the process of atheromatosis is now well established. High levels of total cholesterol, as well as of low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), lipoprotein, and triglyceride concentrations, coupled with decreased levels of high-density lipoprotein (HDL) are responsible for the creation of atheromatous plaque. Of the above factors, LDL cholesterol, and especially the oxidized LDL is considered as the most important contributor of atheromatosis (Elias et al. 2014). Therefore there is a link between cholesterol and stroke risk (Goldstein et al. 2001, Strazzullo et al. 2010, Amarenco et al. 2009) and there is good evidence that lowering blood cholesterol concentrations with statins reduces stroke risk and carotid atherosclerosis, independent of BP, and age (Amarenco et al. 2007).

1.3.2.5. Alcohol.

Controversy exists as to the relationship between alcohol consumption and the risk of stroke (Lindsay et al. 2010). Goldstein et al (2001) found no cause-effect relationship between alcohol use and stroke and studies such as The Northern Manhattan Stroke Study have shown a correlation between a moderate use of alcohol and lower levels of low density lipoproteins (LDL) and lower BP in individuals consuming small, regular amounts of alcohol.

These relations obviously suggest a protective effect of moderate alcohol consumption on stroke.

1.3.2.6. Obesity.

The risk for NCDs including the CVDs stroke and ischaemic heart disease increases progressively as body mass index (BMI) increases (Antipatis et al. 2001). There is a much stronger association of stroke risk with indices of central (abdominal) (waist circumference or waist-to hip ratio) than with indices of general (body-mass index) adiposity (O'Donnell et al 2010, Winter et al. 2008, Yatsuya et al. 2010). Obesity could increase the risk of stroke, not only by increasing the chances of diabetes mellitus or hypertension, but also by promoting excessive inflammation produced by adipose tissue (Daniels et al. 2009, Sriram et al. 2002), and producing associated endothelial dysfunction, insulin resistance, accelerated atherosclerosis, atrial fibrillation, and obstructive sleep apnea. The end result may be progressive thromboembolism that may result in arterial occlusion or rupture (Kernan et al. 2013).

1.3.3. Can conventional risk factors explain all of the risk of CVD?

Although it is well accepted that conventional cardiovascular risk factors account for the large burden of stroke in South Africa (Connor et al. 2009), there is considerable uncertainty as to how best to identify at risk patients. Traditional risk assessment has been shown to predict long-term outcome in large populations (Naghavi et al. 2010). However, the ability to predict risk falls short of predicting near-future events particularly in individuals in clinical practice (Naghavi et al. 2003). In this regard, improving risk prediction using measures (biomarkers) of the pathophysiological changes that account for stroke is an

important approach to prevent stroke. In this regard biomarkers should measure a pathophysiological process responsible for the event. As indicated in previous discussion, atherosclerosis may be a key mechanism responsible for the pathogenesis of a large proportion of strokes.

1.3. Atherosclerosis and the clinical assessment thereof.

1.3.1. Physiopathology.

The arterial wall consists of three anatomically distinct layers: intima, media and adventitia, which are separated by two thin elastic layers, inner and outer, respectively. The endothelial monolayer (intima) is a vital organ of the circulatory system. Its health is essential to normal vascular physiology and its dysfunction is a crucial factor in the pathogenesis of atherosclerosis and its complications (Gimbrone et al. 200). Atherosclerosis is a systemic inflammatory disease of the arterial wall and is the underlying cause of most cardiovascular events (Jashari et al. 2013). Atherosclerotic lesions form predominantly at regions of the arterial tree exposed to low/disturbed blood flow such as branches, bends, and bifurcations, whilst regions exposed to unidirectional high flow are spared (Christina et al. 2011). The process of atherosclerosis begins in childhood with the development of fatty streaks. These lesions can be found in the aorta shortly after birth and appear in increasing numbers in persons aged 8-18 years. More advanced lesions begin to develop when individuals are aged approximately 25 years. Subsequently, an increasing prevalence of the advanced complicated lesions of atherosclerosis exists, and the organ-specific clinical manifestations of the disease increase with age through the fifth and sixth decades of life (Brian et al. 2011).

The mechanisms of atherogenesis remain uncertain. An incompletely understood interaction exists between the critical cellular elements, endothelial cells, smooth muscle cells, platelets, and leucocytes of the atherosclerotic lesion. The "response-to-injury" theory is most widely accepted explanation for atherogenesis (Ross et al. 1993). Probable causes of endothelial injury include risk factors for atherosclerosis, oxidative stressors, angiotensin II, systemic infection and inflammation that inhibit nitric oxide production and stimulate production of adhesion molecules, pro-inflammatory cytokines, chemotactic proteins, and vasoconstrictors (Badimon et al. 2014, Lusis et al. 2000, Jules. 2000). The net effect is the passive diffusion, accumulation and retention of LDLs through the endothelial junction into the intimal layer due to haemodynamic flow stress-induced endothelial cell dysfunction at arterial branches or curvatures (Rupinder et al. 2012). Following endothelial binding of monocytes and T cells, migration of these cells to the sub-endothelial space, and initiation and perpetuation of a local vascular inflammatory response occurs. Low density lipoproteins are oxidised in the sub-endothelium and uptake of oxidised lipids and macrophage transformation into lipid-laden foam cells results in the typical early atherosclerotic lesions called fatty streaks (Badimon et al. 2014). Death of foam cells by apoptosis and necrosis leads to the formation of a soft, destabilizing lipid-rich necrotic core within the intima. Macrophage differentiation and foam cell formation help to bridge the innate and adaptive response to atherosclerosis, and recruited T lymphocytes join macrophages in the intima and direct adaptive immune responses (Rupinder et al. 2012). Macrophages produce pro-inflammatory cytokines that promote smooth muscle cell migration from the media and that further attract and stimulate growth of macrophages. The result is a subendothelial fibrous plaque with a fibrous cap, made of intimal smooth muscle cells surrounded by connective tissue and intracellular and extracellular lipids (Jules, 2000). Atherosclerotic plaques characteristically occur in regions of branching and marked curvature at areas of geometric

irregularity and where blood undergoes sudden changes in velocity and direction of flow (Brian et al. 2011). Stable plaques regress, remain static, or grow slowly over several decades until they may cause stenosis or occlusion (Finn et al. 2010).

In the absence of atherosclerosis, the nourishment of blood vessels is accomplished by adventitial vasa vasorum or oxygen diffusion from the vessel lumen. As the plaque progresses, intimal thickness increases and it exceeds the normal oxygen diffusion threshold. Vasa vasorum proliferate in the inner layers and become the major source for nutrients to the vessel wall. Another common feature of plaque progression is the focal calcification in atherosclerotic plaques which increases with age (Rupinder et al. 2012). Unstable plaques are vulnerable to spontaneous erosion, fissure, or rupture, causing acute thrombosis, occlusion, and infarction long before they cause haemodynamically significant stenosis. Plaque rupture involves secretion of metalloproteinases, cathepsins, and collagenases by activated macrophages in the plaque. These enzymes digest the fibrous cap, particularly at the edges, causing the cap to thin and ultimately rupture. The thrombus may rapidly occlude the vascular lumen and precipitate an acute ischemic event, the thrombus may embolise or the plaque may fill with blood, balloon out, and immediately occlude the artery. In general, unstable coronary artery plaques have high macrophage content, a thick lipid core, and a thin fibrous cap; they narrow the vessel lumen by $< 50\%$ and tend to rupture unpredictably. In comparison carotid artery plaques which have the same composition but typically cause problems through severe stenosis and occlusion or deposition of platelet thrombi, which embolize rather than rupture.

