DUPLEX ULTRASOUND ASSESSMENT OF CAROTID ARTERIAL ATHEROSCLEROTIC DISEASE: INVESTIGATION OF DIRECT STENOSIS MEASUREMENT METHODS AND IMAGE ANALYSIS FOR VULNERABLE PLAQUE IDENTIFICATION

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Dedication

I dedicate this thesis to my parents Loizos and Eirini, who have always been encouraging their children into intellectual pursuit, and my wife Ioanna, who accompanied and supported me throughout this effort. Without their continuous support none of this work would have been possible.

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Declaration

This is to declare that this is my own work and any work of others has been acknowledged.

Savvas Loizos

Abstract

Purpose: Ultrasound assessment of carotid disease is currently based on stenosis haemodynamic effects. The accuracy of direct stenosis measurement remains unclear, while research on atherosclerosis suggests identification of other plaque characteristics beyond size. The aim of the study is to investigate whether direct stenosis measurement and plaque ultrasound image analysis could potentially be used for more accurate diagnostic investigation.

Method and material: Eighty-seven patients with cardiovascular disease had a carotid duplex ultrasound scan and velocity and B-mode measurements data were recorded for direct diameter measurement evaluation. Forty patients were scanned for quantitative plaque analysis and association of several parameters with symptoms was investigated.

Results: For the degree of stenosis, ECST method indicated greater agreement among direct measurement methods with velocity criteria, however in cases of mild stenosis difference was reported. ECST method measurements showed considerably better agreement with MRA stenosis calculations compared with other methods. Intima-media grey scale level was associated with plaque echogenicity but the correlation was not significant, however there was an association with blood cholesterol levels. Percentages of plaque area with grey scale value less than 35 and 40 showed good accuracy in identifying symptomatic patients. As far as fibrous cap is concerned, a thickness less than 300 μm was well correlated with symptomatic disease, however no association was noted for its echogenicity.

Conclusion: ECST direct stenosis measurement method could potentially be used for better stenosis classification in cases with inconclusive haemodynamic estimations. Quantitative plaque analysis, such as fibrous cap thickness measurement and grey level analysis, shows promising results in association with symptomatic disease and particular plaque characteristics.

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BFI-T	B-flow image thickness
CAD	Coronary artery disease
CCA	Common carotid artery
CEMRA	Contrast enhanced magnetic resonance angiography
CEUS	Contrast enhanced ultrasound
CRP	C-reactive protein
CTA	Computed tomography angiography
CVD	Cerebrovascular disease
DSA	Digital subtraction angiography
DUS	Duplex ultrasound
ECA	External carotid artery
FDG-PET	[18F]-fluorodeoxyglucose positron emission tomography
GSM	Grey scale median
HDL	High-density lipoprotein cholesterol
ICA	Internal carotid artery
IMT	Intima-media thickness
LDL	Low-density lipoprotein cholesterol
Lp(a)	Lipoprotein (a)
MACE	Major adverse cardiovascular event
MRA	Magnetic resonance angiography
PET	Positron emission tomography
PSV	Peek systolic velocity
PSVR	Peek systolic velocity ratio
SD	Standard deviation

TIA	Transient ischaemic attack
VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Chapter I

Introduction

1.1 Background

Cardiovascular disease, a group of diseases related with the heart and blood vessels that includes ischaemic heart disease and stroke, is today the leading cause of death worldwide, being responsible for 30% of global deaths.

Stroke specifically stands as a major social and healthcare burden worldwide, being at the same time a significant cause of disability. The overall economic cost of cerebrovascular disease in UK is estimated up to £5 billion per annum and is predicted to increase in the future.

The majority of strokes are of ischaemic etiology, caused by a sudden decrease of blood flow in a part of the brain. This usually is a result of embolization of a cerebral artery, originating either from an atherosclerotic lesion at a more proximal artery, most commonly the carotid artery, or from the heart. Since atherosclerosis is a chronic progressive disease that can be identified, monitored and treated and its association with stroke is well documented, accurate investigation and early diagnosis of carotid atherosclerotic disease is of great importance for stroke prevention and management.

Several imaging modalities are currently used for imaging the carotid arteries. However, it is duplex ultrasound that is predominantly used for routine assessment of carotid stenosis and treatment decisions, as well as patients follow up, depend predominantly on ultrasound findings. Its characteristics, being a quick, relatively cost-effective, noninvasive, patient-safe technique but also its potentials, since it

provides information about the anatomy and the functionality of the vessels explain its superiority over other modalities.

Succeeding carotid angiography as the routine imaging technique for carotid disease assessment, duplex ultrasound investigation mainly focused on plaque size and degree of artery stenosis. Aiming to identify greater than 70% of stenosis, which angiographic studies in the ‘90s have set as a cut-off degree of stenosis for patient selection for endarterectomy, several velocity criteria were published from different centres, leading to confusion among vascular laboratories. The different approaches for stenosis calculation used in those angiographic studies, the American (NASCET) using the distal internal carotid artery diameter and the European (ECST) using the wall-to-wall diameter at the point of maximum constriction as reference, have led to confusion in the scientific community on what “degree of stenosis” represents and how velocity criteria should be interpreted. Meanwhile, direct diameter measurement of stenosis, a method that could help resolve the confusion, has not yet been included in recommendations for carotid imaging with the excuse of variability and overestimation of the stenosis. Despite being an indirect method for stenosis calculation, haemodynamic parameters are still preferred in current imaging protocols over direct stenosis measurement. Whether the latter could and should be used along with, or instead of, velocity measurements, remains unclear.

Furthermore, scientific interest in atherosclerosis has also focused on the identification of characteristics of the vulnerable plaque, i.e. the plaque which is prone to rupture and lead to symptomatic disease. Thereafter, the need for an additional, beyond the degree of stenosis, approach of carotid plaque has become apparent. Over the last decade effort has been made in examining the possible association of ultrasound plaque image characteristics with compositional and morphologic plaque

characteristics with promising results. Despite the fact that several studies have associated several ultrasound image characteristics with histological characteristics of carotid plaque and clinical symptoms, plaque echogenicity being the most representative example, qualitative and quantitative characterization of the plaque has not been yet standardized and hence not included in routine ultrasound imaging practice. Meanwhile, newer characteristics of vulnerable atherosclerotic plaque, such as inflammation, neovascularization and fibrous cap thickness, are being investigated and there is ongoing research on methods for identifying and quantifying those characteristics for a better assessment and treatment of carotid atherosclerotic disease.

1.2 Hypothesis

This study addresses various issues on duplex ultrasound investigation of carotid atherosclerotic disease aiming to recommend potential changes in the routine practice, combining previously suggested methods with modern knowledge on atherosclerosis.

The main hypothesis of the study is that a holistic assessment of carotid plaque which would combine haemodynamic parameters with direct stenosis measurements and would include quantitative details of the plaque derived from image texture analysis and additional measurements could potentially be an accurate and helpful diagnostic tool for identifying the high-risk patient as well as monitor the disease progress.

More specifically:

- Direct stenosis measurement methods will be compared with established velocity criteria for stenosis calculation as well as stenosis measurements using MRA for selection of the most appropriate and accurate for ultrasound use.

- Accuracy of direct stenosis measurement methods will be examined in relation with current velocity criteria in determining degree of stenosis and identifying severe stenosis.
- Association of plaque image characteristics such as echogenicity, heterogeneity and plaque image texture analysis results with symptomatic disease will be investigated.
- Association of plaque fibrous cap and intima-media ultrasound image analysis with symptomatic disease will be investigated

A review of literature on current assessment of carotid atherosclerotic disease and new developments on identifying the vulnerable carotid plaque using ultrasound and other imaging modalities is followed by methods and results of each study addressing the above issues, summarizing with a discussion on study's findings and recommendations for further research.

CHAPTER II

CAROTID ATHEROSCLEROTIC

DISEASE

Chapter II

Carotid atherosclerotic disease

2.1 Introduction

Cardiovascular disease, nicknamed as “the disease of civilization”, consists of a group of pathologic disorders of heart and the blood vessels. The class includes coronary heart disease (acute myocardial infarction, acute coronary syndromes, angina pectoris), cerebrovascular disease (stroke, transient ischaemic attack), peripheral artery disease (e.g. lower limb artery stenosis), rheumatic fever/rheumatic heart disease, hypertensive disease, congenital heart disease, heart failure, cardiomyopathies, heart valve diseases, aortic aneurysms and dissection, arrhythmias, diseases of pulmonary circulation (e.g. pulmonary embolism), disease of veins, lymphatics and lymph nodes etc, however the term usually refers to diseases pathophysiologically related to atherosclerosis. Despite the great incidence and prevalence of cardiovascular disease, particularly in countries of the so-called Western civilization, most of these diseases can be accurately diagnosed and efficiently managed due to the evolution of medical equipment and drug industry.

2.2 Epidemiology

Cardiovascular diseases

Cardiovascular diseases are the leading cause of mortality in developed countries and a rising tendency in developing countries. According to World Health Organization, in 2005 17.5 million people died of cardiovascular disease worldwide, representing 30% of all global deaths. Among all the cardiovascular disorders, coronary artery

disease and cerebrovascular disease were responsible for most of the deaths, causing 7.6 million and 5.7 million deaths respectively on 2005. Over 80% of deaths due to cardiovascular diseases take place in low- and middle-income countries. It is estimated that by 2030 almost 23.6 million people will die from cardiovascular diseases, mainly from heart disorders and stroke, which are projected to remain the leading causes of death worldwide.

The current American Heart Association update on heart disease and stroke in United States, issued annually, estimates that more than 1 per 3 American adults have one or more types of cardiovascular disease; Over 16 million Americans suffer from coronary heart disease and nearly approximately 7 million from stroke. Mortality data are also alarming; on average, in U.S., 1 death every 39 seconds is a result of cardiovascular disease, which was the underlying cause of 813,804 deaths (of total 2,423,712) in U.S. in 2007 (Roger *et al*, 2011) (Fig 2.1).

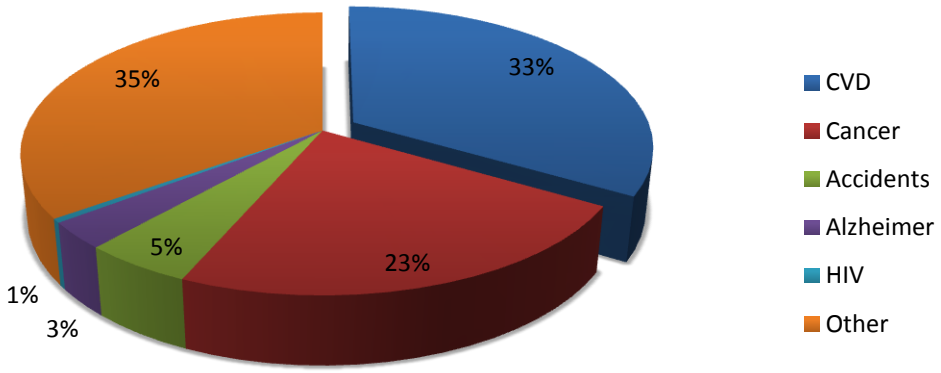


***Fig 2.1**, Percentage breakdown of deaths per underlying cause in U.S.A. in 2007 (Roger et al. 2011)*

It is however noteworthy that the death rates (i.e. number of deaths per 100,000 population) due to cardiovascular disease have declined by 27.8% from 1997 to 2007,

a decrease that, as far as coronary artery disease is concerned, is attributed to increased use of evidence-based medical therapies and changes in modifiable risk factors (Ford *et al*, 2007).

Similarly, the rates in United Kingdom in 2007 indicate that cardiovascular disease was the leading cause of death, responsible for approximately 33% of deaths in men and women (Fig 2.2, Fig 2.3).

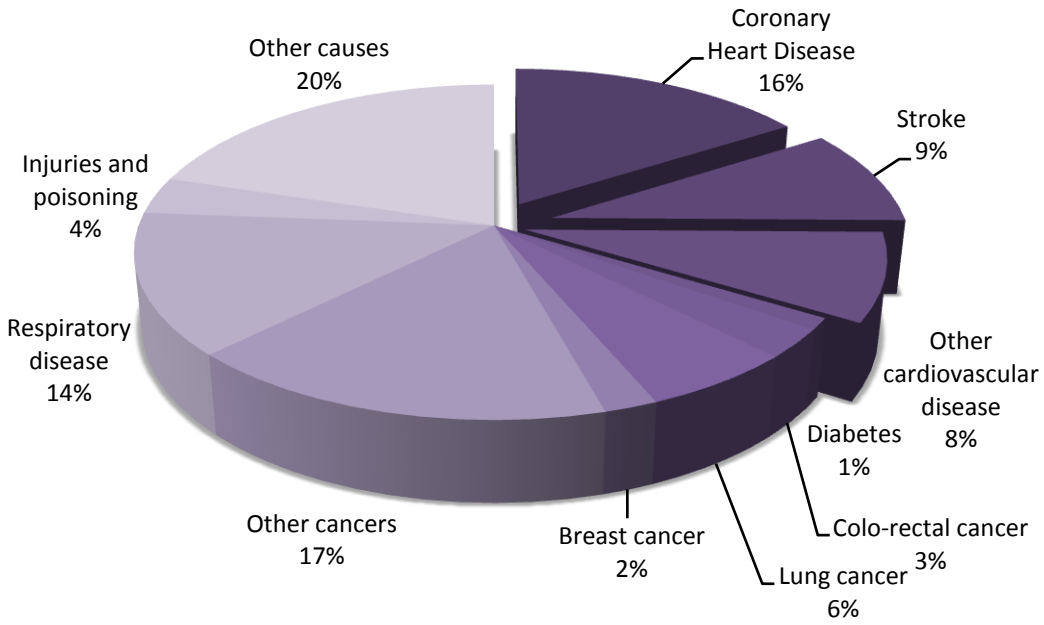
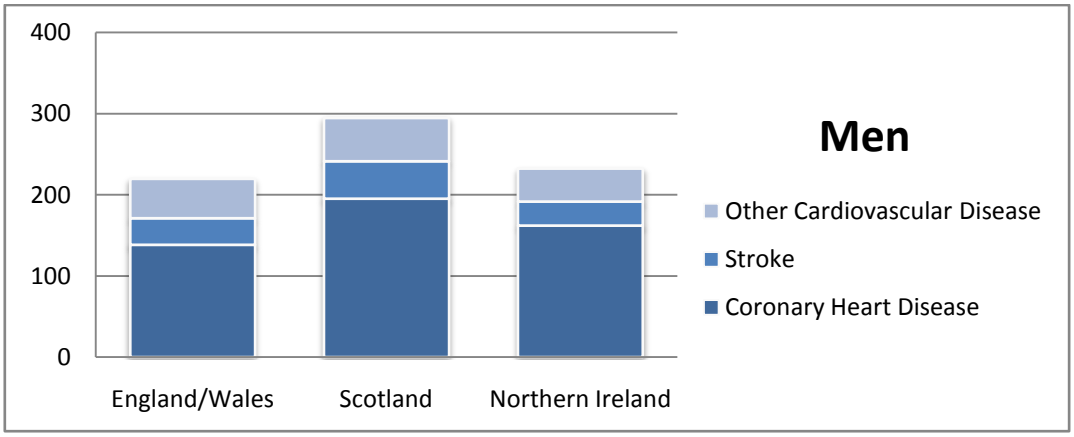


***Fig 2.2**, Percentage breakdown of deaths per underlying cause in men and women across UK in 2007(Scarborough P et al, 2009)*



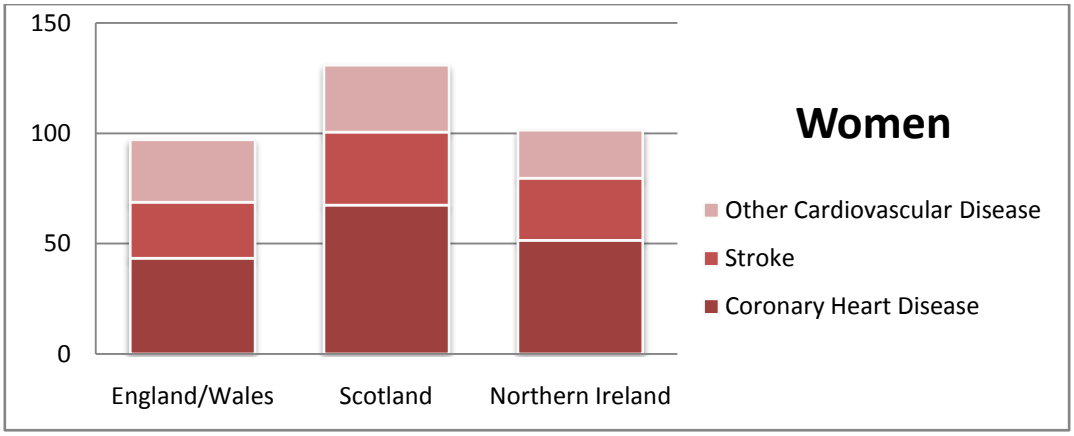


***Fig 2.3**, Death rates (number of deaths per 100,000 population) for coronary heart disease, stroke and other cardiovascular disease for men and women aged 37-74 years old in UK in 2007 (Roger et al, 2011)*

Stroke

Stroke is a major social and healthcare burden worldwide. World Health Organization classifies stroke as the third most common single cause of death, exceeded only by coronary heart disease and cancer (Fig. 2.4). Among the cardiovascular diseases, stroke is the second cause of death, below coronary heart disease (Fig 2.5). Annually 15 million people worldwide suffer a stroke. Of these, 5.5 million die (3 million women and 2.5 million men) and another 5 million are left permanently disabled. In the UK approximately 50,000 people die every year of stroke, while in the U.S. every 40 seconds someone has a stroke and every four minutes someone dies of a stroke (Miniño *et al*, 2009; Bhatnagar *et al*, 2010).

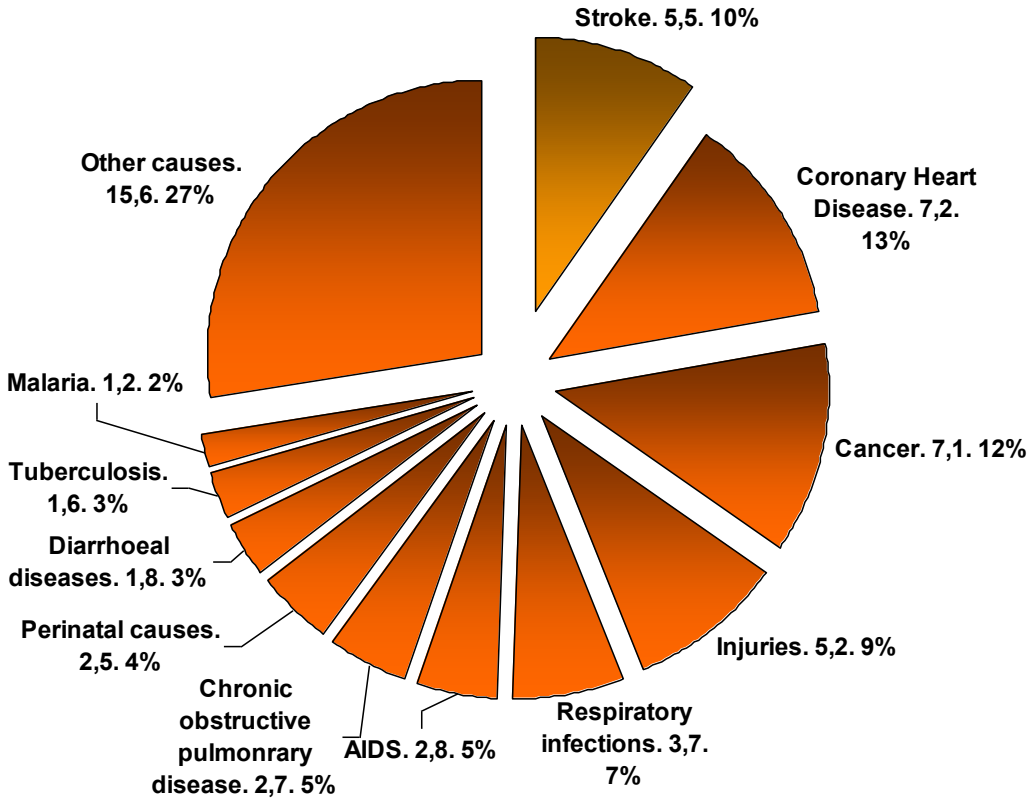


***Fig 2.4**, Number (in millions) and percentage of deaths worldwide from stroke and other leading causes in 2002 (Mackay & Mensah, 2004)*

In UK, stroke is one of the largest health burdens, causing in 2007 around 53,000 deaths (approximately 9% of all deaths, Fig 2.2) and more than 175,000 consultant visits in National Health Service hospitals, whilst it is estimated that each year 111,000 new strokes occur. It is one of the main causes of serious long-term disability, causing 3% of the world's disability burden (Warlow *et al*, 2003) and being the biggest single cause of major disability in UK (Mackay & Mensah, 2004).

Since 1990s the number of inpatient cases of stroke in England has increased by approximately 25% and over 21,000 surgical procedures in England are stroke-related. Its economic consequences are alarming, as the economic cost for the UK health and social care system in 2006/07 was estimated over £2.5 billion for stroke management and £370 million for transient ischaemic attack, adding up to a total cost

of £5 billion, including production losses due to mortality and morbidity (Bhatnagar *et al*, 2010). The burden of stroke is predicted to increase over the years ahead as a consequence of UK's ageing population. Therefore, prevention programs that are aimed at the reduction of risk factors as well as efficient and rapid diagnosis of stroke are crucial.

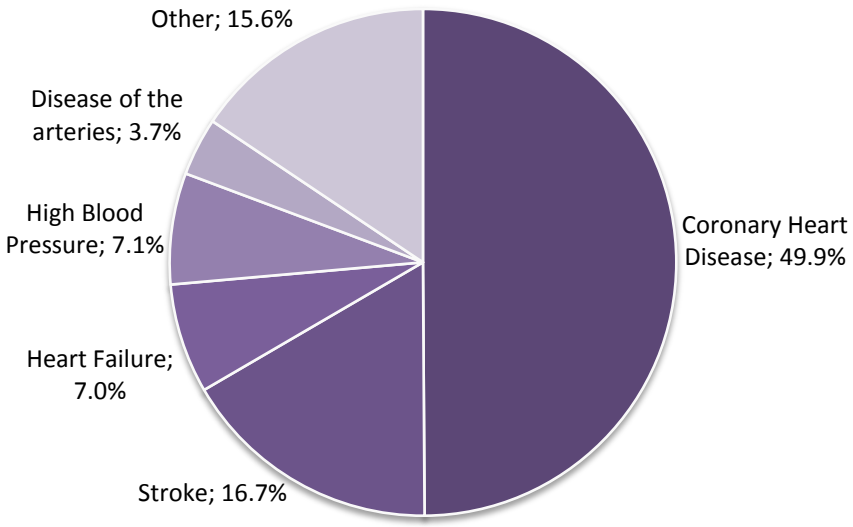


***Fig 2.5**, Percentage breakdown of deaths in U.S. in 2007 due to cardiovascular disease (Roger et al, 2011)*

2.3 Pathological types of stroke

Stroke is the clinical syndrome of rapid onset of focal (or global, as in subarachnoid haemorrhage) cerebral deficit, lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one. If symptoms are temporary and patient recovers in less than 24 hours, the clinical syndrome is called transient ischaemic attack (TIA). There are three pathological types of stroke (Fig. 2.6):

- Ischaemic stroke, caused by vascular infarction (80%)
- Primary intracerebral haemorrhage (15%)

- Other causes, like subarachnoid haemorrhage, tumor etc (5%)

The cause of approximately 50% of strokes is the atherothrombotic disease of the extracranial - or less commonly large intracranial – arteries. About 25% of strokes are caused by occlusion of one of the small perforating cerebral arteries and they are called lacunar infarcts, whether 20% are caused by emboli form the heart, usually because of atrial fibrillation. The remainders are due to rarer causes, like arterial dissection, vasculitis, or remain cryptogenic despite thorough investigation (Warlow *et al*, 2003).

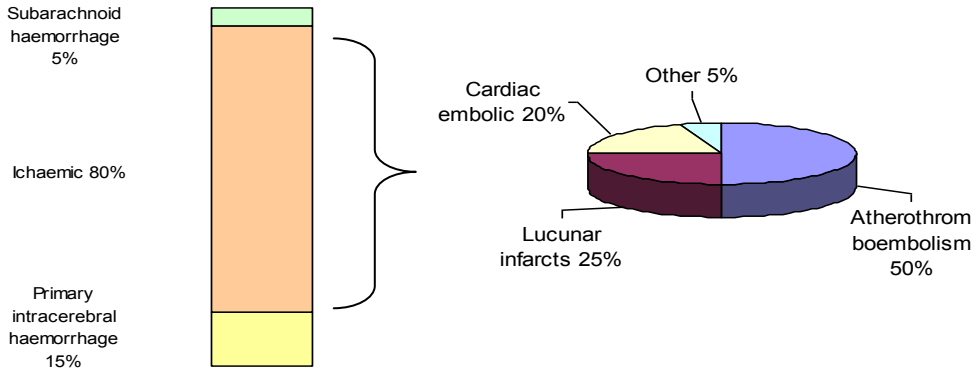


Fig 2.6, *Frequency of main types and subtypes of stroke (Warlow et al, 2003)*

2.4 Anatomy of cerebrovascular system

The brain receives 15% of the cardiac output and is supplied by four vessels, the right and left carotid and vertebral arteries. On the left side the common carotid artery (CCA) and the subclavian artery arise from the aortic arch, whereas on the right side the branchiocephalic artery, also called the innominate artery, arises from the aorta and then divides into the right subclavian artery and right common carotid artery.

The CCA divides then into the internal (ICA) and external (ECA) carotid arteries, the level of bifurcation being highly variable (Fig. 2.7). At that level, the CCA widens to

form the carotid bulb. There are several variations of the carotid bulb morphology, as it may involve the proximal ICA instead of the distal CCA, or may involve both ICA and ECA. In 90% of cases the ICA lays posterolateral or lateral to the ECA and lacks of extracranial branches. By contrast, the ECA branches into the superior thyroid, ascending pharyngeal, lingual, facial, occipital, posterior auricular, maxillary and superficial temporal arteries. In the skull, the ICA follows a curved path, known as the carotid siphon, and finally divides into the middle cerebral artery and the anterior cerebral artery. The intracranial segment of ICA gives branches, the most important of which is the ophthalmic artery, whose terminal branches, the supratrochlear and supraorbital arteries connect with the terminal branches of ECA to form a normal collateralization system. The middle and anterior cerebral artery, along with the posterior cerebral artery, which arises from the basilar artery, form the Circle of Willis (Fig. 2.8). The Circle of Willis provides collateralization between the anterior, posterior, right and left brain circulation.

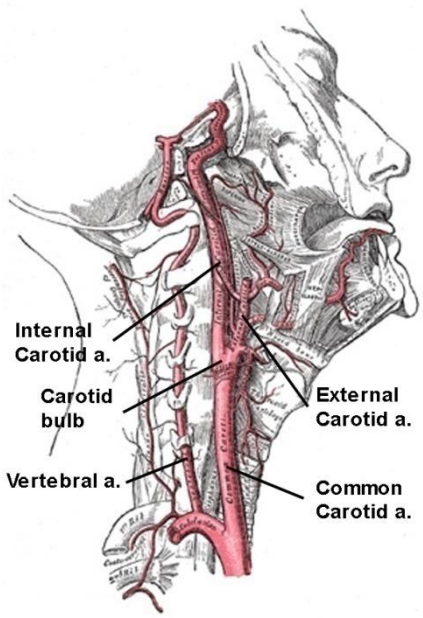


Fig 2.7, *Anatomy of neck arteries (adapted from Gray's Anatomy, edited by T.P.Pick and R.Howden)*

The vertebral arteries are the first branches of the subclavian arteries. They pass upwards through the transverse processes of the spine and join at the base of the skull to form the basilar artery, which then divides to right and left posterior cerebral arteries.

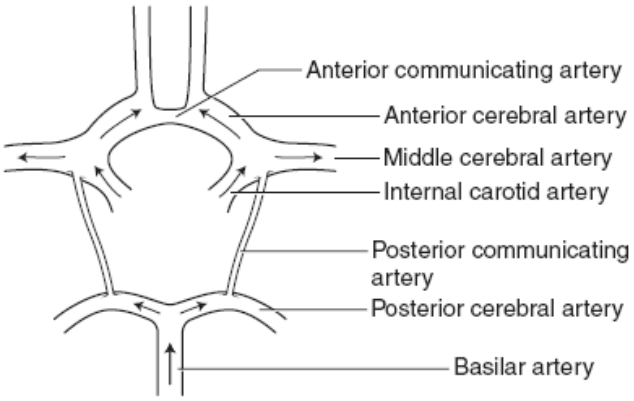


Fig 2.8, *Circle of Willis (Thrush et al., 2004)*

2.5 Atherosclerosis

Atherosclerosis is the most frequent underlying cause of coronary, carotid and peripheral arterial disease and is responsible for a large number of deaths each year worldwide. It is a chronic immunoinflammatory fibroproliferative disease of large and medium-sized arteries. It results in the formation of atherosclerotic lesions and plaques which cause stenoses in the arteries and are potentially prone to rupture.

2.5.1 Formation of atherosclerotic plaque

Although the exact mechanisms of the pathogenesis of atherosclerosis are still uncertain, the "response-to-injury" theory is most widely accepted. According to that theory, the first stage of atherosclerosis is the injury of the endothelium, the inner layer of artery wall, caused by oxidized low-density lipoprotein cholesterol (LDL),

turbulent flow, infectious agents, toxins including by-products of cigarettes smoking, hyperglycemia etc. Sites of disturbed laminar flow in the vascular network, like arterial branch points and bifurcations, are particularly prone to endothelial injury because of wall shear stress.

In lesion-prone areas plasma molecules and lipoproteins extravasate through the leaky and dysfunctional endothelium into the subendothelial space (Fig. 2.9). There, through still unclear mechanisms which include oxidation, lipoproteins are retained and modified and become cytotoxic, proinflammatory, chemotaxic and proatherogenic. Lipid material is then ingested by macrophages which are recruited as an immunologic response to the atherogenesis forming foam cells, so named because of their foamy microscopic appearance. These early and generally asymptomatic lesions that contain only endothelial cells, macrophages and few T-cells are called fatty streaks and can be observed in the arteries of most individuals by the age of 20s.

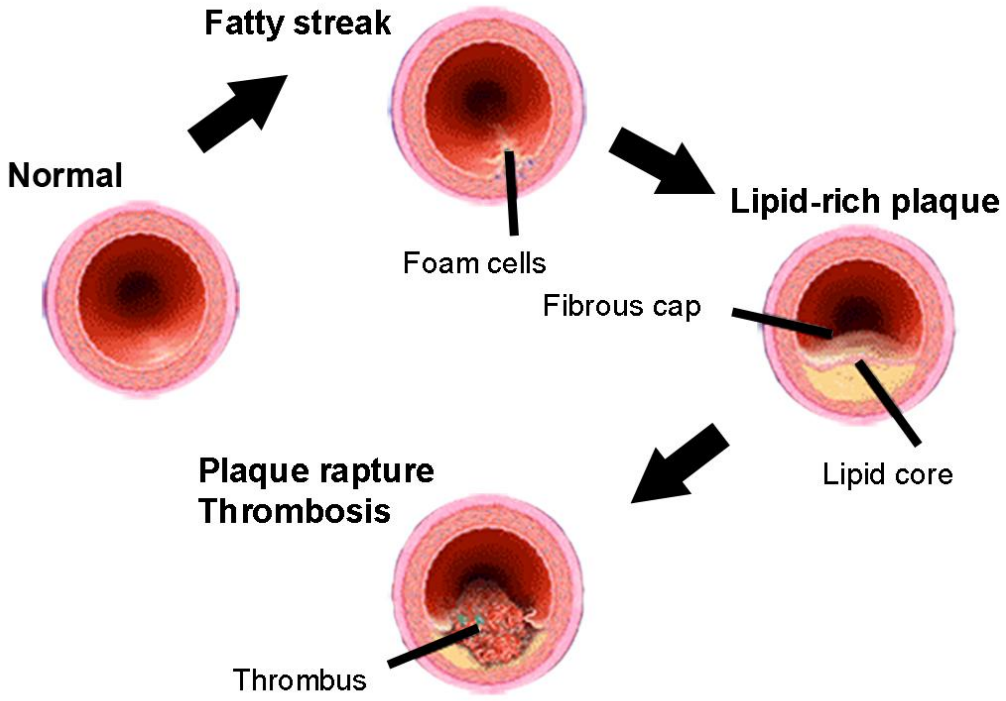


Fig 2.9, *Formation of the atherosclerotic plaque*

The subendothelial foam cells release atherogenic oxidized LDL and reactive oxygen species, further impairing the function of endothelial cells, which apart from forming a barrier between blood flow and underlying tissue, they also produce substances that regulating the vascular tone and arterial health. Nitric oxide, the main endothelial-derived substance, is an important antiatherogenic factor, since it inhibits platelet aggregation, monocyte adhesion and abnormal smooth muscle cell proliferation.

As the disease progresses, smooth muscle cells which are responsible for healing and repair after arterial injury, migrate from the muscular layer to the subendothelial layer and become transformed into fibroblasts. These form a collagenous (fibrous) matrix within the plaque and also a fibrous cap on the luminal side of the plaque, below the intima layer. As a result, lumen and blood flow get reduced causing ischaemia. Nevertheless, smooth muscle cells and collagen-rich matrix confer stability to plaques, protecting them against much more ominous consequences like plaque rupture and thrombosis (Falk, 2006).

2.5.2 Plaque rupture and thrombosis

As atherosclerosis progresses, endothelial cells, macrophages and smooth muscle cells die by apoptosis or necrosis. Disintegration of foam cells and loss of smooth muscle cells may have detrimental consequences, leading to the formation of a destabilizing lipid-rich core and a fragile and rupture-prone fibrous cap. A lesion with these characteristics is called atheroma and despite the fact that it may not significantly increase in size, it is prone to rupture leading to formation of thrombus and embolization. Apoptosis also contributes to the thrombogenicity of the lipid-rich core. Focal calcification and neovascularization as well as intraplaque haemorrhage occurs and increases with age, being more prominent in advanced atherosclerosis.

Inflammation plays a significant role in plaque rupture, as macrophages release lytic enzymes which degrade the fibrous cap. Plaque rupture is also facilitated by vasospasm and increased or oscillating shear stress. Under these circumstances plaque rupture may occur, exposing the thrombogenic lipid-rich atheromatous core to the flowing blood. This can be followed by variable amount of haemorrhage into the plaque and nonfatal or fatal thrombosis of the vessel or embolization.

2.5.3 Risk factors

Since the Framingham Heart Study in 1960s, there is continuous research in atherosclerosis in an effort to identify cardiovascular risk factors and ways they can be modified. The impact of traditional risk factors such as age, sex, hypertension and smoking, has long been demonstrated beyond any doubt. However, new risk factors have emerged, like elevated levels of homocysteine, CRP and triglycerides. The risk factors can be divided into modifiable and non modifiable.

Non modifiable risk factors

Sex

Men are shown to be more prone in atherosclerosis than women. One stated reason is the protective role of estrogens in pre-menopausal women. Between 35 and 55 men are five times more prone in atherosclerosis than women.

Age

Age has a dominant influence in atherosclerosis and is traditionally considered as a risk factor for atherosclerosis, probably due to atrophic and degenerative changes to

the blood vessel wall. Early lesions appear in childhood and disease rises with each decade.

Family history of premature cardiovascular disease

Family history of coronary heart disease, stroke, TIA or peripheral arterial disease is a significant independent predisposing risk factor for atherosclerosis. It is noteworthy that the relative risk is greater in case of disease of mother compared to father. Furthermore derangements in lipoprotein metabolism, like familial hypercholesterolaemia, are strongly correlated with cardiovascular diseases.

Modifiable risk factors

Cholesterol

Hypercholesterolaemia, i.e. high levels of total cholesterol, low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol, is a well established independent risk factor for atherosclerosis and cardiovascular diseases. Low-density lipoproteins can enter the subintimal area of the vasculature and play a significant role in the formation of atherosclerotic plaque. An LDL/HDL ratio over 5 has been correlated with increase probability for cardiovascular disease. Lowering serum cholesterol levels has been proved to significantly reduce the risk of subsequent coronary heart disease events and overall mortality.

According to British Heart Foundation statistics, in England in 2006, 57% men and 61% women had blood cholesterol levels above the target for primary and secondary prevention (5.0 mmol/l) (Scarborough P *et al,* 2009). Centers of Disease Control and Prevention in US suggest that a 10% population-wide decrease in total cholesterol levels could decrease the cardiovascular disease events by 30% (Roger *et al*, 2011).

Smoking

Cigarette habituation is a major risk factor for atherosclerosis and is associated with an increased relative risk of dying from cardiovascular disease. The mechanisms, still not completely clear, include platelets activation, increase of blood viscosity, increase of oxidized LDL and decrease of HDL, resulting in endothelial dysfunction and a relatively hypercoagulable state. The adverse effects of smoking on vascular wall structure have been evaluated by autopsy studies (Solberg & Strong, 1983). A dose-response relationship between the number of cigarettes smoked and the risk for ischaemic stroke has been reported, and the risk is significantly reduced within two years after smoking cessation (Iso, 2011).

Hypertension

Hypertension is a well established risk factor for atherosclerosis and cardiovascular diseases. It is associated with morphologic alterations of the arterial intima that increase its permeability and functional alterations of the endothelium, similar to the changes observed in hypercholesterolaemia and established atherosclerosis. Hypertension has been shown in both epidemiological and experimental studies to accelerate atherosclerotic vascular disease and increase the incidence of clinical complications. Studies have shown that patients with well controlled blood pressure (<120/80 mm Hg) have approximately half the lifetime risk for stroke compared to patients with hypertension (Mancia, 2010).

Metabolic syndrome

The metabolic syndrome encompasses a range of cardiovascular risk factors and is diagnosed when ≥3 of the following risk factors are present: elevated triglycerides

(≥150 mg/dL), low HDL cholesterol (<40 mg/dL in men or <50 mg/dL in women), impaired fasting glucose (≥100 mg/dL), high blood pressure (≥130 mm Hg systolic or ≥85 mm Hg diastolic) and increased waist circumference (≥102 cm in men or ≥88 cm in women). It has been established that it increases a patient's risk factor for atherosclerosis, carotid atherosclerosis and coronary artery disease at any level of LDL cholesterol (Bonora et al., 2003). A recent meta-analysis of 37 prospective studies concluded that metabolic syndrome had at a relative risk for cardiovascular disease of 1.78, showing higher association in women (2.63) and independence from other traditional risk factors (Gami *et al*, 2007).

Diabetes mellitus

Diabetes mellitus is an important risk factor for atherosclerosis and dyslipidaemia and is commonly associated with hypertension, abnormalities of coagulation, platelet adhesion and aggregation, increased oxidative stress and functional and anatomical abnormalities of the endothelium. The Framingham Study, over 30 years ago, established diabetes as an independent risk factor for cardiovascular disease and today diabetes is considered as a coronary heart disease risk equivalent, as diabetes confers a 10-year coronary heart disease risk equal to that of persons with existing coronary heart disease (Fruchart *et al*, 2004). Diabetes has been found to increase stroke incidence at all ages and generally patients with diabetes who suffer an ischaemic stroke are more likely to also suffer from hypertension, coronary heart disease and hypercholesterolaemia (Roger *et al*, 2011).