1.3.2. Indirect assessment of atheromatous lesions.

The technology to assess atheroma using non-invasive indirect approaches has considerably advanced over the past 20 years. This has led to an extensive body of scientific literature evaluating the possible role of the use of this technology to enhance risk prediction. In this regard, the extent of atherosclerotic changes in large arteries may be determined indirectly using high-resolution B mode ultrasound, which can detect two possible changes. In this regard, in the early stages of atheroma, the intima, and media thicken because of atherosclerotic changes and increases in large artery intima-media thickness (IMT) may be detected using ultrasound techniques applied to the carotid artery. Alternatively, with advanced atheroma formation, obvious arterial atherosclerotic plaque may occur and this may also be detected in carotid arteries using high-resolution ultrasound. As long as recommended approaches are employed, as compared to plaque assessments, IMT measurements are relatively simple to perform; due to the edge-detection software are less subject to observer bias; and provide a quantitative rather than qualitative assessment of the degree of atheroma. Moreover, they do not require the observer to be able to accurately identify the presence of atherosclerotic plaque which may take considerable skill, experience and training. Hence, in relatively untrained hands IMT assessments are more attractive than plaque assessment.

A consensus document from the American Society of Echocardiography describes the appropriate approach to intima-media thickness (IMT) assessment (Stein et al. 2008). Although originally both the near and the far wall of the common carotid arteries were initially included in measurement, the current trend is to limit the IMT measurement to the far wall of the common carotid artery. This is based on the fact that far wall measurement of the common carotid artery alone is perpendicular to the ultrasound beam, easily assessable, and reproducible (Wikstrand et al. 2007). Ultrasound systems for IMT assessment now

Mannheim consensus has provided appropriate definitions of plaque. In this regard plaque is defined as a focal thickening that encroaches into the lumen by 0.5 mm or by 50% of the surrounding IMT or where IMT is >1.5 mm (Touboul et al. 2012). Although these indices (IMT and plaque) of subclinical atherosclerosis associate with cardiovascular risk, whether IMT and plaque are distinct phenotypes or single traits at different stages of atherosclerosis development is unclear (Dong et al. 2011 & Della et al. 2012). Moreover, whether plaque assessment is more important than IMT measurements in risk prediction has been a topic of considerable debate.

1.3.3. Carotid intima-media thickness and carotid plaque assessment.

Pathological intimal thickening, which may result in an increase in IMT, represents the earliest manifestation of progressive atherosclerosis. In this regard it is rich in proteoglycans and lipids and lacks smooth muscle cells and collagen. In contrast, medial thickening, which occurs as a consequence of adaptive hypertrophy, and as such is not a true representation of an atherosclerotic lesion, may also cause increases in IMT (Toubol at al. 2004). Carotid IMT may therefore represent adaptive changes to biomechanical parameters with aging and may not be an accurate indicator of atherosclerotic changes (Finn et al. 2010). An increased IMT may also represent "fatty streaks" which consist of foamy macrophages, but these lesions have been shown to regress rather than progress to plaque formation (Wannarong et al. 2013). Hence, there has been considerable uncertainty as to whether IMT or plaque best predict cardiovascular events.

A large number of prospective studies have demonstrated that carotid IMT is predictive of major cardiovascular events, independent of traditional risk factors, with risk ratios ranging from 1.4 to 5.1 for coronary heart disease, and from 2.0 to 3.5 for stroke

(Simon et al. 2007, Ruijter et al. 2012). In addition, Rosvall et al (2005) noted that the presence of increased common carotid IMT was an important risk indicator for stroke, even when information on carotid plaque or cardiovascular risk factors was taken into account. However, Simon et al (2010) suggested that carotid plaque area is more representative of atherosclerosis than IMT. In addition, these authors (Simon et al 2010) indicated that despite much epidemiological data on the prognostic performance of IMT, there is no clear evidence that IMT measurement may improve coronary heart disease risk prediction beyond conventional risk factors and really change the risk of coronary heart disease assigned to an individual. Furthermore, they suggested that there is also insufficient data supporting the possibility that IMT may improve stroke prediction beyond prediction by conventional risk factors (Simon et al. 2010). At a similar time Tatjana et al (2010) suggested that carotid plaque is a distinctive phenotype of atherosclerosis that is most likely not a simple continuum of IMT progression, and predicts stroke and coronary artery disease risk better than IMT. In subsequent meta-analyses the measurement of carotid plaque alone was demonstrated to be more predictive of cardiovascular events than IMT (Inaba et al. 2012, Spence et al. 2012). The recommendation was therefore that IMT assessment should always be supplemented by a thorough scan of the extra cranial carotid arteries for carotid plaque to increase the diagnostic performance of carotid ultrasound (Inaba et al. 2012). The uncertainty as to whether an increase in IMT represents a pathological atherosclerotic or a benign change is highlighted by the lack of ability of increases in IMT to predict atherosclerotic plaque development as recently demonstrated (Rundek et al. 2015).

Naqvi et al (2014) have reviewed the controversy on the issue of whether IMT or plaque best predict CVD. These authors (Naqvi et al 2014) have highlighted that the large clinical studies that measured IMT and plaque to determine their value in predicting incident cardiovascular diseases varied in terms of the site and type of IMT measurement, as well as

the angle of the IMT assessment. Some studies imaged a single segment, and others multiple segments; some studies imaged the far wall of multiple segments, whereas others imaged both near and far walls; some measured the mean or maximum for single measurements; mean of the mean, or mean of the maximum for multiple measurements; some included plaque in the IMT measurements, whereas others considered a plaque-free area. Studies also differed by plaque definition. Some studies defined plaque as a focal thickening of the intima-media >1 mm, protruding into the lumen, which is at least twice as thick as the IMT on either side. Other studies defined plaque as carotid IMT >1.2 mm. Others classified plaque as type 1 (uniformly echolucent), type 2 (predominantly echolucent), type 3 (predominantly echogenic), type 4 (uniformly echogenic) and type 5 (heavy calcification). Hence, there is no clear evidence as to whether IMT, plaque or both add to risk prediction.

1.3.4. Ethnic disparities in IMT and plaque.

Because of ethnic differences in the atherosclerosis process (Bild et al. 2005), the concern of whether IMT is a sufficient risk predictor of CVD may be particularly pertinent for groups of black African descent. Studies conducted in multiple countries have shown that IMT values may differ between distinct ethnic groups, thus indicating the need for regional investigations. In a multi-ethnic study conducted in the United Kingdom, whilst carotid IMT was greater in a group of black African ancestry than in a comparable group of European descent, carotid plaque was less prevalent in black individuals as compared to with white individuals (Mackinnon et al. 2010). This dissociation between IMT and plaque seen in black individuals suggested that an increase IMT in groups of black African ancestry may not directly relate to an increased atherosclerosis. In support of these data, in a recent large community-based study conducted in a Black African sample living in the South West

Township (SOWETO) of Gauteng, our group has noted strong age-related increases in carotid IMT with little evidence of plaque or independent associations between IMT and conventional cardiovascular risk factors (unpublished data). However in the Atherosclerosis Risk in Community Study, no site-specific differences in the prevalence of plaque were noted between African-Americans and Americans of European descent and the positive relations between carotid IMT and plaque were just as strong in African-Americans as they were in Americans of European descent (Li R et al. 1994). Hence the extent to which carotid IMT measurements reflect a pathological atherosclerotic process or a non-pathological process (compensatory medial thickening or fatty streak formation) in black African communities is uncertain. Whilst carotid IMT measurement is conducted using semi-automated border detection software and hence is less reliant on observer error, plaque assessment is entirely observer dependent. Hence, whether carotid IMT or plaque assessment are the more useful measures of stroke prediction in Black African communities is an important question to answer.

What study could further add to our understanding of whether carotid IMT or plaque predicts stroke risk in black African communities? Although several studies have compared the prevalence of an increased IMT and plaque in groups of African versus European descent or relations between IMT and plaque across ethnic groups (Rundek et al. 2015 & Li R et al. 1994) and have reported that IMT is higher in African Americans than in Caucasians (Howard et al. 2005), no study has compared whether IMT or plaque best predict CVD in groups of African ancestry where stroke may occur at a particularly young age on average as compared to other populations. To address this question the best study design would be to evaluate prospectively the ability of IMT and plaque to predict events. However, in South Africa, these studies are limited by poor follow-up and uncertainty as to the cause of hospitalizations or death (50% of death certificates indicate "natural causes"). Hence, the next

best approach is to perform case (stroke)-control (randomly selected individuals) analysis. Therefore, in the present study, I assessed whether IMT or plaque best enhances risk prediction of stroke beyond conventional risk factors in a population of African descent in Gauteng.