Lipoprotein (a)

Lipoprotein(a) [Lp(a)] is the combination of a LDL-similar lipoprotein, apolipoprotein B100, with a carbohydrate-rich hydrophilic protein called

apolipoprotein(a). Despite the fact that the pathophysiological mechanism remains obscure, its dual structure suggests its involvement in the process of both atherogenesis and thrombosis. In vitro studies have shown association of Lp(a) with increased expression of adhesion molecules and increased production of monocyte chemotactic protein and interleukin-8 (Kamstrup, 2010). Elevated levels of Lp(a), particularly plasma levels exceeding 20 to 30 mg dL^{-1}, have been causally associated with increased risk for developing cardiovascular disease. However, no randomized clinical trial has shown that lowering Lp(a) levels decreases cardiovascular diseases risk (Fruchart *et al*, 2004).

Triglycerides

Although it had been a matter of debate, studies have shown that high triglyceride levels should be considered an independent risk factor for cardiovascular disease independent of LDL and HDL cholesterol levels, diabetes or hypertension (Hokanson & Austin, 1996; Yarnell *et al*, 2001).

Homocysteine

Homocysteine is formed during demethylation of methionine. Impaired homocysteine metabolism has been implicated as a factor in atherosclerosis, cerebrovascular disease and peripheral vascular disease. Causes of hyperhomocysteinaemia include genetic causes (i.e. heterozygous cystathionine-β-synthase deficiency), vitamin deficiency (folic acid, B12 vitamin, B6 vitamin) or impaired renal function. The exact mechanism by which high homocysteine levels may be translated into increased risk for atherosclerosis remains speculative, but endothelial dysfunction through oxidation of LDL, production of free radicals and impairment of nitric oxide metabolism, enhancement of platelet aggregation and vascular smooth muscle proliferation are

considered (Fruchart *et al*, 2004; Kullo & Ballantyne, 2005). Recent prospective studies have established the causal association and the independency of increased homocysteine levels as a cardiovascular disease risk factor, indicating that increase in homocysteine level by 5 μmol/l increases the risk by approximately 20% (Humphrey *et al*, 2008).

Fibrinogen

Fibrinogen is a circulating glycoprotein which acts as the final step in the coagulation response to vascular and tissue injury. Apart from that role, it regulates cell adhesion, chemotaxis and proliferation, leads to vasoconstriction at sites of vessel wall injury, stimulates platelet aggregation and determines blood viscosity. Recent studies and meta-analyses support an independent association between elevated levels of fibrinogen and cardiovascular morbidity and mortality as well as between fibrinogen, ischaemic stroke and peripheral vascular disease (Tanne *et al*, 2001).

High-sensitivity C-reactive protein

C-reactive protein (CRP) is a circulating acute-phase reactant increased during the inflammatory response to tissue injury or infection. Despite being a very nonspecific marker, high-sensitivity CRP (hs-CRP) levels have been found to have predictive value for coronary and peripheral atherosclerosis. Hs-CRP constitutes a strong and independent predictor of advanced carotid plaques in dyslipidaemic subjects and early-onset carotid atherosclerosis is associated with elevated serum levels of inflammatory markers (Blackburn R et al., 2001). CRP has been suggested to play a role in the pathogenesis of atherosclerosis, as it could promote low-density lipoprotein cholesterol uptake by macrophages, amplify the inflammatory response and promote endothelial activation (Mosca, 2002). However, the causal association with

cardiovascular disease has not proved yet and recent studies tend to reject the direct role scenario, suggesting its use as a marker rather than a risk factor (Genest, 2010; Elkind, 2010). Recent trials (CARE trial, AFCAPS/TexCAPS trial) have reported greater impact in lowering cardiovascular risk with statin treatment in patients with high hs-CRP levels comparing to low hs-CRP levels. These results suggest using hs-CRP as a criterion for selection of patients for statin treatment even in the absence of dyslipidaemia (Ridker *et al*, 2008; Mora & Ridker, 2006).

Overweight and obesity

Overweight and obesity have long been considered as independent risk factor for cardiovascular disease. The relative risk for cardiovascular disease is increased by 21% and 20% for overweight men and women respectively and by 46% and 64% for obese men and women respectively (Wilson *et al*, 2002). In England from 1994 to 2006 the prevalence of obesity has increased by 50% for men and women and it is predicted that by 2050 60% of UK adult population would be obese (Scarborough P *et al*, 2009). A recent study conducted in U.S. showed that although lifestyle changes like smoking cessation and modern treatments of cholesterol and blood pressure have resulted in gaining 2,770,500 life-years, obesity and diabetes have reduced this gain by 715,000 life-years (Capewell *et al*, 2009).

Sedentary lifestyle

Studies commonly report an inverse association between physical activity and cardiovascular disease risk. Perspective studies have reported decrease of relative risk for cardiovascular events and cardiovascular mortality with physical activity, and although results about dose-response relationship are mixed when risk is adjusted for

other risk factors, most studies agree that vigorous physical activity can significantly reduce cardiovascular relative risk (Carnethon, 2009; Yu *et al*, 2003)

Other risk factors

Other risk factors, such as hypercoagulable states, race, alcohol intake, psychological factors (stress, depression, aggressive behaviour), microalbuminuria, uric acid etc have been correlated with pathogenesis and progression of atherosclerosis. In some cases the exact pathophysiological mechanisms are still unclear.

2.5.4 Clinical features of atherosclerotic disease

The formation of atherosclerotic plaque can be the cause of different cardiovascular diseases, with similar pathophysiologic mechanisms but different symptomatology, diagnostic procedures and treatment, depending on which vessel the plaque is formed in.

Coronary heart disease

In coronary heart disease or ischaemic heart disease the atherosclerotic lesion is formed in one or more coronary arteries of the heart (Fig. 2.10). It is clinically manifested by angina pectoris, precordial chest pain usually precipitated by stress or exertion, relieved at rest or by nitrates, and in late stages by acute coronary syndromes, which include unstable angina and acute myocardial infarction. Coronary heart disease is the leading cause of death worldwide (Fig 2.4).

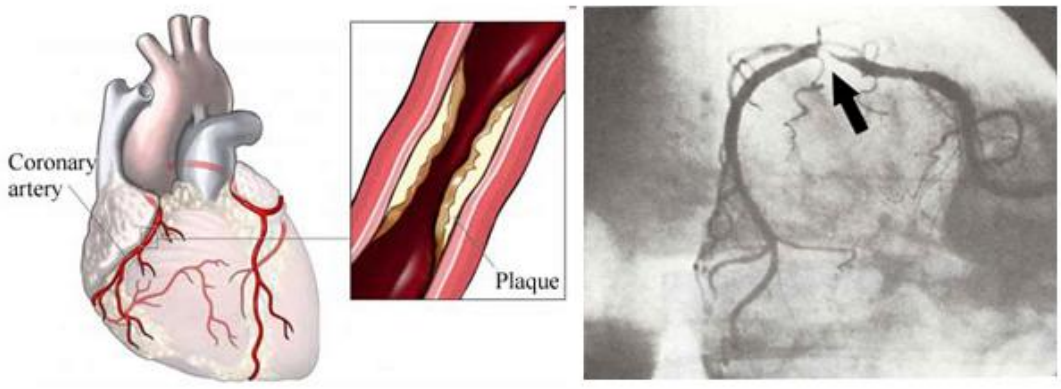


Fig 2.10, *Coronary artery disease in drawing (left) and in coronary angiogram (right)*

Cerebrovascular disease

The presence of atherosclerotic plaque in carotid arteries, usually in proximal internal carotid artery or in the carotid bulb, which may cause either haemodynamic changes in blood flow or, more often, thromboembolism in more distal branches, is stated as carotid artery disease. Its clinical manifestations include contralateral weakness or sensory loss, expressive aphasia or amaurosis fugax (transient partial or complete loss of vision in the ipsilateral eye) and it is considered as a major cause of transient ischaemic attack and ischaemic stroke. Furthermore, formation of atherosclerotic plaque in vertebral artery can cause vertebrobasilar transient ischaemic attack, which is characterized by brainstem and cerebral symptoms including dysarthria, diplopia, vertigo, ataxia and hemiparesis or quadriparesis.

Peripheral vascular disease

Atherosclerosis in limb arteries can cause peripheral vascular disease, also called peripheral arterial occlusive disease. It leads to insufficient tissue perfusion that may acutely be compounded by either emboli or thrombi. Lower limb arterial occlusive disease is more common than upper limb disease. Its main symptom is intermittent

claudication, which is defined as pain or discomfort in limb muscle which occurs after a reproducible amount of exercise and is relieved within a few minutes of rest. In more severe conditions it can cause critical limb ischaemia, that manifests as rest pain which requires analgesia and/or ulceration or gangrene, which if not properly managed can eventually lead to loss of limb or even life.

Renal artery stenosis

Atherosclerotic plaque is the cause of 80-90% of patients with renal artery stenosis (Fig. 2.11). It clinically manifests as renovascular hypertension, which accounts for about 5% of all cases of hypertension, and ischaemic nephropathy.

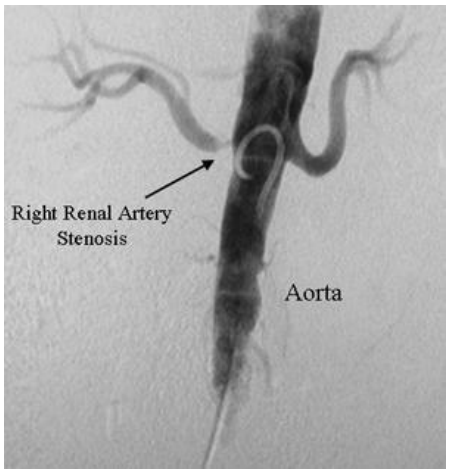


Fig 2.11, *Angiogram of renal artery stenosis*

2.6 Carotid atherosclerotic disease

The formation of atherosclerotic plaque in the carotid artery, usually at the site of the carotid bifurcation, proximal ICA or the carotid bulb, is called carotid atherosclerotic disease, and is a major cause of ischaemic stroke.

2.6.1 Symptoms and indications for investigation

Carotid atherosclerotic disease has been associated with contralateral hemi-paresthesia, dysphasia, ipsilateral monocular blindness, residual blurriness, syncope, confusion and seizure. Major indications for imaging investigation include carotid bruits, amaurosis fugax, hemispheric transient ischaemic attacks and recent stroke. A large number of patients are diagnosed during screening for carotid stenosis, usually prior to coronary artery bypass grafting operation for coronary artery disease treatment, since there is significant risk of stroke postoperative in patients with severe coronary heart disease. It is common practice in many cardiothoracic centres to combine carotid endarterectomy with coronary artery bypass grafting in those patients.

2.6.2 Imaging investigation

The degree of carotid artery stenosis is of great importance in the diagnosis, prognosis and treatment of the carotid atherosclerotic disease and prevention of stroke. Major trials have been conducted showing higher risk of stroke in greater degrees of constriction, defining thresholds for the beneficial results of carotid endarterectomy to patients with symptomatic carotid stenosis (North American Symptomatic Carotid Endarterectomy Trial. Methods, patient characteristics, and progress, 1991, MRC European Carotid Surgery Trial: interim results for symptomatic patients with severe (70-99%) or with mild (0-29%) carotid stenosis. European Carotid Surgery Trialists' Collaborative Group, 1991) and also asymptomatic stenosis (Endarterectomy for asymptomatic carotid artery stenosis. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). In addition to degree of stenosis, other carotid

plaque characteristics, such as composition and inflammation, are now considered as plaque vulnerability factors and research has focused on use of imaging modalities to identify and quantify them. Determination of severity of carotid disease is crucial for the patient management and several imaging modalities are being used, each one with its advantages and limitations.

Digital subtraction angiography

Digital subtraction angiography (DSA) used to be the gold standard for the estimation of carotid artery stenosis. The two major endarterectomy trials (North American Symptomatic Carotid Endarterectomy Trial. Methods, patient characteristics, and progress, 1991, MRC European Carotid Surgery Trial: interim results for symptomatic patients with severe (70-99%) or with mild (0-29%) carotid stenosis. European Carotid Surgery Trialists' Collaborative Group, 1991) for the determination of the threshold percentage of carotid stenosis above which endarterectomy is the favourable treatment, were undertaken during the 1980s up to mid 1990s and used DSA as the gold standard, establishing purely angiographic criteria. Since then, however, newer techniques have become widely available, replacing DSA in the routine practice. DSA has many limitations and it is now rarely used. It is an invasive technique with a relatively high risk of morbidity and mortality, ranging from 1% to 4% in patients with atherosclerosis (Willinsky *et al*, 2003). A risk for iatrogenic stroke of 1.2% has also been reported (Mintz & Hobson, 2000). Even patients without apparent neurological complications have been found to develop minor asymptomatic infarctions resulting from microembolism after DSA (Bendszus *et al*, 1999). It is an expensive procedure, usually performed in hospital, and the use of contrast agent can potentially cause allergic reaction to patient. As far as its diagnostic accuracy is

concerned, DSA can in many cases underestimate the stenosis. The fact that DSA can image only the lumen of the vessel and not the vessel wall, being unable to provide arterial cross-sectional views, leads to different assumptions about the true diameter of the vessel and the diameter reduction. For these reasons DSA has gradually been replaced by other non-invasive, less risky and more advanced modalities.

Duplex ultrasound

Duplex ultrasound (DUS) has replaced angiography in most diagnostic circumstances and is today the first diagnostic tall in doctor's quiver. It is a non-invasive imaging modality which uses the physical principles of ultrasound waves and the Doppler phenomenon for the imaging of subcutaneous tissues and organs. It is safe for the patients and the operator, since it does not expose them to harmful radiation. DUS is a real time imaging modality which allows the visualization of both lumen and wall of the vessel, without the necessity of contrast agent administration. On the other hand, it can only be used for imaging of the extracranial parts of CCA, ICA and ECA, as ultrasound beam cannot penetrate the skull. The operator can insonate the artery in any dimension from any view just by orientating the transducer and can take images of the artery in long and short axis, visualizing the actual vessel and the lumen area. This is a major advantage of the ultrasound compared to other angiographic imaging modalities, either conventional or newer.

DUS can also investigate the morphology of the plaque and provide details about its composition, which has turned out being of great importance for the prognosis of the disease. It is well established that lipid rich ulcerated plaques are more prone to rupture and haemorrhage than calcified fibrous plaques. A lot of studies looking into plaque characterization with DUS have concluded that it can efficiently differentiate

these two categories. In addition, DUS can provide details about the characteristics of the blood flow, such as peak systolic velocity and flow volume, which gives information about the vessel at the insonation point, but also proximally and distally.

A number of studies have tried to determine velocity criteria that represent degrees of carotid stenosis and currently the most widely used parameter for the measurement of the degree of carotid constriction is the peek systolic velocity (PSV). However, the various PSV criteria published, applying several different parameters as well as the great variability among the laboratories, the instrumentations and the operators, has lead to controversy and discussion about which parameter and criteria should be used. No globally accepted criteria have hitherto been established. The fact that DUS is a highly operator dependant modality and the lack of standardized criteria have resulted in the use of different criteria and parameters by laboratories, according to local experience, radiological correlation and surgeons preferences and methods used for the measurement of the size of plaque (Oates *et al*, 2009).

As far as diagnostic accuracy is concerned, several studies have showed DUS to be highly accurate in determining the degree of carotid stenosis. With the application of velocity criteria, DUS can identify greater than 70% stenosis with a sensitivity of 90% and specificity of 85% (Jahromi *et al*, 2005). The threshold of 70% is set by the endarterectomy trials mentioned before, that have shown that patients with greater than 70% stenosis benefited from endarterectomy. A meta-analysis of studies that compared different imaging modalities used for carotid atherosclerotic disease concluded that DUS is very accurate in determining the degree of stenosis when it is more than 70% or less than 50% (Wardlaw *et al*, 2006). The reason is unclear, but one possible explanation could be that the operator subconsciously rounds stenoses falling

between 50% and 69%, categorising up or down into the clinically more definite decision categories.

Currently, DUS is the primary non-invasive imaging procedure for carotid stenosis severity assessment and treatment decision, and only in cases of unclear results a computed tomography angiography or magnetic resonance angiography is suggested for greater diagnostic accuracy (Bates *et al*, 2007). Because of its characteristics and its accuracy, it has also been effectively used for screening the population for carotid and peripheral arterial disease, as well as screening patients prior to CABG for carotid arterial disease (Ballard *et al*, 2007; Bates *et al*, 2007).

Furthermore, new modalities of ultrasound have come in the foreground in carotid plaque investigation with promising results. Contrast enhanced ultrasound, with the use of microbubbles as blood pool agents, has been evaluated for assessing carotid plaque neovascularization, in a similar way that the technique is used for assessing tumour angiogenesis (Feinstein, 2006). Three-dimensional ultrasound has been tested for calculation of plaque volume and its assessment for response-to-treatment evaluation, with good intraobserver and interobserver reliabilities (Landry *et al*, 2004; Makris *et al*, 2011). Recent research has also looked at methods of three-dimensional investigation and analysis of carotid plaque for identification of changes in tissue components (Coleman *et al*, 2005).

Computed tomography angiography

Computed tomography angiography (CTA) is a non-invasive diagnostic modality with much potential, as fast helical CT scanners are now widely available. With the administration of iodine contrast, CTA can be used to grade the severity of stenosis and visualize the arterial lumen and vessel wall. In addition, a number of post-

processing techniques allow the construction of 3D images of carotid arteries, though each has its strengths and limitations. For example shaded surface display reconstructions provide a 3D image of the outer vessel wall but no information about the residual lumen. Multiplanar and curved planar reformations facilitate reconstruction of the tortuous cervical arteries. Studies have reported very high accuracy of CTA in grading carotid stenosis. A meta-analysis of studies that compared CTA with conventional angiography showed sensitivity of 85% and specificity of 93% for detection of a 70% to 99% stenosis and 100% sensitivity and specificity for detection of complete carotid artery occlusion (Koelemay *et al*, 2004). It is noteworthy that a meta-analysis that compared all non-invasive modalities used for the carotid imaging concluded that CTA had the highest specificity of all (95%) for 70-99% stenosis (Wardlaw *et al*, 2006). As far as detection of complete carotid artery occlusion is concerned, CTA is more accurate than DUS, which can misdiagnose tight stenosis with minimal flow as occlusion (Koelemay *et al*, 2004). Since the positive predictive value of DUS in diagnosing total occlusion has been calculated being 86%, falling to 72% in symptomatic patients, it has been suggested that CTA is necessary for the discrimination between total occlusion and trickle flow (Lubezky *et al*, 1998). This is essential because tight stenosis and total occlusion are treated differently.

As far as the plaque compositional information that CTA can provide, studies have shown good correlation between CTA findings and carotid plaque specimen histological analysis for plaque ulceration, calcification and lipid core with overall agreement of 75%. However, there is marked overlap in CT densities associated with haemorrhage, connective tissue and lipids (Wintermark *et al*, 2008).

CTA, when not used for reconstruction of 3D images, suffers the imaging limitations of angiography, as it cannot visualize the vessels in different planes as DUS does. The use of intravenous contrast agent limits its application in patients with renal insufficiency or cardiac failure, while it means potential risk for allergic reaction. It also exposes the patient to higher doses of ionizing radiation compared to conventional angiography. However, machines evolution and introduction of 256 and 512-slice scanners could increase the accuracy, reliability and overall use of CTA for carotid atherosclerotic disease in the future.

Magnetic resonance angiography

Magnetic resonance angiography (MRA) and contrast-enhanced MRA (CEMRA) are increasingly used supplementary to duplex ultrasound or conventional angiography in the diagnosis of carotid artery stenosis. Time of flight (or "inflow") methods, two or three dimensional, are non-invasive techniques which emphasize in blood flow, based on the different signal given by a material that has moved into the imaging volume. The gadolinium based contrast materials which are used in CEMRA are better tolerated than the iodinated media used for conventional angiography. Some of the drawbacks of time of flight techniques, especially signal loss near stenosis, have been overcome in CEMRA (Westwood *et al*, 2002). Among the imaging modalities available, CEMRA has the highest sensitivity (94%) for 70-99% stenosis while MRA has 88%. For that range of stenosis the specificity for CEMRA and MRA is shown to be 93% and 84% respectively. Less accurate but still better than the other techniques, CEMRA has been shown to have 77% sensitivity and 97% specificity for 50-69% stenosis, though the data for that range of stenosis are limited (Wardlaw *et al*, 2006). MRA is superior to DUS for the distinction between less than 70% stenosis and 70-

99% stenosis, whereas for identifying total occlusion there is no significant difference in diagnostic accuracy between MRA and DUS (Nederkoorn *et al*, 2003).

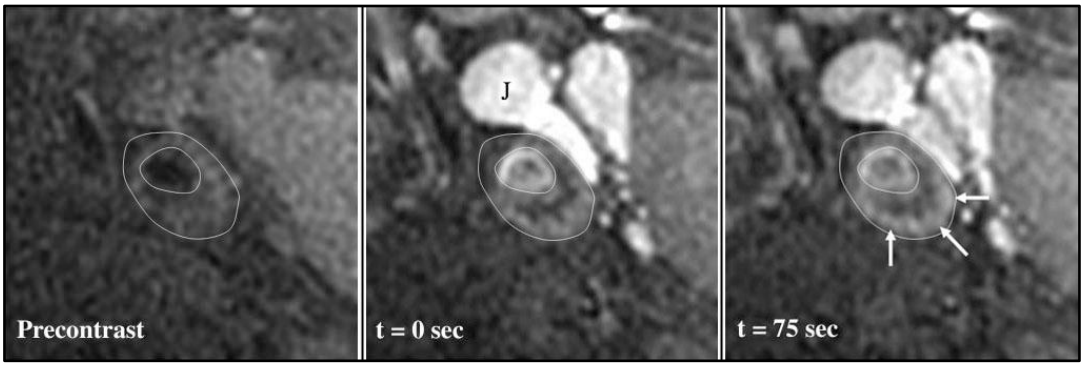


Fig 2.12, *MRI investigation of carotid atherosclerotic plaque using gadolinium-based contrast agent; Precontrast image (left), contrast enhanced at bolus arrival (middle) and 75 sec later (right) (adapted by* Kerwin et al*, 2006)*

There is ongoing research on the carotid plaque compositional details MRI can provide. By combining multiple different contrast weightings, MRI can distinguish different tissues of arterial wall or plaque. Good results have been reported for identification of lipid core, calcification and intraplaque haemorrhage, with satisfying intrareader and interscan reproducibility. Furthermore, dynamic contrast-enhanced magnetic resonance imaging, using gadolinium-based extracellular contrast agents, have been used for morphological and functional analysis of atherosclerotic plaque. This technique has been experimentally tested for atherosclerotic plaque inflammation identification and quantification with promising results (Hur *et al*, 2010). Moreover, increased image enhancement, quantified using kinetic modeling with a vascular component, has been well correlated with macrophage infiltration and neovascularization measured in histological plaque specimens (Kawahara *et al*, 2007; Kerwin *et al*, 2006) (Fig 2.12). However, the mechanism of image enhancement with gadolinium contrast agent remains unclear and the specificity of the technique for inflammation identification is not yet established.

Another suggested technique in magnetic resonance imaging for inflammation assessment is the use of ultrasmall superparamagnetic iron oxide particles (USPIOs) as contrast agents. Studies have shown that such particles are taken up by macrophages and generate a negative contrast in T2-weighted magnetic resonance imaging, which has been significantly correlated with degree of plaque macrophage infiltration (Trivedi *et al*, 2006).

Limitations of magnetic resonance imaging include the inability to perform due to implantable defibrillators and pacemakers and overestimation of carotid stenosis due to movement artifacts. MRA has advanced dramatically but it is still used more as a research tool than a diagnostic one.

Positron emission tomography

Positron emission tomography (PET) is a nuclear imaging technique, relatively new in carotid disease investigation. It can provide qualitative and qualitative information about atherosclerotic plaque, using radiolabeled ligands targeted in particular tissues or molecules. [^{18}F]-fluorodeoxyglucose, a glucose analogue that is taken up by cells proportionally to their metabolism mimicking the biochemical behaviour of the natural glucose molecule, has been used to identify and quantify carotid atherosclerotic plaque inflammation (Rudd *et al*, 2010). In vitro and in vivo studies have reported high correlation between plaque ^{18}FDG uptake compared to background uptake and degree of macrophage infiltration of the plaque (Rudd *et al*, 2002; Tawakol *et al*, 2006). A limitation of PET is its low spatial resolution (approximately 5 mm) and investigation of arteries walls and plaques currently requires the combination with CT or high-resolution MRI, whilst new hybrid PET/CT machines are designed for the modality combination (Kai, 2010) (Fig 2.13).

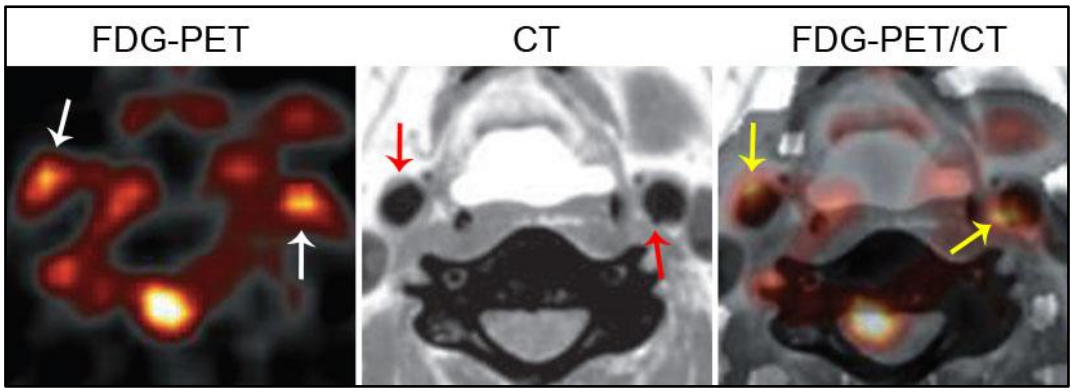


Fig 2.13, *Carotid plaque inflammation detected by [^{18}F]-fluorodeoxyglucose PET (left), CT (middle) and FDG-PET/CT (right) (adapted by Kai, 2010)*

2.6.3 Management of carotid atherosclerotic disease

The choice of appropriate and efficient treatment for carotid atherosclerotic disease depends on the degree of stenosis and the characteristics of the plaque found in the imaging investigation, taking into account the patient symptoms.

Medical treatment

All patients with carotid atherosclerotic disease, regardless of plans for revascularization by carotid endarterectomy or carotid artery stenting, should be treated medically to reduce stroke risk and alter modifiable risk factors. The optimal medical treatment consists of antihypertensive treatment (blood pressure goal is <140/90 mm Hg or for diabetic patients <130/80 mm Hg), smoking cessation, cholesterol level management, predominantly with statins and lifestyle changes (LDL goal <100 mg/dL or <70 mg/dL in coronary heart disease high risk patients), diabetes control (goal HbA1c level <7%), weight reduction and antiplatelet agents (aspirin with or without extended-release dipyridamole or clopidogrel) (Safian, 2011).

Despite the constant debate for the best treatment in border-line stenosis or in asymptomatic patients with severe stenosis, it is common ground that asymptomatic

patients with less than 60% of stenosis and symptomatic patients with less than 50% of stenosis are treated medically and followed up closely, and no revascularization procedure is planned. Furthermore, medical treatment alone is recommended for patients of greater stenosis but with high risk for revascularization procedure. Factors that increase risk of carotid endarterectomy include anatomical factors (bilateral severe carotid stenosis, inaccessible stenosis, hostile neck due to radiation therapy, cervical spine immobility), clinical factors (prior cranial nerve injury, severe cardiopulmonary disease, severe heart failure with ejection fraction <30%), impaired cerebral reserve, chronic kidney failure and age greater than 75 years. Factors that increase risk for carotid artery stenting include anatomical factors (complex aortic arch, brachiocephalic arterial disease, significant carotid plaque calcification, presence of thrombus), clinical factors (need of heart surgery within 6 weeks after stenting), impaired cerebral reserve, chronic kidney failure and age greater than 75 years.

In spite of the fact that the landmark carotid endarterectomy trials have confirmed superiority of surgical revascularization over medical treatment with antiplatelets, it is noteworthy that those studies did not include angiotensin-converting enzyme inhibitors and, most importantly, statins. This optimal medical treatment, which has been shown to efficiently reduce carotid atherosclerotic regression and intraplaque angiogenesis, has been proved to decrease the risk of cerebrovascular events and is currently used for primary and secondary prevention from stoke (Safian, 2011; Kang *et al*, 2004; Koutouzis *et al*, 2007).

Surgical treatment

Carotid endarterectomy is the most common surgical procedure for carotid revascularization. Multicenter trials showed that symptomatic patients with carotid stenosis greater than 70% gain substantial benefit from carotid endarterectomy compared to those treated with best medical treatment. It has also been suggested that carotid endarterectomy should be considered in symptomatic patients with 50-69% stenosis with unstable, ulcerated and vulnerable plaque, where risk for stroke is significant. Furthermore, surgical treatment should be considered if comorbidities such as advanced age, diabetes mellitus and coronary artery disease are present, since they increase risk of stroke in patients treated medically, but not in those treated surgically.

As far as patients with asymptomatic disease are concerned, endarterectomy trials reported that risk for stroke was reduced by 53% in patients with carotid artery stenosis greater than 60% that were treated surgically compared to patients treated medically (Asymptomatic Carotid Atherosclerosis Study, 1995). However, it is noteworthy that the 5-year risk for stroke in the asymptomatic patients that were not operated was only 11%. These results led to major increases in rates of endarterectomy for asymptomatic stenosis in some countries, most notably the United States, where approximate 150,000 endarterectomy are performed each year. Of these at least half are done for stenoses that have never been symptomatic. In contrast, little change for asymptomatic stenosis has been noted in other countries such as the UK, where it was felt that the benefit did not justify the cost, as it was estimated that 40 operations were needed to prevent 1 disabling or fatal stroke after 5 years (Rothwell & Goldstein, 2004).

Current American Heart Association guidelines suggest carotid endarterectomy to be the best treatment for symptomatic patients with stenosis 50% to 99% if the risk for perioperative stroke or death is less than 6% and for asymptomatic patients with 60% to 99% stenosis if the risk is less than 3%, although some would not operate on asymptomatic plaque until it causes more than 80% artery stenosis (Table 2.1). Patient's life expectancy should exceed 5 years and his age should be between 40 and 75 tears (Bates *et al*, 2007). If patient is considered of high risk for endarterectomy because of anatomical and clinical factors mentioned previously, alternative treatment (medical or carotid artery stenting) is recommended.

Cardiovascular	**Wound**	**Neurological**	**Carotid artery**	
Hypertension (20%) Hypotension (5%) Myocardial infarction (1%)	Infection (1%) Hematoma (5%)	Hyperperfusion syndrome Intracerebral haemorrhage Cranial nerve injury (7%) Seizures Stroke (2%-6%)	Carotid artery thrombosis Carotid artery dissection Restenosis (5%-10%)	Death (1%)

***Table 2.1**, Potential complications of carotid endarterectomy*

Carotid endarterectomy has evolved during the last decades from a complex major surgical procedure to a frequently performed intervention with minimal morbidity and mortality and it is proved to be very efficient treatment for severe symptomatic or asymptomatic carotid stenosis.

Carotid angioplasty and stenting

Carotid angioplasty and stenting represents a new alternative revascularization treatment of carotid atherosclerotic disease.

The first balloon angioplasty in carotid stenosis was performed in 1979 but the enthusiasm of medical community was quickly limited by the relatively high incidence of periprocedural embolic stroke. However, improvements in technique and equipment as well as the utilization of embolic protection devices for removal of embolic debris generated during the procedure have made carotid artery stenting a reasonable alternative treatment, especially for patients at high risk for surgery.

With this technique, general anesthesia and its complications are avoided. It is usually performed via a catheter insertion at femoral artery, or in case of an anomalous left carotid artery at brachial or radial artery, avoiding incision in the neck and subsequent cranial and cutaneous nerve damage. Hospital admission and recovery time after endovascular treatment is usually less than with surgery, reducing healthcare costs. However one has to be aware of possible complications of carotid stenting (Table 2.2) as well as of the anatomical and clinical contradictions previously mentioned (tortuosity of aortic arch, heavy lesion calcification, life expectancy < 5 years etc).

Carotid stenting is currently recommended for patients of high risk for surgery with symptomatic stenosis greater than 50% and asymptomatic stenosis greater than 80%.

A randomized trial comparing carotid stenting and carotid endarterectomy in high risk for surgery patients (SAPPHIRE trial) reported less major adverse cardiovascular events (MACE) in 30-day period after revascularization procedure for carotid stenting compared to carotid endarterectomy. MACE incidence after 1 year remained significantly lower after stenting comparing to endarterectomy and stroke after 3

years rate was found around 1.2% per year for both surgery and stenting, nearly 5 times lower than the rate for those treated medically (Safian, 2011).

Cardiovascular	Carotid artery	Neurological	General
Vasovagal reaction (5%-10%) Vasodepressor reaction (5%–10%) Myocardial infarction (1%)	Dissection (<1%) Thrombosis (<1%) Perforation (<1%) ECA stenosis or occlusion (5%–10%) Transient vasospasm (10%–15%) Restenosis (3%–5%)	TIA (1%–2%) Stroke (2%–3%) Intracranial haemorrhage (<1%) Hyperperfusion syndrome (<1%) Seizures (<1%)	Access site injury (5%) Blood transfusion (2%–3%) Contrast nephropathy (2%) Contrast reactions (1%) Death (1%)

***Table 2.2**, Potential complications of carotid artery stenting*

Numerous registries for carotid stenting since then in more than 12,000 patients have reported a MACE in 30 days rate ranging from 3.8% to 8.5%.

A recently completed multicenter randomized trial which compared carotid endarterectomy and carotid stenting in symptomatic and asymptomatic patients with greater than 70% stenosis (assessed by ultrasound) with normal surgery risk (CREST) reported no significant difference in rates of primary trial end point events (i.e. stroke, myocardial infarction or death during the periprocedural period or ipsilateral stroke within 4 years) between surgery and stenting (7.2% for stenting vs 6.8% for endarterectomy). However, a higher risk of stroke for stenting and a higher risk of myocardial infarction for endarterectomy were noted. Particularly in symptomatic patients the risk of stroke and death was significantly higher for stenting (6%) than endarterectomy (3.2%) (Mantese *et al*, 2010).

Further studies are needed and several randomized trials are running to assess long-term therapeutic effect of carotid artery stenting and maybe adjust management guidelines for carotid atherosclerotic disease.

CHAPTER III

CAROTID PLAQUE SIZE ASSESSMENT

WITH ULTRASOUND

Chapter III

Carotid plaque size assessment with Ultrasound

A review of the literature

3.1 Introduction

Among the characteristics of an atherosclerotic plaque in any artery, coronary, carotid or other, its size is the one that has been widely correlated with the severity of the disease and has been assessed with various diagnostic methods even at the time of angiography.

When angiography was the main imaging tool for carotid disease assessment, plaque size and degree of stenosis were the only measurable parameters and every management and therapeutic decision was based on that measurement. Whether a patient would have an endarterectomy operation or would receive medical treatment was decided upon the presence of symptoms and the determination of the degree of stenosis using angiography. Even nowadays that ultrasound has become the routine method for assessing carotid atherosclerotic disease and new ways of assessing the plaque have emerged, the degree of the stenosis remains the primary characteristic of plaque to be assessed for disease classification and progression monitoring. However, there are still ongoing debates and confusion among the vascular surgeons and technologists about the methods used to measure or calculate the degree of the stenosis.

3.2 The angiographic trials and the 70% cut-off

In the decade of the 80s the carotid endarterectomy was one of the most commonly performed operations in the United States. By that time, the overall risk of surgery had not been investigated thoroughly and criticism about the necessity of surgical treatment in certain groups of patients had arisen. In addition to that, there was discussion about the relationship of the degree of stenosis with the risk of symptomatic disease, as it was common knowledge that also small plaques which caused minor or mild stenosis, can also be responsible for TIA or stroke by ulceration and thromboembolism (Warlow, 1984).

In ‘90s, with x-ray angiography still being the main diagnostic method for measuring stenosis at carotid arteries, medical community tried to objectify the way the disease was approached as far as the size of the plaque was concerned. Establishment of criteria that would determine which patient would benefit from surgical treatment and which should be treated medically with antiplatelet and occasionally antihypertensive and antihyperlipidaemic agents was necessary. Two major trials were conducted, one in United States and Canada (North American Symptomatic Carotid Endarterectomy Trial, 1991) and one in Europe (European Carotid Surgery Trial, 1998). In the North American Symptomatic Carotid Endarterectomy Trial (NASCET), 2885 patients were randomized in 106 centres over a period of 9 years to optimal medical treatment of that time and medical plus surgical treatment. The European Carotid Surgery Trial was conducted from 1981 until 1994 in 100 centres in Europe; during that period 3024 patients with symptomatic carotid disease were randomized to best medical treatment and carotid endarterectomy after medical treatment.

The results of these trials regarding the operative risk and the groups most benefiting from surgery were of great importance for the way the disease would be approached the years to follow.

Both trials concluded in favour of surgical treatment in cases of severe stenosis, i.e. 70-99%. According to ECST the highest risk for perioperative stroke was observed in patients with moderate stenosis (9.5%) as compared to those with severe stenosis (3.8%). In more detail, the risk was increased in female (10.4% vs 5.8% in male), in patients with peripheral vascular disease, in hypertensive patients and in patients presented with hemispheric TIA as a sole symptom (9.1% vs 6.3% in hemispheric stoke and 3.2% in retinal events). One third of stokes/deaths in NASCET occurred in the first post-operative day and the 86% of the rest within 7 days of surgery. Furthermore, NASCET reported association of operative risk and morphology of carotid plaque (5.5% for irregular vs 3.7% for smooth plaques). Wound complication (haematoma, infection etc) were reported in 9.3% and cranial nerve injuries in 8.6% in NASCET, though all were mild with full recovery (Naylor *et al*, 2003).

Interestingly, according to NASCET, patients with smaller plaques (50-69%) gained a small but significant benefit from surgery if they match a particular profile (male, presenting with stroke, with hemispheric rather than retinal symptoms). On the other hand, ECST did not report any benefit in cases of mild stenosis. However, it should be stated that the two trials differ in some aspects of methodology, the most significant difference being the method used to measure carotid constriction. In ECST the minimum residual luminal diameter was compared with the estimated artery wall-to-wall diameter at that point, whereas in NASCET the ratio of the smallest residual luminal diameter over the distal disease-free point diameter was used (Fig 3.1). The two methods produce different degree of stenosis for a particular case; for example, a

70% stenosis as measured using the NASCET method is measured as 85% stenosis when ECST method is used (Naylor *et al*, 2003; Nicolaides *et al*, 1996). Likewise, the 50-69% group of NASCET (mild stenosis) is equivalent with the 70-79% group of ECST, so the results about the benefit of surgery are actually the same in both studies.

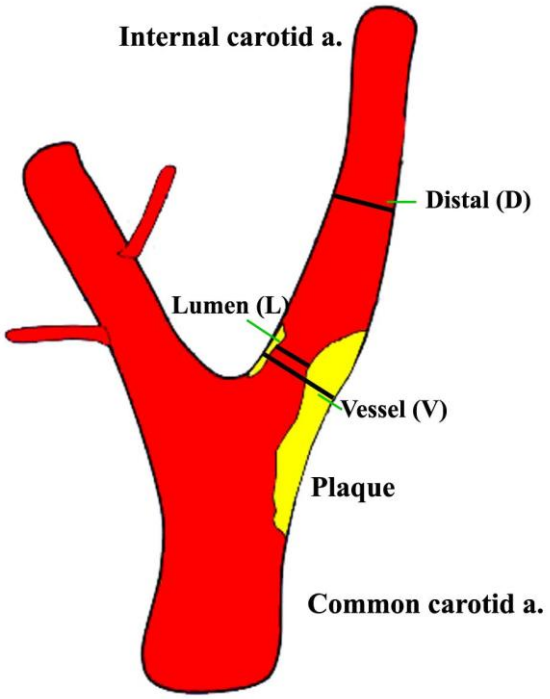


***Fig 3.1**, Different methods for measuring carotid stenosis. NASCET uses the ratio of lumen diameter over distal diameter (L/D) while ECST uses the ration of lumen diameter over the arterial diameter at the point of constriction (L/V)*

At that time, with these methods being applied in angiography, NASCET method seemed to be more accurate and reproducible comparing to ECST method. Since it is the lumen of the vessel rather than the vessel wall that is visualized in angiography, calculation using the ECST method required estimation of the diameter of the artery, whether in the NASCET method one could just measure the luminal diameter of distal disease-free part of the vessel and compare it with the minimum luminal diameter of internal carotid artery. For that reason, NASCET method was more easily adopted for determining the degree of stenosis in a cerebrovascular angiogram.