1.4. Problem statement and aims.

To the best of my knowledge, the uncertainty as to whether IMT or plaque can predict stroke in groups of black African ancestry has not been resolved. Moreover, whether increases in carotid IMT or plaque are able to risk predict for stroke over and above conventional risk factors in populations where stroke occurs at a much younger age than other populations, such as in South Africa, is unknown. Hence, in the present study I aimed to determine the extent to which carotid IMT or plaque add to risk prediction for stroke beyond conventional cardiovascular risk- factors in a group of African ancestry in South Africa where stroke occurs at a relatively young average age.

CHAPTER TWO

METHODS

2.1. Study participants.

The study protocol was approved by the University of the Witwatersrand Committee for Research in Human Subjects (approval number: M140429). The present study was part of on-going studies which are taking place in the Charlotte Maxeke-Johannesburg Academic Hospital, Division of Neurology, and in the School of Physiology, University of Witwatersrand, Medical School. Informed, written consent was obtained from the patient or from the next of skin in case of impaired cognitive functions and from the healthy controls. The minimum age for participation in the study was 18 years, but there was no upper age limit.

2.1.1. Case group

One hundred and thirty seven patients ≥18 years of age of African ancestry (black patients only) consecutively admitted to the Neurology Unit as inpatients with a new stroke, defined as a focal neurological deficit of vascular and ischaemic origin, were evaluated. Participant with cerebral meningitis based on cerebrospinal fluid (CSF) examination, or the presence of intracranial malignancy or intracranial mass lesions on radiographic imaging, vasculitis, and haemorrhagic stroke were excluded. These data were collected in collaboration with the Department of Neurology and specialist neurologists whom decided on the diagnosis based on clinical, laboratory and imaging data. Information on the nature of the stroke was extracted from the patient files and included information on stroke severity, chest roentogram (X-Ray), electrocardiogram, lumbar puncture and brain imaging (CT or MRI Scan) data. The Trial of Org 10172 in Acute Stroke (TOAST) Classification was used to classify each patient (courtesy of Dr Eitzaz Sadiq).

2.1.2. Control group.

Case data were compared with data obtained in 195 age and sex-matched individuals from participants of a community sample older than 16 years of age. The community sample was derived from randomly recruited nuclear families of black African descent (mainly the Nguni [Zulu, Xhosa, Ndebele, Swati], Sotho [South, North Sotho and Tswana] and Venda chiefdoms) with at least two parents or two siblings and living in the South West Township (SOWETO) of Johannesburg, South Africa using the population census figures of 2001. No subjects of mixed, Asian, or European ancestry were recruited. Random recruitment of community participants was based on the following approach: Street names and addresses of households were obtained from the department of home affairs, 2001 census. These households were allocated numbers, and numbers were selected from a random number generator. People residing in informal dwellings or institutions/ homes were not recruited.

2.2. Demographic, clinical and anthropometric measurements.

Demographic and clinical data were obtained using both a questionnaire and by extracting data from the patient files. For the control sample, the questionnaire was explained to the families at the first home visit and then subsequently completed in the presence of trained study assistants at an office visit where questions could be answered. In order to avoid translational errors, the questionnaire was not translated into an African language, but study assistants familiar with all languages spoken in these townships assisted with the completion of each questionnaire when assistance was requested. At a second home visit, answers relative to date of birth, gender, previous medical history, the presence of hypertension, diabetes mellitus, prior and current drug therapy, smoking status (including the number of

cigarettes smoked in the past and at the present time), daily alcohol consumption (beer, traditional beer or other forms of alcohol and the daily quantity) were obtained. For the patients, data obtained on the questionnaire were confirmed from the clinical file or from family members if the patient was uncertain.

Trained observers measured body height and weight with the participants standing and wearing indoor clothes with no shoes using standard approaches. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a body mass index (BMI) $\geq$30 kg/m^2.

2.3. Blood analysis

Data of standard laboratory blood tests of fasting renal function, blood glucose, lipid profiles and percentage glycated haemoglobin (HbA1c), performed as part of the clinical work-up, were extracted from the patient's files. In the control sample, blood analysis was performed for research rather than clinical purposes. Blood samples were obtained on the day of the clinic visit, transported to the laboratory in a cooler box, on ice and sent to the South African National Health Systems Laboratories (NHSL) to obtain a lipid profile (total cholesterol, LDL cholesterol, HDL cholesterol and TG concentrations), a blood glucose measurement, and HbA1c. Lipid profiles were determined by an in vitro test for the quantitative determination of LDL cholesterol in human serum and plasma on Roche/Hitachi cobas **c** system 702, using an automated homogeneous enzymatic colorimetric assay method for direct measurement. The LDL cholesterol standard threshold was determined according to the South African dyslipidemia guidelines 2012 (SAMJ 2012). Participants were considered as having diabetes mellitus or an abnormal glucose control if they had a fasting plasma glucose concentration $\geq$ 7mmol/l, or in whom glucose-lowering agents are prescribed.

Dyslipidemia was identified from standard thresholds of total, LDL or HDL cholesterol concentrations.

2.4. Blood pressure

Blood pressures were determined by trained observers who measured brachial artery BP using a mercury sphygmomanometer according to guidelines. After 10 minutes of rest (patient sitting), five consecutive BP readings followed by a pulse rate count were measured. Standard cuffs with an inflatable bladder with a length of 12 cm and a width of 12 cm were used unless the arm circumference exceeded 31 cm, then larger cuffs with a 31 x 15 cm bladder were employed. The five readings were averaged to obtain single systolic, diastolic and mean arterial BP reading. Participants were considered as having hypertension if the average of the mean values for the clinic readings was ≥140/90 mm Hg or when receiving antihypertensive medication.

2.5. Carotid ultrasound

Carotid IMT (C-IMT) was determined by using high resolution B-mode ultrasound (SonoCalc IMT,Sonosite Inc,Bothell,Washington) employing a linear array 7.5MHZ probe (Figure 2.1). Assessments were performed with the participant lying down and the head turned slightly away from the side on which the measurements were taken. Images of at least 1 cm length of the far wall and near wall of the distal portion of the right and left common carotid artery from an optional angle of incidence (defined as the longitudinal angle of



Figure 2.1. A semi-automated B-mode ultrasound employing a linear array 7.5 MH ultrasound probe.

approach where both branches of the internal and external carotid artery are visualized simultaneously) at least 1cm proximal to the flow divider was obtained. Carotid IMT measurements were determined using semi-automated border-detection and quality control software (Figure 2.2) as the use of this software improves C-IMT reproducibility and reduces observer bias compared with manual techniques. The mean average of five C-IMT measurements from the optimal angle was recorded as the C-IMT for each participant and a report generated. A carotid IMT $\geq$ 0.9 mm was considered to be pathological. Carotid ultrasound was also employed to assess the extent of plaque from both longitudinal and cross-sectional images of the carotid artery (Figure 2.3). Carotid artery plaque was identified as a focal structure that encroaches into the arterial lumen of at least 0.5 mm (Figure 2.4) or 50% of the surrounding intima-media thickness value, or demonstrates a thickness of $\geq$ 1.5 mm as measured from the media-adventitia interface to the intima-media lumen interface (Touboul et al. 2012). All of the assessments were evaluated by an experienced ultrasonographer who assessed the quality of images and identify the presence of plaque independent of myself.

2.6. Data analysis

For database management and statistical analysis, SAS software version 9.4 (SAS Institute) was employed. To assess whether relations between IMT and plaque presence are associated with stroke independent of conventional cardiovascular risk factors, multivariate adjusted logistic regression analysis was performed and regression relations were adjusted for age, gender, hypertension, diabetes mellitus/impaired blood glucose control (HbA1c>7.0%), smoking and HDL cholesterol concentrations. To compare the performance of IMT versus plaque presence for stroke prediction, area under the receiver operating characteristic curves (AUC) was calculated.