3.3 Duplex ultrasound for carotid stenosis measurement

When ultrasound was firstly introduced in clinical practice, in late 1970s and 1980s, it was used predominantly as a screening tool for selection of patients that would then have an angiography for determination of the degree of stenosis. At that time, because of the poor resolution of B-mode, the measurement of the stenosis was based on the spectral broadening and blood flow velocity measurement. Velocity criteria were therefore developed for classification of the stenosis in different categories.

Over the years, as duplex ultrasonography evolved and imaging quality and accuracy of the machines were constantly improving and new modalities like colour Doppler were implemented, it gradually replaced angiography as the main diagnostic tool in the assessment of carotid atherosclerotic disease. The numerous limitations of conventional angiography along with its invasive character and the procedural stroke risk compared with the imaging possibilities, the safety for the patient and the convenience of ultrasound played a decisive role in that change of roles. Duplex ultrasound revealed new ways of approaching carotid plaque. The new modality offered visualization (colour Doppler mode) and quantification (pulse and continuous wave Doppler modes) of the blood flow, visualization of the lumen as well as the wall of the arteries and view of the arteries in cross section, all in a quick non-invasive way.

As a result, by the year 2000, the decision for surgical or medical therapy of carotid disease in most patients was based solely on the results of duplex ultrasound scan as the only preoperative imaging study (Grant *et al*, 2003).

Following the recommendation of the American and European angiographic trials in 1990s for the significance of identification of greater than 50% and greater than 70%

of stenosis, vascular scientists tried to develop new velocity criteria for carotid stenosis grading. These criteria were always compared with angiographic measurement of the stenosis, ignoring the imaging limitations of the technique and the confusion over the angiographic methods of stenosis measurement.

3.4 Controversy and confusion over velocity criteria

Numerous sets of velocity criteria were proposed, giving different interpretations to the various flow velocities parameters (Moneta *et al*, 1995; Staikov *et al*, 2002; Thomas *et al*, 2002; Nicolaides *et al*, 1996; Elgersma *et al*, 1998; Hwang *et al*, 2002; Mittl *et al*, 1994; Nederkoorn *et al*, 2002; Zwiebel, 1987; AbuRahma *et al*, 1998; Filis *et al*, 2002; Robinson *et al*, 1988). The variation of these values is quite remarkable, but at some point it is justified due to the different scanners, protocols, settings like Doppler angle and techniques used in each center (Fig. 3.2).

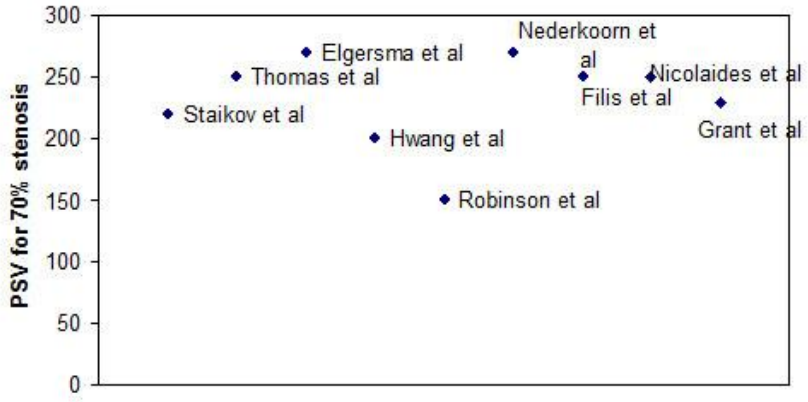


***Fig 3.2**, Variation of PSV cut-off for 70% stenosis (NASCET method) in several published criteria*

Furthermore, the different angiographic methods proposed by the angiographic trials worsened the confusion over the use of velocity criteria among vascular laboratories

and led to different calculation among the diagnostic centres and obstructed the standardization of DUS criteria.

3.5 Consensus criteria

In an effort to standardize criteria and technique for carotid stenosis ultrasound investigation, the Society of Radiologist in Ultrasound tried to arrive at a consensus about several issues (Grant *et al*, 2003). It was decided that Doppler was inaccurate for subcategorizing stenosis less than 50% and recommended that further stratification should apply only in greater constriction (50%-69%, >70% and occlusion). Furthermore, although the direct proportional relationship between increase of Doppler velocity and degree of stenosis was agreed, grading of the stenosis in deciles was considered inaccurate and operators were urged to characterize the stenosis as below or above a cut-off value.

Recommendations were made regarding the parameters used for diagnosing and grading ICA stenosis, suggesting Peak Systolic Velocity (PSV) in ICA as the main one to be used, in addition to the presence of plaque on gray-scale and/or colour Doppler images. In cases of difficulty in diagnosing because of technical or clinical factors, additional parameters, like internal carotid artery / common carotid artery PSV (ICA/CCA PSV) ratio and ICA End Diastolic Velocity (ICA EDV) were agreed to be used. These ratios helped in normalization of proximal or distal effects in peak systolic velocity within the internal carotid artery, such as from any proximal pathology, in the proximal arteries or left ventricular function, aortic valve disease or from distal vascular disease in the distal carotid artery or cerebral vessels. Specific criteria and diagnostic thresholds were also proposed (Table 3.1).

Degree of stenosis	ICA PSV (cm/sec)	ICA/CCA PSV ratio	ICA EDV (cm/sec)
<50%	<125	<2.0	<40
50-69%	125-230	2.0-4.0	40-100
>70%	>230	>4.0	>100
Near occlusion	Variable or undetectable	Variable	Variable
Total occlusion	Undetectable	N/A	N/A

***Table 3.1**, Society of Radiologists in Ultrasound consensus panel Doppler velocity criteria for diagnosis of internal carotid artery stenosis (Grant et al, 2003)*

It is noteworthy that the consensus conference did not make any recommendations regarding direct measurement of the stenosis in B-mode or reporting compositional or morphological details about the plaque.

3.6 UK practice and new recommendations

Despite the fact that in North America most of the vascular laboratories have complied with these consensus criteria, in Europe and particular in United Kingdom it seems that the variability in thresholds used in velocity criteria remained, increased by the continuing confusion and debate over the measurement method (Walker & Naylor, 2006). Furthermore, new velocity ratios were introduced since then, trying to overcome the limitations of PSV, which is not only depended on local constriction but also on any proximal or distal pathology.

An audit for UK practice in diagnosing stenosis greater than 70% with DUS revealed the considerable variability in diagnostic thresholds among the vascular laboratories in UK as well the variation in the measurement method used (Walker & Naylor,

2006). The survey showed that diagnostic centers used different measuring method, between NASCET and ECST, sometimes even without being aware of which one, and thus different DUS thresholds for determining >70% stenosis were applied. It was also clear that there was a remarkable variety in the velocity criteria used to determine this stenosis, with a prevalence of a PSV threshold of >230 cm/s, which represents a >70% stenosis according to the Radiologists consensus panel, measured in the NASCET method (Fig 3.3).

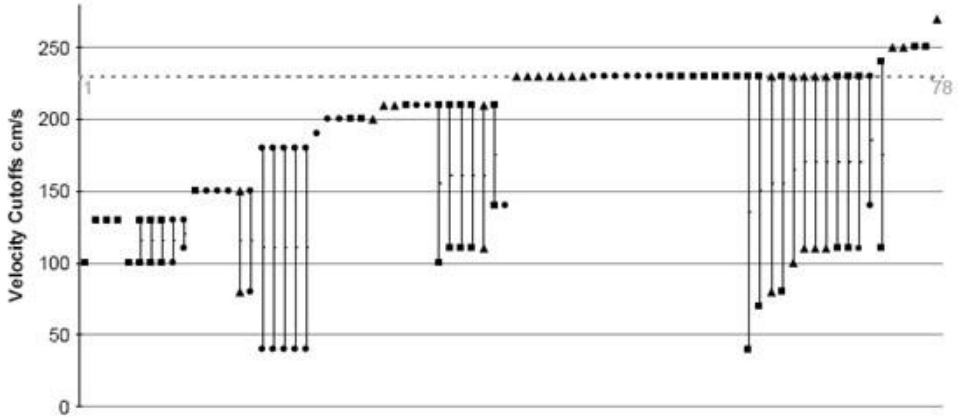


Fig 3.3, *PSV and EDV criteria for diagnosing "stenosis >70%" in the UK; black triangle = NASCET measurement method, black circle = ECST measurement method, black square=do not know (Walker & Naylor, 2006)*

Although methods based on the measurement of blood flow velocities were considered reliable for estimating the degree of constriction of the carotid arteries, universally accepted criteria correlating blood flow velocity with stenosis were still lacking, preventing DUS from being the gold standard in clinical practice and replacing conventional angiography. Meanwhile new imaging modalities like CTA and MRA were rapidly evolving and becoming widely available, while advances in technology of ultrasound further improved B-mode resolution, providing new approaches for assessing the disease.

In 2008 a Joint Working Group was formed between the Vascular Society of Great Britain and Ireland and the Society of Vascular Technology of Great Britain and Ireland for developing new recommendations about ultrasound assessing and reporting carotid atherosclerotic disease (Oates *et al*, 2009).

The group suggested the use of PSV in ICA in conjunction with two ratios, PSVR (PSV in ICA over PSV in CCA) and St. Mary's Ratio (PSV in ICA over EDV in CCA), proposing parameters thresholds for stenosis grading in <50%, 50%-70% and >70% stenosis and classification in deciles using St. Mary's Ratio (Table 3.2). According to the group's recommendation, agreement of at least two of these criteria increases the diagnostic confidence.

Degree of stenosis	**ICA PSV (cm/sec)**	**ICA/CCA PSV ratio**	**St. Mary ratio ICA_{PSV}/CCA_{EDV}**
<50%	<125	<2.0	<8
50-59%	125-230	2.0-4.0	8-10
60-69%	125-230	2.0-4.0	11-13
70-79%	>230	>4.0	14-21
80-89%	>230	>4.0	22-29
>90	>400	>5.0	>30
Near occlusion	Variable	Variable	Variable
Total occlusion	Undetectable	N/A	N/A

***Table 3.2**, Joint Working Group of Vascular Society and Society of Vascular Technology in Great Britain and Ireland velocity criteria for carotid stenosis assessment (Oates et al, 2009)*

As far as the method of measurement is concerned, the group suggested the use of NASCET method, based on the fact that most surgeons in UK are familiar with this

method and is consistent with recommendations in USA and Australia. Furthermore, NASCET method would make comparison with other angiography-like methods, like CTA, easier. However, direct measurement using B-mode was not generally recommended, except for the case of large plaques in carotid bulb with diameter more than 10mm, in which ECST direct measurement method was recommended as NASCET method would falsely underestimate stenosis.

Regarding reporting on plaque compositional and morphological characteristics, the joint group suggested limited qualitative reporting about plaque's exact position, length, morphology and calcification.

3.7 B-mode direct measurement of carotid stenosis

It is apparent that there is no common ground concerning the direct measurement of diameter reduction in B-mode in addition to velocity criteria for a more accurate estimation of the ICA stenosis.

Velocity parameters have important limitations in a number of clinical conditions. Cardiac arrhythmia, aortic valve insufficiency, good collateral circulation, tandem plaques, carotid dilatation or aneurysm may result in underestimation of degree of stenosis. On the other hand, carotid coiling or kink, arteriovenous malformation, carotid-body tumours and contralateral severe stenosis or occlusion may lead to overestimation of stenosis (Zachrisson *et al*, 2001; Krejza *et al*, 2001; Spencer *et al*, 2001). Slight errors in velocity cursor placement or selection of Doppler angle may also result in false measurements and calculations. Furthermore, studies have shown a good linear relationship between actual diameter and area measurements in plaque specimens and direct diameter and area measurements in ultrasound, especially in the transverse section (Jmor *et al*, 1999).

On the other hand, errors in angle determination, tortuous arteries and severe calcification are significant imaging limitations for direct B-mode measuring. Especially the longitudinal view for diameter measurement shows large variations among operators because of the difficulty of reproducing the angle of incidence (Jmor *et al*, 1999).

It has been suggested that there is a strong correlation between B-mode and angiography stenosis measures and that the combination of B-mode stenosis measurement with velocity measurements can provide a more complete estimation of the stenosis and as a result a more accurate diagnosis of the disease (Rotstein *et al*, 2002; MacKenzie *et al*, 2002). However, the accuracy of this suggestion is ambiguous, as other studies have showed that addition of B-mode diameter reduction measurement in DUS did not improve the accuracy of carotid stenosis beyond that from velocity criteria alone, when compared with angiography (Wardlaw & Lewis, 2005). It is noteworthy that the disagreement in results of diameter measurements in DUS and DSA has been attributed to different imaging approach of the stenosis ("best" view of the stenosis on DUS may be different than that on DSA because of technical reasons) and different imaging potentials (DUS also visualizes the vessel wall while DSA only the lumen), which suggests that angiography cannot be a reference for evaluating B-mode measurement (Wardlaw & Lewis, 2005). The issue about the method used for calculating the diameter reduction in ultrasound also remains unclear.

CHAPTER IV

IDENTIFICATION OF THE VULNERABLE PLAQUE USING ULTRASOUND

Chapter IV

Identification of the vulnerable plaque using Ultrasound

A review of the literature

4.1 Looking beyond the size

In the '90s three major multicenter clinical trials, the North American Symptomatic Carotid Endarterectomy Trial (NASCET), the European Carotid Surgery Trial (ECST) and the Asymptomatic Carotid Atherosclerosis Study (ACAS) tried to set the criteria for selection of patients that would be benefited from surgical rather than medical treatment of carotid atherosclerotic disease.

Despite using different methods of assessing carotid stenosis and different patients' categorization, NASCET and ECST studies, which both looked at symptomatic patients, associated stroke risk with the severity of carotid stenosis, suggesting patient selection for endarterectomy based on plaque size. More specifically, they reported an absolute risk reduction of 16% ($p<0.001$) in symptomatic patients with greater than 70% stenosis who were treated surgically after a 5-year follow-up. In patients with mild stenosis (less than 50%) the risk of the surgery itself outweighed its benefits. In the "grey-zone" of moderate disease (50-70% stenosis) the benefit of surgery outweighed the risk, it was considered however more modest, offering a risk reduction of 4-6% (Rothwell *et al*, 2003).

Although NASCET and ECST studies both set the 70% cut-off for endarterectomy patient selection, the data analysis show that classification of carotid atherosclerotic disease based on degree of stenosis enables prediction of one out of four strokes in symptomatic patients and only one out of ten strokes in asymptomatic patients. In the

ECST trial, 43.8% of the 3,018 participants with symptomatic carotid disease had less than 30% stenosis. The 70% cut-off point for surgical benefit for symptomatic patients was later doubted by studies such as the one carried out by Barnett and colleagues. This group reported a significantly lower (p=0.045) 5-year rate of stroke in symptomatic patients with 50% to 70% stenosis that were treated surgically comparing to patients with same degree of stenosis that received medical treatment (Barnett *et al*, 1998). Furthermore, the fact that the benefit from carotid endarterectomy significantly decreased with delayed surgery, whereas it was unlikely that the size of the plaque had changed in the meantime, confirmed that there were other plaque characteristics other than its size or the stenosis they cause that were important in identifying patients at high risk of stroke (Rothwell *et al*, 2004).

In asymptomatic patients, the association of stroke rate with the degree of stenosis was not that clear. Despite the fact that earlier studies suggested slightly greater risk (2-5%) in patients with plaques causing greater than 75% stenosis comparing to less severe disease (risk of stroke 1%) (Autret *et al*, 1987; Meissner *et al*, 1987), in the ACAS study such association was not reported (Endarterectomy for asymptomatic carotid artery stenosis. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995) (Table 4.1). A small stroke risk in severe stenosis in asymptomatic patients was also confirmed by Longstreth et al in 1998. After having scanned 5,441 participants they found a 5% 5-year risk for stroke in patients with greater than 70% stenosis, similar to the stroke risk of patients with moderate stenosis in ACAS study (Longstreth *et al*, 1998). Due to the mixed data, there was much uncertainty about the net benefits of surgical treatment for asymptomatic patients, however, more and more studies tended to encourage surgical treatment in asymptomatic patients with severe disease. The Asymptomatic Carotid Surgery Trial

in UK has reported a significant decrease of the net 5-year stroke risk (6% from 12%) after immediate carotid endarterectomy in asymptomatic patients younger than 75 years old with greater than 70% stenosis comparing to deferral endarterectomy (Halliday *et al*, 2004).

Stenosis	NASCET	ECST	ACAS
60%-69%	13%	11%	6%
70%-79%	21%	9%	5%
80%-89%	27%	21%	NS
90%-99%	35%	32%	NS
80%-99%	31%	24%	3%

***Table 4.1**, Relationship between severity of stenosis and stroke rate (Golledge et al, 2000)*

Recent studies have been looking beyond practice of assessing the diseases strictly based on the size of plaque and have suggested the classification of carotid plaques into two types: a stable plaque that can increase in size and severely decrease the lumen, possibly causing hypoperfusion, but is unlike to rupture and an unstable that, despite possibly being of smaller caliber, is more prone to rupture and is related to a higher risk of embolization and carotid occlusion. This classification is consistent with the fact that the majority of strokes appear to have resulted from embolization from atherosclerotic plaques causing only mild or moderate artery stenosis or acute occlusion and propagation of thrombus distally rather than hypoperfusion due to lumen stenosis (Seeger *et al*, 1995; Golledge *et al*, 2000). It is noteworthy that in the early stages of atherosclerotic plaque formation, the carotid artery wall can remodel so the luminal size is not compromised (Pasterkamp *et al*, 2004). This can lead to

significant plaque growth, likely to rupture and lead to embolization, without significant stenosis of the artery, hence not treated as a high-risk plaque by the traditional, degree-of-stenosis based assessment of the disease.

Early identification of the unstable plaque in patients with less than 70% of stenosis, the common cut-off in patients' selection for revascularization procedures, could improve patient selection and prevent future strokes (Grønholdt *et al*, 2001). Furthermore, screening patients with arterial disease risk factors or patients suffering from cardiovascular disease but asymptomatic for cerebrovascular disease and classification of plaque as unstable or high-risk could potentially lead in better management of the disease, more aggressive medical treatment and possibly surgical treatment for more efficient stroke prevention.

4.2 The vulnerable plaque

Vulnerable or unstable plaque is the atherosclerotic plaque that has a high possibility to cause thrombotic complications such as stroke or a plaque that tends to progress rapidly (Saam *et al*, 2007). Studies have identified certain morphological and compositional characteristics that classify a plaque as vulnerable (Fig 4.1).

Fibrous cap

In studies looking at histological analysis of post-endarterectomy specimens, plaques associated with cerebrovascular symptoms have been found having a much thinner fibrous cap, significantly infiltrated by macrophages and lymphocytes and with decreased smooth muscle cell content (Golledge *et al*, 2000; Devuyst *et al*, 2005). A thin fibrous cap (<650 μm) has been shown to increase the circumferential stress on the plaque and thus make it more prone to rupture and lead to thrombosis and

embolism (Waki *et al*, 2003). As atherosclerosis progresses, foam cell disintegration, smooth muscle cell apoptosis and increased metalloproteinase release by activated leukocyte, result in collagen degradation and fibrous cap thinning (Falk, 2006). T-lymphocytes also play significant role in fibrous cap thinning, by inducing the macrophage metalloproteinase secretion and promoting smooth muscle cell apoptosis via interleukin-1 production.

Lipid-rich core

Lipoproteins play an important role in atherosclerotic plaque formation, accumulating in the subendothelial area in the early stages, following endothelium dysfunction and injury, where they are ingested by macrophages, creating the foam cells. The apoptosis of foam cells leaves extracellular lipid, cholesterol crystals and necrotic debris, which are the main component of the necrotic core of the atheromatic plaque (Virmani *et al*, 2000; Thim *et al*, 2008). Lipoproteins can also contribute to the necrotic core formation by direct accumulation in the lesion without being ingested by macrophages or by the cholesterol-rich erythrocyte membranes in case of intraplaque haemorrhage (Thim *et al*, 2008).

Lipid and cholesterol laden plaques with low levels of collagen have indicated greater instability and higher probability for rupture (Seeger *et al*, 1995). In coronary arteries plaques, the significance of the size of the lipid core has been well established by postmortem and atherectomy studies and several endarterectomy studies have reported increased lipid-rich core (usually more than 25%) in symptomatic patients (Davies *et al*, 1993; Grønholdt *et al*, 2002; Virmani *et al*, 2000). The necrotic core's proximity to the lumen has also been associated with increased risk of ischaemic events, as it makes it easier for the core to be exposed after the fibrous cap thinning,

leading to rupture and creation of thrombus and possibly embolization (Bassiouny *et al*, 1997).

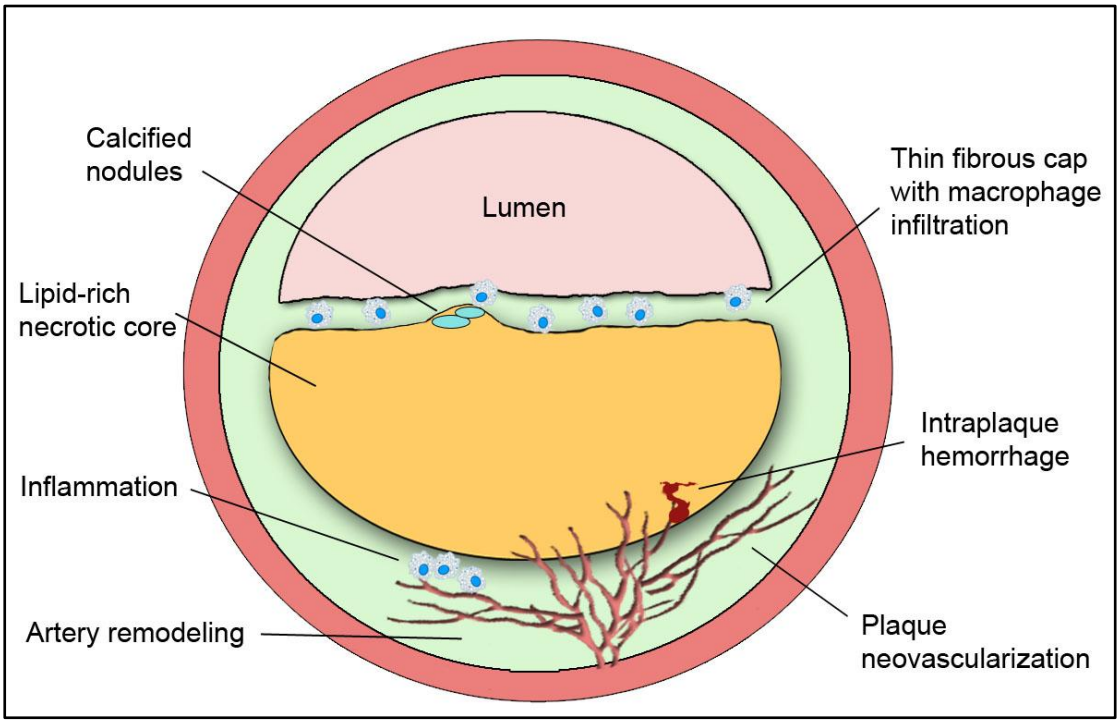


***Fig 4.1**, Characteristics of the vulnerable atherosclerotic plaque*

Intraplaque haemorrhage

Subintimal intraplaque haemorrhage, occurring from cracks or fissures that originate from the luminal surface of the plaque or from the rupture of vasa vasorum, has been suggested to predispose to plaque instability (Bassiouny *et al*, 1997; Alexander-Sefre *et al*, 2003; Saam *et al*, 2007). Haemorrhage is directly connected with plaque neovascularization, as new immature microvessels are more fragile and prone to lead to intraplaque haemorrhage.

Inflammation

Macrophage infiltration of the atherosclerotic plaque has been seen as a feature of vulnerable atherosclerotic plaque for over a decade, predominantly for coronary

arteries. Macrophages cause the release of lytic enzymes such as collagenase, gelatinase and stromelysin which degrade fibrous cap matrix proteins and make it more prone to rupture (Moreno *et al*, 1994). Furthermore, plaque macrophage take up the atherogenic low-density lipoprotein, forming foam cells laden that constitute plaque's core and lead on lesion progression (Kai, 2010). Carotid plaque specimens from patients with symptomatic cerebrovascular disease have been reported indicating significantly greater inflammatory activity than specimens from asymptomatic patients (Spagnoli *et al*, 2004). Leukocyte recruitment depends on the expression of adhesion molecules on the intimal surface as well as the release of soluble factors. It requires interaction between endothelial selectins and leukocyte ligands and it is further promoted by monocyte chemotactic protein and binding of leukocyte CD18 and endothelial adhesion molecules such as ICAM-1 and VCAM-1. Whether leukocytes enter the plaque via the intima or the vasa vasorum of the vessel wall or the new vessels created within the plaque remains unclear. However, studies on coronary plaques have suggested neovessels being a major source of inflammatory factors, reporting high leukocyte adhesion factors' expression in those immature vessels (O'Brien *et al*, 1996).

Plaque neovascularization

Vasa vasorum are small vessels responsible for the nourishment and the draining of the outer components of vessel wall of most muscular and conduit arteries and veins. They vary in size and structure depending on the size and the oxygen need of the vessel they nourish. In large arteries and veins, or in cases of atherosclerotic arteries, the vasa vasorum extend into the outer layer of the media (Williams & Heistad, 1996). In non diseased arteries the intima is nourished by diffusion from the lumen.

However, as atherosclerotic disease progresses and the intima becomes thicker, oxygen diffusion is impaired and vasa vasorum become the main source of nourishment for the whole vessel wall. Neovascularization is the creation of new blood vessels resulting in the formation of a neovascular network particularly in atherosclerosis of capillaries (Fig 4.2). Angiogenesis occurs when intima thickens more than 500 μm, mainly because of the hypoxic state caused by the atherosclerotic lesion which triggers the production of angiogenic factors like vascular endothelial growth factor (VEGF). Apart from hypoxia, hypertension and hypercholesterolemia have also been characterized as angiogenic stimuli (Langheinrich *et al*, 2007).

***Fig 4.2**, Photomicrograph display of CD31-stained tissue samples. Red arrows show plaque microvessels (adapted by Shah et al, 2007)*

Atherosclerotic plaque neovessels, alternatively named as vasa plaquorum in recent literature, originate predominantly from the adventitial vasa vasorum and secondarily (< 5%) from vessel lumen (Galis & Lessner, 2009). Neovasculature created by spouting growth of adventitial vasa vasorum has been correlated with increased

inflammation, great lipid core and severe stenosis, whereas lumen originated neovascularization has been correlated with smaller plaques and intraplaque haemorrhage (Moreno *et al*, 2006).

Carotid plaque neovascularization has been associated with plaque vulnerability. In a study in 1999 by McCarthy and colleagues, the histological analysis of plaques taken from symptomatic and asymptomatic patients showed significantly higher neovessel density for plaques from symptomatic patients (4 vessels/mm^2) compared with those from asymptomatic (0.7 vessels/mm^2, $p<0.0004$). Apart from the number of plaque microvessels, their morphology also differs in symptomatic and asymptomatic patients. Symptomatic lesions have been associated with larger, irregular and immature (lacking smooth cell coating) neovessels comparing to asymptomatic plaques neovasculature. Adjacent to microvessels macrophage infiltration and high VEGF expression have also been correlated with symptomatic patients (Dunmore *et al*, 2007). Furthermore, the number of new vessels within the plaque fibrous cap has been reported significantly larger in symptomatic lesions comparing with asymptomatic (McCarthy *et al*, 1999).

Artery remodeling

Remodeling of coronary arteries in the site of an atherosclerotic plaque, namely compensatory enlargement that permits growth of the plaque without reduction of luminal area, has been associated with plaque's likelihood to rupture and thus plaque vulnerability. The artery remodeling has been associated with higher lipid content and macrophage count in the plaque, both markers of plaque vulnerability as has been mentioned (Varnava *et al*, 2002). Carotid artery remodeling is commonly seen in sites of atherosclerotic plaque creation.

Focal calcification

Atherosclerotic plaques with large calcific deposits are considered stable and unlikely to rupture. A recent systematic review of studies looking at characteristics of vulnerable plaque concluded that clinically symptomatic carotid plaques had a lower degree calcification volume, weight or percentage (Kwee, 2010). However, it should be noted that studies on coronary atherosclerotic plaques have suggested that calcified nodules near the fibrous cap that protrude into the lumen can result in plaque rupture and therefore focal calcification should be seen as a minor criterion for plaque vulnerability (Schaar *et al*, 2004; Falk, 2006; Saam *et al*, 2007).

Shear stress

The fact that atherosclerotic plaques are not evenly distributed in the arterial system, but are formed in areas such as inner curvatures of non-branching arteries or opposite to the flow divider of bifurcations, has lead to the association of atherosclerosis occurrence with local haemodynamic factors and particularly wall shear stress. Shear stress is the drag force arising from the friction between two layers in a fluid, induced by the difference in movement of the two layers and the fluid's viscosity, but it also arises at the interplay between blood and endothelial layer (Helderman *et al*, 2007). Observational studies in human and animals have shown that regions in the arterial network with low (< 1.5 N/m^2) or oscillating shear stress are more susceptible to atherosclerosis compared to regions with normal or high shear stress, which seems to act atheroprotectively to artery wall. Low shear stress has been associated with many plaque vulnerability characteristics, such as greater lipid cores, fewer smooth muscle cells and collagen in lesion cap, intraplaque haemorrhage and outward remodelling. It has been suggested that shear stress alters the inflammatory gene expression of

endothelial cells and increases the permeability of endothelium to lipoproteins and macrophages. Studies have confirmed increased expression of VCAM-1 and other atherosclerosis markers such as IL-6 and CRP in lowered shear stress regions (Cheng *et al*, 2006; Li *et al*, 2008).

4.3 Assessment of plaque composition using Duplex Ultrasound

In the DUS imaging an atheromatic plaque can be classified according to certain parameters, such as echogenicity (echolucent or echogenic), texture (homogeneous and heterogeneous) and surface (regular, ulceration) (Hennerici *et al*, 1998). There have been efforts over the years for correlation of these sonographic characteristics with the actual composition of the plaque in a way that duplex ultrasound would provide compositional information for better vulnerable plaque identification.

Plaque echogenicity

Plaque echogenicity (i.e. how "bright" or "dark" a plaque appears in the ultrasound) is the most commonly used imaging characteristic of the carotid plaque since Duplex Ultrasound began to be used for the assessment of plaque composition. In 1988, Gray-Weale classification of carotid plaques according to their echogenicity was established and has widely been used for plaque description (Gray-Weale *et al*, 1988). According to that classification, carotid plaques were divided into four groups; echolucent, mainly echolucent, mainly echogenic and echogenic. A great number of studies have since then looked at the clinical manifestation of carotid atherosclerotic disease with plaques of different echogenicity. Most of them have reported significantly greater relevant risk for cerebrovascular symptoms in patients with

echolucent plaques, regardless of the degree of the stenosis caused by the plaque (Polak *et al*, 1998; Mathiesen *et al*, 2001). It has been suggested that plaque echolucency should be considered as an independent risk factor for ischaemic stroke, regardless of other cardiovascular risk factors (Grønholdt *et al*, 2001). However, in asymptomatic patients the association of plaque echogenicity and risk for stroke has been reported to be less significant (Halliday *et al*, 2004).

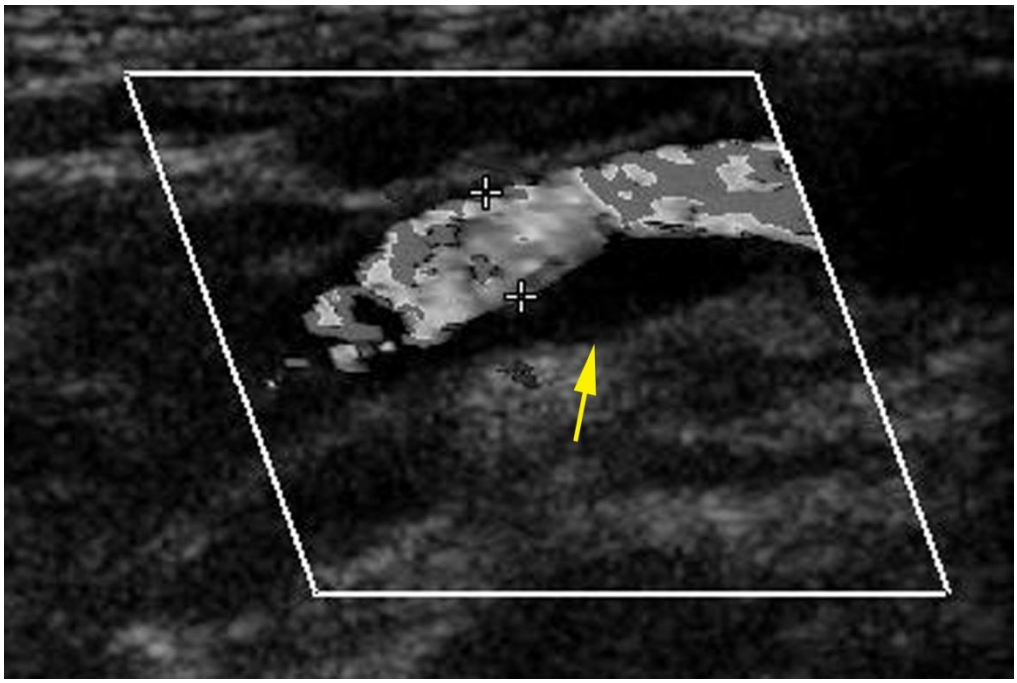


***Fig 4.3**, Echolucent carotid plaque (arrow)*

Histological analysis of plaque specimens have reported that echolucent plaques contain more soft tissue (lipid and haemorrhage) while echogenic are primarily composed of fibrous tissue and calcification (Grønholdt *et al*, 1997). However, the poor reproducibility of subjective echogenic classification among studies, possibly because of the inter-observer variability of the methods, the high operator dependence and the limited soft tissue contrast, has led to conflicts over the accuracy of ultrasound in plaque composition assessment (Montauban van Swijndregt *et al*, 1998; Saam *et al*, 2007). This subjectivity obstacle was partially overcome by development of methods

that normalized ultrasound images and objectively characterized the plaque, like the grey scale median method (GSM) (Elatrozy *et al*, 1998b). Since the introduction of the grey scale median method as an objective computerized way for the characterization of plaque echogenicity, studies began correlating symptomatology and histology with this value in an effort to establish a cut-off point that would identify vulnerable plaque.

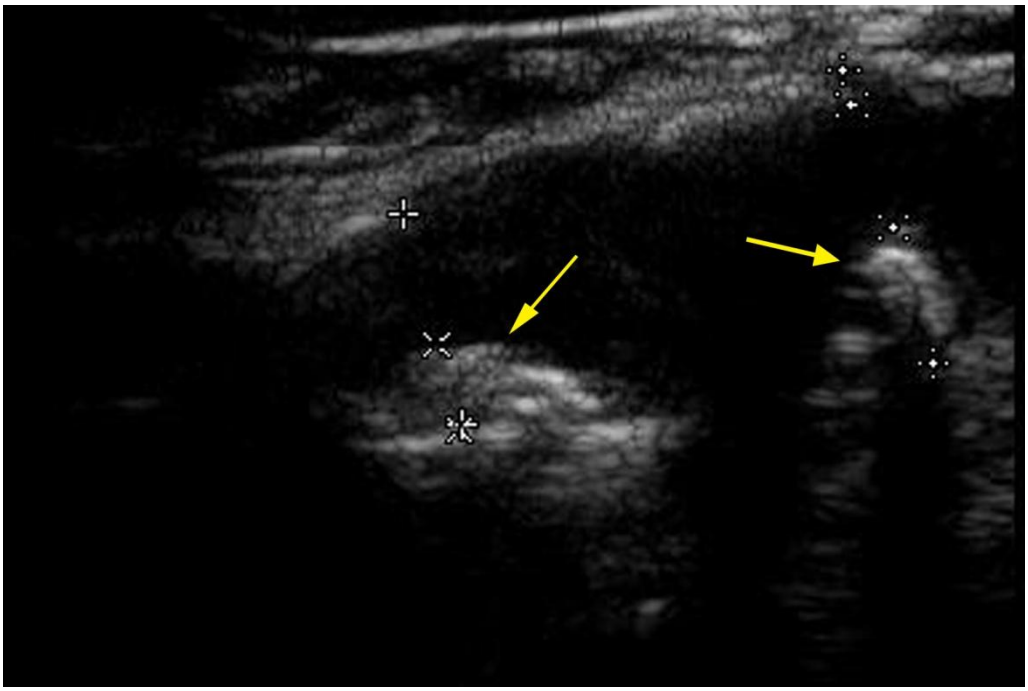


***Fig 4.4**, Two echogenic plaques (arrows)*

Grey scale median

High correlation was reported by numerous studies between low GSM value (lower than 32, a cut-off many studies have used) and symptomatology, in many cases confirmed with cerebral infarction identification in CT scans (el-Barghouty *et al*, 1996; Falkowski *et al*, 2004; Wijeyaratne *et al*, 2003; Grogan *et al*, 2005). The relative risk for ischaemic stroke was reported to be higher for low GSM comparing to degree of stenosis (Grønholdt *et al*, 2001). With time, more sophisticated methods

for computerized analysis of plaque echogenicity were developed, calculating the grey scale median for small regions of interest on carotid plaques with similar results regarding the relationship of GSM value and symptomatology (Sztajzel *et al*, 2005). Another grey scale image parameter that has been investigated, the percentage of pixels with gray scale value less than 40 (P_{40}), has also been found to be significant for distinguishing symptomatic and asymptomatic plaques (Pedro *et al*, 2000).

GSM value was also calculated in 3D duplex ultrasound images, showing good correlation with symptomatology for plaques causing less than 70% stenosis (Heliopoulos *et al*, 2008).

Regarding the ability of ultrasound to distinguish tissue components of atherosclerotic plaque using computerized analysis of plaque echogenicity, results were mixed for different tissues. Initial results showed only little agreement between plaque GSM and histology findings, however further studies associated plaque low echogenicity with intraplaque haemorrhage and high echogenicity with calcification, (Aly & Bishop, 2000; Denzel *et al*, 2003; Tegos *et al*, 2000). New techniques, using stratified grayscale measurement or pixel distribution analysis have been suggested good correlation with both symptomatology and histology (Lal *et al*, 2006; Sztajzel *et al*, 2005). Until today, there is no consensus about specific GSM threshold for vulnerable plaque identification or other method for assessing plaque vulnerability based on echogenicity.

Plaque homogeneity

Heterogeneous plaques have been associated with higher incidence of symptoms than homogeneous plaques, regardless the severity of stenosis (AbuRahma *et al*, 2002). Visual characterization of plaque homogeneity as well as characterization based on

computerized methods for assessing grey scale images has shown prevalence of heterogeneous plaques in symptomatic patients (el-Barghouty *et al*, 1996; Wijeyaratne *et al*, 2003). Among the heterogeneous plaques the echolucent area is usually juxtaluminal in symptomatic patients and basal in asymptomatic patients, whereas in homogeneous plaques the echogenic cap has been well correlated with asymptomatic patients and negative CT while plaque surface disruption has been related with symptomatic patients and positive CT results (Pedro *et al*, 2000).