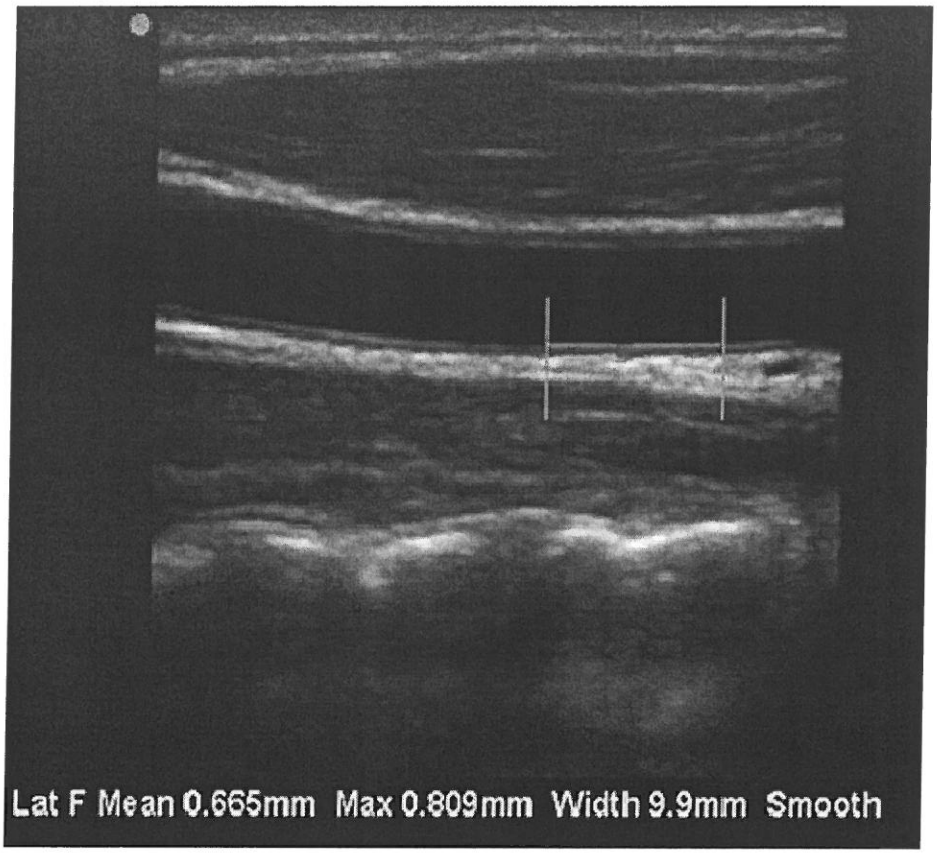

Figure 2.2. An image of the common carotid segment and IMT measurements represented by the two yellow lines using semi-automated border-detection and quality control software.

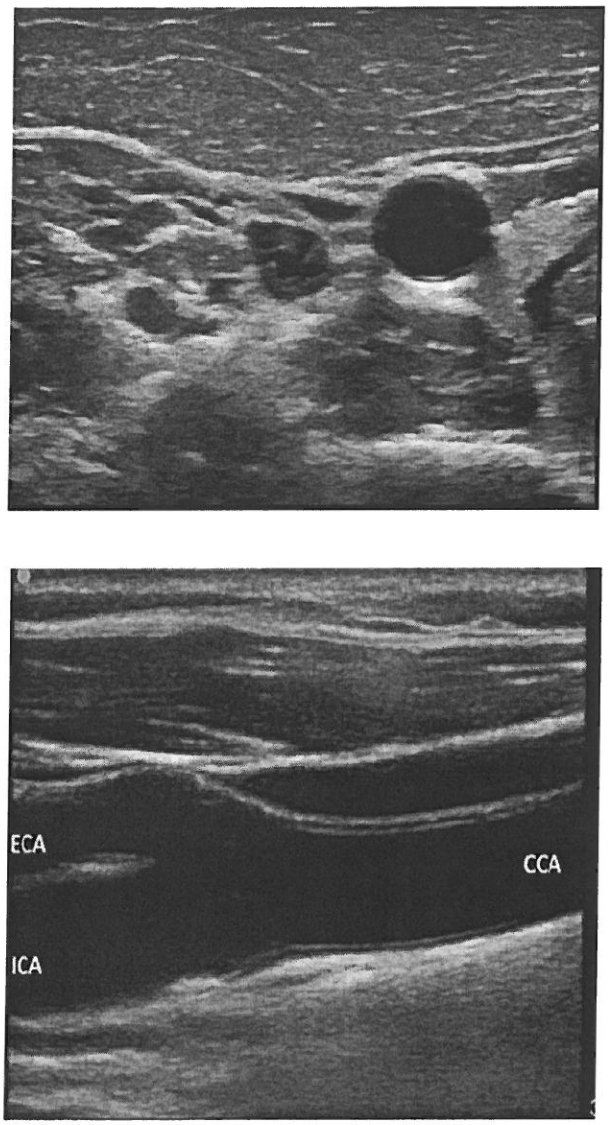

Figure 2.3 A transverse view (upper panel) and a longitudinal view (lower panel) of the carotid artery. (CCA: Common carotid artery, ICA: Internal carotid artery, ECA: External carotid artery)

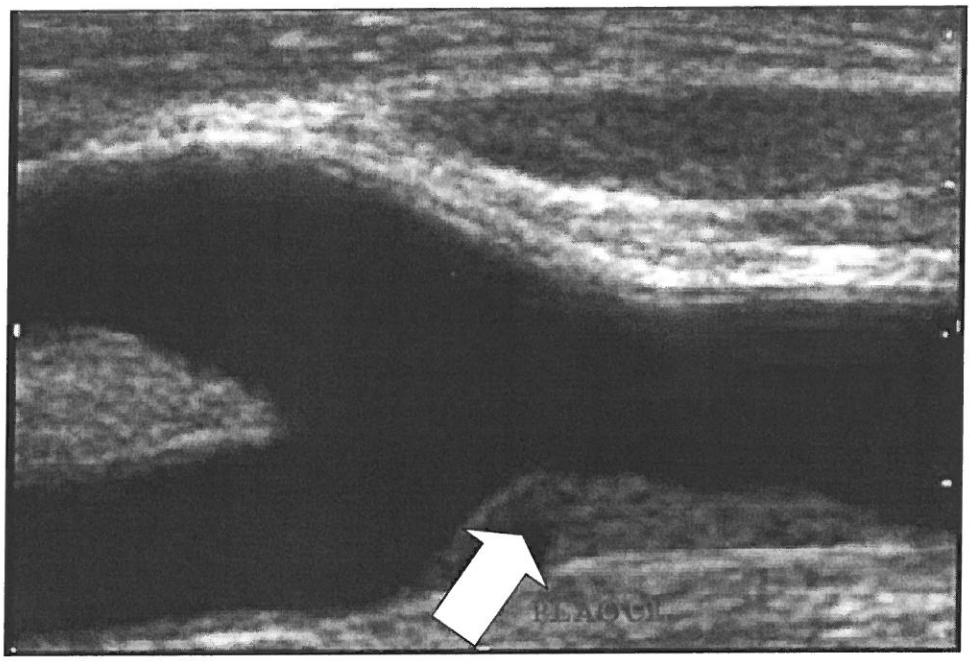

Figure 2.4. A longitudinal view of the carotid artery showing the presence of plaque.