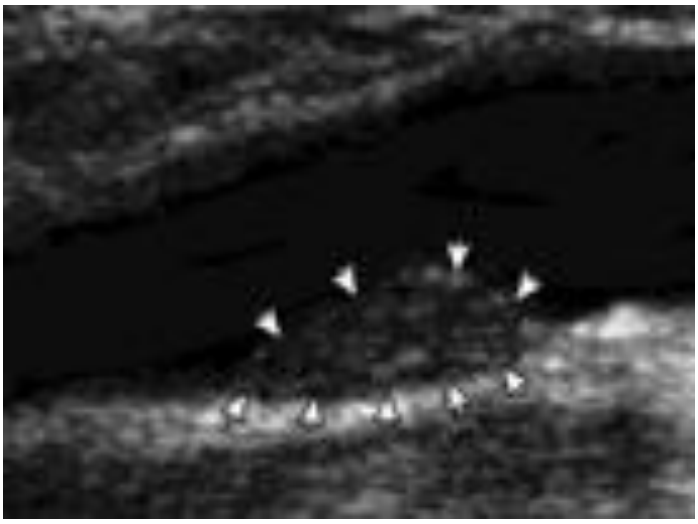


***Fig 4.5**, An homogeneous carotid plaque with regular surface*

Plaque irregularity

Plaque irregularity has been shown to be another risk factor for cerebrovascular symptomatology and particularly ischaemic stroke (Kwee *et al*, 2008). In 2006, Prabhakaran and colleagues assessed with ultrasound more than a thousand carotid plaques, and after following up the patients for more than 6 years they reported nearly threefold stroke risk in patients with irregular plaques (hazard ration 2.7) (Prabhakaran *et al*, 2006). A similar study in Japan in elderly people showed high correlation between plaque irregularity and risk for ischaemic stroke (Kitamura *et al*, 2004).

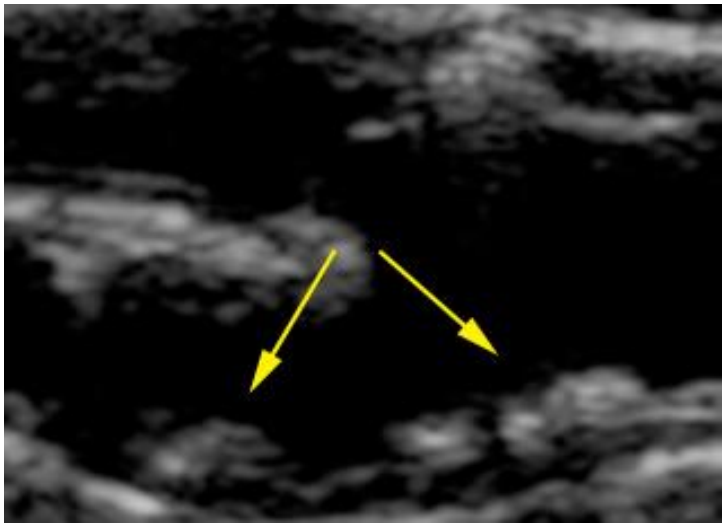


***Fig 4.6**, An irregular, heterogeneous carotid plaque*

4.4 Fibrous cap thickness

Fibrous cap thinning has been suggested to increase the circumferential stress of fibrous cap and thus make the plaque more prone to rupture. There is ongoing research on ultrasound methods for fibrous cap thickening measurements but initial results using both B-mode ultrasound and integrated ultrasound backscatter (IBS) analysis show good correlation with histological analysis (Waki *et al*, 2003; Devuyst *et al*, 2005). However, the axial resolution which ranges between 200 to 600 μm for most ultrasound instruments is an important limitation for measurements of such small calibre.

4.5 Intima-media thickness

Carotid intima-media thickness (IMT) is considered a significant marker of atherosclerotic burden and, despite not being a characteristic of the plaque per se, it provides useful information about patient risk stratification. Carotid IMT has been associated with cerebrovascular symptomatology and hence plaque vulnerability. The

Rotterdam study, in which 6,913 participants previously asymptomatic of cerebrovascular disease were followed-up for more than 6 years, reported strong relationship between ultrasound measured IMT and risk of stroke, independently of other cardiovascular factors (Hollander *et al*, 2003). Several follow-up studies have reached similar results concerning IMT importance (Kitamura *et al*, 2004).

4.6 Plaque angiogenesis

Since the establishment of atherosclerotic plaque neovascularization as a feature of vulnerable and symptomatic plaque there have been efforts to visualize and quantify plaque angiogenesis. Ultrasound, particularly contrast enhanced ultrasound, as well as magnetic resonance imaging, has been used for assessing plaque angiogenesis.

Carotid B-mode ultrasound has predominantly been used for plaque identification and carotid stenosis and intima-media thickness measurement. For plaque angiogenesis identification and quantification, contrast enhanced ultrasound (CEUS) has been suggested showing good results compared to plaque histology analysis and patient symptomatology. Acoustically reflective microbubbles have been used as contrast agents for enhancement of carotid lumen and plaque morphology, improvement of IMT measurement and identification of adventitial vasa vasorum and plaque neovasculature (Feinstein, 2006). A variety of contrast agents have been used for neovascularization imaging, including perflutren protein type-A microspheres (Optison, GE Healthcare, Little Chalfont, Buckinghamshire, UK), perflutren lipid microspheres (Definity, Bristol-Myers Squibb Medical Imaging, Billerica, Massachusetts), and phospholipidstabilized microspheres of sulfur hexafluoride (SonoVue, Bracco Altana Pharma, Konstanz, Germany) (Staub *et al*, 2010). For comparison with histology, microvessels in post-endarterectomy carotid plaque

specimens are identified using several immunohistochemical markers, like CD31, CD34 antibody and VEGF antibodies (Shah *et al*, 2007).

Histological analysis of post-endarterectomy plaque specimens has showed significantly greater neovasculature in plaques that were enhanced during CEUS examination comparing to those that were not (Coli *et al*, 2008). Visual classification of plaque contrast enhancement (none, limited, moderated and significant) has showed agreement with the extent of plaque neovascularization (Shah *et al*, 2007).

There is ongoing research on methods that would objectively quantify contrast enhancement for plaque neovascularization quantification. B-flow imaging technique, a method that enhances signal coming from moving ultrasound reflectors and visualizes blood flow with high frame rate and resolution, has been used to objectively quantify contrast enhancement by the measurement of B-flow image thickness (BFI-T). Although BFI-T has been found to be significantly higher in patients with atherosclerotic disease, no histological validation for neovascularization has been reported yet (Magnoni *et al*, 2009).

Another suggested method uses time-signal intensity curve analysis software for calculation of the curve of the ultrasound energy reflected by tissue and microbubbles over time. Plaque enhanced intensity was calculated by subtracting the baseline plaque intensity (i.e. before injection of contrast agent) from the plaque peak intensity during contrast enhancement. The ratio of plaque enhanced intensity over the lumen enhanced intensity was also calculated. Results indicated significantly greater contrast enhancement for symptomatic patients, but no comparison with histological data was performed for correlation with angiogenesis (Xiong *et al*, 2009). Another group suggested a similar enhancement quantification method for the calculation of the maximum level of plaque dB-Enhanced at the peak contrast agent's concentration and

reported significantly higher dB-Enhancement in plaques with greater neovessel density, as detected using CD34 immunostaining (Faggioli *et al*, 2011).

As far as the standard ultrasound ability to assess carotid plaque neovascularization is concerned, in a study very recently published, Feinstein's group in Chicago compared standard duplex ultrasound plaque assessment for echogenicity and degree of stenosis with neovascularization identified using CEUS. Results indicate inverse correlation for plaque echogenicity and good correlation for degree of stenosis with plaque neovascularization. No histological confirmation of neovascularization was performed as angiogenesis was decided solely upon CEUS examination (Staub *et al*, 2011).

4.7 Plaque inflammation

Lately there has been increasing interest in establishing techniques for imaging atherosclerotic plaque inflammation. Contrast enhanced ultrasound is one of the modalities that have showed satisfying results, along with magnetic resonance imaging, positron emission tomography and thermography.

In vitro studies have shown that microbubble contrast agents are phagocytosed by macrophages and remain active, reflecting ultrasound beam for up to 30 minutes later. In vivo studies have also shown sufficient detection of microbubbles in carotid plaques 6 minutes after injection, reporting significant difference in image enhancement between symptomatic and asymptomatic patients (Owen *et al*, 2010). Despite the fact that the mechanism of microbubble adhesion to the atherosclerotic plaque as well as whether it is inflammation-specific with histological validation remains unclear, contrast enhanced ultrasound is a promising modality on plaque inflammation assessment.

CHAPTER V

CAROTID PLAQUE SIZE ASSESSMENT WITH ULTRASOUND USING B-MODE MEASUREMENT METHODS

Chapter V

Carotid plaque size assessment with Ultrasound using B-mode measurement methods

Introduction

Since the introduction of duplex ultrasound in clinical practice for the assessment of carotid atherosclerotic disease, velocity criteria have been used to calculate the degree of the stenosis. However, the variety of velocity criteria used nowadays in vascular laboratories across the world as well as the fact that velocity measurement remains an indirect way to estimate stenosis based on its haemodynamic effects, has urged societies of vascular scientists to come up with a universal method of accurate calculation of carotid stenosis using duplex ultrasound.

A method which has been used in a few vascular laboratories alongside the velocity parameters as well as in several studies looking on carotid stenosis is the direct measurement of the stenosis in internal carotid artery. However, there is no consensus yet regarding direct measurement methods accuracy or which among the suggested methods should be used when appropriate.

Using current velocity criteria employed in most vascular laboratories in UK for carotid atherosclerotic assessment with duplex ultrasound as reference, methods of direct diameter and area measurement applied in ultrasound were investigated for agreement and correlation for carotid stenosis calculation. Their accuracy on stenosis classification in categories was also investigated in reference to current velocity criteria. Lastly, magnetic resonance angiography stenosis measurement was compared with direct measurement ultrasound measurements.

Method and materials

5.1 Selection of patients

Patients presenting at the vascular clinic, at Hammersmith Hospital, over a period of 12 months, from February 2008 to February 2009, had a duplex ultrasound scan with the investigation protocol for assessing carotid artery disease. The indications for carotid investigation were either symptomatic carotid disease, such as prior TIA or stroke, or asymptomatic patients with peripheral arterial disease or coronary artery disease. The study was approved from the local research ethics committee.

5.2 Patient assessment

Patients were interviewed before the scan and a full patient history was taken, including:

a. Current medical condition

b. Previous vascular or cardiovascular surgeries

c. Current medication

d. Presence of any risk factors for cerebrovascular disease described in chapter II (smoking, hypertension, diabetes, peripheral vascular disease, coronary artery disease, family history of arterial disease)

e. Presence of any symptoms for cerebrovascular disease described in chapter II (aphasia, dysphasia, visual disturbances, vertigo, carotid bruits, dysarthria, weakness or paralysis of extremities)

Furthermore laboratory results were gathered from patient notes, mainly about risk factors associated with atherosclerosis, including levels of:

a. Cholesterol

b. Lipoproteins (HDL, LDL)

c. Triglyceride

d. Hs-CRP

e. Fibrinogen

f. Homocysteine

5.3 Duplex Ultrasound investigation

5.3.1 Instrumentation

For the carotid scanning an HDI ATL 5000 scanner was used, with a linear transducer of 5–12 MHz. All images were stored on CD and then transferred and processed in an in-lab PC. For diameter and area measurements the software ImageJ (National Institute of Health, Maryland, USA), a public domain Java-based image processing and analysis software, was used.

5.3.2 Investigation protocol

Patients were examined lying supine and carotid arteries were scanned both longitudinally and transversely. After the identification of basic landmarks, like the common carotid artery (CCA), the carotid bifurcation, the bulb and the internal carotid artery (ICA), any atherosclerosis or other pathology present was noted.

Maximum peak systolic (PSV) and end diastolic velocities (EDV) in the CCA were measured, as well as the intima-media thickness, preferably 20mm below the bifurcation (Fig 3.1, point A). In the ICA and ECA the diameter of the vessel (wall-to-wall) and the minimum residual lumen perpendicular to the longitudinal axis of the vessel at the position of maximum constriction, the maximum peak systolic and end diastolic velocities in that point, as well as the arterial cross-sectional area of the vessel and the residual lumen in the transverse axis were measured (Fig 5.1, point B and C). Peak systolic velocity and end diastolic velocity, lumen diameter and area in a non-diseased part of ICA distally to the stenosis were also measured (Fig 5.1, point D). For all the Doppler investigations and measurements of the flow small sample gate at centre steam position was used, in the longitudinal axis of the vessel, at an angle preferably at 60^{o} or less.

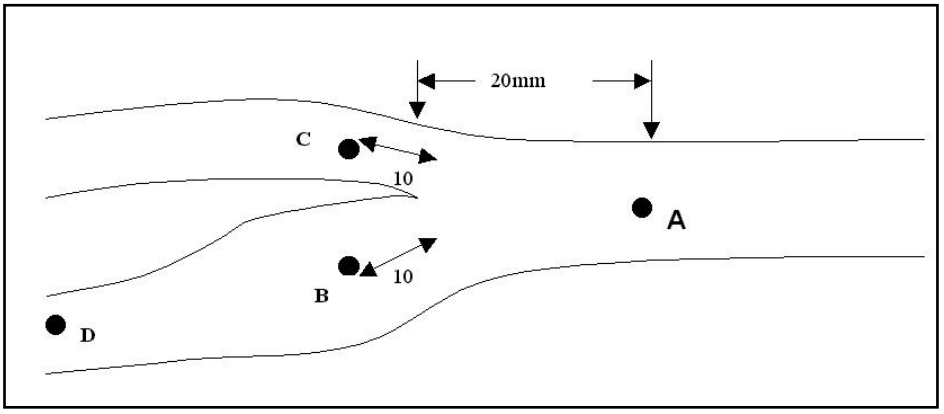


***Fig 5.1**, Points of measuring velocity and B-mode data*

5.4 Data processing and analysis

5.4.1 Haemodynamic parameters

Flow velocity parameters were calculated, including ICA peak systolic velocity, ICA end diastolic velocity, peak systolic velocity ratio (PSVR, maximum peak systolic

velocity within constriction in internal carotid artery over maximum peak systolic velocity in common carotid artery measured at set distance from bifurcation), peak systolic velocity ratio ic/icd ($PSV_{ICD}R$, maximum peak systolic velocity within constriction in internal carotid artery over maximum peak systolic velocity in distal internal carotid artery) and St. Mary's ratio (StM R, maximum peak systolic velocity within constriction in internal carotid artery over end diastolic velocity in common carotid artery) (Table 5.1).

PSV R	Peak systolic velocity ratio ICA/CCA	PSVic/PSVcc
$PSV_{ICD}R$	Peak systolic velocity ratio ICA/ICA distally)	PSVic/PSVicd
StM R	Peak systolic velocity ICA/End diastolic velocity CCA	PSVic/EDVcc

***Table 5.1**, Velocity parameters calculated. PSVic: PSV within constriction in ICA, PSVcc: PSV in CCA, PSVicd: PSV in distal ICA, EDVcc: EDV in CCA*

5.4.2 B-mode parameters

Using diameter and area measurements in different points of CCA and ICA, diameter reduction was calculated using NASCET, ECST and CCA methods (Table 5.2, Fig. 5.2). Area reduction at the point of maximum constriction in ICA was also calculated.

% DST-C	Diameter reduction comparing diameter in CCA	(Dcc-Dmr)/Dcc x 100
% DST-N	Diameter reduction with NASCET method	(Dicd-Dmr)/Dicd x 100
% DST-E	Diameter reduction with ECST method	(Dv-Dmr)/Dv x 100
% AST	Area reduction	(Av-Amr)/Av x 100

***Table 5.2**, B-mode parameters calculated. Dcc: diameter in CCA, Dmr: diameter of minimum residual lumen, Dv: diameter of vessel, Dicd: diameter of lumen in distal ICA, Av: area of vessel, Amr: area of minimum residual lumen*

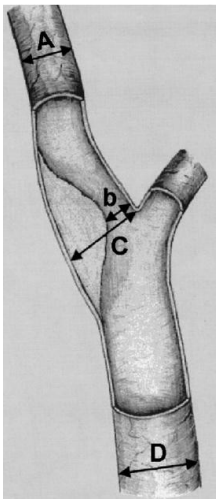


***Fig 5.2**, Schematic drawing depicting NASCET, ECST and CC methods for angiographic assessment of carotid stenosis (Staikov et al., 2002). DST-N: (A-b)/A x100; DST-E: (C-b)/C x 100; DST-C: (D-b)/D x 100*

5.4.3 Statistic analysis

The data were processed and analysed using Microsoft Excel (Microsoft, Washington, USA) and the statistical software SPSS v19 (IBM, New York, USA). The level of significance was set at $p < 0.05$. Correlations coefficients were used to compare different method's results. Also t-tests and nonparametric tests were obtained for comparison tables.

Results

5.5 Descriptive statistics

In total 87 patients were scanned and images were taken from both carotids of each patient. Therefore 174 cases were analyzed.

The mean age of patients was 70.9 ± 9.3 years. Sixty six (75.9%) patients were men (mean age 70.6 ± 8.6 years) and 21 (24.1%) were women (mean age 71.6 ± 11.3 years). Forty five (54.7%) patients were current or past smokers, 65 (74%) suffered from hypertension and were treated with medication, 35 (30%) were diabetic, 51 (59%) had hypercholesterolemia, 47 (54%) had family history of cardiovascular disease, 13 (15%) had history of ischaemic heart disease and 10 (11%) had history of atrial fibrillation. The mean body mass index was 26.2 ± 4.3 for men and 28.7 ± 7.8 for women.

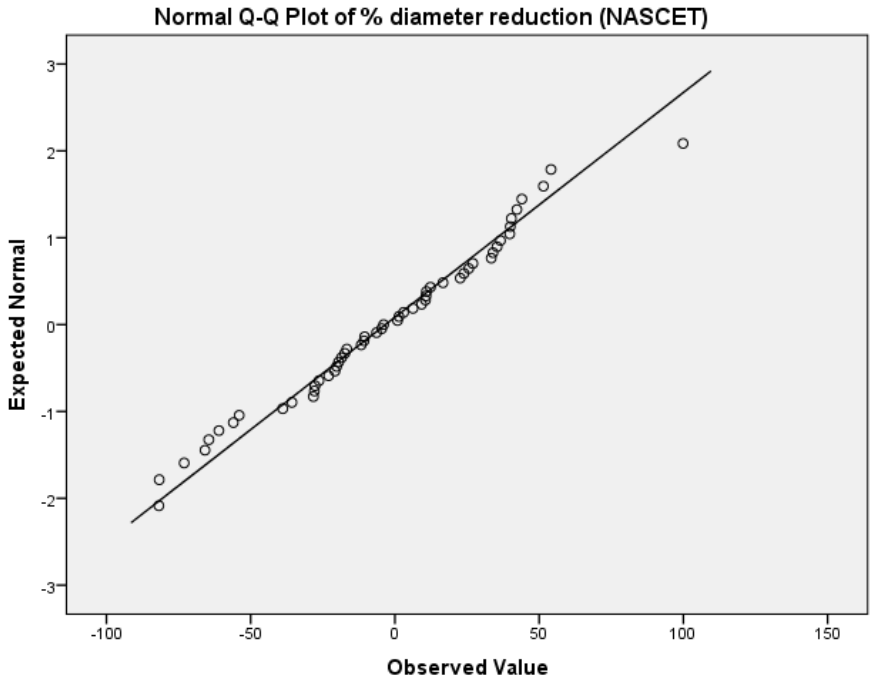
Among the enrolled patients, 52 (60%) had suffered an ischaemic stroke, 19 (22%) had suffered a transient ischaemic attack and 16 (18%) reported other cardiovascular but not cerebrovascular symptoms in the past.

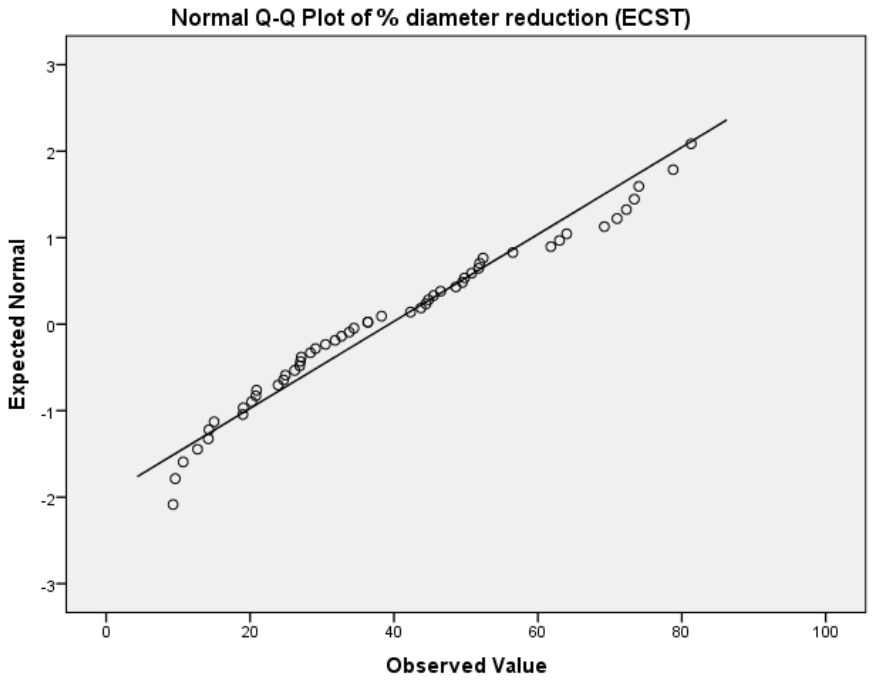
The mean internal carotid artery stenosis measured in B-mode using ECST method was 39.2% ± 19.2 and the mean internal carotid artery area stenosis was 39.6% ± 21.7.

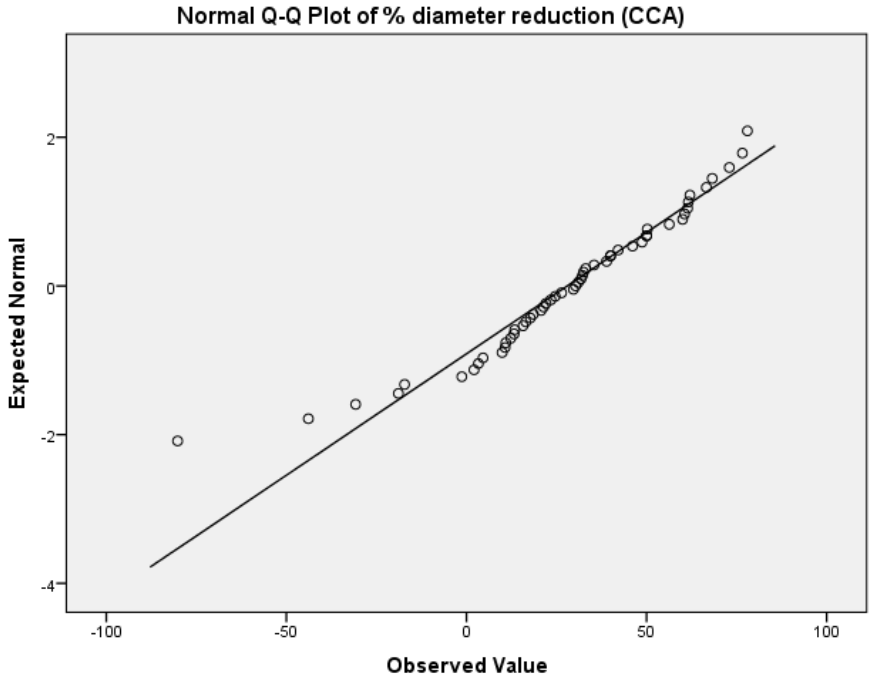
5.6 Statistic analysis

5.6.1 Normal distribution

Velocity and B-mode parameters were tested for normal distribution using Kolmogorov-Smirnov test for the whole sample. Diameter stenosis parameters using NASCET, ECST and CCA method were found to be normally distributed, while the area stenosis and the velocity parameters did not show normal distribution. Below the Q-Q plots for the B-mode diameter and area stenosis parameters are sited (Fig 5.3).




Normal Q-Q Plot of % diameter reduction (ECST)
Expected Normal
Observed Value
3
2
1
0
-1
-2
-3
0
20
40
60
80
100


Normal Q-Q Plot of % diameter reduction (CCA)
Expected Normal
Observed Value
2
0
-2
-4
-100
-50
0
50
100

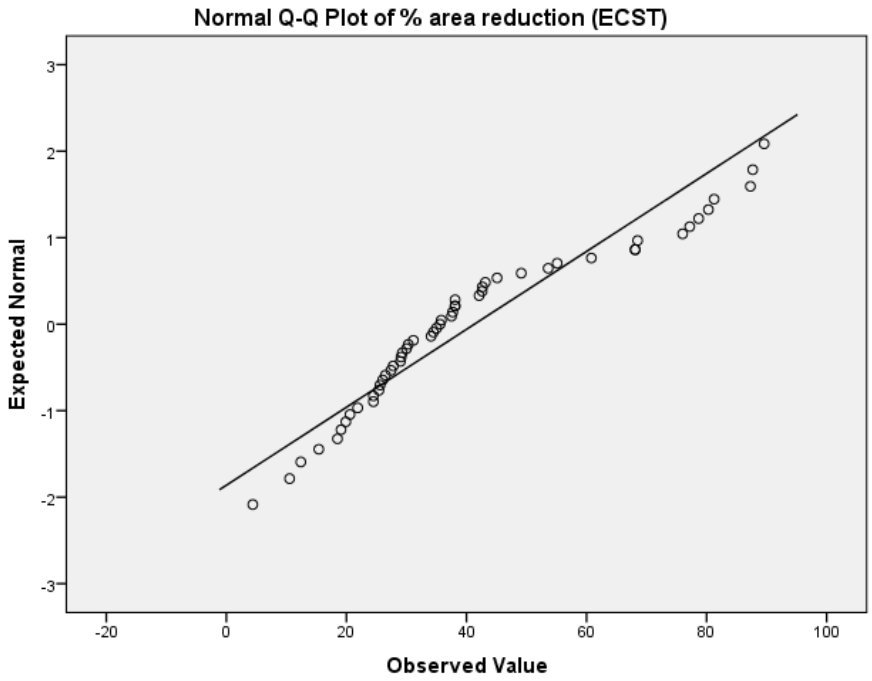


***Fig 5.3**, Normal Q-Q Plots of area stenosis and diameter stenosis using NASCET, ECST and CCA methods of direct measuring in duplex ultrasound*

5.6.2 Direct diameter and area measurements

5.6.2.1 Correlation with velocity parameters

Spearman's rank correlation coefficient was used for investigating the correlation between PSV in internal carotid artery and direct diameter and area measurement using the methods previously described. Test results indicated good correlation (p value <0.01) for all direct measurement methods, among which the ECST method of diameter measurement showed the highest correlation (correlation coefficient .645, p<0.01) (Fig 5.4).

The good correlation between systolic velocity and direct stenosis measurement is explained by the principle of continuity of incompressible fluid motion. According to this principle a fluid's volume flow rate (i.e. the volume of fluid that passes through a

tube per second) is constant and thus fluid velocity is higher in points of tube with smaller cross-sectional area. However, it is noteworthy that the ECST method of diameter measurement appears to express more accurately this flow velocity and vessel diameter correlation.

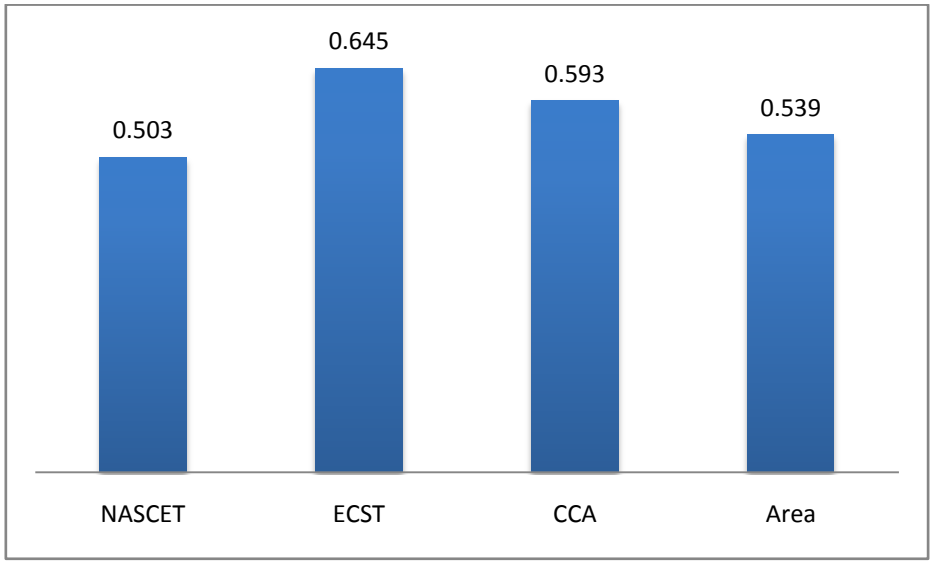


***Fig 5.4**, Spearman's correlation coefficient between different methods for direct diameter and area measurement in duplex ultrasound and PSV at internal carotid artery*

5.6.2.2 Classification of stenosis in three categories

Vascular scientists usually stratify carotid atherosclerotic disease in categories for various degrees of stenosis. As mentioned previously, several velocity criteria are used to categorize carotid stenosis and this variation has contributed to the confusion there is among vascular laboratories and surgeons. The most commonly used velocity criteria across UK are Grant et al criteria, named also Consensus of Radiology velocity criteria, which were introduced in 2003. Using these criteria, PSV is mainly used to grade carotid stenosis in three categories, less than 50%, between 50% and 69% and greater than 70%. Using the consensus criteria for carotid stenosis

classification, direct measurement methods (diameter and area) were investigated for agreement with the established PSV method.

Spearman's rho was used to investigate the relationship and correlation between stenosis classification results using PSV and Grant et al criteria and direct measurement methods. Good correlation was reported for all three methods of diameter measurement and the area measurement method (p value <0.05). The correlation coefficient was notable greater for area measurement and ECST method of diameter measurement. Similar results were produced when the classification using direct measurement methods was compared with classification using the Peek Systolic Velocity Ratio (PSVR) method of Grant et al criteria. The following graph shows the correlation coefficients of all four methods comparing with PSV and PSVR Grant et al criteria (Fig 5.5).

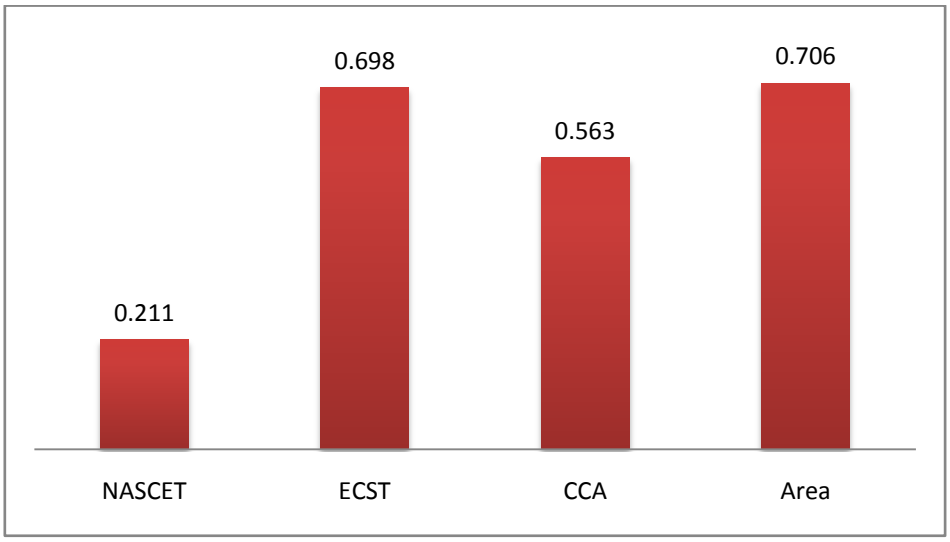


***Fig 5.5**, Spearman's correlation coefficient between classification of stenosis in three categories (<50%, 50-69%, >70%) using different methods for direct diameter and area measurement in duplex ultrasound and Grant et al criteria*

Chi test were used to investigate the significance of relationship between the stenosis classification calculated using direct diameter and area measurement methods and the classification derived by the application of Grant et al velocity criteria. Results

indicated significant relationship between classification calculated by PSV criteria and direct stenosis measurement classification, reporting a greater Pearson chi square value for ECST method and a lower for NASCET method (Table 5.3).

Method	Value	Sig. (p value)
NASCET diameter	14258	0.007
ECST diameter	84296	0.000
CCA diameter	60661	0.000
Area	62246	0.000

***Table 5.3**, Chi square test results for classification of stenosis in three categories using PSV Grant et al criteria and direct diameter and area methods*

Sign Test reported no significant difference between overestimation and underestimation of grading in ECST, CCA and area methods comparing with PSV Grant et al criteria, whereas NASCET criteria underestimated 28% of the investigated cases. Agreement with PSV criteria was higher for area measurement and lower for NASCET diameter method (Table 5.4, Fig 5.6).

Method	Percentage of cases
NASCET	70.1
ECST	77.0
CCA	74.6
Area	80.4

***Table 5.4**, Percentage of cases classified in the same category using direct diameter and area measurement methods and Grant et al velocity criteria*

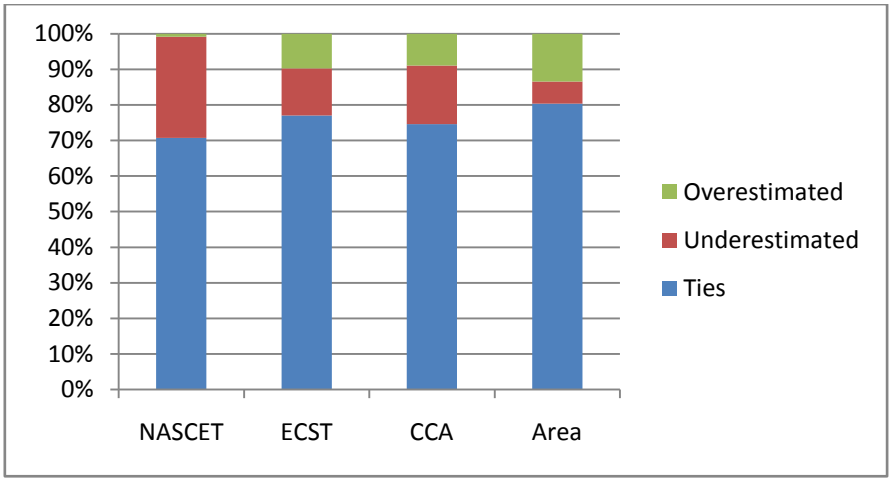


***Fig 5.6**, Percentage of cases tied, overestimated and underestimated using direct diameter and area methods comparing with classification of the stenosis using PSVR Grant et al criteria*

The agreement of stenosis classification using direct diameter and area measurement methods with the classification using Grant et al velocity criteria was investigated using Gamma test. NASCET method showed poorer agreement with Grant et al classification, whereas ECST and area measurement methods showed greater agreement (gamma coefficient was 0.908 and 0.927 respectively) (Fig 5.7).

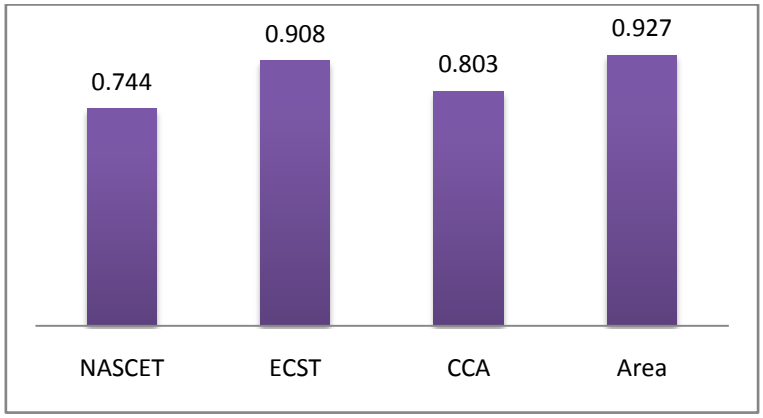


***Fig 5.7**, Gamma coefficient for agreement investigation of calculated stenosis using direct diameter and area methods and Grant et al velocity criteria*

These results confirm better correlation of ECST and CCA diameter method and area measurement method with established velocity criteria used for the classification of carotid atherosclerotic disease comparing with NASCET diameter method, which

shows great variation and disagreement, mainly because of underestimation of the stenosis.

5.6.2.3 Classification of stenosis in four categories

Another set of velocity criteria widely used in the literature and applied by some vascular laboratories across UK is the Sabeti et al criteria which, contrary to consensus criteria, classifies the stenosis in four categories by splitting the lower than 50% group in two (0-29% and 30-49%). Sabeti et al criteria use mainly the PSV in internal carotid artery in addition to the Peek Systolic Velocity Ratio, using different cut-off values comparing to Grant et al criteria.

Carotid artery stenosis calculated by direct diameter and area methods were classified in four groups (0-29%, 30-49%, 50-69% and >70%) and the results were tested for correlation with classification using Sabeti et al velocity criteria using Spearman's rho (Fig 5.8). Test reported high positive correlation between classification results ($p < 0.01$ for all methods). Among B-mode measurement methods, ECST diameter measurement method had a greater correlation coefficient comparing to velocity criteria with both PSV and PSVR (0.655 and 0.636 respectively), while NASCET diameter measurement method had the lowest (0.605 and 0.524 respectively).

Chi test was used for investigation of the significance of relationship between the stenosis classification in four categories using direct diameter and area measurement methods and the classification by applying the Sabeti et al velocity criteria.

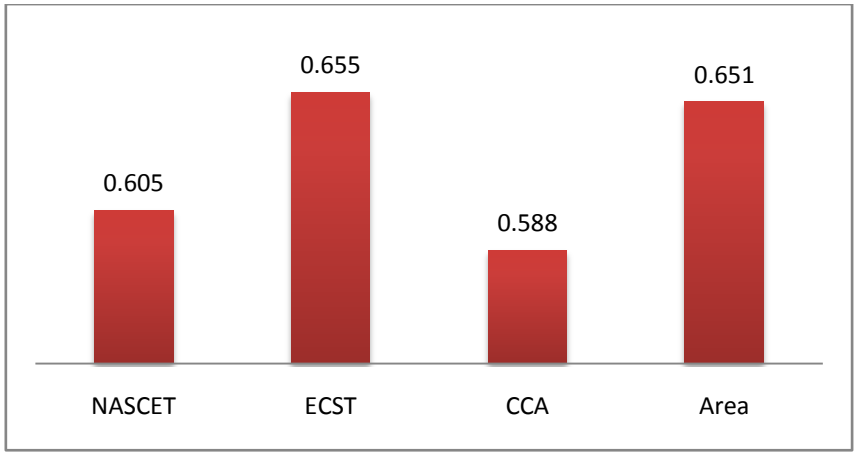


***Fig 5.8**, Spearman's correlation coefficient between classification of stenosis in four categories (0-29%, 30-49%, 50-69%, >70%) using different methods for direct diameter and area measurement in duplex ultrasound and Sabeti et al velocity criteria*

Results indicated association between classification using direct measurements and Sabeti et al velocity criteria (p value < 0.05) for all four methods. The percentage of agreement in stenosis grading was lower comparing to that when stenosis was classified in three categories for ECST, CCA and area measurement methods (Table 5.5). Test results indicated that NASCET diameter method underestimated 25% of the investigated cases whereas ECST, CCA and area methods overestimated 38%, 31 % and 39% of cases respectively (Fig 5.9).