CHAPTER THREE

RESULTS

3.1. Characteristics of the participants.

The characteristics of the patients with stroke and the age-sex matched control participants are shown in Table 3.1. As per the study design, age and sex were not different between the two groups. There were also no difference in BMI, systolic blood pressure and the proportion of participants who smoked. More patients with stroke were hypertensive and had diabetes mellitus. As a consequence of therapy being initiated soon after admission, 70% of patients with stroke received antihypertensive medication, whilst 26% of control participants received therapy. Hence, diastolic blood pressure was lower in patients with stroke. Also as a consequence of therapy being initiated soon after admission, 88% of patients with stroke received lipid-lowering treatment, whilst none of control group were receiving lipid lowering treatment. Hence total and LDL cholesterol concentrations were lower in patients with stroke as compared to the control group. In addition, however, an effect that cannot be attributed to therapy, HDL cholesterol concentrations were lower in the patients with stroke as compared to controls. Data on antihypertensive and lipid lowering therapy prior to admission for stroke was not available. Average IMT was higher in patients with stroke as compared to the control group. Hence, a greater proportion of patients with stroke had IMT values greater than the threshold of 0.9 mm as compared to the controls and there was a tendency for patients with stroke to have more carotid plaque as compared to the control group. The frequency of TOAST stroke subtypes in our study sample was: 39% undetermined, 19% small vessel, 18% cardio-embolic, 10% bleeding, 9% others, 6% atherosclerotic. Only 25% of atherosclerotic stroke patients had plaque.

3.2. Risk factors independently associated with stroke.

Table 3.1. Characteristics of patients with stroke and of age and sex-matched controls

Characteristic	Controls	Stroke	p-value
Sample size	195	137	
Males (%)	40.5	43.0	=0.64
Age (years)	48.7±18.1	49.4±14.5	=0.69
Body mass index (kg/m^2)	29.5±8.4	29.8±7.9	=0.76
Hypertension (%)	38.0	64.2	<0.0001
Diabetes mellitus (%)	10.8	18.3	=0.050
Antihypertensive treatment (%)	26.0	70.0	
Lipid lowering therapy (%)		88.0	
Smoking (%)	19.0	25.6	=0.15
Systolic blood pressure (mm Hg)	133±24	128±19	=0.067
Diastolic blood pressure (mm Hg)	84±11	79±14	<0.005
Total cholesterol (mmol/l)	4.94±1.01	4.14±1.07	<0.0001
LDL cholesterol (mmol/l)	2.86±0.73	2.54±0.89	=0.0005
HDL cholesterol (mmol/l)	1.40±0.42	1.07±0.36	<0.0001
Triglycerides (mmol/l)	1.47±0.82	1.21±0.64	<0.005
Carotid intima-media thickness (mm)	0.677 ±0.15	0.743±0.17	<0.0005
Intima-media thickness >0.9 mm (%)	7.2	16.8	<0.01
Carotid plaque (%)	17.4	25.6	=0.073

LDL, low density lipoprotein, HDL, high density lipoprotein

Table 3.2 shows the risk factors independently associated with stroke. Independent of alternative risk factors, hypertension was positively associated with stroke and HDL cholesterol concentrations were negatively associated with stroke. As diastolic blood pressure and LDL cholesterol concentrations were lower in stroke patients (patient measurements were obtained after initiating therapy, which included an angiotensin-converting enzyme inhibitor, and a statin) as compared to the controls, diastolic blood pressure and LDL cholesterol concentrations were negatively associated with stroke. Although age, sex, smoking and diabetes mellitus were not independently associated with stroke, as they are well-recognised conventional risk factors normally associated with stroke, in subsequent multivariate adjusted models they were forced into the models. This is particularly important for diabetes mellitus which showed trend effects on unadjusted analysis (Table 3.1) for more patients with stroke to have this condition ($p=0.05$).

Table 3.2. Risk factors independently associated with stroke.

Risk factors	β ± SEM	OR	(95% CI)	Wald X^2	p-value
Age	0.006 ±0.010	0.994	(0.975-1.014)	0.353	=0.55
Sex	0.279± 0.287	1.322	(0.753-2.320)	0.945	=0.33
BMI	-0.004± 0.0201	0.995	(0.957-1.035)	0.058	= 0.809
Smoking	0.487 ±0.339	0.615	(0.316-1.196)	2.056	=0.15
Hypertension	1.321± 0.321	3.745	(7.042-1.996)	16.93	<0.0001
Diabetes	0.004± 0.404	0.995	(0.451-2.199)	0.0001	= 0.99
Systolic BP	0.003±0.009	1.003	(0.985-1.022)	0.140	=0.71
Diastolic BP	-0.045±0.015	0.956	(0.927-0.985)	8.714	<0.005
HDL cholesterol	-2.279 ±0.435	0.102	(0.044-0.241)	27.356	<0.0001
LDL cholesterol	-0.411±0.194	0.663	(0.453-0.969)	4.494	<0.05

OR, Odds ratios; BP, blood pressure, BMI, body mass index. See table 3.1 for other abbreviations.

3.3. Unadjusted and multivariate adjusted carotid intima-media thickness.

Figure 3.1 shows the unadjusted and the multivariate adjusted IMT values in patients with stroke and in controls. Without adjustments, carotid IMT was significantly greater in patients with stroke as compared to controls. Similarly after adjusting for age and sex, carotid IMT was significantly higher in stroke patients compare to the control. Moreover the increased IMT in the patients with stroke was not attributed to conventional risk factors as after further adjustments for these risk factors (hypertension, diabetes mellitus, smoking and HDL cholesterol concentrations), carotid IMT remained higher in patients with stroke as compared to the control participants.

3.4. Odds of ischemic stroke associated with carotid intima-media thickness or plaque.

Figure 3.2 shows the unadjusted and conventional risk factor adjusted odds ratio of stroke associated with carotid plaque, carotid IMT or pathological carotid IMT. Carotid IMT, considered as a continuous trait and IMT considered as a categorical trait (above threshold of 0.9 mm) were both associated with increased odds of stroke before adjusting as well as after adjusting for age and sex and even after further adjustments for risk factors (hypertension, diabetes mellitus, smoking and HDL cholesterol concentrations). In comparison however plaque failed to show significant associations with stroke either before or after adjustments for conventional risk factors.

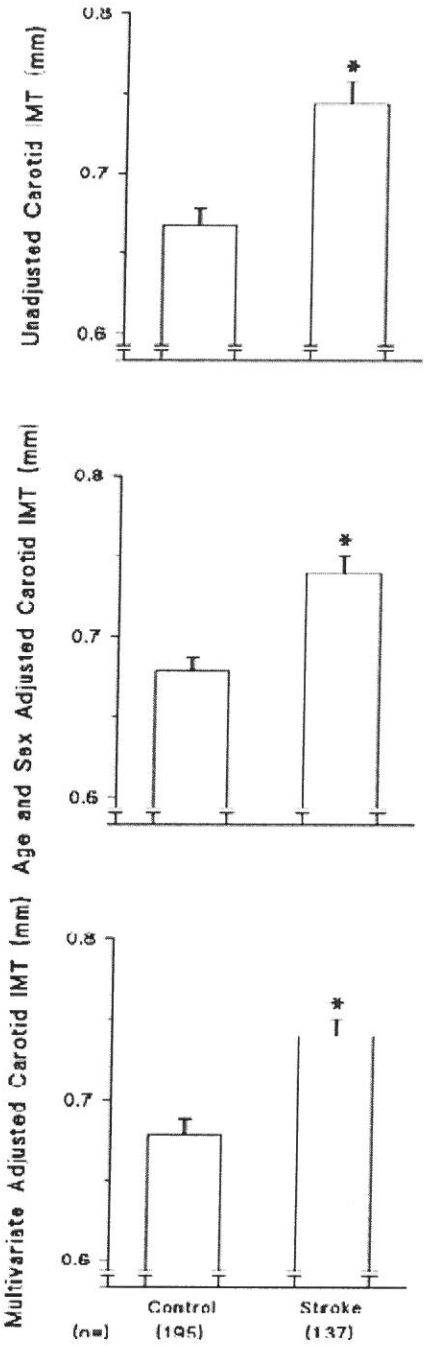

Figure 3.1. Unadjusted, age and sex adjusted, and multivariate adjusted (age, sex, hypertension, diabetes mellitus, smoking and HDL cholesterol concentrations) means of carotid intima-media thickness (IMT) in stroke patients (Stroke) compared to age and sex-matched controls (Control). *$p<0.0005$ versus controls.

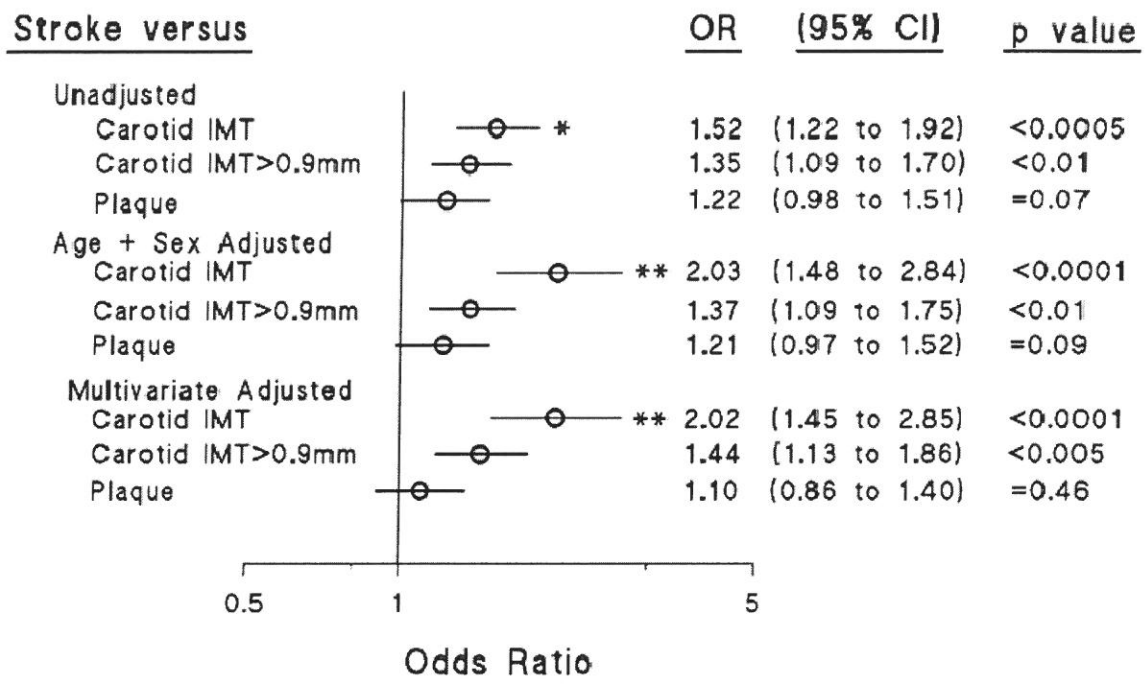

Figure 3.2. Odds of ischemic stroke associated with one standard deviation of carotid intima-media thickness (IMT) (0.162), carotid IMT>0.9mm (0.315) or plaque (0.406). *p<0.05, **p<0.005 versus carotid IMT>0.9mm and plaque. Multivariate adjustments are for age, sex, hypertension, diabetes mellitus, smoking and HDL cholesterol concentrations.

3.5. Performance of carotid intima-media thickness or plaque for detecting stroke.

Carotid IMT, considered as a continuous ($p<0.0005$) and as a categorical (IMT values greater than the threshold of 0.9 mm) ($p<0.01$) trait showed significant performance (area under the receiver operating curve [AUC]) for the detection of stroke (Figure 3.3). However plaque failed to show significant performance for stroke detection (AUC, $p=0.07$) (Figure 3.3). Moreover, carotid IMT considered as a continuous trait showed a significantly greater performance (comparison of AUC values) than plaque ($p<0.02$) or IMT values greater than the threshold of 0.9 mm ($p<0.005$) for the detection of stroke (Figure 3.3).

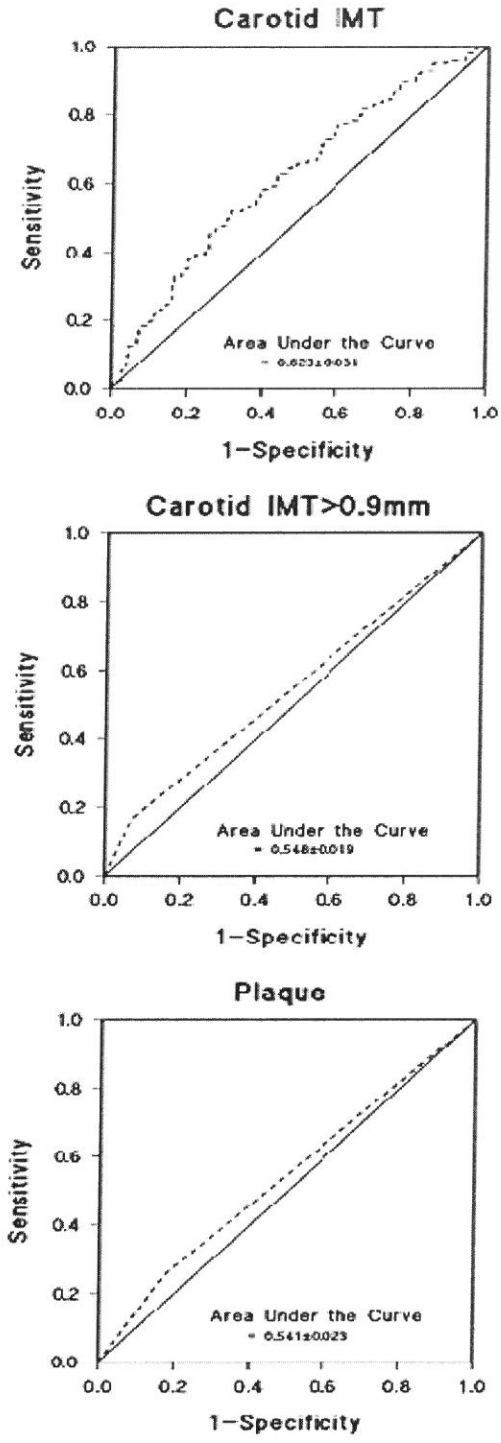

Figure 3.3. Performance (area under the receiver operator characteristic curves) of carotid intima-media thickness (IMT), carotid IMT>0.9mm and plaque for detecting stroke.

CHAPTER FOUR

DISCUSSION

4.1. Primary results of the present study.

The main findings of the present study are as follows: In a group of African ancestry, where hypertension and lower HDL cholesterol concentrations were independently associated with stroke, stroke was also associated with carotid IMT considered as a continuous and as a categorical (above threshold of 0.9 mm) trait independent of hypertension and HDL cholesterol concentrations (and other risk factors forced into the model), but not with carotid plaque. Furthermore, carotid IMT, considered as a continuous and as a categorical trait, but not carotid plaque, showed significant performance for stroke detection.

4.2. Conventional risk factors and stroke.

In the present study, the average age of patients with stroke (49.4 years) indicates that a significant proportion of individuals of African descent experience a stroke in the Gauteng region at a remarkably young age. Importantly, several studies have reported on a high proportion of stroke in younger persons living in developing countries (Radhakrishnan et al.1986, Jacobs et al. 2002). What are the possible reasons for this finding? In this regard, in the present study hypertension was strongly and independently associated with stroke, whilst other than HDL cholesterol concentrations, alternative risk factors contributed little to the risk of stroke. Most studies conducted in various populations suggest that hypertension is the single most important conventional risk factor for the development of stroke (WHO, 2006, Willey et al. 2014). South Africa is no exception to these findings and prior studies have similarly demonstrated that hypertension is the most important risk factor for stroke in black patients, whilst white patient are 44% less likely to have hypertension than blacks even after adjustments for age and gender (OR 0.66, 95% CI: 0.59 - 0.74), with a strong association

with systolic hypertension (Connor et all. 2005). However, for a stroke to have occurred at such a young age in the present study and for hypertension to be a major risk factor, suggests several possibilities to explain these data. In this regard, hypertension may occur at a young age in groups of black African ancestry in Gauteng. Indeed, African-Americans have a higher prevalence of hypertension and develop hypertension at a younger age as compared to Americans of European ancestry (William et al. 2014). Secondly, hypertension may have been undetected or poorly managed and uncontrolled for prolonged periods. In this regard, individuals of African descent may only attend hospital when sick and attend less frequently for general check-ups. However, whether conventional risk factors can fully account for stroke in the young has not been determined. Indeed, in the Helsinki Young Stroke registry, where the authors examined the aetiology of stroke in adults less than 49 years of age, these authors reported that the underlying aetiology was unclear (Putaala et al. 2009).