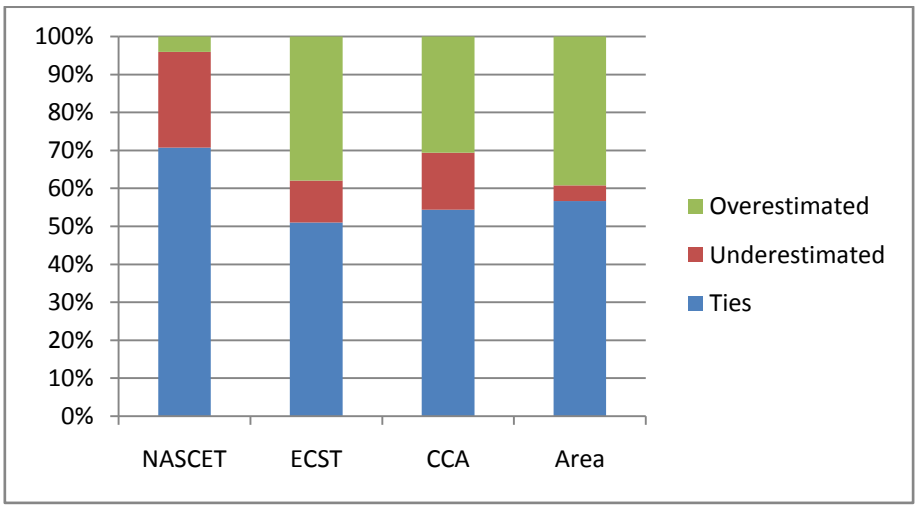


***Fig 5.9**, Percentage of cases tied, overestimated and underestimated using direct diameter and area methods comparing with classification of the stenosis using PSV Sabeti et al criteria*

Method	Percentage of cases
NASCET	70.7
ECST	51.0
CCA	54.4
Area	56.7

***Table 5.5**, Percentage of cases classified in the same category using direct diameter and area measurement methods and PSV Sabeti et al criteria*

The agreement of stenosis classification using direct diameter and area measurement methods with the classification using Sabeti et al velocity criteria was investigated by calculating the Gamma coefficient. All four direct measurement methods showed poorer agreement with Sabeti et al velocity criteria comparing with Grant et al velocity criteria apart from NASCET method whose agreement with velocity criteria was slightly increased. Area measurement method had again the highest gamma coefficient (0.898) and CCA method had the lowest (0.769) (Fig 5.10).

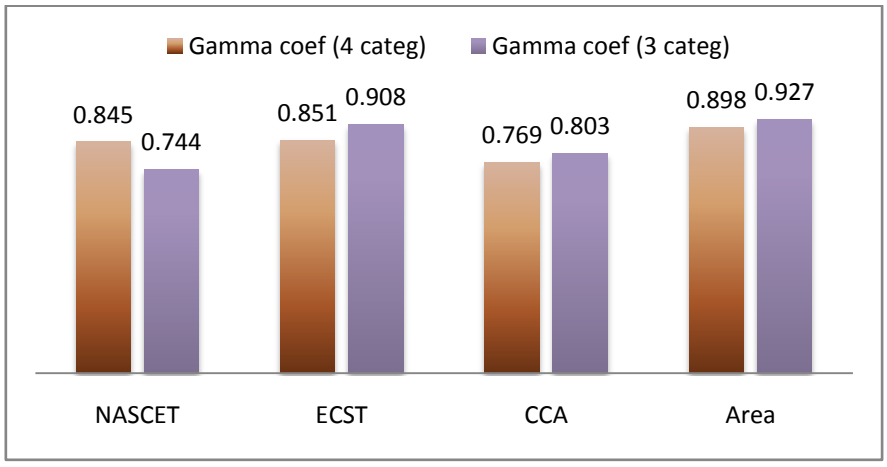


***Fig 5.10**, Gamma coefficient for agreement of calculated stenosis using direct diameter and area methods and Sabeti et al velocity criteria in relation with gamma coefficient for agreement using Grant et al velocity criteria*

Summing up, the statistic analysis of the classification data reveals a difference in the agreement of velocity criteria and direct measurement methods when stenosis is

classified in three and four categories. More specifically, the ECST, CCA and area measurement methods showed better agreement with Grant et al velocity criteria in three-category classification, as a larger number of cases was overestimated by direct measurement methods (or underestimated by velocity criteria methods) when stenosis was classified in four categories.

5.6.2.4 Identification of severe stenosis

Direct diameter measurement methods were tested for ability to identify greater than 70% stenosis, comparing with velocity criteria currently used. Fisher's exact test was used to investigate the significance of relationship between direct measurement methods and velocity criteria and gamma values were calculated for investigation of the strength of association.

Fisher's exact test indicated significant relationship between Grant et al PSV velocity criteria and ECST and CCA diameter measurement methods and area measurement methods ($p < 0.001$), while there was no agreement with results using NASCET diameter measurement method ($p > 0.05$) (Table 5.6).

	NASCET	**ECST**	**CCA**	**Area**
Sig. (p value) Fisher's test	0.911	**0.000**	**0.000**	**0.000**

***Table 5.6**, Fisher's exact test results for identification of greater than 70% stenosis using direct diameter and area methods comparing with Grant et al PSV velocity criteria*

Gamma coefficient was higher for area measurement method, followed by ECST diameter method. CCA method had the weakest agreement among the three methods (Fig 5.11).

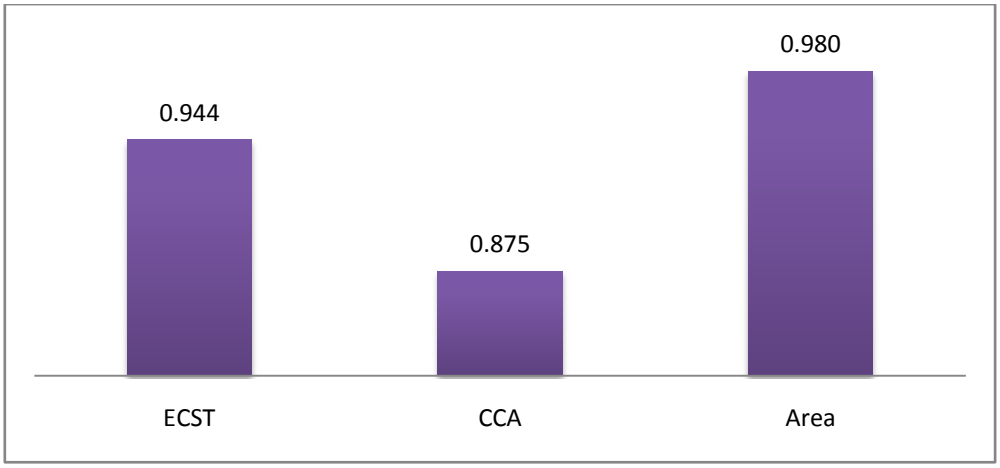
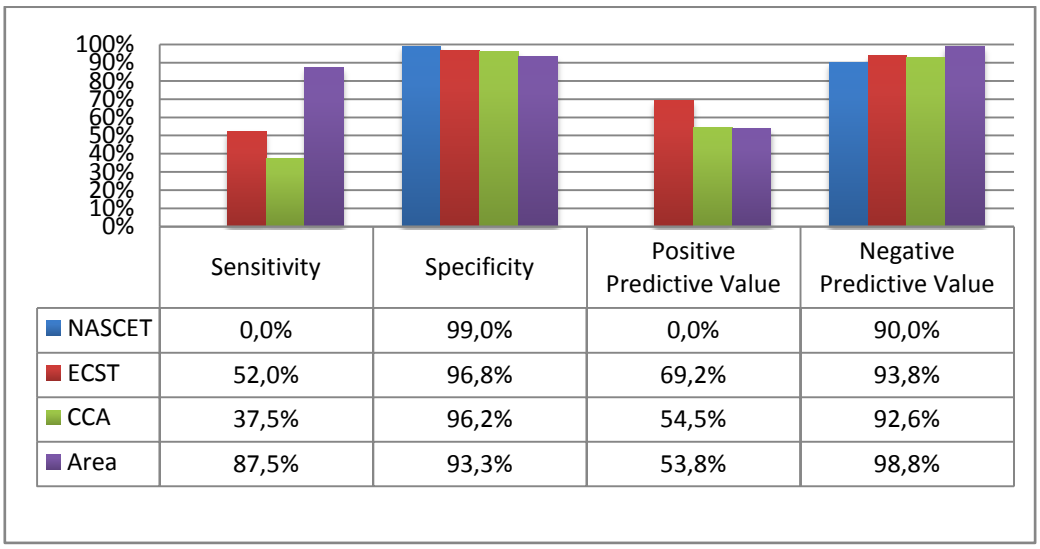


***Fig 5.11**, Gamma coefficient for agreement of identification of greater than 70% stenosis using direct area and ECST and CCA diameter methods and Grant et al PSV velocity criteria*

As far as the sensitivity and specificity is concerned, using Grant et al velocity criteria as reference, area measurement method appeared to have the highest sensitivity (87.5%) and NASCET diameter measurement method the highest specificity (99%). ECST method had the highest positive predictive value (69.2%) and area measurement the highest negative predictive value (98.8%) (Fig. 5.12).



	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
NASCET	0,0%	99,0%	0,0%	90,0%
ECST	52,0%	96,8%	69,2%	93,8%
CCA	37,5%	96,2%	54,5%	92,6%
Area	87,5%	93,3%	53,8%	98,8%

***Fig 5.12**, Sensitivity, specificity, positive predictive value and negative predictive value of direct measurement methods for identifying greater than 70% stenosis using Grant et al PSV velocity criteria as reference*

The same statistical analysis was performed comparing identification of greater than 70% stenosis using direct measurement parameters and the combination of PSV and PSVR Grant et al criteria. Velocity ratios are used in cases where PSV itself does not correspond, according to the operator, to the stenosis visualized, so as a stenosis would be considered severe only if it fulfills both PSV and PSVR Grant et al criteria.

Fisher's test results were same as previously, indicating significant relationship between Grant et al velocity criteria and ECST and CCA diameter measurement methods and area measurement methods ($p < 0.001$), while there was no agreement with results using NASCET diameter measurement method ($p > 0.05$). Interestingly, gamma coefficients for ECST and CCA diameter measurement methods and area measurement method were increased, while sensitivity significantly increased for direct diameter methods apart from NASCET. ECST method appeared to have 63.6% and area measurement 100%. Specificity remained in high levels and there was a slight increase in negative predictive value, being 100% for area measurement and 96.9% for ECST method (Fig 5.13, Fig 5.14).

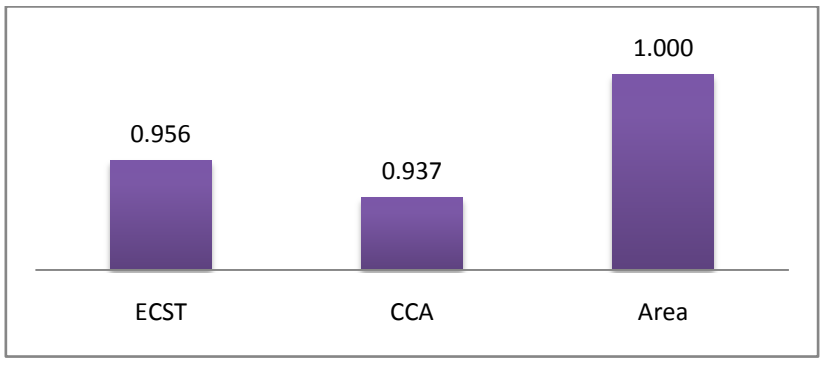
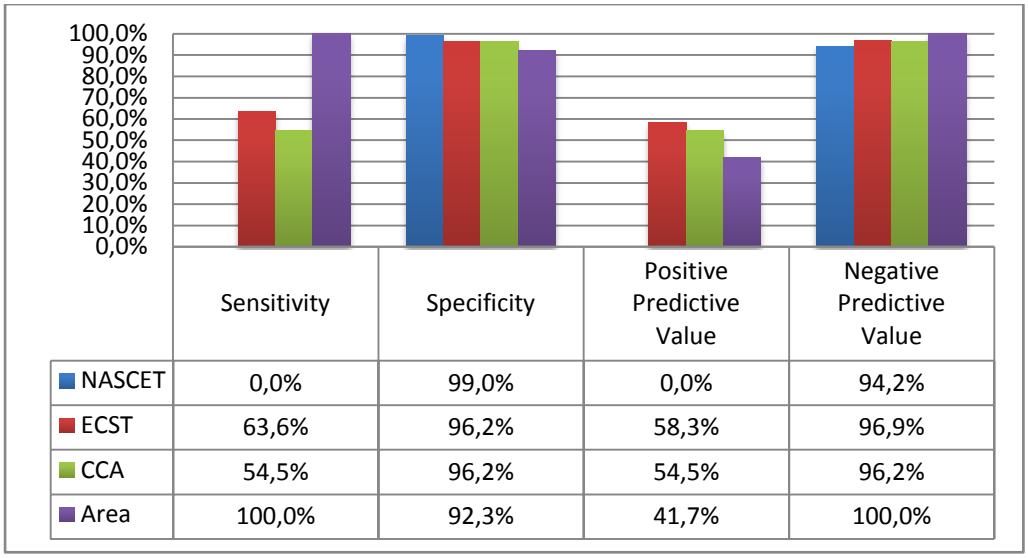


***Fig 5.13**, Gamma coefficient for agreement of identification of greater than 70% stenosis using direct diameter and area methods and Grant et al PSV and PSVR velocity criteria*



	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
NASCET	0,0%	99,0%	0,0%	94,2%
ECST	63,6%	96,2%	58,3%	96,9%
CCA	54,5%	96,2%	54,5%	96,2%
Area	100,0%	92,3%	41,7%	100,0%

***Fig 5.14**, Sensitivity, specificity, positive predictive value and negative predictive value of direct measurement methods for identifying greater than 70% stenosis using Grant et al PSV and PSVR velocity criteria as reference*

5.6.2.5 Comparison with MRA

Among the patients enrolled in the study, few had their duplex ultrasound test followed by a magnetic resonance angiography, so as the treatment route could be decided by their doctor. Due to the fact that MRA was not routinely used for carotid atherosclerotic plaque assessment the number of study participants that had an MRA was limited (N=8).

The degree of the stenosis based on velocity criteria and direct diameter and area measurement methods was compared with MRA measurement of the stenosis for agreement. Despite the small number of data, results could offer some useful information about the variation and accuracy of direct diameter measurement methods as results were compared with measurements of another modality.

MRA measurement and duplex ultrasound direct measurement data were normally distributed (Shapiro-Wilk test p value > 0.05 for all measurements) and paired sample t-test was used for investigation of agreement between estimation of carotid stenosis

using the different methods. Test results showed that t-test hypothesis could not be rejected and that there was no significant difference between all direct measurements and MRA measurements. Because of the small number of data, Wilcoxon Sign Rank Test was also performed, which confirmed the statistic insignificance of difference (Table 5.7).

	NASCET	ECST	CCA	Area
Paired sample t-test	.089	.280	.545	.596
Wilcoxon test	.109	.465	.465	.465

***Table 5.7**, Paired sample t-test and Wilcoxon Sign Rank Test p value for carotid stenosis calculation using direct diameter and area measurement methods compared with MRA stenosis calculation*

It is noteworthy however that the p value of both tests in NASCET method was considerably lower comparing to the p value of the other methods.

The deviation of the measured stenosis for each method from the MRA estimated stenosis was calculated and the means were compared. NASCET method showed a considerably higher mean deviation compared with the rest (28.7% with maximum of 47% underestimation) while ECST method measurement deviated only by 4.5 % (maximum 9% underestimation) (Fig 5.15). These results indicate a much greater agreement between ECST, and secondarily CCA and area, measurements and MRA measurements. On the other hand NASCET method appears to significantly underestimate the stenosis comparing with MRA measurements.

***Fig 5.15**, Mean deviation (percentage) of calculated carotid stenosis using direct diameter and area measurements from the MRA measurement of the stenosis*

5.6.3 Corrected NASCET measurement

Following the difference in results in the two angiographic trials that used different stenosis approach, it was suggested that there was a linear relationship between NASCET and ECST measurement results, summarized in the following formula:

$$\boldsymbol{ECST}\% = \mathbf{0.6}\ \boldsymbol{x}\ \boldsymbol{NACSET}\% + \mathbf{40}\%$$

(Rothwell *et al*, 1994).

Using NASCET method measurements, a "corrected NACSET" (corNASCET) stenosis measurement was calculated using the formula above, and the agreement with ECST method measurement and velocity criteria results was investigated.

Paired sample t-test rejected significance of difference between the two stenosis calculation methods (p value 0.998), confirming that corNASCET can produce results similar to ECST measurement. Fisher's exact test was performed for investigation of agreement between stenosis classification results of the two methods and velocity criteria. The relationship of velocity criteria was found statistically significant for both

diameter measurement methods. However, the gamma coefficients confirmed the stronger agreement of ECST with the velocity criteria comparing with corNASCET measurements (Fig 5.16).

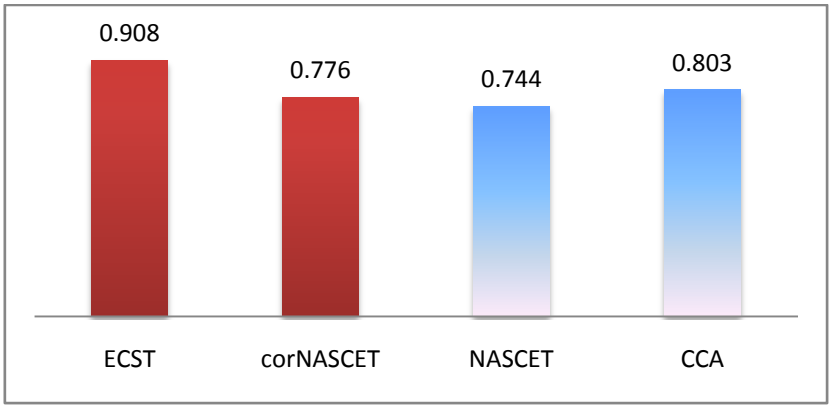


***Fig 5.16**, Gamma coefficient for agreement of calculated stenosis using direct diameter and area methods and Grant et al velocity criteria*

ECST method superiority comparing with corrected NASCET measurement was confirmed in the accuracy to identify greater than 70% stenosis. Fischer's exact test indicated significant but moderate relationship between corNASCET measurement and velocity criteria comparing to strong relationship for ECST method. Again, gamma coefficient was greater for ECST method (0.956) comparing with corNASCET's (0.937).

5.6.4 Other statistic results

5.6.4.1 PSV_{ICA}/PSV_{ICD} velocity ratio

Apart from the calculation of PSV_{ICA}/PSV_{CCA} ratio (PSVR) and the St. Mary's ratio (PSV_{ICA}/EDV_{CCA}), the ratio of PSV in ICA over the PSV in distal ICA ($PSV_{ICD}R=PSV_{ICA}/PSV_{ICD}$) was calculated for each patient and investigated for differences between groups of different carotid stenosis. Interestingly, robust test of

equality of means (Welch test) indicated significant difference in PSV_{ICD} ratio values for patients with <50%, 50%-70% and >70% stenosis (Welch value 4.271, p value <0.05) (Table 5.8, Fig 5.17).

ICA Stenosis	Mean	Std. Deviation
<50%	0.942	0.298
50-69%	1.829	0.757
>70%	3.357	1.659

***Table 5.8**, Means and Standard Deviation of PSV_{ICD} ratio for groups of different ICA stenosis*

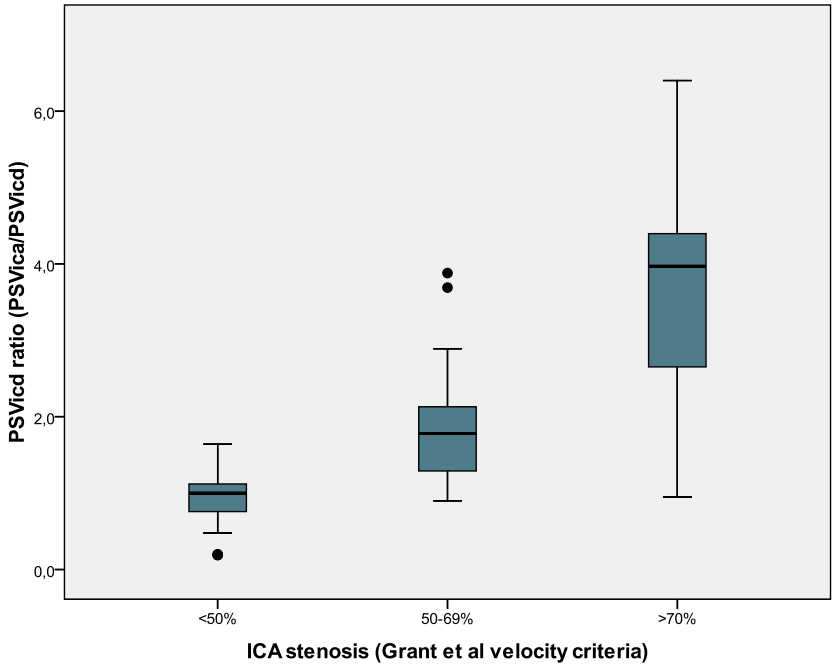


***Fig 5.17**, Boxplot of PSV_{ICD} ratio values comparing with degree of ICA stenosis*

ROC curve for PSV_{ICD} ratio as well as PSVR and St. Mary's ratio was calculated for investigation of accuracy for identification of greater than 70% stenosis. Despite the fact that the area under the curve for PSV_{ICD} was the lowest among the velocity ratios

(0.891), it represented very good accuracy as a diagnostic test. A PSV_{ICD} ratio value of 2.0 indicated 80% sensitivity and 91% specificity for identifying greater than 70% stenosis, which is comparable with the accuracy of the other two ratios used in velocity criteria today (Fig 5.18). In less than 70% stenosis, ratio's accuracy to distinguish greater and lower than 50% stenosis was lower; a value of 1.0 showed 76% sensitivity but only 50% specificity in identifying 50-69% plaques. Nevertheless, sensitivity and specificity results were similar with those of the other ratios using the suggested thresholds.

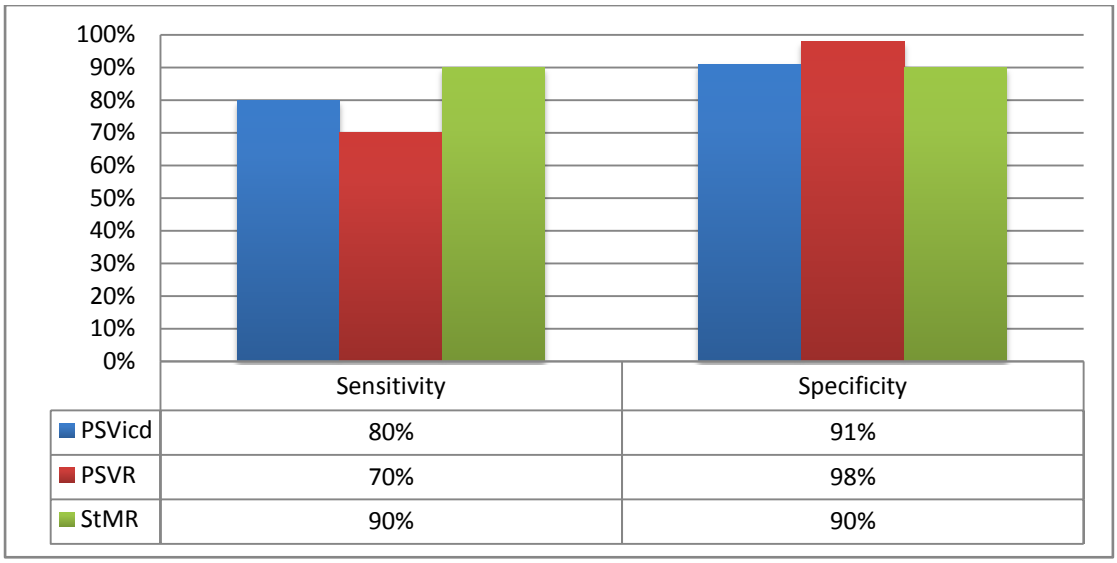


***Fig 5.18**, Sensitivity and specificity for $PSV_{ICD}R$ (cut-off value = 2), PSVR (cut-off value = 4) and St. Mary's ratio (cut-off value = 14) for identifying greater than 70% stenosis*

5.6.4.2 Intima-media thickness

Intima-media thickness is a significant marker for generalized atherosclerosis as well as a specific risk factor for cerebrovascular disease.

Statistical analysis of the study's data indicated a significant difference between symptomatic and asymptomatic patients, which agrees with literature regarding IMT.

Mean IMT for symptomatic patients was 0.097 ± .02 cm while for asymptomatic patients was 0.079 ± .02 cm (Mann-Whitney U value 902.500, p value < 0.01).

IMT was significantly higher in current or past smokers (0.093 ± 0.03 cm) comparing to no smokers (0.086 ± .03 cm, Mann-Whitney U value 1218.500, p value < 0.05). No significant difference was found regarding hypertension or diabetes.

CHAPTER VI

INVESTIGATION OF CAROTID PLAQUE GREY SCALE CHARACTERISTICS

Chapter VI

Investigation of carotid plaque grey scale characteristics

Introduction

Since the determination of the characteristics of the vulnerable atherosclerotic plaque, identification of the vulnerable plaque has become a major research field for all imaging modalities.

Ultrasound is the main diagnostic tool for assessing carotid plaque size and has also been used as a screening method. Although computed tomography and magnetic resonance imaging have showed promising results on compositional investigation of carotid plaque and more and more research focuses on those modalities, studies have shown that ultrasound can also provide useful information on plaque morphology and composition. However, no consensus has been reached regarding qualitative and quantitative characterization of carotid plaque in routine ultrasound investigation.

The aim of the project is to investigate ultrasound image texture analysis and greyscale level of the carotid plaque, the intima-media and the fibrous cap of the plaque and examine association with symptomatology and risk factors, looking into the possibility of inclusion of such methods in clinical practice in future protocols.

Method and materials

6.1 Selection of patient

Patients presenting at the vascular laboratory at Hammersmith Hospital, over a period of 6 months, from June 2008 to December 2008, were tested with the investigation protocol for assessing the composition of carotid plaque, after having a thorough ultrasound scan of the carotid arteries following the standard protocol for the investigation of the carotid atherosclerotic disease. The indications for investigation were either current symptomatic carotid disease, such as TIA or Stroke, history of cerebrovascular symptoms in the past or asymptomatic patients with severe peripheral arterial disease or coronary artery disease. The study was approved from the local research ethics committee.

6.2 Patient assessment

Patients were interviewed before the scan and a full patient history was taken, including:

a. Current medical condition

b. Previous vascular or cardiovascular surgeries

c. Current medications

d. Presence of any risk factors for cerebrovascular disease described in chapter II (smoking, hypertension, diabetes, peripheral vascular disease, coronary artery disease, family history of arterial disease)

e. Presence of any symptoms for cerebrovascular disease described in chapter II (aphasia, dysphasia, visual disturbances, vertigo, carotid bruits, dysarthria, weakness or paralysis of extremities)

Furthermore laboratory results were gathered from patient notes, mainly about risk factors associated with atherosclerosis, including levels of:

a. Cholesterol

b. Lipoproteins (HDL, LDL)

c. Triglyceride

d. Hs-CRP

e. Fibrinogen

f. Homocysteine

6.3 Duplex ultrasound investigation

6.3.1 Instrumentation

An HDI ATL 5000 scanner was used for the carotid scanning, with a 5–12 MHz linear array probe. All images were stored in Compact Discs and then transferred and processed in an in-lab PC. For diameter and area measurements the software ImageJ (National Institute of Health, Maryland, USA), a public domain Java-based image processing and analysis software, was used. For image editing and normalization Adobe Photoshop CS 5 for Microsoft Windows (Adobe Systems, California, USA) was used. For texture analysis, Adobe Photoshop CS5, Mazda (Institute of Electronics, Technical University of Łód, Poland) and Image Pro Plus 6.0 (Media Cybernetics, Maryland,

USA) were used. Numeric data were typed into Microsoft Excel Spreadsheets (Microsoft, Washington, USA) and the statistical package SPSS v19 (IBM, New York, USA) was used for statistical analysis.

6.3.2 Examination protocol

The composition assessment scan was performed after a standard carotid ultrasound scan for identification and measurement of carotid atherosclerotic disease. Patients were examined lying supine and carotid arteries were scanned both longitudinally and transversely using the anterolateral projection (positioning the transducer in front of the sternocleidomastoid muscle), while the scanner settings returned to machine's default for cerebrovascular vessels examination (32 Hz, Depth 4cm, TI 0.1, MI 0.7, Dynamic Range 60 dB). After the identification of the plaque, depth of image, focus, total gain and time gain compensation settings were altered to achieve better visualization. If internal carotid artery could not be clearly visualized due to vessel calcification or tortuosity, lateral projection (positioning the transducer over sternocledomastoid muscle) or posterolateral projection (positioning the transducer behind the sternocleidomastoid muscle) were used.

Consecutive pictures of the carotid bifurcation and the internal carotid artery were taken in transverse plane in B-mode, acquired by sliding the transducer from the point of the bifurcation up the neck following internal carotid artery until the end of the plaque. Pictures were taken every approximately 1 cm, so for each plaque a mean of 4 images were taken, depending on extend of the plaque. The same procedure was repeated using colour Doppler imaging, taking care that the colour box was shortened to highlight just the vessel so the best image resolution could be achieved. Finally, a short loop video (duration of 3 seconds) was recorded while insonating the plaque and

sliding the transducer from the bifurcation level going upwards to the end of the plaque.

Then, transducer was rotated head-to-toes and the plaque was identified in longitudinal plane. Depth setting was adjusted so as the whole region of plaque could be viewed and images were recorded in B-mode. Transducer was slided and tilted so as near and far wall adventitia was imaged in right angles and preferably the artery intima could be clearly visualized. In cases where plaque could not be outlined easily, Colour Doppler images were recorded for identification of the plaque. Last, images of common carotid artery intima-media were recorded in longitudinal plane.

6.4 Image analysis

6.4.1 B-mode image normalization

Since images were recorded using different ultrasound scanner settings for the optimal visualization of the arteries and plaque by the operator, for the analysis of the images to be comparable and reproducible, images needed to be standardized. Images for texture analysis were standardized using a method described in the literature based on grey scale value of point references, which has shown good reproducibility (Elatrozy *et al*, 1998b).

After images were transferred to a personal computer, Adobe Photoshop CS 5, an image editing software, was used to normalize the images. In the original image, the grey scale median (GSM) of two reference areas, blood and artery adventitia, was calculated using the histogram function of the software. Then the Curves function was used to algebraic linear scale the grey scale of the whole image so as the resulting

blood GSM would be between 0 and 5 and adventitia GSM would be between 185 and 195 (Fig 6.1). The new image was saved in the same format for further analysis.

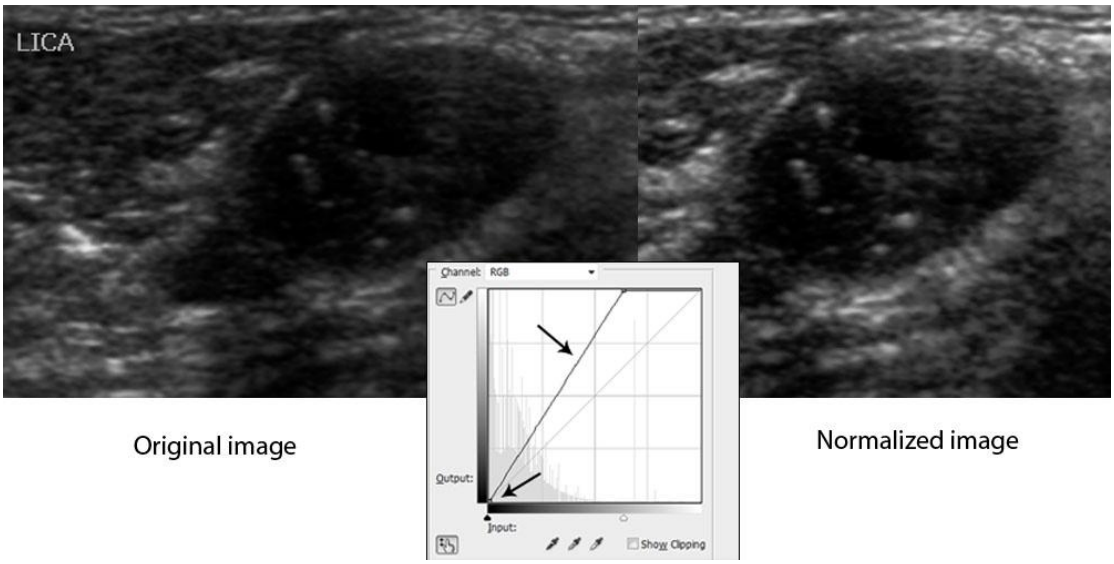


***Fig 6.1**, An example of B-mode image normalization by algebraic linear scaling using Adobe Photoshop curves. The left image is the original one of a plaque in internal carotid artery. The right image is the resulted one, after the linear scale using curves function (central image). The black arrows indicate the grey scale values of reference point*

6.4.2 Intima-media grey scale median calculation

In normalized common carotid artery images, histogram function of Adobe Photoshop software was used for calculation of the grey scale median of artery's intima-media. In all cases, the far vessel wall intima-media was analyzed. While zoomed at the vessel wall, a part of intima-media was selected using the Polygonal Lasso Tool. The histogram function was then used for calculation of the GSM (Fig 6.2).

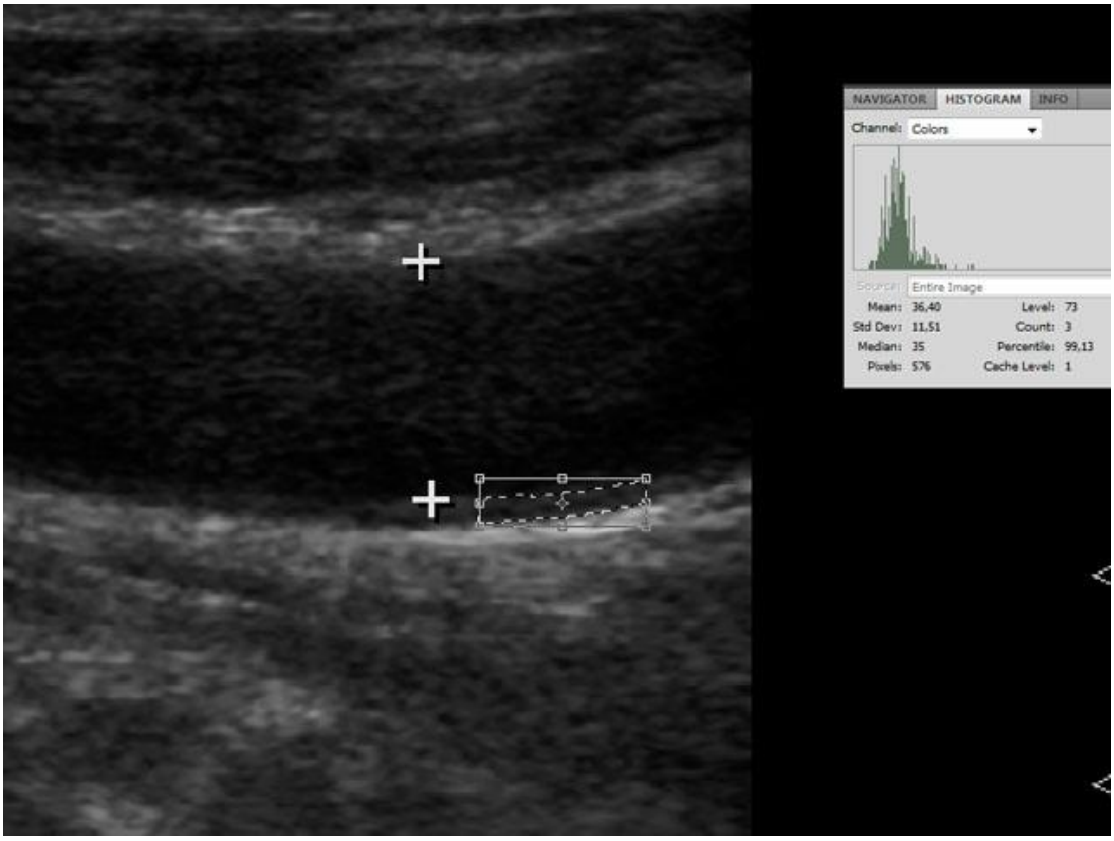


***Fig 6.2**, An example of common carotid artery intima-media selection and GSM calculation using Adobe Photoshop*

6.4.3 Carotid plaque grey scale median calculation

Using the same technique, carotid plaque's grey scale median was calculated. After loading the standardized image in Adobe Photoshop, Polygonal Lasso Tool was used to select the whole plaque. Both longitudinal and transverse images were used. Grey scale median was calculated using the histogram function for all the images (Fig 6.3).

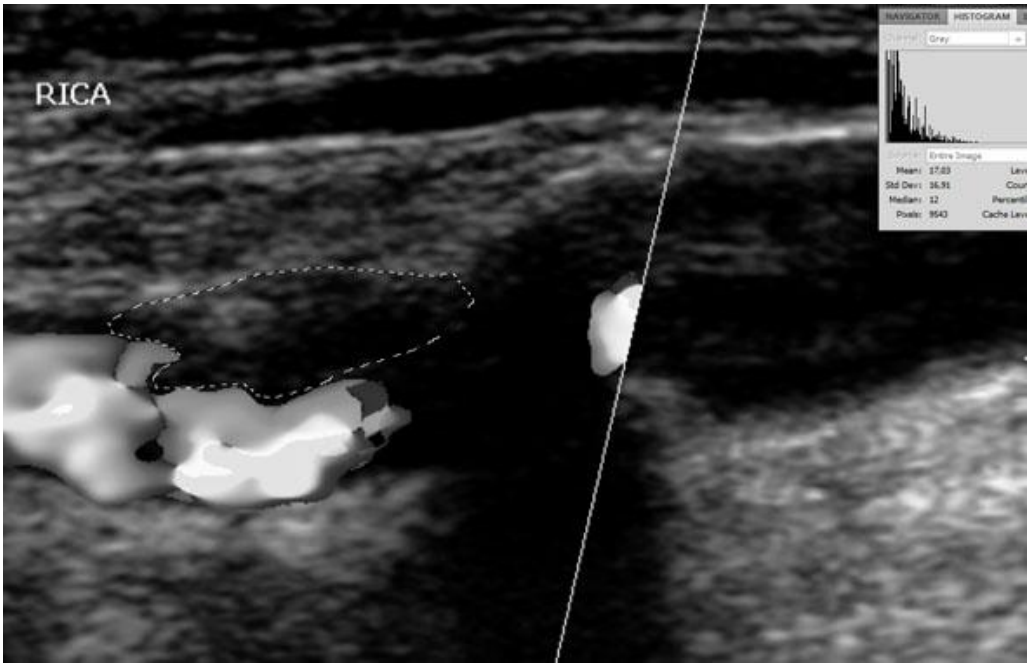


***Fig 6.3**, An example of internal carotid plaque selection and GSM calculation using Adobe Photoshop on a colour Doppler greyscale normalized image*

6.4.4 Fibrous cap thickness and GSM

Using the same technique, carotid plaque fibrous cap's grey scale median was calculated, using Adobe Photoshop and the Polygonal Lasso Tool. Both longitudinal and transverse images were used. Grey scale median was calculating using the histogram function for all the images (Fig 6.4). Fibrous cap thickness was measured using ImageJ software.