With respect to cardiovascular risk factors other than hypertension and the association with stroke, in the South African Stroke Risk in General Practice study, an overall prevalence of cigarette smoking of 21% was reported (Steyn et al 1986), a finding not dissimilar to our findings where 25.6% of stroke patients were noted to be smokers. However, in the present study cigarette smoking was not associated with stroke. Nevertheless, 19% of controls were noted to smoke and hence a much larger study sample may have revealed an association between smoking and stroke. In this regard, South Africa has been a global leader in adopting legislation for tobacco control, and this has had a major impact on smoking in younger age groups and decreased passive smoke exposure to children in the home environment (Connor et al 2005). Nevertheless, South Africa is facing a transition process with regards to habitual behaviours where many of the young are shifting from cigarette smoking to smoking of illicit drugs (Madu et al.2003). Whether illicit drug use is responsible for the incidence of stroke among Black South Africans requires further study.

With respect to diabetes mellitus as a risk factor for stroke, patients with diabetes mellitus are at a greatly increased risk of ischaemic stroke at all ages, but especially in the under 65 year age group (Hughes et al. 2013). However, the excess stroke risk associated with diabetes mellitus appears to be decreasing over time in the black population, whereas it is remaining stable among whites (Hughes et al. 2013). Nevertheless, age-specific risk ratios by race show a decrease over time in the black population older than age 65 years, but not in those less than 65 years of age (Hughes et al. 2013). In the present study, although this failed to achieve statistical significance, a trend for more patients with stroke to have diabetes mellitus (18.3%) as compared to the control group (10.8%, p=0.05) was noted. Although on multivariate analysis this trend effect was eliminated, a much larger study sample may have revealed a significant association. Importantly, in developing countries such as South Africa, type 2 diabetes mellitus is often undiagnosed, and related cardiovascular disease risk may therefore exceed that of known cases owing to the unmanaged glycaemic state (Matsha et al. 2012).

With respect to blood cholesterol concentrations as a risk factor for stroke, data from developed countries clearly demonstrate that elevated serum cholesterol concentrations are strongly associated with ischaemic stroke and have been noted to be a significant risk factor among those of African ancestry (Curb et al. 2004). However, in the present study I failed to note a significant association between stroke and total or LDL cholesterol concentrations, although reduced HDL concentrations were significantly associated with stroke. This finding of the lack of significant association between stroke and total or LDL cholesterol concentrations may nevertheless have been confounded by statin use at the time of admission. Importantly, urban black professionals in South Africa have been reported to have significantly higher total serum cholesterol levels than their rural counterparts; suggesting that increasing exposure to lifestyle risk factors may contribute to cardiovascular risk

(Vorster et al 2002). With urbanisation and westernisation, blood cholesterol concentrations may be increasing within the black South African population and there is indeed an enhanced admission rate for ischaemic heart disease within this group (Raal et al.2011). Nevertheless, patients admitted to hospital in the public sector in South Africa are far less likely to occupy professions, and hence the lack of association between total or LDL cholesterol concentrations and stroke in the present study may reflect the fact that LDL cholesterol plays little role in the risk of stroke in the patients reported on in the present study.

Although in the present study I show a clear relationship between lower HDL cholesterol concentrations and stroke, there still some controversy as to the association between HDL cholesterol concentrations and stroke risk. Several prospective and other studies have found higher HDL cholesterol concentrations associated with a lower stroke risk (Tanne et al.1997, Lindenstrom et al.1994, Hu et al. 2011, Bowman et al. 2003, Assmann et al.1996, Sridharan et al. 1992), whilst others have found no relation or even an increased risk (Berger et al. 2012 , Shahar et al. 2003, Bots et al. 2002). Although earlier studies noted that blacks tend to have a lower prevalence of low HDL cholesterol concentrations than other ethnic groups, and that HDL cholesterol concentrations do not appear to be protective for cardiovascular disease (Powell et al 1987), in the Multi-Ethnic Study of Atherosclerosis, the authors noted that higher HDL cholesterol concentrations are indeed associated with a lower risk of stroke in blacks (Reina S et al. 2015). Although the relationship between HDL cholesterol concentrations and stroke is to some extent controversial, there is nevertheless a well-established inverse relation between serum concentrations of HDL cholesterol and the risk of coronary heart disease (Hu et al. 2011, Bowman et al. 2003, Assmann et al.1996, Sridharan et al. 1992).

4.3. Carotid intima-media thickness or plaque and stroke: comparison with previous studies.

Several studies clearly indicate that carotid IMT and carotid plaque add to conventional risk factors in the prediction of stroke, but the extent to which they add to risk prediction and the extent to which carotid plaque improves on risk prediction over and above carotid IMT is controversial. As indicated in the introduction to the present dissertation, a large number of prospective studies have demonstrated that carotid IMT is predictive of major cardiovascular events, independent of traditional risk factors, with risk ratios ranging from 2.0 to 3.5 for stroke (Ruijter et al. 2012). From an overall perspective of the role of carotid IMT alone, in a meta-analysis of 14 population-based cohorts consisting of 45 828 individuals, during a median follow-up of 11 years, where 4007 first-time myocardial infarctions or strokes occurred, the authors examined the ability of carotid IMT to enhance risk assessment (Ruijter et al. 2012). After first fitting the risk factors of the Framingham Risk Score and then extending the model with common carotid IMT measurements to estimate the absolute 10-year risk of developing a first-time myocardial infarction or stroke in both models, the net reclassification improvement was small (0.8%; 95% CI, 0.1%-1.6%)(Ruijter et al. 2012). In those at intermediate risk, the net reclassification improvement was 3.6% in all individuals (95% CI, 2.7%-4.6%) and no differences between men and women were noted (Ruijter et al. 2012). The authors therefore concluded that the addition of common carotid IMT measurements to the Framingham Risk Score was associated with small improvement in 10-year risk prediction of first-time myocardial infarction or stroke (Ruijter et al. 2012). In this regard, the present study is in-part in agreement with this meta-analysis in that I show an independent relationship between carotid IMT and stroke. However, the impact of carotid plaque over and above carotid IMT on risk prediction is more controversial.

Importantly, as described in the introduction to the present dissertation, Rosvall et al (2005) noted that the presence of increased common carotid IMT was an important risk indicator for stroke, even when information on carotid plaque or cardiovascular risk factors was taken into account. However, Simon et al (2010) suggested that carotid plaque area is more representative of atherosclerosis than IMT. In addition, these authors (Simon et al 2010) suggested that there is insufficient data supporting the possibility that carotid IMT may improve stroke prediction beyond prediction by conventional risk factors (Simon et al. 2010). In subsequent meta-analyses the measurement of carotid plaque alone was demonstrated to be more predictive of cardiovascular events than IMT (Inaba et al. 2012, Spence et al. 2012). Moreover, increases in carotid IMT fail to predict atherosclerotic plaque development (Rundek et al. 2015). The recommendation has therefore been that IMT assessment should always be supplemented by a thorough scan of the extra cranial carotid arteries for carotid plaque to increase the diagnostic performance of carotid ultrasound (Inaba et al. 2012). Importantly, however, Naqvi et al (2014) highlighted that the large clinical studies that measured carotid IMT and plaque to determine their value in predicting incident cardiovascular diseases varied in terms of the site and type of IMT measurement, as well as the angle of the IMT assessment. Some studies imaged a single segment, and others multiple segments; some studies imaged the far wall of multiple segments, whereas others imaged both near and far walls; some measured the mean or maximum for single measurements; mean of the mean, or mean of the maximum for multiple measurements; some included plaque in the IMT measurements, whereas others considered a plaque-free area. Studies also differed by plaque definition. Some studies defined plaque as a focal thickening of the intima-media >1 mm, protruding into the lumen, which is at least twice as thick as the IMT on either side. Other studies defined plaque as carotid IMT >1.2 mm. Others classified plaque as type 1 (uniformly echolucent), type 2 (predominantly echolucent), type 3 (predominantly

echogenic), type 4 (uniformly echogenic) and type 5 (heavy calcification). Hence, at this time there was no clear evidence as to whether IMT, plaque or both add to risk prediction.