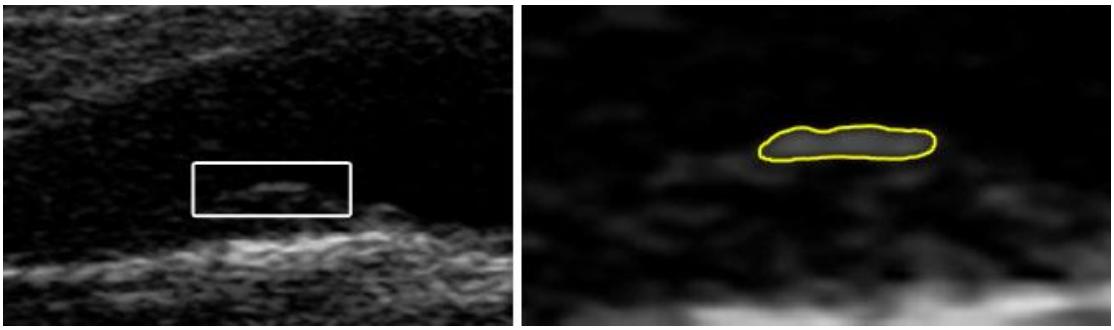
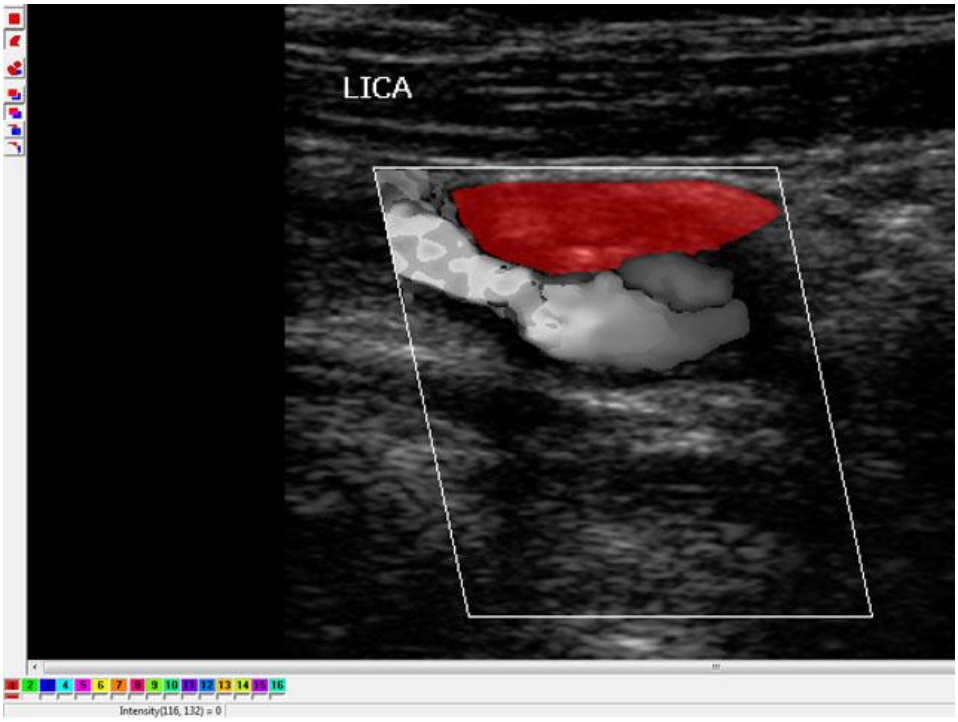


***Fig 6.4**, An example of fibrous cap selection and GSM calculation using Adobe Photoshop*

6.4.5 First order grey level parameters calculation

For the calculation of first order grey level parameters, variance, skewness and kurtosis, for the carotid plaque, Mazda software was used. Normalized greyscale image was loaded in the software and the polygon draw tool was used for the selection of the whole plaque as the region of interest to be analyzed. Then analysis was run and results were typed in Microsoft Excel Spreadsheets. In cases where there was plaque identified in both near and far artery wall, both regions were selected and analyzed (Fig 6.5).



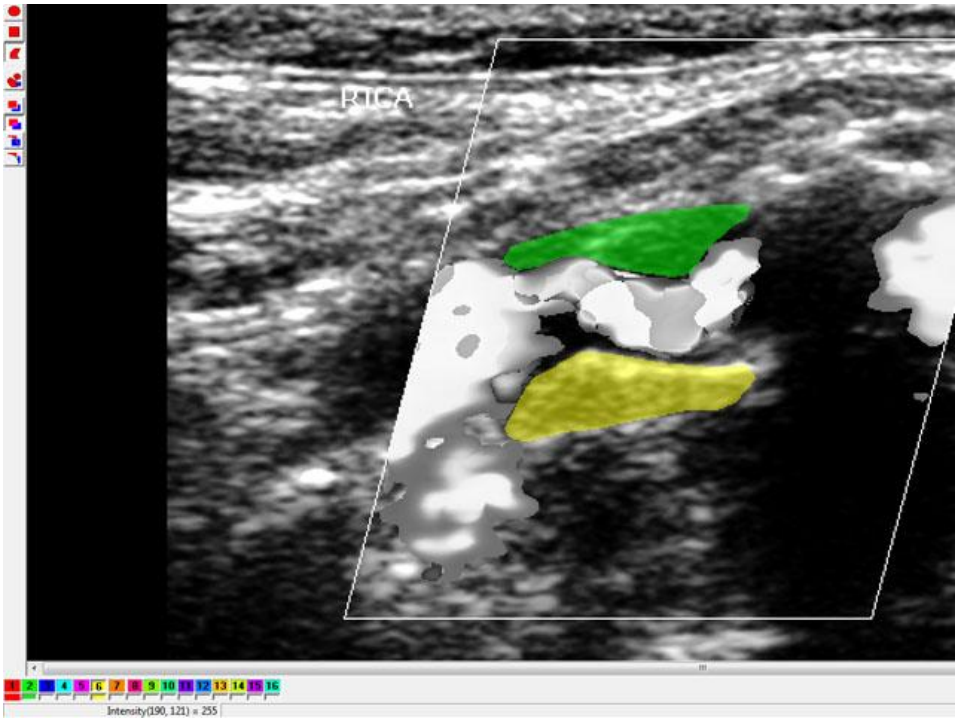


***Fig 6.5**, Examples of internal carotid plaque selection for analysis using Mazda software*

Variance

Variance is a measure of variability of data and describes their distribution around the mean. For univariate data Y_1, Y_2, ..., Y_N, variance is defined by the formula: $\frac{\sum_{i=1}^{N}(Yi-\bar{Y})^2}{N}$, where $\bar{Y}$ is the mean and N is the number of data points.

Skewness

Skewness is a measure of asymmetry from the normal distribution. It can come in the form of positive or negative skewness, depending on whether the data are skewed to the right or to the left of the data average. For univariate data Y_1, Y_2, ..., Y_N, skewness is defined by the formula: $\frac{\sum_{i=1}^{N}(Yi-\bar{Y})^3}{(N-1)s^3}$, where $\bar{Y}$ is the mean, s is the standard

deviation and N is the number of data points. Skewness is zero in normal distribution and near zero in symmetric data.

Kurtosis

Kurtosis is a measure of whether data are peaked or flat comparing to normal distribution. Positive kurtosis means data tend to have a sharp peak near the mean, whereas negative kurtosis data tend to have a flat top. For univariate data Y_1, Y_2, ..., Y_N, kurtosis is defined by the formula: $\frac{\sum_{i=1}^{N}(Yi-\bar{Y})^4}{(N-1)s^4} - 3$, where $\bar{Y}$ is the mean, s is the standard deviation and N is the number of data points.

6.4.6 Percentiles calculation

Using Image Pro Plus 6.0 software, the percentage of pixels (percentiles) with grey scale value lower than 20 (P_{20}), 25 (P_{25}), 30 (P_{30}), 35 (P_{35}) and 40 (P_{40}) were calculated in normalized grey scale ultrasound pictures of plaques.

Plaque area was selected using Adobe Photoshop as described previously (6.4.3) and a new image, containing only the plaque, was loaded in Image Pro Plus. A macro was created that used Count/Size function and Histogram based selection for identification and calculation of percentage of area with grey scale value ranging 0-25, 0-30, 0-35 and 0-40 (Fig 6.6).

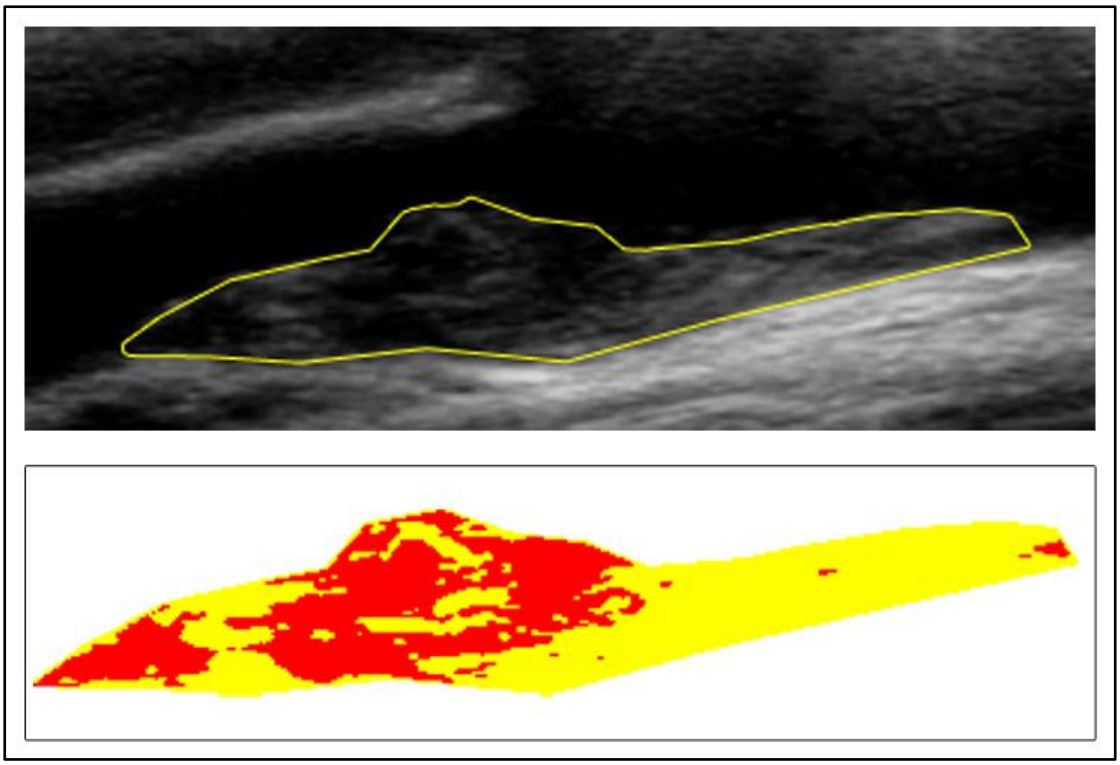


***Fig 6.6**, Example of plaque selection and percentile calculation using Image Pro Plus*

6.5 Subjective plaque classification

Cases were classified subjectively using standardized images as far as echogenicity (echogenic or echolucent), homogeneity (homogeneous or heterogeneous) and morphology (regular or irregular) is concerned.

Results

6.6 Descriptive statistics

In total, 40 patients were scanned and images were taken from both carotids of each patient. Therefore 80 cases were analyzed.

The mean age of patients was 68.7 ± 9.2 years. Twenty nine (72.5%) patients were men (mean age 69.5 ± 8.5 years) and 11 (27.5%) were women (mean age 66.64 ± 11.0 years). Thirteen (32.5%) patients were current or past smokers, 32 (80%) knew to have hypertension and were treated with medication, 13 (32.5%) were diabetic, 20 (50%) had hypercholesterolemia, 8 (20%) had history of ischaemic heart disease and 5 (17.5%) had history of atrial fibrillation.

Among the enrolled patients, 34 (85%) had a recent cerebrovascular event (stroke or transient ischaemic attack and 6 reported other recent cardiovascular symptoms or cerebrovascular-like symptoms (vertigo etc).

Thirty two cases with no or minimal carotid stenosis were excluded from further analysis. In 8 cases marked carotid stenosis was identified using velocity criteria and no good B-mode image of the plaque could be recorded due to technical difficulties and were excluded from the study. From forty cases that were further analyzed, 4 cases had greater than 70% stenosis, 6 cases had 50% to 69% stenosis and 30 cases had 20-49% stenosis.

6.7 Statistic analysis

6.7.1 Normal distribution

Image texture analysis grey level parameters were tested for normal distribution using Shapiro-Wilk test. Common carotid intima-media GSM, GSM of plaque's fibrous cap in short axis, mean plaque GSM from long and short axis views, plaque GSM in best view (long or short axis view) and grey scale values standard deviation in best (long or short axis) view, Variance in long and short axis, Skewness in long and short axis and in best view were reported to be normally distributed (Table 6.1). Kurtosis in long or short axis, mean grey scale values standard deviation from long and short axis views, and fibrous cap GSM in long axis view reported no normal distribution .

Parameter	**Sig. (p value)**	**Parameter**	**Sig. (p value)**
Common Carotid Artery Intima-media GSM	**.966**	Grey scale value standard deviation in best view	**.095**
Fibrous Cap GSM Short axis	**.392**	Variance in best view	0.001
Fibrous Cap GSM Long axis	.004	Skewness in best view	.048
Mean plaque GSM (long & short axis)	**.246**	Kurtosis in best view	0.000
Plaque GSM in best view	**.072**	Mean grey scale value standard deviation in long and short axis	.022

***Table 6.1**, Shapiro-Wilk test p-values for normality of distribution of image texture analysis grey level parameters*

6.7.2 Common carotid intima-media GSM

The grey scale median of the intima and media layers of common carotid artery far wall was compared with internal carotid artery plaque grey scale median (long and short axis mean GSM and GSM in best view) for correlation using Pearson's Correlation test since all parameters were normally distributed. Test indicated no significant correlation between parameters (p value > 0.05).

Common carotid intima-media GSM was compared for difference between cases with internal carotid plaque with high or low GSM value, using 32 as a cut-off point. The parameter was distributed normally in the two groups (Shapiro-Wilk test p value > 0.05) and t-test was used for investigation of difference's significance. Despite the fact that common carotid intima-media GSM was reported to be slightly higher in cases with more echogenic plaque (Fig 6.7), there was important overlap and the difference was found insignificant according to t-test results (p value > 0.05)

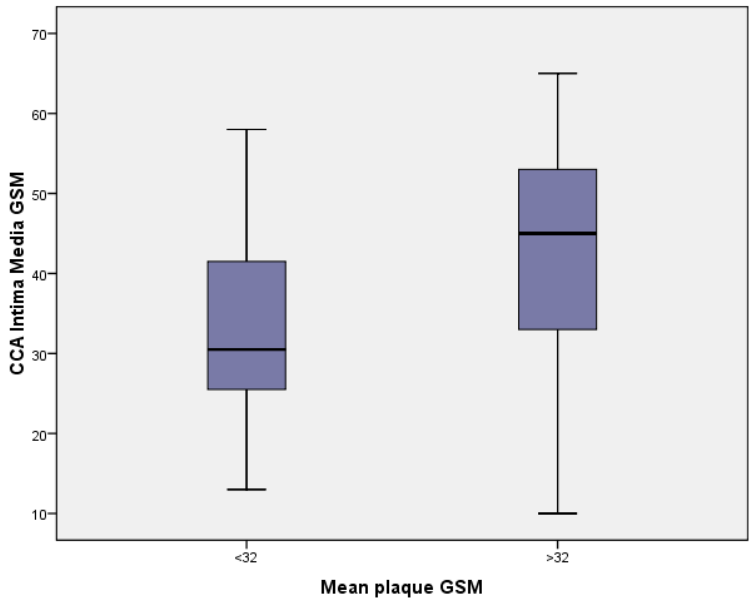


***Fig 6.7**, Boxplot of common carotid intima-media GSM in patients with internal carotid plaque with GSM less or greater than 32*

Common carotid intima-media GSM was then investigated for difference between cases with severe (greater than 70% of diameter stenosis using ECST method) and milder stenosis (less than 70% of diameter stenosis) in internal carotid artery. Parameter was normally distributed in the two groups (Shapiro-Wilk test p value > 0.05) and t-test was used for significance examination. The GSM of common carotid intima-media was found to be less in patients with severe stenosis (mean GSM 27.7 ± 5, N=5) comparing with patients with less than 70% stenosis (mean GSM 40.68 ± 13, N=53) (Fig 6.8). However, t-test was marginally negative for statistical significance of that difference (p value 0.095, equal variances assumed).

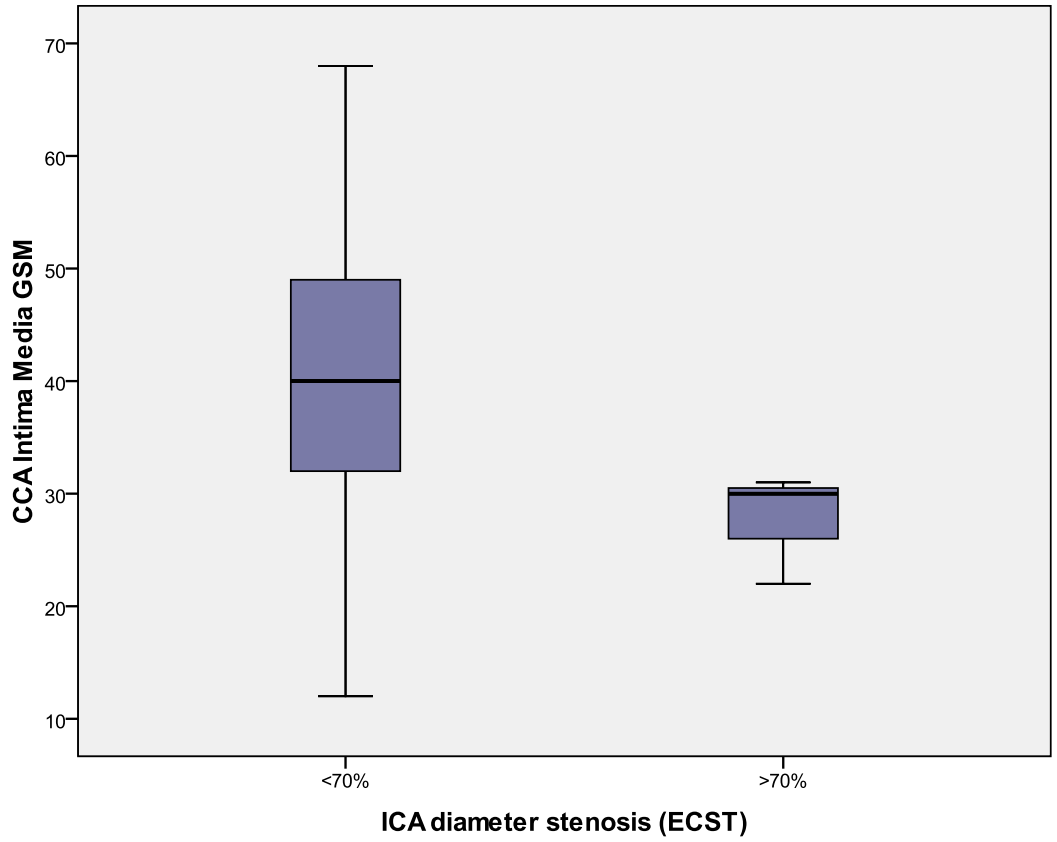


***Fig 6.8**, Boxplot of common carotid intima-media GSM in patients with severe (>70%) or milder (<70%) internal carotid artery stenosis based on ECST method diameter measurement*

There was no difference found in common carotid artery intima-media GSM between symptomatic or asymptomatic patients (t-test p value > 0.05). However, significantly lower GSM values were reported in patients with high blood cholesterol levels (mean 35.2 ± 13.5) comparing with patients with normal blood cholesterol levels (mean 43.5 ± 13.3, Mann-Whitney U 324.5, $p < 0.05$) (Fig 6.9).

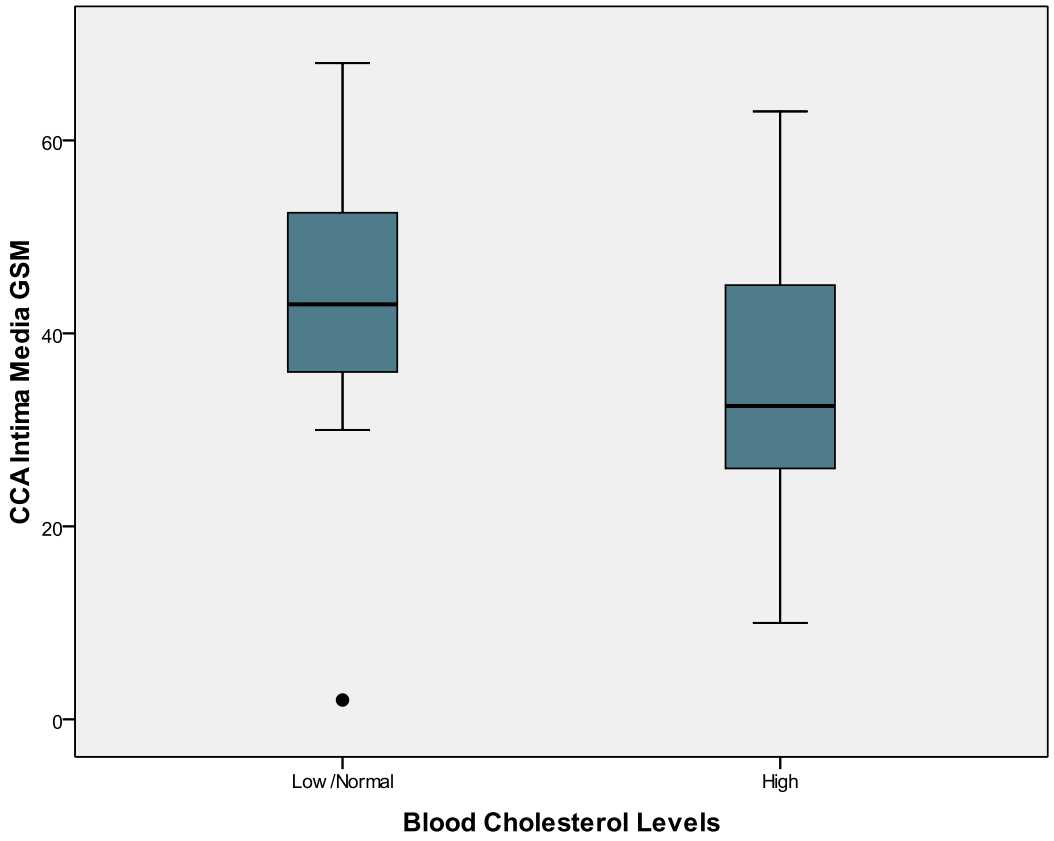


***Fig 6.9**, Boxplot of common carotid intima-media GSM in patients with high and low/normal blood cholesterol levels*

No correlation was reported between common carotid intima-media GSM and other cardiovascular risk factors.

6.7.3 Plaque texture analysis and subjective classification

Carotid plaque subjective classification concerning echogenicity and homogeneity was compared with computerized texture analysis results.

6.7.3.1 Echogenicity

Among the parameters investigated for difference in plaques classified subjectively as echogenic and echolucent, grey scale median of the plaque in all views separately and the mean grey scale median of the plaque as well as variance in best view were found to be statistically different between groups (Table 6.2, Fig 6.10). Parameters were normally distributed in two groups (Shapiro-Wilk test p value > 0.05) and therefore t-test was used for investigation of difference significance.

	Echolucent	**Echogenic**	**Sig. (p value)**
Mean plaque GSM	16.1 ± 12.8	54.8 ± 20.1	0.000
Plaque GSM in best view	16.3 ± 5.7	54.7 ± 23.9	0.000
Plaque GSM in short axis	13.6 ± 13.4	57.5 ± 18.5	0.000
Plaque GSM in long axis	16.8 ± 11.5	53.5 ± 22.1	0.000
Variance	664.2 ± 318.2	1480.3 ± 845.8	0.005

***Table 6.2**, Mean values and t-test results for grey scale parameters for echolucent and echogenic plaques*

ROC curves were calculated for mean plaque GSM for identification of echolucent plaque. Results indicate that GSM value of 35 had 100% sensitivity and 72.2% specificity in echolucent plaque identification. The area under the curve was 0.954. Similar were the results from the ROC curve calculation for plaque GSM in best

view. For GSM value of 32, sensitivity was 100% and specificity was 72 %, with the area under the curve being 0.931. Both plaque GSM measurements reported excellent accuracy for separating the two groups.

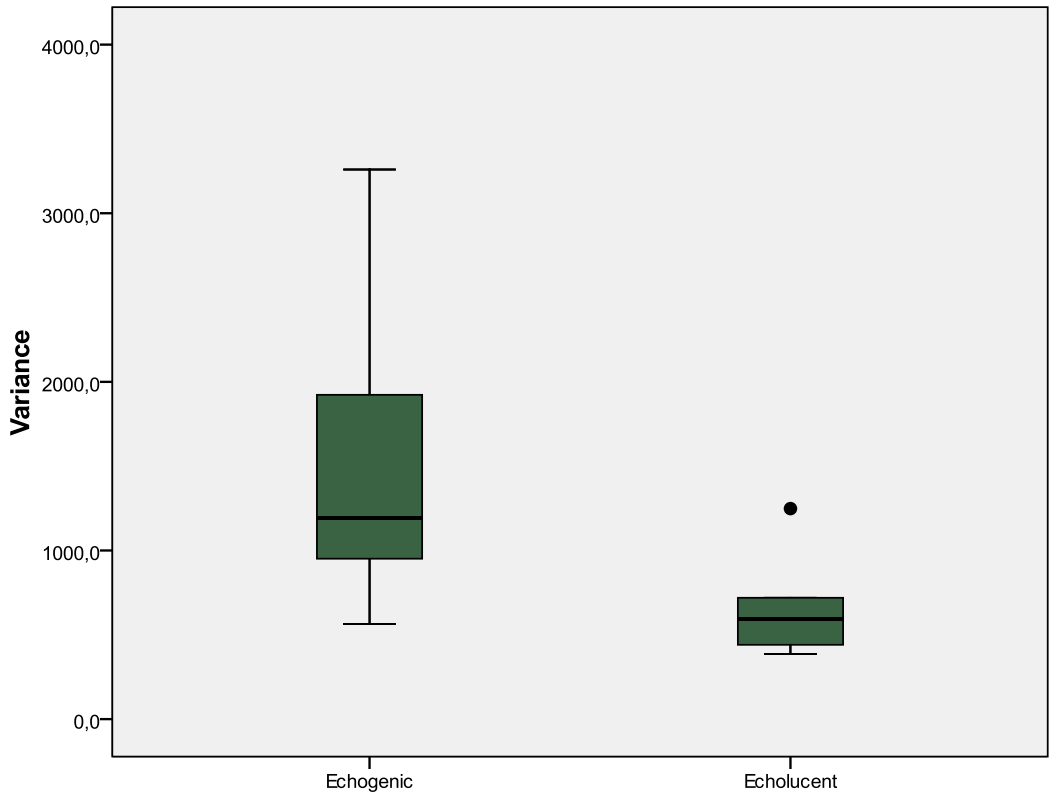


***Fig. 6.10**, Boxplot of plaque variance in echogenic and echolucent plaques as they were classified visually*

ROC curve was calculated for plaque variance for investigation of ability to distinguish echolucent plaque. The area under the curve was 0.833 which stands for good accuracy and a variance threshold of 830 showed 83% sensitivity and 71% specificity for identifying echolucent plaques.

Rest of the parameters that were significantly different in echogenic and echolucent plaques showed a smaller under the curve area.

6.7.3.2 Homogeneity

Among the parameters investigated for difference in plaques classified subjectively as homogeneous or heterogeneous, grey scale value standard deviation seemed to have different values in the two groups (Fig 6.11); homogeneous plaques had a mean grey scale value standard deviation of 28.9 ± 10.0, lower than that of heterogeneous plaques which had a mean value of 37.8 ± 10.7. Parameter showed normal distribution in both groups (Shapiro-Wilk test p value > 0.05) and t-test was used for investigation of significance. Test results were marginally negative for significance of the difference (p = 0.064, equal variances not assumed).

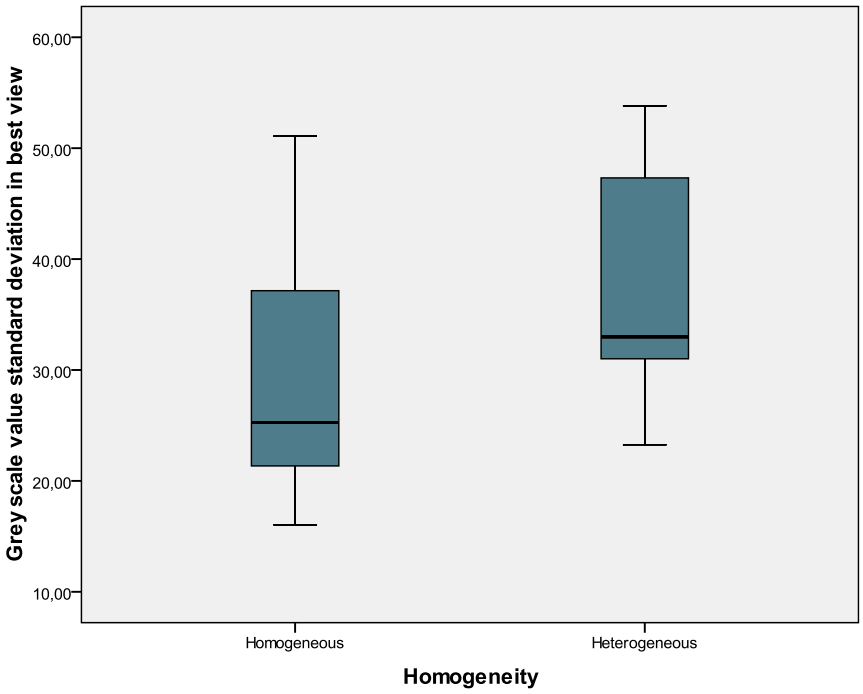


Fig 6.11, *Boxplot of grey scale value standard deviation in best view (long or short axis) in homogeneous and heterogeneous plaques as they were classified subjectively*

ROC curve was calculated for grey scale value standard deviation in best view so the ability of this parameter to identify heterogeneous plaques could be investigated. Area under the curve was 0.741, indicating fair accuracy for group separation. A standard

deviation value of 30.4 reported a sensitivity of 77.8% and specificity of 66.6% for identifying heterogeneous plaque.

6.7.4 Plaque GSM and degree of stenosis

Plaque GSM was investigated for difference in patients with different degree of internal carotid artery stenosis. Plaques causing greater stenosis had lower GSM in best view than plaques causing milder stenosis (Table 6.3, Fig 6.12). It was normally distributed in all three groups (Shapiro-Wilk test p value > 0.05) and ANOVA test was used for examination of difference significance. Test results indicated no significance in the difference of plaque grey scale median among the groups (Table 6.4).

Degree of stenosis	Plaque grey scale median
<50%	50.5 ± 25
50-69%	34.0 ± 17
>70%	18.0 ± 8

***Table 6.3**, Plaque GSM in best view mean values in patients with different degree of stenosis measured using ECST diameter measurement method*

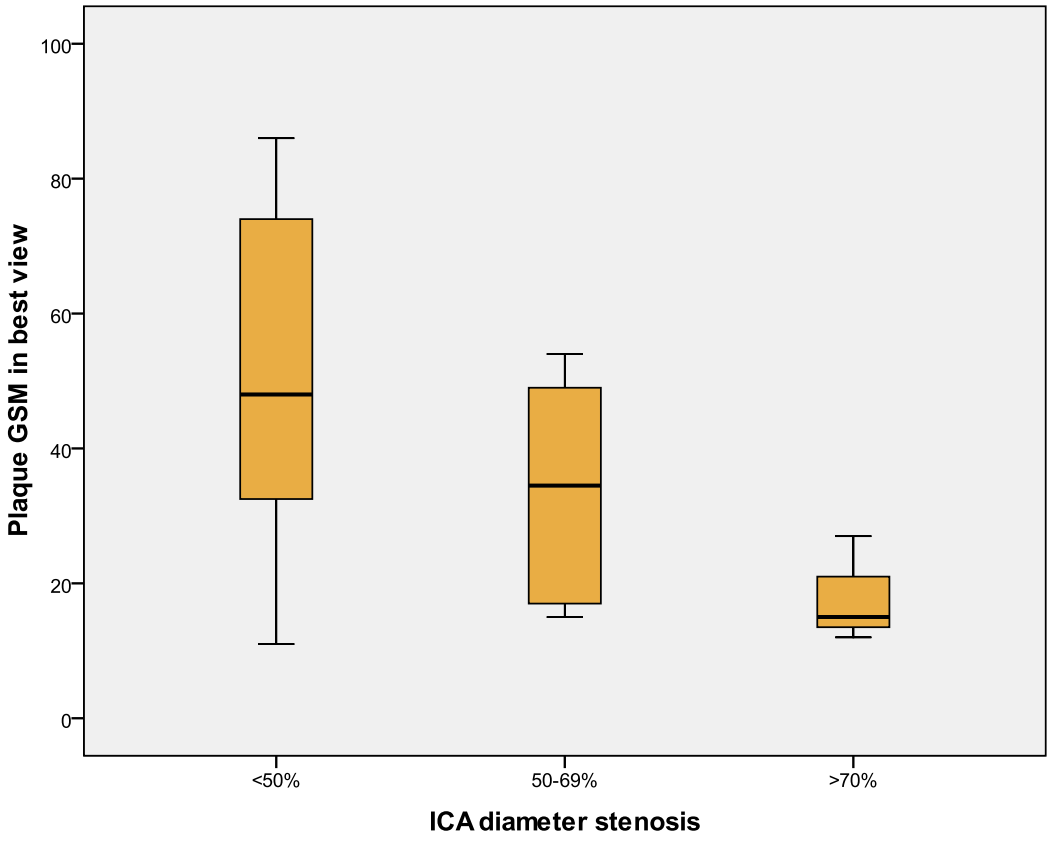


***Fig 6.12**, Boxplot of plaque GSM in best view mean values in patients with different degree of stenosis measured using ECST diameter measurement method*

Groups of patients according to degree of stenosis		Mean difference	Std Error	Sig. (p value)
< 50%	50-69%	16.5	10.9	0.306
	>70%	32.5	14.2	0.081
50-69%	< 50%	-16.5	10.9	0.306
	>70%	16.0	15.9	0.583
>70%	< 50%	-32.5	14.2	0.081
	50-69%	-16.0	15.9	0.583

***Table 6.4**, ANOVA test results for plaque grey scale median in groups patients with <50%, 50-69% and >70% stenosis*

6.7.5 Grey scale percentiles

The percentage of plaque area (percentiles) with grey scale value ranging from 0 up to particular cut-off values were compared between symptomatic and asymptomatic patients for investigation of difference. Indeed, all five percentiles were lower in plaques from symptomatic patients comparing to those from asymptomatic patients (Table 6.5, Fig 6.13).

Percentiles were normally distributed for both symptomatic and asymptomatic patients (Shapiro-Wilk test p value > 0.05) and t-test was used for investigation of significance of difference.

T-test results indicated significant difference for all percentiles between symptomatic and asymptomatic patients, with p value ranging from 0.003 to 0.034

	Symptomatic	**Asymptomatic**	**Sig. (p value)**
P_{20}	41.90 ± 21.0	22.44 ± 22.9	0.034
P_{25}	49.21 ± 21.1	27.77 ± 24.1	0.022
P_{30}	55.55 ± 20.4	33.22 ± 25.5	0.017
P_{35}	61.17 ± 19.6	38.07 ± 25.4	0.012
P_{40}	66.27 ± 18.2	42.50 ± 24.9	0.007

***Table 6.5**, Percentile mean values and standard deviation for symptomatic and asymptomatic patients and t-test p value for significance of difference*

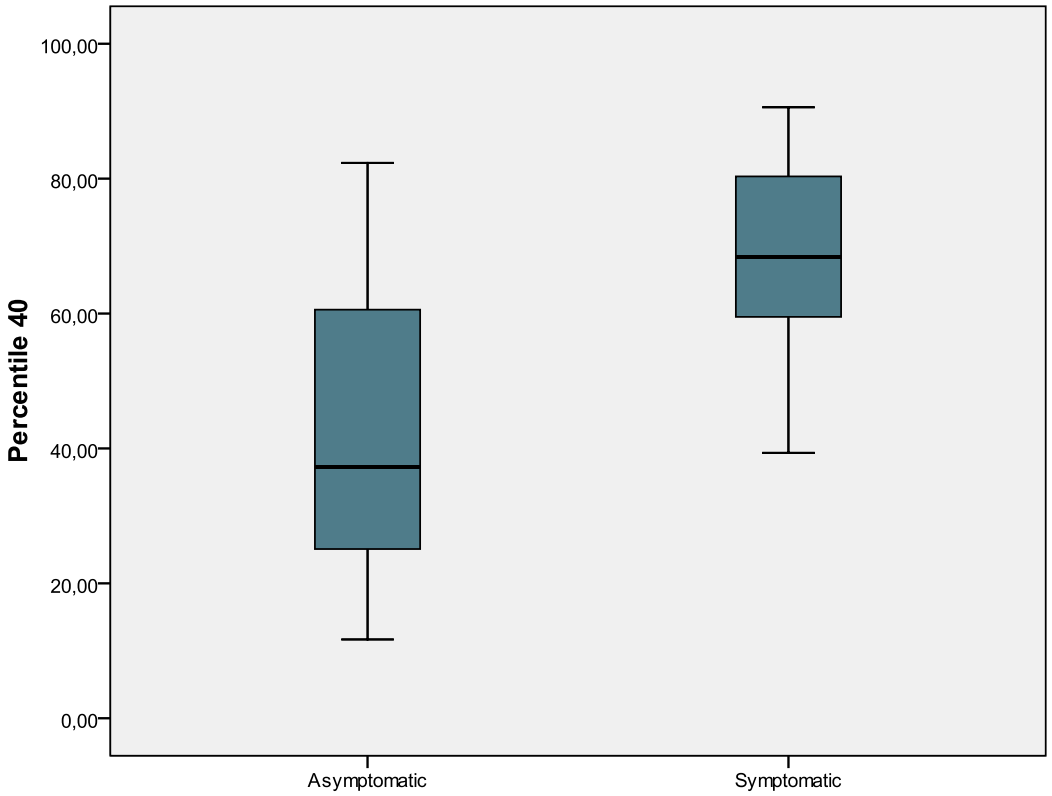


***Fig 6.13**, Boxplot of P40 for symptomatic and asymptomatic plaques*

ROC curves were calculated for Percentiles for investigation of accuracy in identifying symptomatic plaques. P40 and P35 had the greatest area under the curve (0.768) and P20 had the smallest (0.745), which describes fair diagnostic accuracy. A P_{40} value of 50, which is interpreted as 50% of plaque pixels having grey scale value less than 40 in a 0 to 256 grey tones scale, had a sensitivity of 80% and specificity of 75% for identifying potentially symptomatic plaque.

6.7.6 Fibrous cap investigation

Plaques of patients with cerebrovascular symptomatology had a significantly thinner fibrous cap comparing to asymptomatic patients (Fig 6.14). Mean fibrous cap thickness for symptomatic patients was 0.29 ± 0.05 mm while in asymptomatic

patients it was 0.38 ± 0.08 mm and t-test confirmed statistical significance of this difference between the groups (t-test p value < 0.05 with equal variances). No difference was found between patients with a history of stroke and patients with history of TIA.

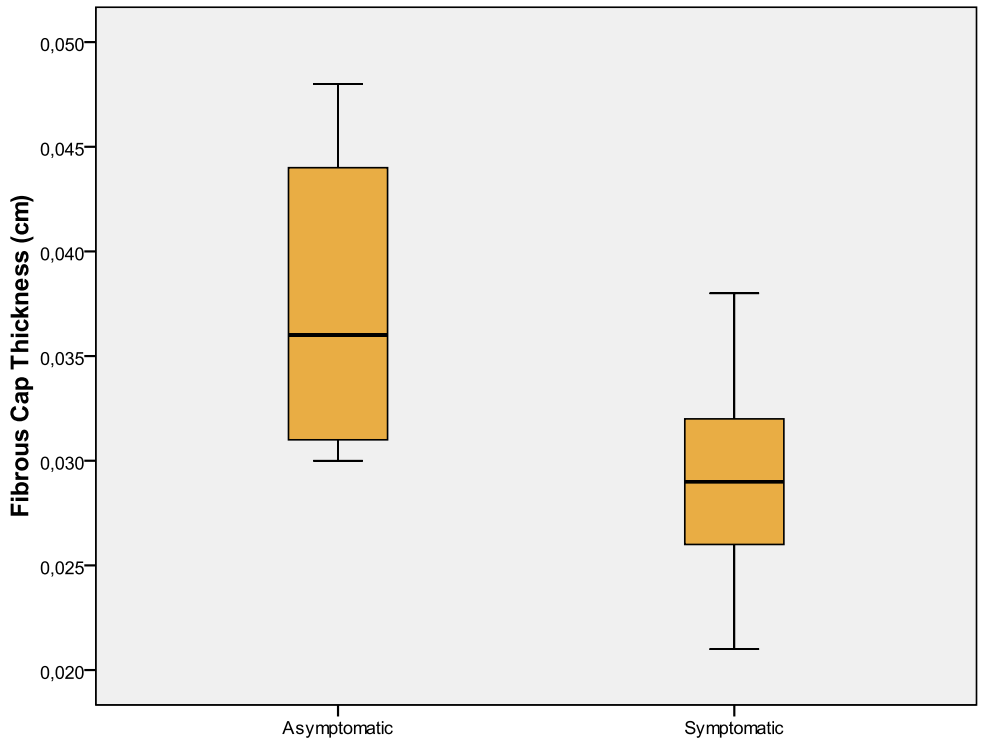


***Fig 6.14**, Boxplot of fibrous cap thickness in symptomatic and asymptomatic patients*

Plaque fibrous cap was also found significantly thinner in plaques whose morphology was characterized as irregular (Fig 6.15). Plaques with regular morphology had a mean fibrous cap thickness of 0.32 ± 0.05 mm while irregular plaques had a mean fibrous thickness of 0.26 ± 0.05 mm, a difference which was found statistically significant (t-test p value <0.05 with equal variances).

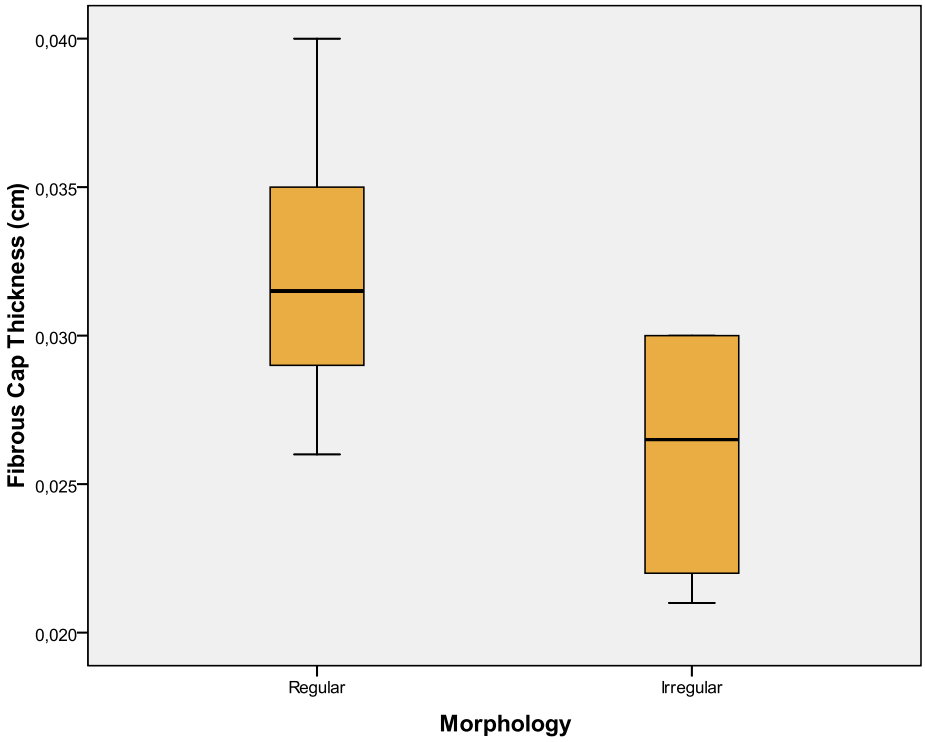


***Fig 6.15**, Boxplot of fibrous cap thickness of regular and irregular plaques*

No association was found between fibrous cap thickness and plaque's echogenicity or size. As far as cardiovascular risk factors are concerned, fibrous cap was slightly thinner in plaques of smokers and diabetic patients but there was overlap and difference was found statistically insignificant.

Regarding fibrous cap GSM, it was well correlated with plaque GSM (Spearman coefficient 0.379, p value <0.5) and was slightly higher in irregular plaques and plaques causing severe stenosis. However, none of these differences were statistically significant.

6.7.6 Grey level parameters and symptomatic plaque

The comparison of plaque Variance, Skewness and Kurtosis between symptomatic and asymptomatic patients did not indicate any significant difference between the two groups. However, it is noteworthy that asymptomatic plaques had a higher Kurtosis value comparing to symptomatic. Particularly, Kurtosis value of less than 0.6 had 100% specificity in distinguishing symptomatic and asymptomatic plaques.

Grey scale median was lower in symptomatic patients (mean 40) than asymptomatic (mean 49) patients; however there was a considerable overlap and statistical analysis rejected significance.

6.7.7 Image texture analysis and cardiovascular risk factors

Image grey scale parameters results were compared with patient cardiovascular risk factors for correlation. Kurtosis of internal carotid plaque was reported having significantly lower values in patients that used to be or currently were smokers (mean 1.03 ± 2.8) comparing with no smokers (mean 2.16 ± 2.2, Mann-Whitney U 41.000, p value < 0.05). No other correlation was observed.

6.8 Combination of plaque ultrasound characteristics for estimation of risk for stroke

Following the statistical analysis of image texture parameters in symptomatic and asymptomatic patients, counting in the significance of the degree of stenosis, a number of ultrasound characteristics of the carotid plaque were combined in an effort

to create a scoring system that would provide information upon plaque vulnerability and possibility for symptomatic disease. The variables that were used and the score points for each are cited in Table 6.6. Minimum total score for a plaque was 0 and maximum score was 6.

	Score points		
DUS variables	**0**	**1**	**2**
Percentile 40 (P_{40}) (%)	0-34	35-59	>60
Diameter stenosis (ECST method) (%)	0-50	50-70	>70
Fibrous cap thickness (mm)	>0.3	<0.3	
Intima-media thickness (cm)	<0.11	>0.11	

***Table 6.6**, Duplex ultrasound variables and score points for carotid plaque assessment*

Three categories for risk of stroke were defined; low risk for score 0-1, increased risk for score 2-3 and high risk for score 4-6.

***Table 6.7**, Score system risk stratification*

Distribution of study's patients using the proposed score indicated that almost 95% of symptomatic plaques fell within increased or high risk group, while all no asymptomatic plaques had a score greater than 3 (Fig 6.16).

The odds ratio (OR) of symptomatic disease for a plaque that had a total score equal to or greater than 3 was 13.714 (95% CI 1.381 to 136.212). The relative risk for symptomatic disease for plaques in increased risk comparing with plaques in low risk group was 3.93 (p value < 0.05) while the relative risk for plaques in increased or high risk comparing with plaques in low risk was 5.5 (p value <0.05).

The relative risk for each score system component separately was significantly lower compared with scoring system relative risk. Particularly, for Fibrous cap thickness relative risk was 1.28, for IMT was 1.75, for stenosis greater than 70% was 1.41 and for P_{40} was 1.64.

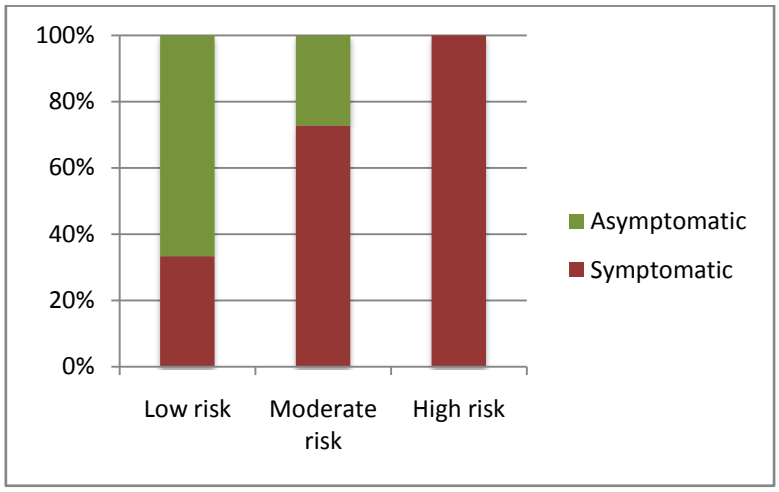


***Fig 6.16**, Stratification of symptomatic and asymptomatic plaques in risk groups using the proposed scoring system*

CHAPTER VII

DISCUSSION

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Discussion

7.1 Introduction

In the last decades, the advances in science and medicine have lead to a better understanding of pathophysiology of cardiovascular disease and specifically atherosclerosis. Twenty years ago, identification of high cholesterol as a risk factor for cardiovascular diseases was a major breakthrough and quickly became the main target for both prevention and treatment research. Since then, a number of risk factors have been found, many of the mechanisms have been explained, sets of markers for predicting risk have been suggested and new ways of investigation and treatment are being validated (Fig 7.1). However, despite the advances in knowledge and expertise, prevention of cardiovascular disease has not reached its full potential and cardiovascular disease remains the leading cause of deaths worldwide.

***Fig 7.1**, TIME magazine covers illustrating cholesterol and inflammation as risk factors for cardiovascular disease and the importance of prevention*

Today, stroke is a major cause of death and disability, causing in UK around 53,000 deaths each year and being responsible for approximately 175,000 hospital visits, while only in England around 21,000 surgical procedures are stroke related. The overall economic cost of cerebrovascular disease goes up to £5 billion, predicted to increase in the future due to the ageing population.

Approximately half of the ischaemic strokes are due to emboli from atherosclerotic plaques located in carotid arteries and thus, early diagnosis and accurate investigation of carotid atherosclerotic diseases is of great importance for stroke prevention and management. Duplex ultrasound is currently the main diagnostic tool for assessment of carotid stenosis and treatment decisions as well as patients follow up depend predominantly on ultrasound findings. Computed tomography angiography and magnetic resonance angiography have also been introduced in carotid stenosis investigation with promising results, however in clinical practice they are solely used in cases where ultrasound assessment is inconclusive.

Despite the fact that carotid ultrasound is the mostly used stand-alone imaging modality for carotid disease investigation, there is still debate regarding methods and criteria for calculating the degree of stenosis as well as techniques for acquiring quantitative information about the carotid plaque itself. Ongoing research aims to provide answers and standardize techniques and protocols, however the remaining confusion leads to the risk of ultrasound being held back with the excuse of obsolescence and unreliability, while other techniques are constantly improving and new techniques come into practice.

7.2 Carotid stenosis measurement

The degree of stenosis of internal carotid artery by the atherosclerotic plaque has been the first and most assessed characteristic of carotid disease and its accurate determination is very important for any diagnostic modality, even after the identification of additional characteristics which add in plaque vulnerability.

Velocity criteria are predominantly used for carotid stenosis estimation with ultrasound, based on the haemodynamic effects of the stenosis. A number of different sets of velocity parameters and criteria have been published and despite the consensus recommendations in 2003 which tried to standardize investigation protocol, confusion remains in vascular laboratories across European countries, including UK. Recent joint recommendations from the Vascular Society and the Society of Vascular Technology in Great Britain and Ireland addressed and tried to resolve the issue, suggesting the use of velocity criteria and introducing direct diameter measurement in case of bulb stenosis in cases of carotid bulb diameter exceeding 10 mm. Controversy still remains on the ability of ultrasound to accurately calculate the stenosis in B-mode as well as on the methods for diameter measurement, since suggested velocity criteria are based on NASCET method but instead for bulb stenosis measurement the ECST method is recommended.

The inclusion of direct stenosis measurement in the carotid ultrasound protocol alongside velocity parameters could potentially increase modality's accuracy and extend its potentials for assessing the disease. The size of carotid plaque and the degree of stenosis need to be accurately measured for patient monitoring in consecutive follow-ups in order to note possible disease progress or regress due to treatment. For that purpose, classification of stenosis in categories of 20% is barely

useful and since haemodynamic parameters can hardly distinguish smaller differences, addition of direct measurement could provide more precise stenosis determination. Furthermore, velocity parameters cannot accurately assess less than 50% stenosis because of limited haemodynamic effect. Therefore, most reports classify any mild stenosis as a "less than 50%", regardless if it is 40% or 20%. Moreover, direct measurement results remain independent from cardiac function, contralateral disease or proximal or distal lesions, factors that have haemodynamic impacts and affect velocity parameters. However, there are also limitations for their use, like the acoustic shadowing of severe plaque calcification which prevents plaque visualization or significant plaque echolucency which makes the plaque hardly visible and the size calculation almost impossible. Nevertheless, a combination of velocity parameters and direct measurements could counter those limitations and increase the accuracy of the investigation protocol.

Using current velocity criteria used in vascular laboratories across UK as reference, different methods of direct stenosis measurement were applied for this study and investigated for accuracy. The number and the variety of cases examined allows for quite safe conclusions. Both symptomatic and asymptomatic patients were scanned as occurs in every vascular laboratory's routine practice.

Among direct diameter measurement methods, ECST method indicated significantly better correlation with velocity criteria comparing with CCA and NASCET methods (Fig 5.4). Area measurement method also showed very good correlation with velocity criteria. These results are in accordance with published findings that suggest agreement of ECST and CCA angiographic methods with velocity criteria comparing with NASCET method (Staikov *et al*, 2000).

Stenosis grading could not be compared directly since velocity criteria only classify the stenosis in categories rather than giving a precise percentage of stenosis, so direct stenosis measurement were used for classifying the stenosis and results were examined.

In general, agreement between direct stenosis measurement and velocity criteria was greater when the stenosis was graded in three (0-49%, 50-69% and >70%) rather than in four (0-29%, 30-49%, 50-69% and >70%) categories (Fig 5.10). In three categories classification, the ECST method had the best correlation with velocity criteria comparing with the rest, with an agreement that reached 80% of results. It is noteworthy that ECST method almost equally underestimated and overestimated some cases, whereas NASCET method underestimated 30% of the cases and area measurement tended towards overestimation of the stenosis. However, when stenosis was classified with different criteria in 4 categories, all methods severely overestimated the stenosis except for NASCET which still underestimated it (Fig 5.9).

These results showed significant disagreement between direct stenosis measurement methods and velocity criteria in mild carotid stenosis. Considerable disagreement was found in cases of plaques in the bulb, where velocity criteria significantly underestimated the stenosis comparing with direct measurement findings (Fig 7.2). Keeping in mind that various studies have reported inaccuracy of velocity criteria to further classify stenosis less than 50%, the disagreement could be caused by this inaccuracy and the false underestimation of the stenosis by velocity criteria rather than by false overestimation of direct stenosis measurement methods. Another reason for the deviation between stenosis grading with velocity criteria and direct measurement methods could be cases of inability to correctly align the probe with the

vessel and use a Doppler angle of 45-60^{o} due to technical difficulties (e.g. distal bifurcation), in which case the velocity measurements could be incorrect.

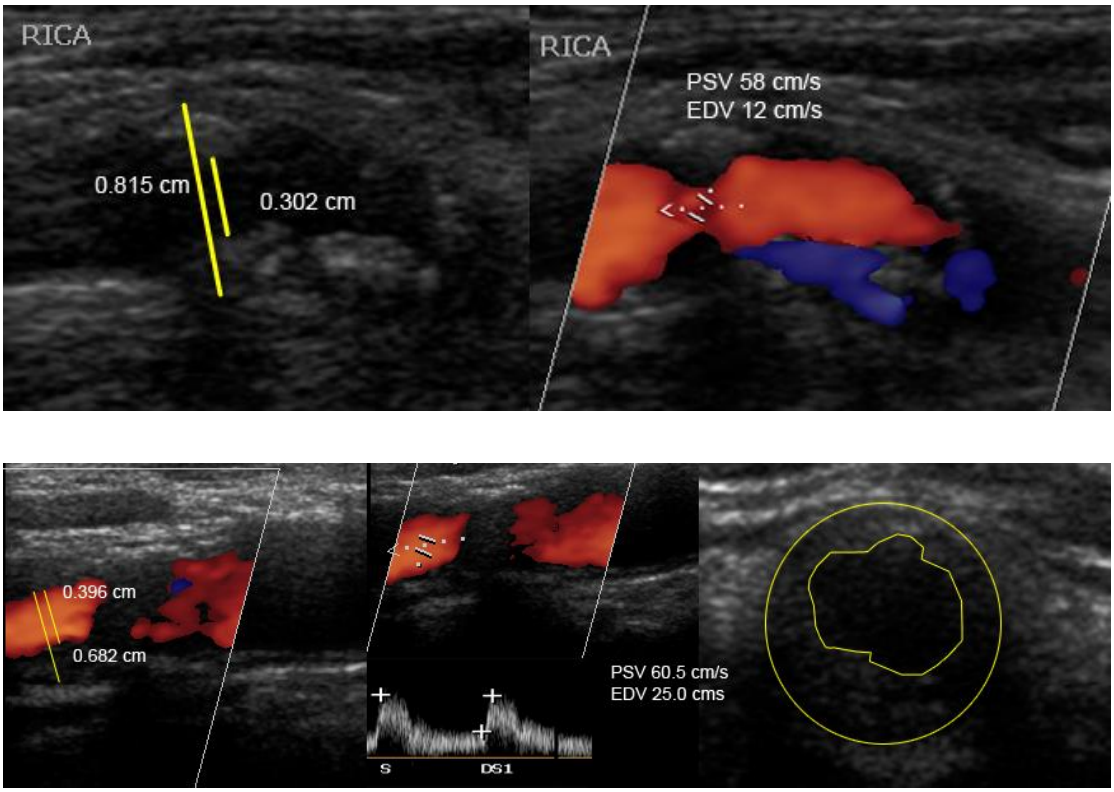


***Fig 7.2**, Examples of stenosis underestimation with velocity criteria; in the first picture, a plaque in the bulb causes more than 50% stenosis measured with ECST diameter measurement method but velocity criteria classifies it as a less than 50% stenosis, even less than 30% if Sabeti et al criteria are applied. In the second picture, a plaque in proximal internal carotid artery causes approximately 40% of stenosis measured with ECST diameter measurement method, however velocity criteria classify it as less than 30% stenosis. In the transverse plane the area stenosis of the plaque can be visualized.*

Similar comparison was executed to investigate the ability of the methods to identify severe (greater than 70%) stenosis, using current velocity criteria as reference. Among the diameter measurement methods, ECST method had the highest sensitivity, positive predictive value and negative predictive value, followed closely by CCA method. NASCET method reported minimal sensitivity but high specificity (Fig 5.14). This is explained by the fact that NASCET method severely underestimated stenosis, classifying as severe only a fraction of cases with actual severe stenosis. It is

noteworthy that area measurement method reported great accuracy in identification of severe stenosis.

In a number of cases, NASCET method resulted in a paradox of negative stenosis, because distal disease-free part of internal carotid artery was of smaller caliber than the residual lumen of proximal internal carotid artery. This was more common in cases of mild stenosis, where lumen was not severely decreased, or in case of significant artery remodeling at the point of plaque, in which case the residual lumen was larger than the implied stenosis because of the vessel enlargement. This is a major limitation for NASCET method which increases the variation of its results and explains up to a degree the disagreement with other direct measurement and velocity criteria findings.

Magnetic resonance angiography was also used as reference for direct measurement method investigation. Despite the limited number of patients, the agreement with ECST diameter measurement method was apparent compared with the rest. The mean deviation of ECST diameter measurements from MRA measurements was only 4.5%, while NASCET diameter method's measurements deviated by 28.7% (Fig 5.15). Although MRA is not a gold standard in carotid diseases investigation, it is used in case of inconclusive results from ultrasound assessment and the significant agreement with ECST method demonstrates the accuracy of the method. Comparison with digital subtractive angiography and computed tomography angiography could also provide useful information regarding the comparison of ultrasound B-mode measurement methods. Furthermore, comparison of B-mode measurement methods with other imaging modalities in greater number of patients could allow investigation of their accuracy in determining the exact degree of mild and moderate stenosis, since such an

investigation cannot be performed using velocity parameters as reference due to their low accuracy.

Retrospective analysis of the angiographic trials that introduced NASCET and ECST methods of stenosis measurement suggested that ECST and NASCET results are related with a particular formula. Using that formula, a corrected NASCET estimation for degree of stenosis was calculated and results were compared with ECST method's using velocity criteria as reference. The aim was to investigate whether NASCET method could be applied in ultrasound and provide a more accurate stenosis estimation using the particular formula rather than reporting the initial estimation as the degree of stenosis. Although the agreement of corrected NASCET estimation with velocity criteria was improved, it remained inferior to ECST method regarding grading the stenosis and identifying greater than 70% stenosis. Taking into consideration than this would be an indirect and complicated method for stenosis calculation, there is no point for such a suggestion, despite the relatively accurate results.

In summary, ECST direct diameter measurement method indicated very good agreement with velocity criteria and excellent agreement with magnetic resonance angiography in grading stenosis, particularly in cases of moderate and severe stenosis. The marked deviation in cases of mild stenosis should be further analyzed but could be attributed to the milder haemodynamic effect of this degree of stenosis which makes velocity criteria relatively inaccurate. CCA method also showed significant agreement with velocity criteria for diameter stenosis calculation, however it was inferior compared with ECST in every test. NASCET method showed major limitations and the variation of stenosis estimations is prohibitive for ultrasound application. Area stenosis could provide useful information in case of discrepancy

between velocity and diameter measurement results as it offers a different view of stenosis anatomy, but care should be taken in its application as wrong placement of the probe could lead to overestimation of the stenosis.

7.3 Plaque grey scale characteristics

Following the description of the vulnerable plaque, the plaque which usually results in symptomatic disease regardless its size, identification of such plaques using imaging modalities has been the aim of much research in the field of stroke management. Despite the fact that new modalities such as MRA, CTA and PET have shown promising results on plaque characterization, most of them are still used in research institutions rather than in clinical practice. Moreover, modern ultrasound scanners with higher resolution probes can now provide information about the plaque morphological characteristics. Given the fact that duplex ultrasound is the first and most of the times the only imaging examination of a carotid plaque, such information can prove very useful for the further patient management and risk stratification.

Plaque echolucency has been associated with soft tissue components, like lipid and haemorrhage, as well as with a high risk for symptoms, comparing with increased plaque echogenicity. Since its introduction, grey scale median has been investigated in a great number of studies but despite its positive results, no thresholds and criteria have been established, preventing it from being implemented in routine ultrasound carotid assessment protocol. Recently, other measurements, like grey scale percentile calculation or fibrous cap thickness, have been studied with mixed results. To date, quantitative characterization of the plaque is not included in the standard ultrasound carotid investigation procedure.

Common carotid intima-media thickness is a well established atherosclerosis marker, with particular significance for carotid atherosclerotic disease. Investigating intima-media grey scale characteristics, intima-media GSM showed an association with plaque GSM, as mean intima-media GSM was higher in plaques of high echogenicity, however the difference was not found significant in this study (Fig 6.6). In a recent much larger study conducted in Uppsala, Sweden, which enrolled more than 1,000 patients, a good correlation between plaque and intima-media GSM was reported (Lind *et al*, 2007). Such an association seems logical, since deposition of lipoproteins, which is a major factor for plaque echogenicity, occurs along the whole artery and is responsible for intima-media thickening and plaques forming in other points of arterial network. This is confirmed by the significantly lower intima-media GSM found in patients with high blood cholesterol levels (Fig 6.8). Recent studies have also reported association of intima-media echolucency with mortality, independently from its thickness (Wohlin *et al*, 2009). Considering that intima-media selection using ultrasound is much quicker and simpler comparing with plaque outline and selection and can be done automatically nowadays in newer ultrasound software, intima-media GSM could be further investigated as a technique for lipid subintimal deposition assessment and as a potential cardiovascular risk marker.

Looking at the plaque itself, first order grey level parameters were tested for association with visual subjective characterization regarding echogenicity and homogeneity. Several studies have showed the ability of plaque GSM to objectively distinguish echolucent and echogenic plaques and results from this study are similar with literature findings. A GSM value of 35 indicated excellent accuracy in identifying echolucent plaques, which agrees with previously published GSM thresholds. Moreover, plaque variance was significantly lower in echolucent plaques

and showed good accuracy in distinguishing echolucent from echogenic plaques. It is noteworthy that an association, however not statistically significant, was found between plaque size and echogenicity, showing lower GSM in larger plaques. This could be mean that plaques with lower echogenicity tend to increase in size and result in more severe stenosis or that the plaque echogenicity characteristics change as it grows. The latter explanation agrees with recent studies which suggest association of plaque shear stress, which is depended on plaque size and morphology, with plaque development and composition (Cheng *et al*, 2006; Li *et al*, 2008). A study that would follow up patients with mild stenosis and compare plaque ultrasound characteristics as the disease progresses would provide more details about this association.

As far as homogeneity is concerned, grey scale value standard deviation was found higher in plaques visually classified as heterogeneous, however the difference was reported marginally insignificant. Nevertheless, a higher pixel grey scale value standard deviation can be expected in heterogeneous plaques, since grey scale heterogeneity means greater variation of pixel grey scale values, hence greater deviation from the mean. If such an association is established in future studies, this parameter could be potentially used for objectively commenting on plaque homogeneity, which would be useful for assessing risk for symptoms.

Calculating the percentage of pixels with grey scale value up to or over a threshold has been suggested as another way to assess plaque echogenicity in addition to overall plaque GSM calculation (Elatrozy *et al*, 1998a). Percentages of plaque area (percentiles) with grey scale value less than a number of thresholds, ranging from 20 to 40, were calculated and compared between symptomatic and asymptomatic plaques. All investigated percentiles were significantly higher in symptomatic plaques, but P35 and P40 (percentages of area with grey scale value up to 35 and 40

respectively) indicated great accuracy in distinguishing symptomatic patients. Considering the ease of the method, which requires just the outline of the plaque from the operator, percentile calculation could potentially be used for further characterization of the echogenicity of carotid plaque.

Thin fibrous cap is one of the characteristics of the vulnerable plaque and ongoing research investigates methods to assess it. Although ultrasound lacks the imaging resolution of magnetic resonance imaging, studies have shown that fibrous cap thickness (FCT) can be accurately measured in ultrasound. Plaque fibrous cap was found significantly lower in symptomatic patients (Fig 6.12) and a FCT value of 300 μm could accurately distinguish symptomatic from asymptomatic plaques (area under the curve 0.831). Recent studies have concluded in similar findings and have confirmed ultrasound measurements with histological analysis, suggesting that a vulnerable plaque has a minimum FCT < 200 μm and a FCT at the most representative part lower than 500 μm (Redgrave *et al*, 2008). Despite the fact that measurements of such caliber can be very challenging in ultrasound and could suffer from interobserver variability, advances in image resolution and ultrasound software could allow easier, even automated, and accurate measurement of fibrous cap thickness.

Regarding fibrous cap echogenicity, a correlation was found with plaque echogenicity and plaque regularity in morphology, however not statistically significant. As far as symptoms are concerned, in theory, a fibrous cap with diffuse calcification, hence more echogenic would correlate with more stable and asymptomatic plaque, comparing with fibrous cap with only nodules of calcification, a characteristic that has been associated with plaque vulnerability. However, no difference was found in

fibrous cap echogenicity between symptomatic and asymptomatic patients, but further research could shed more light on the subject.

Correlation of plaque echogenic characteristics with plaque tissue components has been established in studies comparing ultrasound images with histological analysis of post endarterectomy specimens. However, it appears that other characteristics of the vulnerable plaque, like fibrous cap thickness and plaque heterogeneity can also be assessed with ultrasound with promising results. Advances in ultrasound will offer the possibility of more accurate plaque composition and morphology assessment, providing information that would help decide upon disease prognosis and management.

Further studies, comparing ultrasound findings with histological analysis could confirm associations and establish methods for ultrasound investigation of vulnerable plaque. Furthermore, reproducibility as well as intraobserver and interobserver agreement of these methods should be examined, because plaque and fibrous cap selection can be very challenging and result in great variation between operators. Last but not least, all the grey level analysis is performed in previously normalized images, which is also done manually, lurking the danger for variability and irreproducibility. In case future ultrasound softwares implement techniques for image texture analysis, they should also implement automated normalization method, possibly by storing a duplicate image in the default machine's settings, additionally to the image using the current settings operator has set for optimal visualization. This way the manual normalization could be avoided.

7.4 Secondary results

7.4.1 The PSV_{ICD} ratio

Despite the fact that the investigation of velocity parameters was not a primary aim of the study, the collected data allowed the statistical analysis of the various measured velocity parameters and ratios. Apart from the PSVR and St. Mary's ratio, a third ratio was calculated by dividing the PSV in internal carotid artery with the PSV in distal non diseased internal carotid artery (PSV_{ICA}/PSV_{ICD}), named PSV_{ICD} ratio. The principle behind this ratio is the comparison of blood flow velocities at different points of the same vessel, where the vessel caliber is similar and the accommodated blood volume is the same. Like all ratios, it would be independent of more proximal, distal disease or contralateral disease and heart disease, as well as independent of external carotid artery disease, which can affect the systolic and diastolic velocity at common carotid artery and hence the other two ratios.

The PSV_{ICD} ratio showed good correlation with the degree of stenosis calculated with direct measurement methods as well as current velocity criteria. Comparing with PSVR and St. Mary's ratio using established threshold values, a PSV_{ICD} value of 2.0 showed greater sensitivity comparing to the first and greater specificity than the latter, indicating almost excellent accuracy in identifying greater than 70% stenosis. Although accuracy was significantly lower in distinguishing mild (<50%) from moderate (50-69%) stenosis using value 1.0 as a threshold, sensitivity and specificity indices were similar to those of the two currently used ratios.

The reproducibility and accuracy of the PSV_{ICD} ratio remains to be tested in further studies, favorably using other angiographic like methods as reference for degree of

stenosis estimation. Its accuracy in categorizing stenosis in 10% ranges should also be investigated and should it proves accurate enough, it could potentially be used in the future in revised velocity criteria as an additional parameter. However it should be noted that the recording of flow in distal internal carotid artery is sometimes technically difficult due to anatomical reasons, such as high carotid artery bifurcation or vessel tortuosity. This is a limitation of this parameter comparing to the other ratios which compare proximal internal carotid artery velocities with common carotid artery velocities.

7.4.2 Score system for plaque vulnerability

Having scanned both symptomatic and asymptomatic plaques and measured several plaque characteristics, from haemodynamic effects of stenosis to plaque grey scale median and fibrous cap thickness, a combination of a number of characteristics was investigated for ability to distinguish potentially symptomatic from asymptomatic plaques. Four characteristics were tested and included in a scoring system; percentile 40, diameter of stenosis (using ECST direct measurement method), fibrous plaque thickness and intima-media thickness. Threshold values were set using ROC curves and crosstabs analysis for each characteristic and score points were assigned (Table 6.6).

Three categories were defined, according to plaque total score; low, increased and high risk for stroke. Statistical analysis indicated greater odds ratio and relative risk for stroke for increased and high risk category comparing to low risk plaques, showing good accuracy for the score system in identifying potentially symptomatic plaques.

It is noteworthy that the relative risk for each plaque characteristic separately was significantly lower compared to score system relative risk, which shows that the combination of vulnerability characteristics in a score system might have greater diagnostic value in the identification of the vulnerable plaque.

A limitation of this score system is the inclusion of fibrous cap thickness measurement, which can be challenging in many cases, whereas in some plaques cannot be visualized at all. However, in the cases where it could be measured, it significantly increased the specificity and the accuracy of the score system.

Also, the particular score system uses percentile 40 rather than other more sophisticated image texture analysis parameters. The reason for that is that in this study other grey level parameters failed to show significant difference regarding patient symptomatology. Should such difference be proved in further studies for other image analysis parameters associated with plaque vulnerability, they could be introduced in a more complex score system.

Furthermore, the score system was created retrospectively, looking on patients with known clinical status, but was not tested prospectively in patients. Further research that would include patient follow up is necessary to assess score system accuracy and diagnostic value. Ideally, future ultrasound software will be able to calculate several plaque and stenosis parameters and provide a possibility for plaque vulnerability and patient risk.

7.5 Preliminary immunohistochemistry study for identification of ultrasound image characteristics of carotid plaque inflammation and neovascularization

Plaque neovascularization and inflammation are today considered as major factors for atherosclerotic plaque vulnerability and ways to assess those using imaging modalities are investigated. Magnetic resonance imaging and positron emission tomography have shown good results in identification of both factors, while contrast enhanced ultrasound has also been tested for assessing predominantly plaque neovascularization and secondary plaque inflammation with promising results. Duplex Ultrasound, without the use of contrast agents, has not been used to date for assessing inflammation and angiogenesis and grayscale characteristics of plaques regarding those two factors have not yet been identified.

In order to define grey scale characteristics of plaque inflammation and neovascularization, a study has been designed to compare ultrasound image texture analysis with immunohistochemistry analysis of post endarterectomy specimens using CD68 and CD31 for inflammation and neovascularization assessment respectively. The study protocol and preliminary data are presented below.

7.5.1 Patient enrolment

Patients with diagnosed severe carotid atherosclerotic disease who were considered for endarterectomy by vascular surgeons at Charing Cross Hospital, Imperial College London NHS Trust, were enrolled to the preliminary study and had a carotid duplex ultrasound scan prior to decision for optimal treatment. Study inclusion criteria were

symptomatic patients (recent TIA or stroke episode) with previously diagnosed severe carotid artery stenosis.

In total 20 patients were scanned and images were taken from one carotid artery of each. From those, eight patients underwent carotid endarterectomy (median 15.5 days after the ultrasound scan, mean carotid stenosis 67.8 ± 10.1%) and plaque specimens were collected and analyzed.

7.5.2 Examination protocol and image analysis

A Philips IU 22 ultrasound scanner was used for carotid scanning, with a 5-12 MHz linear array probe. Patients were examined lying supine and the carotid artery that was known to have severe stenosis was scanned using the anterolateral projection. After the identification of the plaque, depth of image, focus, total gain and time gain compensation settings were altered to achieve better visualization.

Applying the standard carotid duplex ultrasound scan protocol, images of common, internal and external carotid were taken and velocity tracings were recorded for degree of stenosis estimation. Following that, the protocol described in chapter 6.3.2 was followed and several pictures of the carotid bifurcation and the internal carotid artery were then taken in longitudinal and transverse plane in B-mode and Colour Doppler mode.

Images were transferred in in-lab PCs for editing and analysis. Using Adobe Photoshop CS5 and Curves function, images were standardized using the method described in chapter 6.4.1. Then, using histogram function GSM and grey scale standard deviation was calculated for the whole plaque (method described in chapter

6.4.3). Mazda software was then used for calculation of grey level parameters (Variance, Skewness and Kurtosis) using the method described in chapter 6.4.5.

7.5.3 Carotid endarterectomy surgery

For some of the patients participated in the study carotid endarterectomy surgery was selected as the optimal treatment.

The operation usually is performed under general anaesthesia. It involves an incision along the anterior border of sternocleidomastoid muscle and lateral retraction of the muscle and internal jugular vein for carotid artery exposure. Distal internal carotid artery, well after the plaque point, is mobilized and clamped off and a shunt is put to maintain cerebral blood flow. If patient is under local anaesthesia, artery can be temporarily occluded rather than placing a shunt and adequacy of compensated cerebral blood flow can be checked by patient's ability to move hands or feet or talk. Common carotid artery and distal external carotid artery are also clamped and artery is dissected, starting from internal carotid artery and continuing circumferentially. Dissection is then continued in proximal external carotid artery and any plaque is removed. In the end, vessel wall is closed using sutures or vein patches, and cerebral blood flow is restored after clamp removal.

7.5.4 Specimen immunohistochemistry analysis

Following patients' carotid endarterectomy surgery, plaque specimen was collected and analyzed using immunohistochemistry methods at the Histopathology Department of Charing Cross Hospital.

Tissue embedding and cryosectioning

Plaque regions were placed within optimised cutting temperature embedding matrix upon 3mm thick cork discs and were rapidly frozen using Frostbite (Surgipath) before inserting into a bath of 100% isopropanol surrounded by dry ice to snap freeze.

Using Leica CM1900 UV cryostat (Leica Microsystems) at -25 ± 1 ^{o}C for cryosectioning, 5μm sections of plaque region were transferred in slides. Slides were labeled depending if the section belonged to the top edge, medium or bottom part of the plaque.

Immunohistochemistry analysis

Immunohistochemistry protocol, based on the Standard Operating Procedures and implemented within Charing Cross Hospital Department of Histopathology, was followed for the analysis of the plaque specimens.

Frozen tissue sections were fixed in acetone at room temperature for 10 minutes. Standard Avidin-Biotin Complex immunohistochemistry technique was used. This method involves three layers of antibodies; the primary antibody is the study maker, CD31 for endothelium and CD68 for macrophages. The second is the biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase. Avidin is a glycoprotein that can be labelled with peroxidase or fluorescein (chromogen) with high affinity to biotin. Due to that affinity, the first layer is amplified at the second and third layer, increasing the sensitivity of the method

Chromogen diaminobenzidine SK-4100 (Vector Laboratories) was added for antibody/antigen complex visualization. Diaminobenzidine produces a brown precipitate which is insoluble in alcohol in the presence of hydrogen peroxide.

Five millilitres of distilled water, 2 drops of buffer stock solution, 4 drops of diaminobenzidine stock solution and 2 drops hydrogen peroxidase were mixed and added to each slide for 5 minutes. Slides were then immersed in running tap water. Surgipath Harris's haematoxylin followed by 0.3% acid was applied so the cell nuclei were stained blue.

The slides were then subjected to serial 70% and 90% alcohol baths and finally completely dehydrated with 99% Industrial Methylated Spirits (IMS). The alcohol was removed using xylene and the sections were mounted in Pertex mountant with 50 x 35 mm glass coverlips on Leica Auto-coverslipper.

Pictures of the stained slides were taken for further analysis. For each plaque, three to four pictures from bottom, mid and top parts were taken.

CD31-Platelet Endothelial Cell Adhesion Molecule-1

Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), also referred as CD31, is a transmembrane glycoprotein adhesion molecule with a molecular mass of 130 kD which is highly expressed at the lateral borders of vascular endothelial cells and weakly expressed on peripheral leukocytes and platelets. Along with vascular endothelial cadherin (VE-cadherin) and vascular endothelial growth factor receptor 2 (VEGF-R2) forms a shear stress responsive complex, activated by acute onset of laminar blood flow. CD31 expression has been detected on endothelial cells of atherosclerosis-prone aorta and within neovascularisation regions of atherosclerotic plaques (Li *et al*, 2006; Galkina & Ley, 2007). CD31 immunohistochemistry staining has been used for identification of vasa vasorum in arterial wall (Burtea *et al*, 2008). In the study, monoclonal mouse anti-human antibody clone JC70A (DAKO, Glostrup, Denmark) was used.

CD68

Monoclonal antibodies against CD68 are widely used specific markers for macrophage immunotyping (Kunisch *et al*, 2004; Micklem *et al*, 1989). CD68 is a heavily glycosylated transmembrane protein of 354 amino acids with molecular mass of 110 kD, located mainly in lysosomal membranes of human monocytes and tissue macrophages (Holness & Simmons, 1993). Antibodies against CD68 have been used for identifying macrophages in a variety of human tissues, including the red pulp of spleen, the lamina propria of the gut, the lung alveoli and the bone marrow. In the study, monoclonal mouse anti-human CD68 antibody clone PG-M1 (DAKO, Glostrup, Denmark) was used.

Immunohistochemistry image analysis

The images of the immunohistochemistry analysis were edited and processed for CD31 and CD68 expression using Image Pro Plus 6.0.

Images were loaded into the software and were enhanced using contrast enhancement function (Fig 7.3) for the best differentiation between marker expressions (dark brown due to diaminobenzidine chromogen), cell nuclei (blue spots due to haematoxylin staining), plaque calcification (dark blue areas due to haematoxylin staining) and matrix (Fig 7.4).

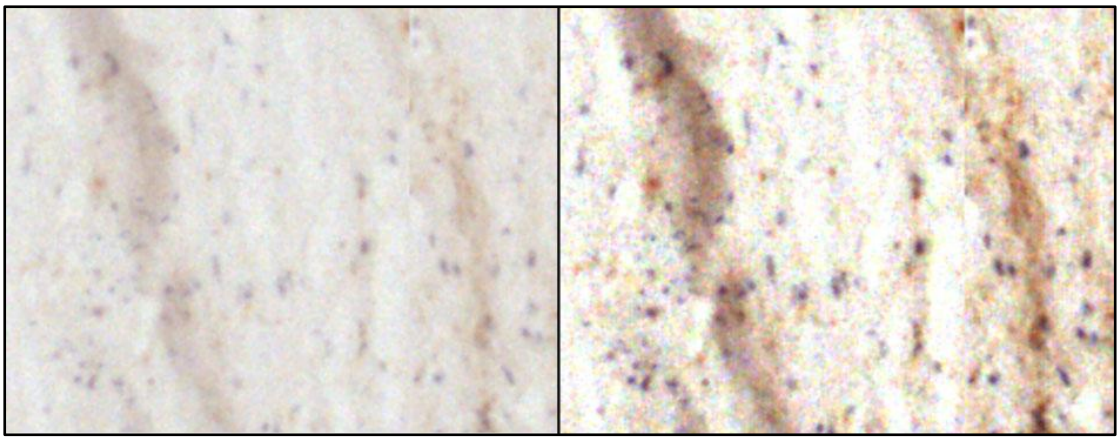


***Fig 7.3**, Original (left) and post enhancement (right) image using Image Pro Plus*

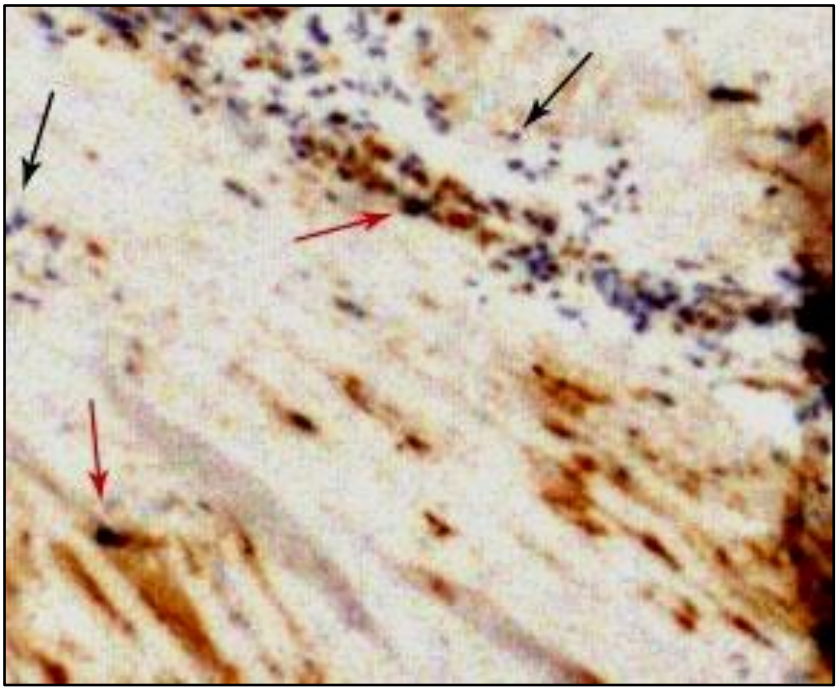


***Fig 7.4**, Immunohistochemistry image of plaque specimen using CD68 antibody and haematoxylin staining. Cell nuclei are stained blue (black arrows) and antibody/antigen complex is stained dark brown (red arrows)*

Macrophage infiltration quantification

Six regions of interest (dimension 200x200 pixels) were randomly selected for each CD68 slide. Using the Count/Measure function and histogram and eye drop tools, areas of marker expression were highlighted and selected for object and area measurement (Fig 7.5). Means were calculated from all regions of interest from all

slides of each plaque and plaques were classified between low and high marker expression.

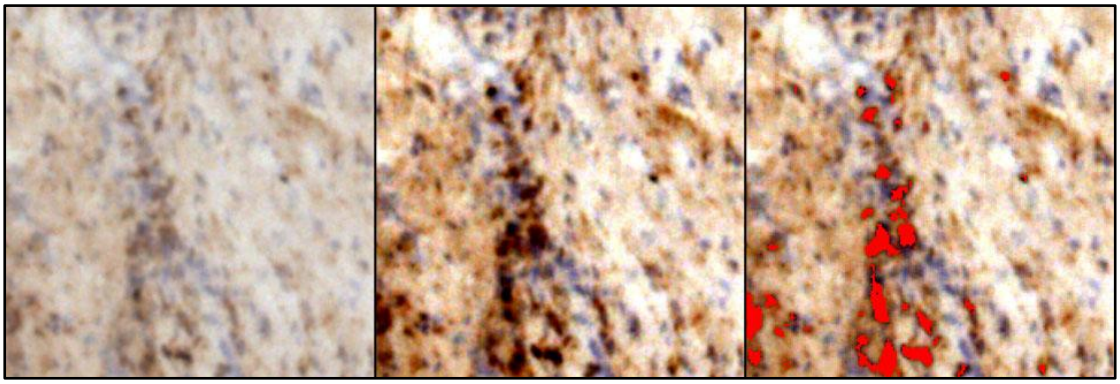


***Fig 7.5**, Macrophage infiltration quantification method in plaque specimen using CD68 antibody and immunohistochemistry technique and Image Pro Plus for image enhancement and analysis. Left: region of interest (ROI) of 200x200 pixels of the original picture captured from the microscope; Middle: contrast and brightness enhanced picture of the same ROI; Right: selection and measurement of antibody/antigen complex area at the same ROI*

Plaque angiogenesis quantification

For CD31 slides, microvessels were identified as round holes in the plaque matrix with high marker expression in the perimeter (Fig 7.6). The presence and number of microvessels identified in each slide was measured and each plaque specimen was classified between low and high neovascularisation.

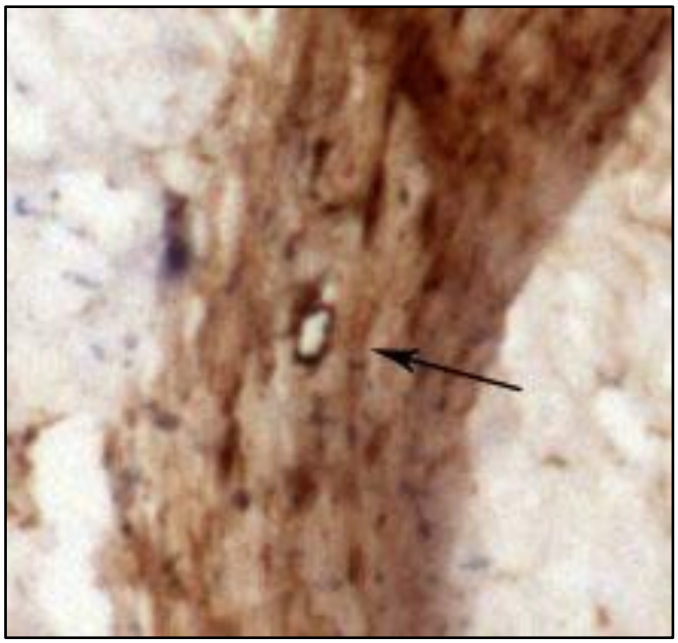


***Fig 7.6**, Microvessel (black arrow) identified in an immunohistochemistry analysis image of carotid plaque specimen with use of CD31 antibody*

Calcification quantification

In both CD31 and CD68 slides, calcified areas at plaque specimens were identified as dark purple areas because of haemartoxylin staining (Fig 7.7). Count/measure function and histogram tool were used for selection and measurement of these areas and plaques were classified for absence or presence of calcification.

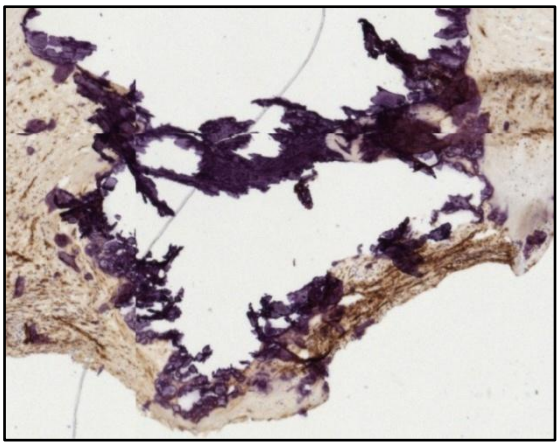


***Fig 7.7**, Calcified areas at a plaque specimen stained with haematoxylin*

7.5.5 Comparison of histoimmunochemistry analysis results with ultrasound image texture analysis

Inflammation assessment

Plaques specimens with increased macrophage infiltration were identified with immunohistochemistry methods using CD68 antibody as a marker. Two groups were formed, for low and high antibody/antigen expression. Grey scale parameters for ultrasound images were compared between the two groups and examined for significant difference.

Among the parameters examined, plaque's ultrasound image kurtosis was found to be greater (mean 0.49 ± 1.03) in patients whose plaque specimen indicated higher CD68 expression than in patients whose plaques showed lower expression (mean -0.30 ± 0.28) (Fig 7.8).

Kurtosis was normally distributed between the two groups (Shapiro-Wilk test p value > 0.05) and independent-samples T-test was used for investigation of the significance of difference. Test rejected statistical significance of the difference (p value > 0.05).

Other grey scale parameters showed no difference between plaques with high and low macrophage infiltration.

It is noteworthy that patients whose plaques indicated a higher CD68 expression on the immunohistochemistry analysis had slightly higher, though not statistically significant, values for White Cell Count and Neutrophile Count. No other difference as far as blood test results or medication was traced between the two groups.

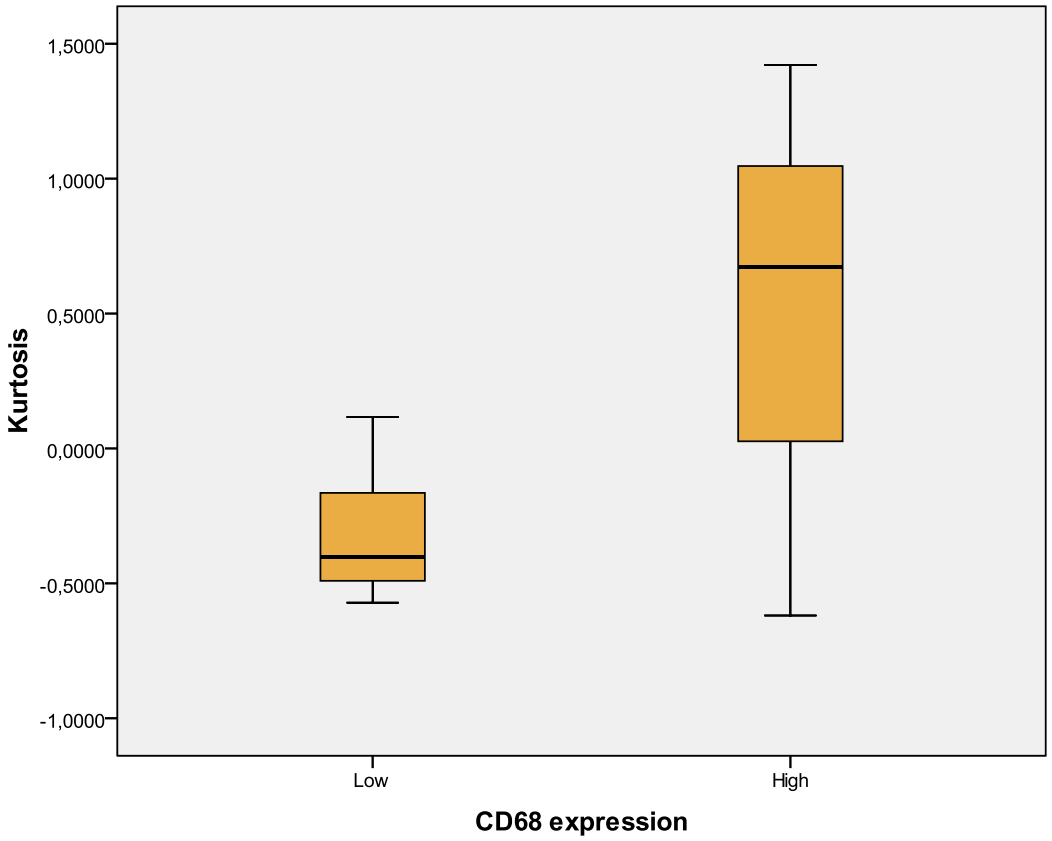


***Fig 7.8**, Kurtosis of ultrasound image of plaques showing high and low CD68 expression in immunohistochemistry analysis*

Neovascularization

Plaque specimens were classified in two groups for low or high neovascularization using CD31 antibody and immunohistochemistry techniques. Ultrasound image analysis grey scale parameters were computed and compared between the two groups.

Plaques with greater degree of neovascularization were found to be significantly more echolucent (mean GSM 50 ± 29.4) comparing to those with low angiogenesis (mean GSM 77 ± 2.8, Mann-Whitney test p value < 0.05) (Fig 7.9).

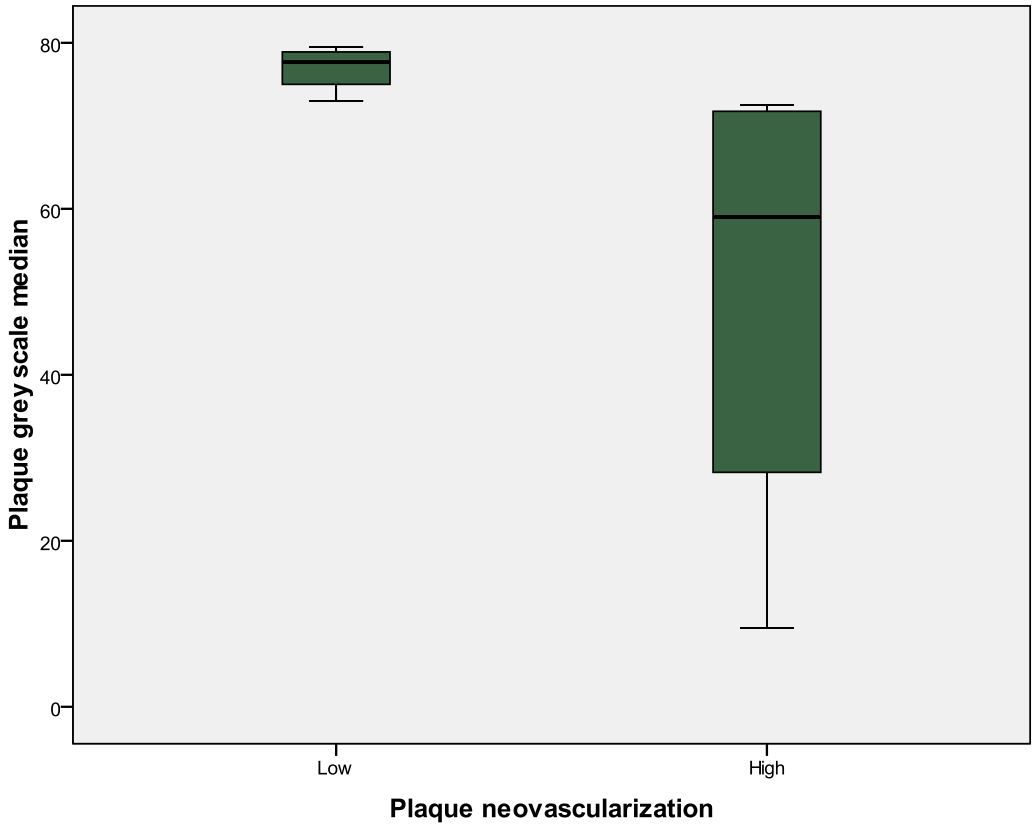
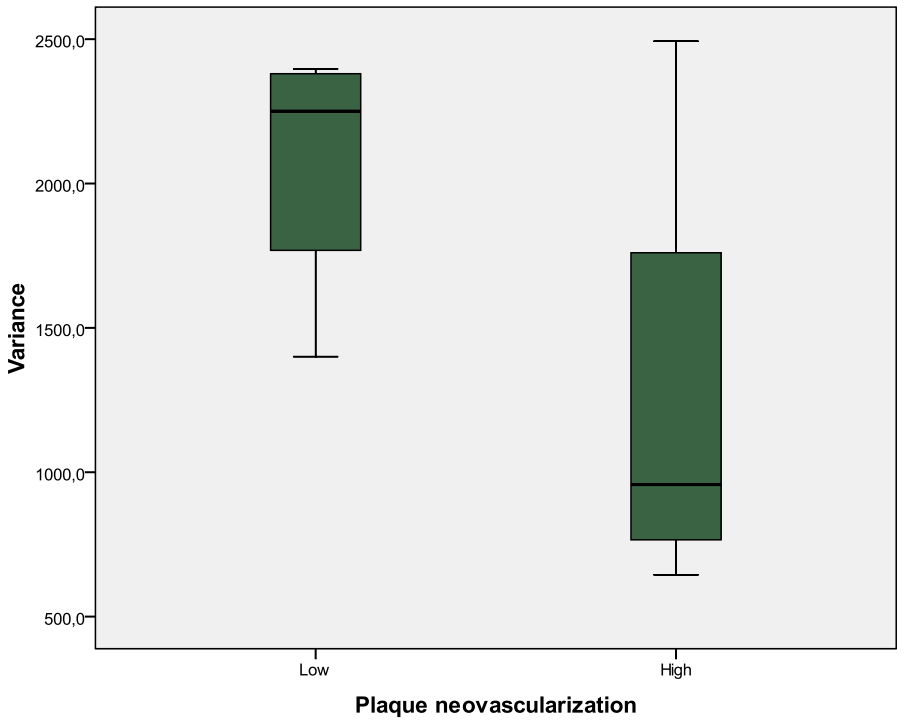


Fig 7.9 *Boxplot of plaque grey scale median in plaques with high and low neovascularization, as they were classified from immunohistochemistry analysis*

Among the other grey level parameters investigated for the two groups, plaque variance was found lower (1263 ± 835) and plaque skewness higher (0.699 ± 0.48) for plaques with more significant neovascularization plaques with greater neovascularization than for plaques with mild angiogenesis (2074 ± 464 and 0.453 ± 0.1 respectively) (Fig 7.10). However, the differences were reported statistically insignificant (Mann-Whitney U test p value > 0.05).



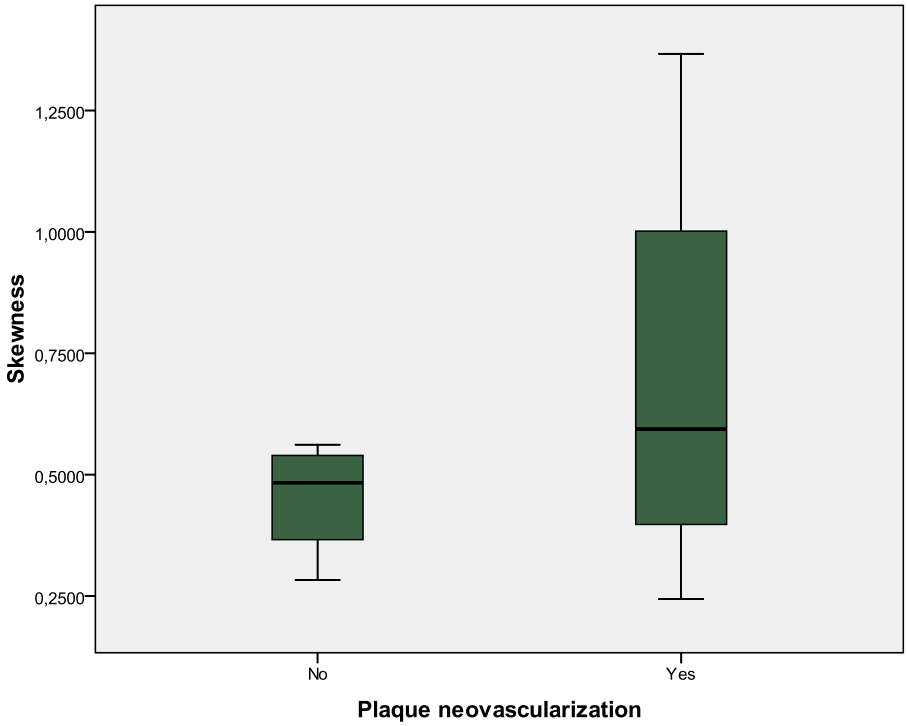


Fig 7.10, *Boxplots of variance and skewness computed from ultrasound image analysis for plaques with high and low angiogenesis as they were classified using immunohistochemistry techniques and CD31 antibody*

No association was found between degree of diameter and area stenosis caused by the plaque and degree of plaque neovascularization. Furthermore, no significant difference was found regarding blood test results and medication between the two groups.

Calcification

Plaque calcification was identified in carotid plaque specimens using haematoxylin staining. Grey level parameters computed from ultrasound images were compared between patients with calcified and non calcified plaques for significance of difference.

Calcified plaques were found to be more echogenic (mean GSM 74.4 ± 2.6) than non calcified plaques (mean GSM 32.3 ± 25.6) (Fig 7.11). Mann-Whitney test results indicated significance of difference (p value < 0.05).

ROC curve was calculated for GSM value to investigate the ability to differentiate calcified and non calcified plaques. Area under curve was 1.000 (excellent) and GSM cut-off value of 65 indicated 100% sensitivity and 100% specificity for identifying calcified plaques.

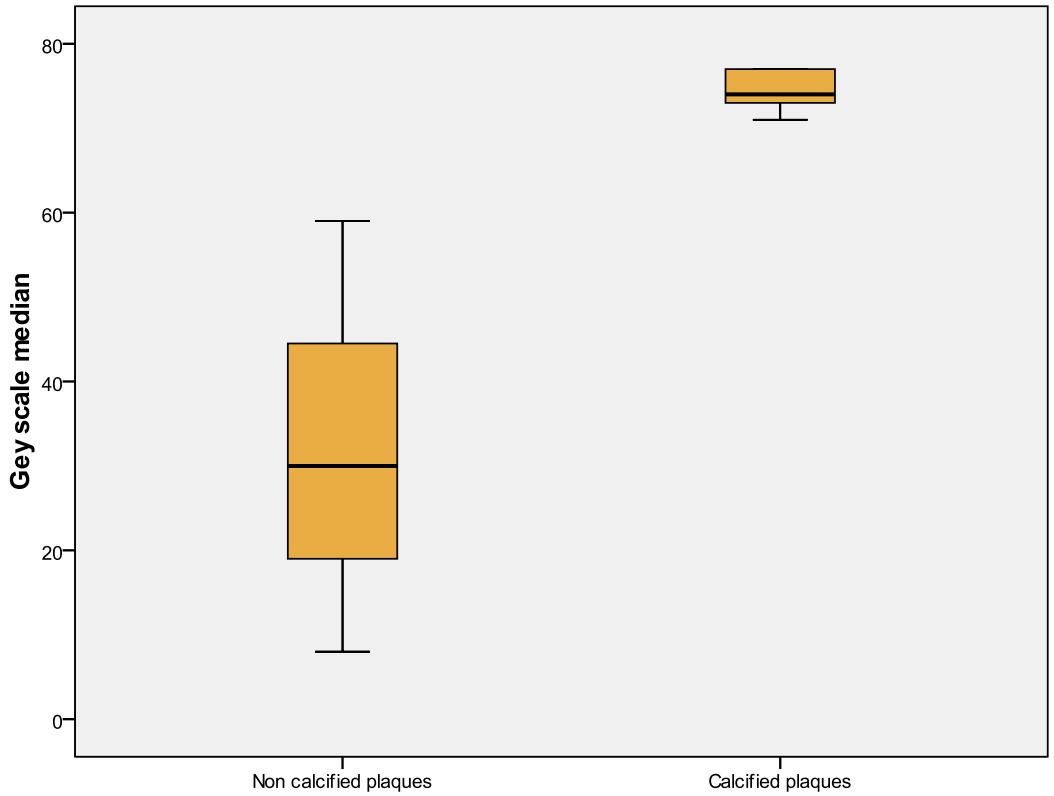
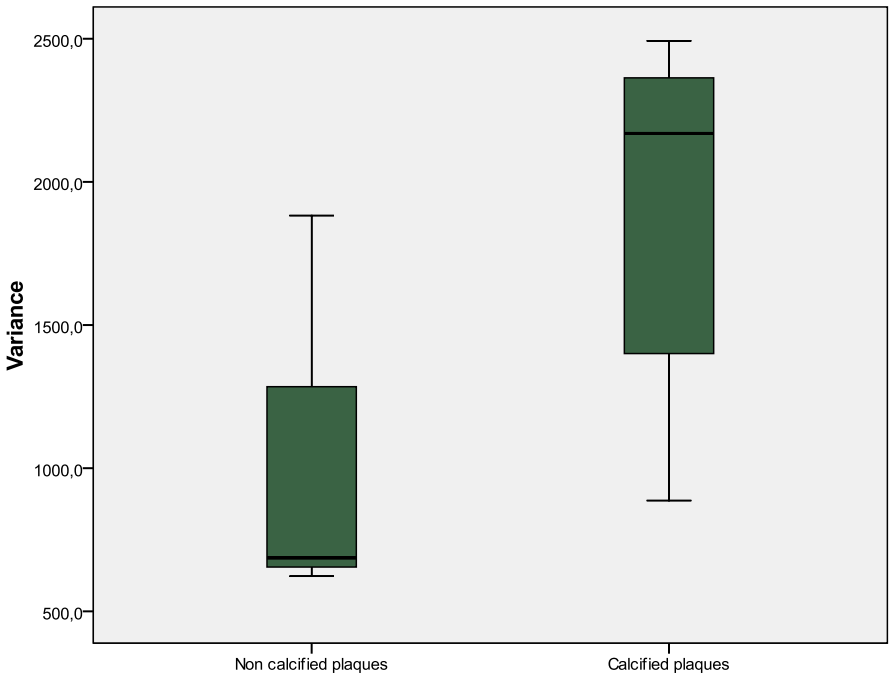


***Fig 7.11**, Boxplot of grey scale median value of ultrasound image of calcified and non calcified plaques, as they were classified from immunohistochemistry analysis*

Images of calcified plaques were also found having greater variance and lower skewness than those from non calcified plaques (Fig 7.12) but nonparametric tests rejected statistical significance of difference.

No difference was reported for other calculated image parameters.



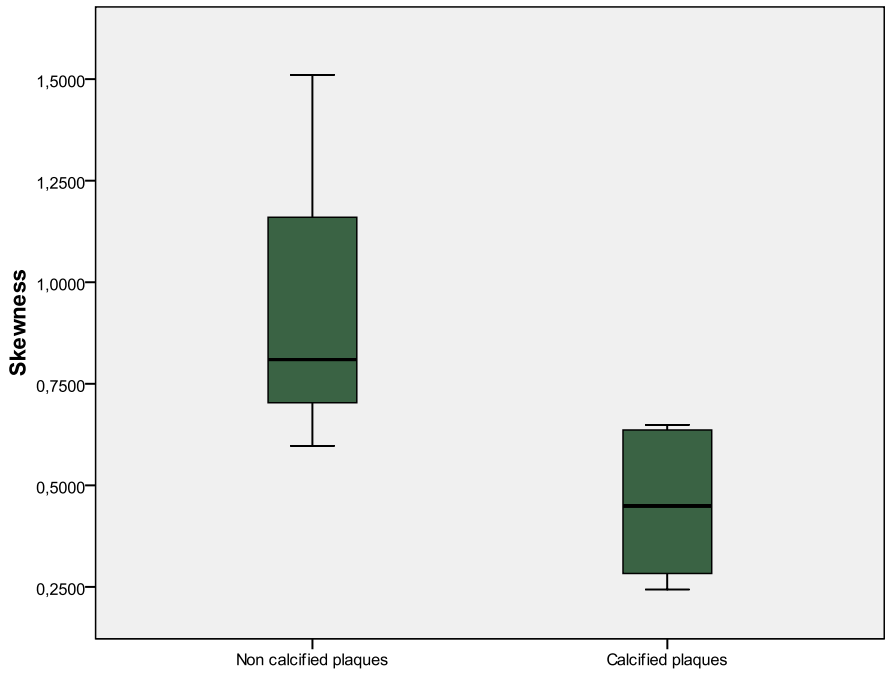


***Fig 7.12**, Boxplots of variance and skewness of ultrasound image of calcified and non calcified plaques, as they were classified from immunohistochemistry analysis. Nonparametric tests rejected significance of difference.*

7.5.6 Conclusions

Plaque inflammation and neovascularization have both been associated with plaque vulnerability in coronary and carotid atherosclerotic plaques and there is ongoing research on imaging and quantifying those characteristics for early and accurate identification of unstable plaque. The aim of this preliminary study was to investigate ultrasound image grey scale characteristics of carotid plaque inflammation and neovascularization, by comparing immunohistochemistry analysis of post endarterectomy specimens with ultrasound image texture analysis of carotid plaque. Despite the limited number of data, some useful conclusions can be drawn regarding plaque inflammation and neovascularization grey scale characteristics that could potentially lead in further research.

Plaque angiogenesis was well associated with plaque echogenicity and plaques with greater number of microvessels had a significantly lower plaque grey scale median (Fig 7.8). It is noteworthy that both low and high angiogenesis groups had no significant difference in cholesterol and triglyceride blood levels or in cholesterol treatment, so the assumption that increased angiogenesis contributes in plaque echolucency independently of plaque lipid deposition might be solid. The association of plaque echolucency with angiogenesis has also been recently suggested by studies performing both standard and contrast enhanced carotid ultrasound, in which plaques which in standard ultrasound appeared echolucent showed increased enhancement in contrast enhanced ultrasound comparing with the echogenic plaques (Staub *et al*, 2011). However, histological analysis that would indicate the equality of lipid core and hence the independent role of angiogenesis in plaque echolucency remains to be done in future studies.

Despite the fact that the difference of the other grey level parameters between plaques with high and low neovascularization was not found statistically significant in the preliminary data analysis, it is noteworthy that plaques with greater neovascularization were characterized by lower variance and higher skewness in ultrasound. A larger study could establish or reject such associations.

Regarding the inflammation of carotid plaques, ultrasound image texture analysis reported a greater kurtosis in plaques whose histological analysis showed higher CD68 expression, hence greater inflammation. Specifically, kurtosis was predominantly positive in inflamed plaques and negative in plaques with low degree of inflammation. However statistical analysis rejected the statistical significance of the difference and research is needed for further investigation. No association was reported between degree of inflammation and other grey level parameters.

Histology studies that examine carotid plaque composition usually require plaque decalcification in order to further assess the specimen for lipid core and intraplaque haemorrhage quantification. In those studies plaque calcification cannot be accurately measured but only estimated from the volume of missing tissue. Cryosectioning technique used in this study enabled cut of plaque specimen without preceding decalcification, allowing plaque calcification identification and quantification using haematoxylin staining. Generalized plaque calcification is considered as a stability marker for carotid atherosclerotic plaque, comparing to spotty calcification that has showed association with plaque vulnerability. Furthermore, degree of plaque calcification plays an important role on selection of revascularization treatment, since heavy lesion calcification is considered as a contra-indication for carotid angioplasty and stenting.

Comparing ultrasound image analysis results with specimen histological analysis for calcification assessment, plaque grey scale median was found significantly higher in calcified plaques. Using a value of 65 as a threshold, this technique showed excellent sensitivity and specificity in distinguishing calcified from non-calcified plaques. Should this method be evaluated in larger studies, this easily acquired parameter could be used to decide upon plaque calcification deposition of atherosclerotic plaque. The preliminary data analysis also indicated an association between calcified plaques and lower skewness and greater variance of ultrasound images; however the association was not statistically significant. A larger study could provide the necessary data for confirmation or rejection of such an association.

7.6 Future research recommendations

Over the last decades there has been considerable evolution in ultrasound technology and great amount of research on the potential of ultrasound in providing more accurate stenosis assessment as well as qualitative and quantitative details about carotid plaque. However, clinical evidence suggests that carotid atherosclerotic disease approach with ultrasound has not significantly changed, failing to incorporate modern research results.

The haemodynamic effect of stenosis is valuable clinical information and current ultrasound investigation is predominantly based on this by estimating degree of stenosis through velocity parameters. However, there are cases where detailed measurements regarding the size of plaque in longitudinal and transverse views as well as the exact stenosis measured directly in B-mode and Colour Doppler images could more accurately describe the extent of the disease, alongside haemodynamic information. This study has indicated that ECST method of direct measurement when

applied in ultrasound could potentially be an accurate alternative method for stenosis measurement, showing good correlation with velocity criteria and MRA measurements. Larger studies, that would enroll a greater number of patients and assess the stenosis with more imaging modalities, such as digital subtractive angiography and three-dimensional ultrasound, could allow a better evaluation of direct measurement approach for deciding upon its inclusion in the standard carotid ultrasound imaging protocol.

Regarding plaque composition, numerous studies have shown correlation of grey level analysis results with particular tissue components, whereas modern research has looked on the use of second order statistics for plaque characterization and changes monitoring related to treatment. Further research is needed for confirming these results and incorporating them in ultrasound software, in a way that future ultrasound machines can offer plaque mapping with compositional details.

This study's results suggest potential association of grey level statistics with inflammation and neovascularization. However, the small number of patients prevents any solid conclusions. Future studies which would enroll greater number of patients, apply more sophisticated image texture analysis and use immunohistochemistry analysis for plaque inflammation and neovascularization assessment of post-endarterectomy specimens are required for establishment or rejection of the association. In case of confirmation of such association, incorporation of this texture analysis in ultrasound software would allow even better identification of vulnerable plaque.

Modern research has also focused on fibrous cap assessment for plaque risk of rupture stratification. This study's results indicate a strong association of fibrous cap thickness measured in ultrasound and symptomatic disease, which has been suggested

by histological studies. However, the reproducibility of the method was not assessed, while the number of plaques that allowed measurement of fibrous cap thickness was limited. A small number of published studies recommend methods for ultrasound assessment of fibrous cap, however there are doubts about the accuracy of measurement due to technical limitations (Devuyst *et al*, 2005). Evolution of ultrasound technology and improvement of image resolution could allow accurate identification and measurement of fibrous cap and further research is recommended for establishment and evaluation of methods to assess it. Moreover, analysis of the grey level of fibrous cap could potentially be another way to assess plaque vulnerability and more studies are required to elucidate this aspect.

Intima-media thickness has long been considered a useful marker of atherosclerosis in the arterial network and is routinely monitored for disease progression and treatment evaluation. Only recently have researchers investigated the association of its echogenicity with symptomatic disease and plaque characteristics (Wohlin *et al*, 2009; Lind *et al*, 2007). This study's results agree with recently published data, suggesting an association of intima-media grey level with carotid plaque grey level, though not statistically confirmed, as well as a strong correlation of intima-media echolucency with patient's blood cholesterol levels. Further studies that would look into more in-depth intima-media image texture analysis and investigate association with more cardiovascular risk factors, carotid plaque characteristics, clinical symptoms and disease progress could provide useful information for intima-media assessment and disease management.

The preliminary immunohistochemistry study's results suggest potential association of grey level statistics with inflammation and neovascularization. However, the small number of patients prevents any solid conclusions. Future studies which would enroll

greater number of patients, apply more sophisticated image texture analysis and use immunohistochemistry analysis for plaque inflammation and neovascularization assessment of post-endarterectomy specimens are required for establishment or rejection of the association. It is suggested that additional markers for neovascularization assessment, such as CD34 and VEGF antibodies, could make the analysis easier and more accurate in future studies. Furthermore, a future study could look into histology analysis of the same specimens regarding both compositional tissues and inflammation and angiogenesis, so as the contribution of each plaque characteristic in ultrasound image texture could be independently investigated. In case of confirmation of association between ultrasound image characteristics and plaque inflammation, neovascularization and calcification, incorporation of texture analysis in ultrasound software would allow even better identification of vulnerable plaque.

Apart from duplex ultrasound, there is ongoing research on methods of carotid plaque assessment using contrast enhanced ultrasound. Numerous studies have shown promising results regarding identification of plaque neovascularization, whereas only a few have looked into the assessment of plaque inflammation using late-phase contrast enhanced ultrasound (Feinstein, 2006; Owen *et al*, 2010). More research is needed for standardization of methods and evaluation of their accuracy, while methods for computerized quantification of plaque contrast enhancement need to be established.

Scientific interest has also been turned towards three-dimensional ultrasound, which has been simplified by the development of 3D ultrasound probes and modern image-analysis software and offers the advantage of volumetric vascular imaging. Recent studies have concluded on very high reproducibility in plaque volume measurement and good reproducibility in plaque morphology and composition assessment.

However, it should be noted that 3D ultrasound can be more technically challenging comparing with 2D ultrasound (Makris *et al*, 2011). Furthermore, three-dimensional ultrasound may allow a simplified assessment of wall shear stress, which appears to be of great importance in the progress of atherosclerotic disease. More research is needed for method establishment and accuracy evaluation of this new promising modality.

7.7 Summary

Duplex ultrasound stands today as the main imaging modality for assessing carotid atherosclerotic disease. Despite research results on new methods and techniques for ultrasound investigation of carotid plaque, carotid stenosis assessment in clinical practice is still based on velocity criteria, providing information about the haemodynamic effects of the stenosis and indirectly estimating its degree. To date, direct stenosis measurement or quantitative characterization of plaque have not been included in the standard investigation protocol. Meanwhile, other imaging modalities like computed tomography angiography and magnetic resonance angiography have enabled assessment of parameters beyond that of luminal stenosis.

Summarizing the study results, regarding direct stenosis measurement, ECST method superiority among the direct methods for carotid stenosis measurement is apparent and its inclusion in carotid ultrasound investigation protocol could improve the estimation of degree of stenosis, especially in cases where velocity criteria give inconclusive results or seem inconsistent with visualized stenosis. Furthermore, grey scale texture analysis of plaque ultrasound image appears able to provide information about plaque heterogeneity and vulnerability, since some parameters show good correlation with lipid and calcification deposition and are related with increased risk

for stroke. Regarding plaque neovascularization and inflammation, association with grey scale parameters seems promising, however it remains to be proved in future studies. Plaque fibrous cap thickness measurement and intima-media grey scale analysis could also provide information about potentially symptomatic plaque that could help identify high risk patient.

Research on atherosclerosis constantly updates plaque formation mechanisms adding new factors related to plaque growth and risk of rupture. Taking into consideration current knowledge around carotid atherosclerotic disease, current carotid plaque ultrasound assessment that looks only on the degree of stenosis using velocity parameters seems obsolete. A lot of ultrasound potentials remain unexploited and duplex ultrasound may be held back by the excuse of inaccuracy. A revised holistic ultrasound approach of carotid plaque, which will combine accurate stenosis measurement using uniformly accepted methods, stenosis haemodynamic effect with velocity parameters as well as quantitative details about plaque morphology, composition, inflammation and neovascularization could increase duplex ultrasound efficacy in the investigation of carotid atherosclerotic disease, making it a powerful diagnostic tool for patient screening, assessment and following up.

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