From a more contemporary perspective in a multi-ethnic study conducted in 4 384 participants of the Cardiovascular Health Study (61% women, 14% black; mean baseline age, 72 ± 5 years) without CVD at baseline, a higher carotid IMT category and the presence of plaque were both associated with higher incidence rates for stroke and CVD (Gardin et al. 2014). Importantly, however, the addition of carotid IMT improved the ability of Framingham risk score-models to discriminate cases from non-cases of incident stroke and CVD ($p=0.015$ and $p<0.001$, respectively), but there was no further improvement by adding plaque (Gardin et al. 2014). For both stroke and CVD, the ability to improve risk stratification (net reclassification improvement) was driven by down-classifying those without incident disease (Gardin et al. 2014). Although the addition of plaque to carotid IMT did not result in a significant improvement in risk stratifying for either outcome, it was significant among those without incident disease (Gardin et al. 2014). The authors concluded that in older adults, the addition of carotid IMT modestly improves 10-year risk prediction for stroke and CVD beyond traditional risk factor models, mainly by down-classifying risk in those without stroke or CVD disease (Gardin et al. 2014). However, the authors also concluded that the addition of plaque to carotid IMT adds no statistical benefit in overall risk prediction, although there is evidence of down-classification in those without events (Gardin et al. 2014). In part in-keeping with the authors of that study (Gardin et al. 2014), in the present study I show that carotid IMT is independently associated with stroke in a group of black South Africans, but that carotid plaque is not. With regards to subtypes of ischaemic stroke, small-vessel strokes are more common among blacks, as demonstrated in several studies (Gross et al. 1984, Gan et al. 1997), and this may limit the ability of plaque, which is an index of the extent of carotid atherosclerosis, to associate with stroke. In this regard, in the

Northern Manhattan Study, the incidence of lacunar, intracranial and extracranial atherosclerotic strokes was higher in black and Hispanic subjects than in whites (Halina et al. 2005). However, in a large population-based study comparing the incidence of ischaemic stroke subtypes among blacks and whites, Alexander et al (2004), noted that Blacks have nearly twice the incidence of small-vessel stroke and stroke of undetermined cause. Furthermore, large-vessel stroke is 1.4-times more common and cardio-embolic stroke trends toward a higher incidence rate among blacks (Alexander et al. 2004). This finding underscores the importance of large-vessel and cardio-embolic stroke among blacks. Using a TOAST classification, our study demonstrated a high proportion of undetermined, small vessel and cardio-embolic stroke, and low proportion of atherosclerotic stroke. Moreover, only 25% of atherosclerotic (large vessel) stroke had plaque. Therefore, the low frequency of atherosclerotic strokes may explain the inability of plaque to predict stroke beyond conventional risk factors.

4.4. Potential relevance of the present findings.

In a multi-ethnic study conducted in the United Kingdom, whilst carotid IMT was greater in a group of black African ancestry than in a comparable group of European descent, carotid plaque was less prevalent in black individuals as compared to white individuals (Mackinnon et al. 2010). This dissociation between IMT and plaque seen in black individuals suggests that an increase IMT in groups of black African ancestry may not directly relate to an increased atherosclerosis. Furthermore, in a recent large community-based study conducted in a black African sample living in the South West Township (SOWETO) of Gauteng, our group has noted strong age-related increases in carotid IMT with little evidence of plaque or independent associations between IMT and conventional cardiovascular risk

factors (unpublished data). However in the Atherosclerosis Risk in Community Study, no site-specific differences in the prevalence of plaque were noted between African-Americans and Americans of European descent and the positive relations between carotid IMT and plaque were just as strong in African-Americans as they were in Americans of European descent (Li et al. 1994). Hence the extent to which carotid IMT measurements reflect a pathological atherosclerotic process or a non-pathological process (compensatory medial thickening or fatty streak formation) in black African communities is uncertain. Although several studies have compared the prevalence of an increased IMT and plaque in groups of African versus European descent or relations between IMT and plaque across ethnic groups (Rundek et al. 2015 & Li R et al. 1994) and have reported that IMT is higher in African Americans than in Caucasians (Howard et al. 2005), no study has compared whether IMT or plaque best predict stroke in groups of African ancestry. In this regard the present study is the first to suggest that carotid IMT in black Africans is indeed a pathological process with a similar ability to enhance risk prediction for stroke beyond conventional risk factors as in other ethnic groups, but that the measurement of carotid plaque may have limited application. Hence, carotid IMT, which is conducted using semi-automated border detection software and hence is less reliant on observer error than plaque assessment, may be a useful measure of stroke prediction in black African communities.

The present study also adds to prior studies in an additional and quite unique manner. In this regard, the present study demonstrates that even in a study sample where the average age reflects the fact that at last half of those patients experiencing stroke were less than 50 years of age, that carotid IMT, which is well recognised to be largely determined by increasing age, is independently associated with stroke. In this regard, in the present study, the study sample was too small to categorise those into younger or older individuals. Hence, it is possible that the ability of carotid IMT to independently associate with stroke occurred

mainly in the older age group. However, using a carotid IMT of 0.9 mm failed to show as strong an ability to add to risk prediction as carotid IMT considered as a continuous trait. This finding suggests that the relatively high thresholds which mainly apply to older individuals, may be too high to add to risk prediction. In this regard, lower thresholds in younger individuals may have improved on the strength of the association with stroke. Hence, further studies are required to identify appropriate IMT thresholds for risk prediction in younger individuals in South Africa.

4.5. Limitations of the present study.

There are several limitations to the present study which may affect the interpretation of the data. In this regard, the present study was a case-control study which may be limited by population stratification (where the controls may not be completely representative of the case sample). However, the controls were age and sex matched to the case sample and obtained from a randomly selected community sample representative of patients admitted with stroke. Secondly, we may have missed cases that were not admitted to hospital (who died before admission or refused hospitalization) and hence whether cases represent all patients with ischaemic stroke is uncertain. Third, in the present study, because of a lack of availability of the software to perform these assessments, we failed to quantify the area, volume, density or character of the carotid plaque and hence we may have missed important characteristics of plaque which may have improved on risk prediction. Fourth, we may have missed the presence of plaque higher up in the internal carotid artery, a site of predilection for plaque, which in some patients may have been difficult to image. Fifth, all of our measurements were performed after admission, when most patients were already receiving antihypertensive and lipid lowering therapy. This could explain the negative relations between LDL cholesterol

concentrations or diastolic BP and stroke. Sixth, carotid ultrasound was performed in B-mode only as the portable device employed does not have the software for colour Doppler imaging. Hence, we could have underestimated the proportion of participants with carotid plaque.

4.6. Conclusions

In conclusion, the current study suggests that in a group of African ancestry where the average age of stroke reflects at least half of all patients being under 50 years of age, that carotid IMT may add to conventional risk factors in identifying the risk of stroke. However, the presence of carotid plaque may add little to conventional risk factors in identifying the risk of stroke.

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R14/49 Dr Eitzaz Sadiq and Dr Harold Majane et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140429

NAME: (Principal Investigator)	Dr Eitzaz Sadiq and Dr Harold Majane et al
DEPARTMENT:	Neurosciences Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Risk Predictors for Stroke in Human Immundeficiency Virus Infected South Africans
DATE CONSIDERED:	25/04/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	06/06/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES