

# *Vascular ultrasound for the assessment of carotid atherosclerosis*

**Davide Vanoli, MD**



**Department of Public Health and Clinical Medicine**  
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# List of papers

This thesis is based on the following articles, which will be referred to in the text by the corresponding Roman numerals (Studies **I-IV**):

**I:** Vanoli D, Lindqvist P, Wiklund U, Henein M, Näslund U. Fully Automated On-Screen Carotid Intima-Media Thickness Measurement: A Screening Tool for Subclinical Atherosclerosis. *J Clin Ultrasound*. 2013 Jul-Aug; 41 (6): 333-39

**II:** Vanoli D, Wiklund U, Lindqvist P, Henein M, Näslund U. Successful novice's training in obtaining accurate assessment of carotid IMT using an automated ultrasound system. *Eur Heart J Cardiovasc Imaging*. 2014 Jun; 15 (6): 637-42

**III:** Vanoli D, Grönlund C, Näslund U, Wiklund U, Johansson E, Wester P, Lindqvist P. Ultrasound-based plaque texture characteristics are not useful in risk stratification in patients with symptomatic carotid stenosis waiting for carotid surgery. *Manuscript submitted*.

**IV:** Vanoli D, Nyman E, Wiklund U, Näslund U, Lindqvist P. Quantification of atherosclerotic plaque area by vascular ultrasound: reproducibility and comparison between conventional B-mode imaging and contrast-enhanced ultrasound. *Manuscript*.

# Abstract

**Background:** Atherosclerotic cardiovascular disease (CVD) is a common cause for mortality globally, and is expected to remain the single leading cause of death as the global population ages and as life expectancy increases.

Cardiovascular screening models have been developed, and continue to be developed, to identify individuals at high-risk of CVD. This is necessary to establish prevention strategies to decrease the occurrence of an acute event or debilitating syndrome. These strategies largely take the form of lifestyle modification and pharmacological intervention. The Framingham risk score, NCEP/ATP II guidelines and SCORE risk assessment protocol are widely used screening models. Ultrasound based methods are non-invasive, affordable and have been suggested to improve assessment and definition of individual patients potential risk of CVD. Increased carotid artery intima-media thickness (cIMT) is a known and established sign of early atherosclerosis. The presence of plaque and the overall burden of atherosclerosis seems to have even greater value in predicting cerebrovascular and cardiovascular events. Ultrasound based texture characteristics of plaques such as entropy, grey scale median, discrete white area, coarseness and juxtaluminal hypoechoic black area have been proposed as useful possible predictors of plaque vulnerability. Moreover, ultrasound contrast agents have been developed to improve visualization and subsequent quantification of an atherosclerotic plaque. Considering the current available data and research, the importance of developing tools and techniques for detection and characterization of atherosclerotic changes, to indicate risk for CVD and the subsequent early intervention and prevention, appears clearly as an effort to improve public health. In this present collection of studies (4 papers) we aim to contribute to the development of clinically useful ultrasound methods and tools for the accurate assessment, understanding and management of atherosclerosis.

**Methods:** To validate ultrasound-based methods for assessment of early signs of atherosclerosis, measured as cIMT, a total of 144 subjects underwent bilateral carotid ultrasound. In study I, the performance of an ultrasonography software capable of fully automated on-screen cIMT measurements was tested and compared with the traditional manual measurement approach. The coefficient of variation and the intraclass correlation coefficient for both methods were compared to verify the reliability and reproducibility of results generated by the new ultrasound software. To test the accessibility and possible clinical applications of this new technology tested in study I, the new software was used by novice's scanners in study II, and the intraobserver variability of the cIMT measurements were assessed and compared with that of an expert operator.

In study III, ultrasound texture characteristics of 327 plaques including entropy, grey scale median, discrete white area, coarseness and juxtaluminal hypoechoic black area were assessed as possible predictors of future cerebrovascular events in a cohort of 133 patients with symptomatic carotid stenosis waiting for carotid surgery. The reproducibility of measuring plaque area (expressed as intraclass correlation coefficient) using conventional ultrasound and contrast enhanced ultrasound was tested in study IV in an attempt to find a simple and reproducible parameter for monitoring changes in atherosclerotic burden.

**Results:** The technology tested in study I was found to have good inter- and intra-system reproducibility compared with conventional methods. Moreover, it was found to produce reproducible results when used by expert and novice operators after a short period of training (study II), confirming the possibility for the employment of this technology in a large screening public health programs. Although such technology may have immediate practical application, other and more sophisticated ultrasound based plaque characteristics (such as grey scale median, entropy, coarseness, juxtaluminal hypoechoic black area) were not shown to be beneficial in predicting plaque vulnerability (study III). Contrast enhanced ultrasound technic tested in study IV did not improve quantification of atherosclerotic plaque burden.

**Conclusion:** Medical ultrasound technology by using a automatically measure of carotid intima media thickness can be used with high reproducibility and also possible to be transferred to primary care by a well designed training program. Plaque characteristic using carotid ultrasound was not found to be useful in risk stratifying symptomatic patients with severe carotid stenosis. Furthermore, contrast enhanced ultrasound technique was found to have high reproducibility in plaque area assessment but not better then conventional b-mode based method in quantifying the atherosclerotic burden. Therefore, more sophisticated ultrasound based methods for assessment plaque characteristics was not found to be beneficial in predicting plaque vulnerability.

# Abbreviations

ATP: adult treatment panel

CAC score: coronary artery calcium score

CCA: common carotid artery

CEA: carotid thromboendarterectomy

CEUS: contrast enhanced ultrasound

cIMT: carotid intima media thickness

CT: computer tomography

CVD: cardio vascular diseases

CVE: cardio vascular events

DWA: discrete white area

GSM: grey scale median

ICA: internal carotid artery

IMT: intima media thickness

JBA: juxtaluminal hypoechoic black area

LDL: low density lipoprotein

MR: magnetic resonance

NASCET: North American Symptomatic Carotid Endarterectomy Trial

NCEP: national cholesterol education program

PET: positron emission tomography

ROI: region of interest

SCORE: systematic coronary risk evaluation

TGC: time gain compensation

TIA: transitory ischemic attack

TPA: total carotid plaque area

VIP-VIZA: Västerbotten Intervention Programme - VIZualization of Arterial plaque

3D: three-dimensional

2D: two-dimensional

# Sammanfattning på svenska

En ledande orsak till hjärt-kärlsjukdom och död är förträngningar i kärlen, såsom åderförkalkning (ateroskleros). Hjärtkärlsjukdomar förväntas även i framtiden vara en av de ledande orsakerna i samma takt som befolkningen åldras och livslängden ökar. Metoder för att förebygga akuta hjärtkärlsjukdomar och för att hitta individer med ökad risk utvecklas därför kontinuerligt.

För att kunna identifiera individer med ökad risk för hjärtkärlsjukdom har man börjat använda ultraljudsundersökningar av halspulsådern, såsom vaskulärt ultraljud. I denna undersökning utgår man från olika förutbestämda värden för att göra en analys över risken att bli sjuk.

Det som leder till en förträngning är plack som uppstår i kärlen. Med en ultraljudsundersökning av halspulsådrar (carotisartärer) så kan man analysera plackens olika egenskaper och utifrån dessa göra en bedömning över hur stor risken är att bli sjuk. Metoderna för detta har förbättrats av allt mer utvecklad teknologi och genom att man börjat använda kontrastmedel för att synliggöra placket i kärlen bättre.

Plack kan finnas i våra kärl utan att ge upphov till hjärtinfarkt, stroke eller förtida död. En utveckling av metoden med ultraljudsundersökningar är därför viktig för att i tidigt skede kunna göra rätt bedömning av vilka individer som, utöver plack, också har en ökad risk att bli sjuk. För dessa personer ordinerar sedan en livsstilsförändring tillsammans med medicinsk behandling.

I studie 1 undersöktes vid Klinisk Fysiologi, Hjärtcentrum, Norrlands universitetssjukhus 144 patienter med vaskulärt ultraljud. IMT, intima-media wall thickness (kärlväggstjocklek) mättes med både den traditionella metoden för riskbedömning, vilket betyder att en specialist gör varje värdering och bedömning manuellt, och en annan metod där mätning av värden och bedömningen görs automatiskt av ultraljudsmaskinen. Studien visade att den automatiserade metoden var jämförbar med den traditionella metoden för riskbedömning.

I studie 2 visades även att mindre erfarna användare av ultraljud kan lära sig att göra undersökningar med avseende på kärlväggstjocklek på ett lika reproducerbart sätt som en van undersökare.

I studie III bedömdes om plackens egenskaper (såsom coarseness, entropy, grey scale median, discrete white areas and juxtaluminial black areas) kan vara användbara för att förutse så kallad plackinstabilitet. 133 patienter med

aterosklerotiska plack i åtminstone en halspulsåder undersöktes med vaskulärt ultraljud. Alla dessa patienter hade haft neurologiska symtom som TIA och/eller stroke medan de väntade på kirurgisk åtgärd. Trettiofyra av patienterna fick en ny TIA/stroke vilken tyder på instabil plack.

Resultatet av studien visar att ingen av de ovan nämnda metoderna för bedömning av plackegenskaper var användbara i syfte att förutse plackinstabilitet. Finndet är i motsats till vad som tidigare rapporterats i litteraturen och talar för en mer försiktig syn på användning av dessa nya ultraljudsmetoder för att bedöma plackinstabilitet i klinisk praxis.

I studie IV studerades om ultraljudsteknik baserad på contrast enhanced ultrasound technique (CEUS) kunde vara användbar för att bättre bedöma aterosklerotiska plack med plack area jämfört med bedömning utan CEUS. 86 patienter undersöktes från den pågående VIP-VIZA trial ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT01849575). Resultatet visar att CEUS tekniken producerar pålitliga och reproducerbara resultat men jämförbara med konventionella metoder.

Dessa studier analyserar användningen av ultraljud i utredning av ateroskleros. Ytterligare forskning fordras för att förbättra metoden ytterligare och för att öppna möjligheter att genomföra stora allmänna undersökningar av befolkningen med hjälp av vaskulärt ultraljud och på så sätt göra en insats för folkhälsan.

# Introduction

Atherosclerotic cardiovascular disease (CVD) is the number one cause of mortality globally, and is expected to remain the single leading cause of death as the global population ages and as life expectancy increases (1). The incidence of myocardial infarction in Sweden has decreased since the 1980s, however CVDs remain the leading cause of death in almost 40% of the Swedish population, followed by cancers (approximately 25%) (2). Multiple CVD risk markers have already been identified, and studies suggest 90-95% of acute events, i.e. myocardial infarction (MI) and stroke, are caused by modifiable risk factors(1, 3-6).

Atherosclerosis is the most common form of CVD and it generally begins in early childhood and remains silent or asymptomatic for decades. A large proportion of atherosclerotic heart disease (40-60%) in later life presents as potentially life-threatening syndromes including acute coronary syndrome, stroke, and in some cases unexpected or sudden death.

Therefore, cardiovascular screening models have been developed, and continue to be developed, in an attempt to identify high risk individuals in order to establish prevention strategies to decrease and/or prevent the occurrence of an acute vascular event or debilitating syndrome. These strategies largely take the form of lifestyle modification and pharmacological intervention (7, 8). The Framingham risk score, the NCEP/ATP II guidelines and the SCORE risk chart are currently the most widely used screening models (2). A fundamental problem with these models is that scoring does not directly assess for the individual's risk, but rather their risk based on the individual as part of a group classification.

## *Pathophysiology of atherosclerosis*

The etymology of “atherosclerosis” is: “athero” indicating “gruel” and “skleros” meaning hardening.

The examination of atherosclerotic lesions using modern techniques of molecular and cellular biology has revealed that each lesion contains different elements of chronic inflammatory fibroproliferative response. This process includes monocytes (macrophages and T lymphocytes) followed by the proliferation of smooth-muscle cells, the accumulation of connective tissue and connective matrix molecules (such as collagen, proteoglycans and elastic fibers) as well as intra- and extracellular deposition of lipids.

Atherosclerotic lesions have been analysed at different stages (9) :

- 1 Adaptive intimal thickening: This stage is characterized by accumulation of smooth-cells in a proteoglycans-rich matrix. The

lesion is also characterized by the absence of lipid deposition and inflammatory processes.

2. Intimal xanthoma or more commonly called “fatty streak”: Low density lipoprotein (LDL) particles accumulate into the intima layer, undergo a process of oxidation, and this in turn leads to chronic stimulation of the immune response. The oxidised LDL particles induce the endothelial cells to express adhesion molecules, which in turn induce the adhesion and migration of monocytes into the arterial wall (intima layer) that then differentiate into different phenotypes of macrophages (M1 and M2-like phenotypes).
3. Pathological intima thickening: This is represented by the thickening of the intima due to the underlying accumulation of lipids and inflammatory cells. This lesion is characterized by the absence of necrosis and by the presence of a fibrous cap that covers the area of lipid accumulation.
4. Fibroatheroma: Foam cells, macrophages and smooth muscle cells accumulate with lipids in the arterial wall. Histologically, this lesion is characterized by the presence of a necrotic core. Neovascularization and hemorrhage may also be present.
5. Fibrocalcific plaque: This represents an evolution of the previous lesion. Components that can undergo calcification, usually the necrotic core constituted by apoptotic cells and extracellular matrix, calcify.

## *Carotid Atherosclerosis*

Atherosclerosis is a systemic disease that has focal manifestation. One common area of focal manifestation is the common carotid arteries.

Atherosclerotic lesions in the carotid vessels are one of the most significant causes of stroke and transient ischemic attack. Recognised risk factors for atherosclerosis are obesity, smoking, familiarity, hypertension, diabetes, and hypercholesterolemia (10-13).

Atherosclerotic plaque can be found in any segment of the carotid vessels, but is mainly seen in the bulb, at the bifurcation of the common carotid artery, and in the proximal 2 - 3 centimeters of the internal carotid artery (ICA).

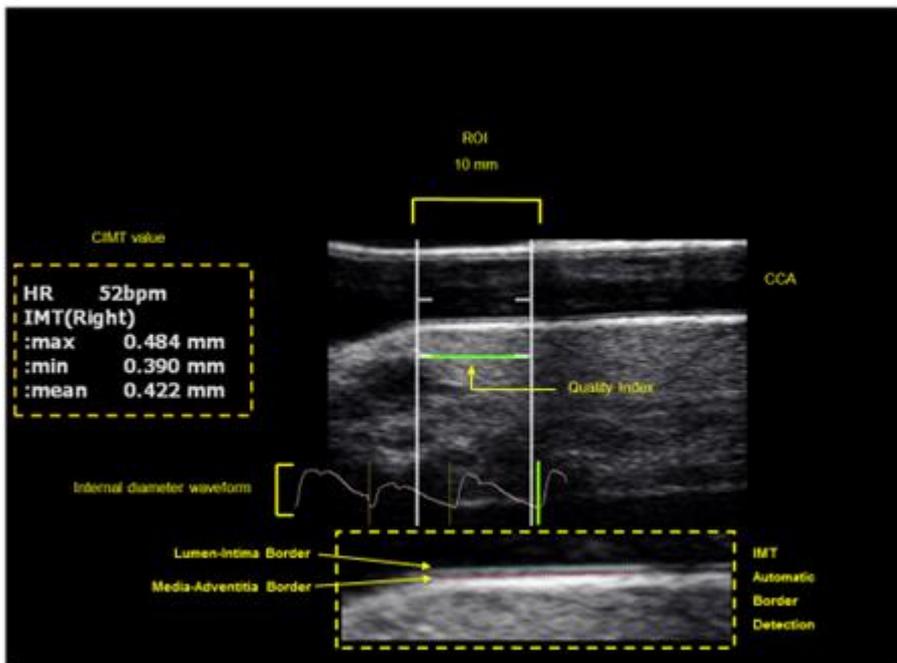
The increased susceptibility of these segments to develop atherosclerosis can be explained by different mechanisms that include abnormal wall artery shear stress and perturbation interrupting the normal laminar blood flow (14, 15).

Pro-atherogenic stimuli can trigger endothelial dysfunction and activate the immune response, which is responsible for the progression of the atherosclerotic lesions. Strategies for identifying clinical and subclinical plaques have been proposed and debated; a unanimous consensus seems to be gathered around ultrasound, as ultrasound represent a non-invasive method sensitive enough to detect early sign of atherosclerosis.

*Carotid intima-media thickness (cIMT) and ultrasound.  
Non-invasive assessment of the early atherosclerosis*

Three layers compose the wall of an artery. From the lumen outwards these are the tunica intima (or intima), the tunica media, and the tunica adventitia. The vessel wall nearest to the transducers is called the “near wall” and the farthest one the “far wall”. The interfaces between the three layers and between the lumen and the intima will reflect an ultrasound beam because of the difference in acoustic impedance.

After processing these signals, a typical two layered image will be formed as shown in figure 1.



**Figure 1. Ultrasonographic measurement of intima-media complex.**

On the far wall the distance between the interface lumen-intima and adventitia-media is called the intima-media complex of the far wall. This complex has been extensively used to study, non-invasively, early atherosclerotic changes of the vessels wall.

Pignoli et al. (16, 17) and Wendelhag et al. (18) successfully demonstrated that there were no significant differences between measurements of the intima-media thickness of the far wall measured in histological specimen and those measured by B-mode ultrasound images (19, 20). This introduced

the concept that ultrasound based measurements of the intima-media thickness of the far wall can be used as surrogate for in vivo measurements (21).

For the near wall, comparison between measurements obtained by B-mode images and those taken on histological specimen reveal that B-mode measures were approximately 20% less than those obtained histologically (22). Interference in the reflection of the ultrasound from the leading edge of the adventitia to the trailing edge of the intima of the near wall can account for this difference (23).

The early ultrasound measurements of the intima–media complex were manually obtained using a simple “3 points” measuring system. Basically, with a caliper tool, three measurements of the cIMT were taken along the last centimeter of the far wall of the common carotid artery. The average of those three measurements was considered the average thickness of the intima-media complex.

In the last 20 years, improvements in ultrasound technology have produced semiautomatic and automatic systems for the measurements of the intima–media complex based on algorithms for the segmentation of vessels wall (24, 25).

The most performed techniques for carotid wall segmentation are listed below:

- Edge tracking and gradient-based techniques: The edge-tracking technique is based on the idea that the common carotid artery (CCA) could be thought of as a dark region (the lumen) surrounded by two double line interfaces. cIMT could be assessed by measuring the intensity profile of a section of the image from a centerline (lumen) to the vessels borders (walls). Gradient base methods divide the B-mode imaging of the CCA into columns and measure the gradient of the intensity profile for each column (25);
- Dynamic programming techniques: These methods require integration of multiple measurements of echo intensity, intensity gradient and boundary continuity (25);
- Active contours – based segmentation: A highly computational cost technique that uses a parametric two-dimensional contour (called “snake”) defined as a set of vertices connected by line segments to assess the cIMT. This technique is sensitive to pre-processing standardization of images (25);

- Local statistics and “snakes”: This technique implements algorithms that locate the CCA in the ultrasound frame and uses algorithms (snakes based) to measure the cIMT.

From a technical point of view computerized measurement of the cIMT should be accurate, unaffected by speckle noise or ultrasound artefacts, repeatable in different clinical scenarios, operator independent and possibly validated in multicenter trials. However, a robust standardization of cIMT assessment is still yet to be achieved.

### *Ultrasound-based plaque texture characteristics*

Atheromatous plaques are ever-changing structures at the microscopic level. This phenomenon is reflected by the macroscopic changes in texture characteristics of the plaques that may be observed by vascular ultrasound (26). A smooth surface, high echogenicity and a homogenous texture are characteristics of stable plaques, whereas an irregular surface, low echogenicity and a heterogeneous texture are characteristics of potentially unstable plaques (27). A significant association between plaque morphology, low echogenicity and neurological events has been reported (27).

Plaque echogenicity assessed by B-mode ultrasound has been extensively studied and was correlated with the content of soft tissue and the amount of calcification in carotid plaques (28). Plaque echogenicity can be quantitatively expressed as the distribution of the “grey value” of the pixels within the plaque by utilizing dedicated computer-assisted software. The median value, instead of the mean value, of the grey scale is usually utilized. The grey scale median (GSM) of the ultrasound plaque image is used for characterising plaques as echolucent ( $GSM \leq 32$ ) or as echogenic ( $GSM > 32$ ) (27). GSM is defined as the median of the grey values of all pixels within a cropped plaque image. Plaques with low echogenicity (low GSM) on B-mode are richer in lipid content than those with higher GSM (29-31). It has been reported that plaques with high lipid content, thus low GSM, are more prone to rupture (32-36). Elatrozy et al. (37) reported that a carotid plaque with  $GSM < 40$  is significantly associated to ipsilateral ischemic stroke or trans ischaemic attack (TIA). Plaque texture other than GSM, such as juxtaluminal hypoechoic black area (JBA), was reported to have a positive correlation with ipsilateral neurological events (38). Coarseness and entropy are additional texture features that may play a role in identifying vulnerable plaques (27).

### *Contrast enhanced ultrasound (CEUS) and plaque imaging*

Plaque burden has been acknowledged as a predictor of cardiovascular events (CVE) and efforts have been made to accurately measure and quantify this (39). Although plaque volume assessed with three-dimensional (3D) ultrasound is a reliable and sensitive method, it has not been widely employed because the technique is time consuming and requires a skilled operator trained in 3D image acquisition and analysis techniques (40). In the Tromsø study, the total carotid plaque area (TPA) was shown to be a predictor of CVE (41, 42), where it was established that the greater the TPA, the higher is the risk of a CVE. Thus, clinical quantification of carotid plaque area has become an attractive tool to predict CVE risk. Evaluation of plaque area can also be performed using conventional B-mode images and contrast enhanced ultrasound images (CEUS) (5).

CEUS has been reported to provide detailed visualization of carotid atherosclerotic lesions, and has a number of distinct advantages over other imaging modalities, such as CT and MRI (43, 44). The CEUS technique is a safe procedure, does not require preliminary laboratory testing, can be ECG-triggered, even in patients with cardiac arrhythmias, and can be performed at lower cost than CT and MR in a variety of scenarios. CEUS is carried out in real-time, allowing rapid changes to be recorded (4). CEUS has also been used to visualize morphological features of vulnerable plaque (5-8). Moreover, CEUS imaging improves the delineation of the hypoechoic intima-media, whereas the adventitia appears as a bright echogenic layer. This facilitates a more precise detection of the inner and outer margins of the carotid arterial wall, thus improving the visibility, and in turn the assessment of carotid plaque ulcerations and lumen irregularities. The capability of CEUS to quantify plaque burden and size, in comparison with conventional methods such as B-mode, remains an issue that has been poorly addressed.

## *Controversial issues in ultrasound assessment of atherosclerotic burden*

Assessment of atherosclerotic burden is still a challenging issue. The most important problems may be categorised as follows:

- Definition problems
- Standardization of ultrasound protocols
- Ultrasound artefacts
- Quantification issues
- Reproducibility of results

### *Definition problems*

The American Society of Echocardiography and European Mannheim consensus defined intima-media thickness as a double-line pattern visualized by echotomography on both walls of the CCAs in a longitudinal image (45). The intima-media is formed by two parallel lines consisting of the leading edges of two anatomical boundaries – the lumen-intima and media-adventitia interfaces. An atherosclerotic plaque is defined as focal structure encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value, or demonstrated as a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface (45, 46). These definitions are very broad and will include the majority of carotid lesions. These standard definitions permit comparisons between findings and surely improve the reproducibility of results.

Critiques (47) have been moved to the Mannheim consensus regarding overestimation of small plaques defined as “at least 0.5mm or 50% of the surrounding IMT value” which requires a good image quality in order to be applied. In the presence of poor image quality, resulting in less than optimal definition of the plaque, the Mannheim consensus cannot be realistically used as it would lead to an underestimation of the number of the plaque present in a given vessel (47). Another important issue, not addressed in the Mannheim consensus, is the growing intima-media thickness around a plaque defined as the “focal structure”. If just the intima-media thickens around a plaque, the value of the thickness of the intima-media will match that of a plaque, thus “the plaque” will no longer be defined as “plaque”, leading again to an underestimation of the atherosclerotic burden.

## *Standardization of ultrasound protocols*

Standardization of ultrasound protocols are required to ensure that two fundamental questions, “how to measure” and “where to measure”, are answered when assessing atherosclerotic lesions with ultrasound. In 2008, The American Society of Echocardiography (46) addressed this issue and published a consensus statement describing in detail the long process that is required in order to achieve reproducible accurate measurement of atherosclerotic burden. In fact, the results of none of the previous conducted prospective studies of carotid intima-media thickness and risk for CVE in individuals without known CVD (ARIC Study, CAPS Carotid Atherosclerosis Progression Study, CHS Cardiovascular Health Study, Rotterdam study, MDCS Malmö Diet and Cancer Study) (48-52) could be classified as reliable and accurate because of the discrepancies in “where” the measures of the cIMT were taken (far or near wall of the carotid vessels or even in with vessels common carotid artery or internal carotid artery or both). It is still under debate whether the mean or maximum value of the cIMT should be used as the marker of early atherosclerosis (46, 49-54). Some suggests that none of the actuarial values of cIMT have a prognostic value, but instead the rate of thickening over time may have a higher predictive value (54-57). “Long” and “short” protocols have been suggested and investigated in order to address which one could be most predictive for future CVE (58). Currently published results suggest that “long” protocols with multiple cIMT measurements should be performed (58).

## *Ultrasound artefacts*

Artefacts are an inherent problem to the medium of ultrasound. Ultrasound artefacts are a result for the physical nature of ultrasound and its interaction with tissues and structures of the body. Alternating cycles of air compression and decompression assume the name of “sound” and, when considered in a specific range of frequencies, “ultrasound”.

Reflection, attenuation, and diffraction of the “interrogating” beam by the tissues and structures of the body, influence negatively the “returning” beam creating distortions and pitfalls in the reconstructed B-mode images.

An infinitely thin interface between two tissues with different acoustic impedances will reflect ultrasound with amplitude that is not proportional to the thickness of the interface but to differences in acoustic impedance (the greater the difference the greater the reflection from the interface). Thus, the thickness of the echoes returning from that interface is not proportional to the thickness of the interface but to the difference of acoustic impedance between the two-mediums/ tissues. This phenomenon is at the base of some the issues encountered attempting the quantification of the cIMT, a highly

reflective very thin interface.

Difference in acoustic impedance, attenuation and diffraction are also responsible for perhaps the most frequent artefact in imaging of atherosclerosis – “shadowing”, defined as “an abrupt, discrete diminution of the ultrasound signal”(59)

Shadowing artefacts often occur on clinical ultrasound B-mode images distal to smooth, rounded cavities, such as cysts, aneurysms and blood vessels, which contain a fluid with a speed of sound different to the surrounding tissue. Geometry itself has been indicated as a possible explanation for the artefacts; in fact when the fluid in a cavity has a speed of sound lower than the surrounding tissue, the cavity would act as a lens bending the lateral aspect of the ultrasound beam inward leaving a shadow region distal to the edges of the cavity (59).

More complex structures containing different tissues with different acoustic properties generate shadowing by simply attenuating and reflecting, almost entirely, the interrogating ultrasound beam leaving behind a region of signal “silence”. The latter is the most frequently encountered type of shadowing in atherosclerosis ultrasound imaging. The “silence” left behind does not allow analysis of the morphology of the edge or echogenicity of the structure, swallowing up boundaries between anatomical structures and atherosclerotic lesions. Absence or altered signal does not permit delineation of boundaries thus, correct quantification of areas or volumes. The operator must be aware of the nature of ultrasound in order to do an accurate assessment and recognise artefacts for what they are and be able to avoid them.

### *Quantification issues*

Atherosclerotic plaques are 3D structures. B-mode ultrasound imaging is still based on 2D images depicting plaques in relation of the 2D planes used to insonating plaques. Mono- or dimensional measures are used routinely to quantify plaques assuming that a certain parameter (for example, an area or a distance) represents the whole volume of the structure. However, “assuming” is not “measuring”.

Quantification of a 3D structure requires a volumetric parameter. Volumetric assessment of plaque has been achieved with currently available ultrasound systems, but ultrasound artefacts affect the reconstructed volumes negatively and are responsible for the large variability of results in term of precision and inter- as well intraobserver variability (60-64). True 3D assessment of plaque is still an ongoing process that may not yet be ready for clinical use.

### *Reproducibility of results*

Combining all of the previously described issues results in a large variability when using ultrasound to assess the atherosclerotic burden at the individual level. A variety of protocols used by different sonographers may produce various results because of, for example, the different angle of insonation used to visualise plaque. At an individual level, different artefacts may be encountered in serial ultrasound controls producing different challenges in quantifying the same plaque in the same patient. Attempts to quantitatively assess the same plaque may not be possible using 2D parameters. Volumetric measures, due to the above-mentioned problems, are not yet suitable for routine use.

# Objectives

The general aim of this project was to evaluate the feasibility and reproducibility of ultrasound measurements of atherosclerotic markers such as: carotid intima media thickness (study I and II), plaque texture characteristics (study III) and the atherosclerotic plaque burden tested by using contrast enhanced technique (study IV).

The measurements of carotid intima media thickness were performed by systems with different levels of automation (study I) and by operators with different levels of expertise (study II). The reproducibility of ultrasound measurements (plaque area) performed with and without contrast enhanced technique was evaluated in study IV. Beside the reproducibility of ultrasound measurements, in study III we evaluated if some of the new ultrasound based characteristics of atherosclerotic plaque could be useful as marker of vulnerability.

The specific aim of each study is listed below;

**Study I:** To evaluate the feasibility and reproducibility of cIMT measurement obtained with an automated on-screen carotid ultrasonograph and with conventional, B-mode images based, system.

**Study II:** To assess the feasibility and reproducibility of training a small group of novice ultrasound operators in acquiring the skill of obtaining cIMT measurements using the automated ultrasound system tested in study I.

**Study III:** To evaluate if some plaque texture characteristics can be useful in predicting recurrent ipsilateral ischemic cerebrovascular events in patients with symptomatic carotid stenosis that are waiting for carotid endarterectomy.

**Study IV:** To evaluate the feasibility and reproducibility of plaque area measurements obtained from contrast enhanced ultrasound images (CEUS) compared to those obtained with conventional B-mode images, and verify if the reproducibility is affected by the time frame in which the CEUS images are acquired.

# Materials and methods

## Subjects

For all the participants in the studies an informed consent form was obtained. All studies were accepted by the ethical approval board at Umeå University.

In the studies we analysed three different groups of subjects. In study I and II we investigated groups of subjects that were referred for carotid ultrasound examination at the vascular ultrasound laboratory of the Department of Clinical Physiology, Heart Centre, University Hospital, Umeå, Sweden. The Heart Centre is the referral centre for tertiary care in a region with a population of 881,000 inhabitants. In study III we investigated a group of patients with symptomatic carotid stenosis awaiting carotid endarterectomy. In study IV a subgroup of the ongoing VIP-VIZA trial ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT01849575) were investigated.

### Study I

Between January 2010 and December 2010 50 patients with a mean age of  $62 \pm 5$  years (range: 23-84 years; 36 males) were enrolled for the study. The underlying clinical indications for referral were: peripheral arterial disease (n=20), screening for early diagnosis of abdominal aortic aneurysm (n=10) and suspected deep vein thrombosis (n=20). Twenty patients had previous neurological symptoms. Exclusion criteria were: previous diagnosis of stenotic carotid disease, previous carotid surgery and carotid aneurysms. Patients with atrial fibrillation or pacemaker were also excluded.

### Study II

This study included 96 subjects that were recruited from those referred to the Clinical Physiology department, Heart Centre, Umeå University Hospital, from other clinics or general practitioners for a carotid ultrasound examination. The 96 patients were enrolled in a period of four weeks (24 patients per week). The underlying clinical indications for the referral were the same as study I. Five operators, four novices and one expert operator performed the ultrasound examination. The novices were persons without any previous experience in performing carotid examinations that were enrolled from among the personnel of the department. After the examination performed by the novices, all patients/subjects underwent a second reference scan by an expert operator. The subjects were divided in groups of 6 individuals per novice per week. Before the carotid examination,

the study protocol was explained to the patients, who all gave informed consent prior to participation in the study. Candidates risk assessment for atherosclerosis was carefully assessed including weight, height, smoking habits, family history of heart/vascular disease, diabetes and dyslipidaemia. Body mass index (BMI) was calculated as weight in kilograms divided by the square height in meters. Participants with BMI > 35 Kg/m<sup>2</sup> were excluded. Subjects with previously diagnosed carotid stenosis, carotid endoarterectomy, carotid aneurysms, atrial fibrillation or pacemaker treatment were excluded.

### **Study III**

Between May 2008 and December 2009, 162 patients were admitted to the Stroke Centre at the University Hospital in Umeå, Sweden, and were scheduled for carotid thromboendarterectomy (CEA). All patients were symptomatic and had had a cerebrovascular event (CVE) such as transient ischemic attack (TIA) or ischemic stroke within the last 6 months prior to admission. Stroke was defined according to the World Health Organisation as a clinical syndrome typified by rapidly developing signs of focal or global disturbance of cerebral functions lasting more than 24 hours or leading to death, with no apparent causes other than of vascular origin. TIA is defined as a sudden, focal neurologic deficit that lasts for less than 24 hours, and it is presumed to be of vascular origin and is confined to an area of the brain or eye perfused by a specific artery. Retinal embolism was considered as a stroke, and amaurosis fugax was considered as a TIA. Asymptomatic patients and those with carotid occlusion, irrespective of the side, were excluded. All patients had an internal carotid artery stenosis > 70% on at least one side. The severity of the internal carotid artery stenosis was defined according to Hansen et al. (65) The patients' general characteristics are shown in Table 1 in the corresponding manuscript. An ipsilateral CVE was defined as the appearance of new signs and symptoms compatible with the side of a previously detected atherosclerotic plaque.

### **Study IV**

The ongoing VIP-VIZA trial (NCT01849575) is a large pragmatic randomized controlled study in primary prevention of CVE's. The study consists of 3620 participants aged 40, 50 or 60 years old - all undergoing a carotid vascular ultrasound at baseline. From this population 86 subjects were referred to the Department of Clinical Physiology at the Norrlands University Hospital, Umeå, Sweden, for a second, more extensive ultrasound examination. Indications for additional ultrasound assessment were: Presence of atherosclerotic plaque leading to a  $\geq 40\%$  reduction in lumen

diameter in one of the carotid vessels, calculated by the NASCET formula (7, 66), poor image quality of the first ultrasound screening, and suspicion of other carotid pathologies (for example, aneurysm). From the initial cohort of 86 subjects, 53 were found to have no significant plaques (41 subjects were assessed because of poor image quality in the first examination, 12 were found to have additional pathologies that included atherosclerosis). The remaining 33 subjects were recruited for this study. The inclusion criteria for this study were one or more plaques in the carotid vessels with a lumen area reduction of  $\geq 40\%$ , no signs of ulceration or rupture of the plaques, no history of previous TIA or stroke, and no history of previous allergic reaction to any ultrasonographic contrast agent.

# Ultrasound system, ultrasound contrast agent and off-line software

In **study I** a conventional ultrasound system (Acuson Sequoia®, Siemens Medical Solutions, Mountain View, CA) equipped with a 9.0-MHz (L4) linear-array transducer was used in study I and III. An automated ultrasonograph (Panasonic Healthcare Corporation of North America, Newark, NJ, USA) equipped with a 9.0-MHz linear-array transducer was used in **studies I and II**. The latter, in distincton to the conventional ultrasound system, is capable of a truly full automatic measurement of the carotid intima-media thickness according to a segmentation algorithm based on a radiofrequency signal.

In **studies III and IV** a system capable of contrast enhanced ultrasound examination was used (Philips iU22 xMATRIX® ultrasound system, Philips Healthcare, Bothell, Washington) with a L9-3 linear array probe (160 elements, 9.0 to 3.0 MHz frequencies spanning, 15 degrees of trapezoid imaging with 38 mm aperture length). For study III a mechanical index of 0.12 was used in all subjects. The image depth was adjusted to 3-4 cm according to the size of the carotid vessels. The focus was positioned just below the carotid vessel in order to avoid contrast bubble destruction. The time gain compensation (TGC) was adjusted in order to achieve homogeneous signal intensity.

In **study IV** sulphur hexafluoride with a phospholipid shell was used as the contrast agent (SonoVue® Bracco Spa, Italia). Descriptions of the pharmacokinetic and pharmacodynamics properties of this contrast agent have already been published (5-8).

In study III, a research software package, developed at the Department of Biochemical Engineering - Norrlands University Hospital, Umeå, Sweden, was used for plaque texture extraction and analysis.

## Definitions used in the studies

**Carotid Intima-Media thickness** was defined, according to the Mannheim consensus (45), as a double-line pattern visualized by echotomography on both walls of the CCAs in a longitudinal image. It is formed by two parallel lines consisting of the leading edges of two anatomical boundaries, the lumen-intima and media-adventitia interfaces.

**An atherosclerotic plaque** was also defined according to the Mannheim consensus (45) as a focal structure encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding intima media thickness

value, or demonstrates a thickness of 1 - 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface.

**Grey Scale Median (GSM)** is defined as the median of the grey values of all pixels within a cropped plaque image. A GSM value of  $\leq 32$  defines echo lucent plaque; a GSM value of  $\geq 32$  defines an echogenic plaque (27). In case of multiple plaques, the averaged GSM was calculated. The GSM area represents the averaged GSM weighted for the averaged areas.

**Entropy** was defined as a measure of the random nature of the grey tone values within the plaques (27). In other words, entropy is the grade of dissimilarity of the grey tone in a given structure. A large value indicates a non-homogenous plaque without a recognizable pattern.

**Coarseness** was defined as the “granularity” of the plaque’s grey tone (27). A low coarseness can be seen in areas with a large variation of grey tone uniformity, conversely a high coarseness corresponds to small variations in grey tone uniformity.

**Juxtaluminal black area (JBA)** is defined as any black area or areas close to the lumen of the carotid vessels without a visible echogenic cap (27).

**Discrete white areas (DWA)** was defined as areas with pixels having greyscale values  $\geq 124$  without producing shadowing (27). Despite the name, those areas were depicted in red by the extraction software adopted in study III.

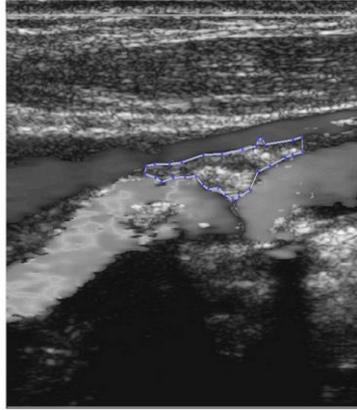
## Plaque texture feature extraction and analysis

A research software package, developed at the Department of Biochemical Engineering - Norrlands University Hospital, Umeå, Sweden, was used for plaque texture extraction and analysis (67-69).

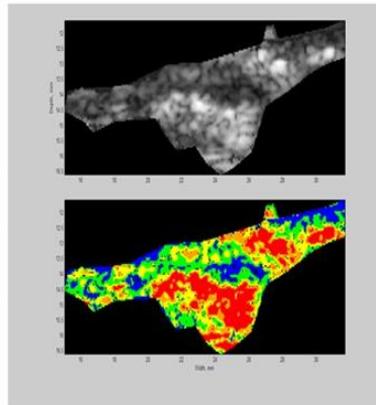
The ultrasound image was normalized by selecting a region of interest (ROI) within the darkest spot of the vessels lumen avoiding areas of background artefacts (“noise”). Another ROI was selected including the brightest part of the adventitia. In a B-mode image for the plaques emerging from the near wall, the near walls adventitia was chosen. For the plaques of the far wall the corresponding adventitia was chosen. The image pixels were then normalized using the ROIs and the intensity of the blood was set at 0

(corresponding to the darkest pixel) and the adventitia's ROI was set at 190 (the brightest pixel). The pixel density was standardised to 20 pixel/millimetre. Finally, the plaque in the normalised B-mode frame was visualised and the outer contour manually outlined and cropped prior to feature calculation. The frame for the analysis was chosen from a B-mode cine loop; this was best for plaque visualisation.

Figures 2 and 3 show examples of the analysis process.



**Figure 2. A plaque is manually outlined.**



**Figure 3. Colour-encoded areas with different echogenicity.**

# Ultrasound examination protocols

## *Assessment of Carotid-Intima Media Thickness*

In general, the ultrasound examination of the carotid vessels was performed according to the current guidelines (1, 53). Special protocols were adopted in each study according to the specific end-point. For the manual measurement all subjects were examined in a semi-supine position with the head turned 45 degrees away from the side of the neck being scanned. The sonographers obtained a longitudinal frozen B-mode image of the distal 10 mm of the right and left common carotid arteries, immediately before the bifurcation, according to the guidelines of the Mannheim IMT consensus. The far wall of the carotid vessels was insonated from a single angle view, which was the best for intima-media visualization. A three lead ECG recording was obtained simultaneously with the images. From the frozen frame, the cIMT was measured according to the Mannheim consensus. The mean value of the cIMT was used. The focus and the general gain were adjusted in order to maximize the signal/noise ratio in each subject, however, dynamic range, persistence and edge enhancement were kept unchanged.

For the automated measurement of the cIMT three separate scans of the right and left target segments of the common carotid artery were performed. The distal centimetre of the common carotid artery was visualized. The vessel motion detector system identified end-diastole based on the change in arterial diameter during the cardiac cycle. The auto-ROI tool automatically identified the interface lumen-intima on the far wall of the vessel where it was positioned. Finally, mean, maximum and minimum cIMT values were produced. The mean cIMT values were then recorded. The protocol for the automated measurement of the carotid intima-media adopted in study I was also adopted in study II.

## *Plaque Area quantification; Conventional B-mode images and contrast enhanced ultrasound images*

In study IV, plaque area was analysed off-line using two different methods – outlining the plaque area with a calliper tool based on conventional B-mode and based on contrast enhanced ultrasound (CEUS) images (44, 70, 71).

For conventional B-mode images, a standard carotid ultrasound was performed according to published guidelines (1, 3, 50, 53). After achieving the optimal insonation angle for visualization of a presenting carotid plaque,

an ECG-triggered B-mode image loop (3 heart cycles) was acquired and digitally stored in DICOM format.

CEUS were acquired following the conventional B-mode image loop. Peripheral vein access, using the cubital vein of the right arm, was prepared for injection of the sonographic contrast agent (SonoVue® Bracco Spa, Italia) (72). A commercially available software for CEUS ultrasound was preinstalled on the Philips iU22 xMATRIX© ultrasound system used in this study. The contrast agent was prepared according to the manufacturer's guidelines and administered with a bolus injection. The bolus dose was arbitrarily calibrated based on the subjects' weight: 1.0 ml for subject's  $\geq 80$  kg, 0.75 ml for those  $< 80$  kg. Using the same angle of insonation used for acquiring the conventional B-mode images, a 20 second ECG-triggered image loop was acquired for each plaque. The first frame of each image loop contained the first bubbles to appear in the lumen of the carotid vessel and was indicated as time zero ( $T_0$ ). Using  $T_0$  as reference, three additional time frames were defined:  $T_5$  was defined as  $T_0 + 5$  seconds,  $T_{10}$  as  $T_0 + 10$  seconds,  $T_{15}$  as  $T_0 + 15$  seconds. For subjects with bilateral plaques, a second injection of contrast was performed. All ultrasound image loops were digitally stored in DICOM format.

For off-Line analysis, all plaques were analysed using a commercially available ultrasound analysis software (EchoPAC™ Dimension, General Electric, Milwaukee, Wisconsin). The optimal frame for plaque visualization was selected from the stored image loops for analysis. Using the calliper tool, the contour of the plaque was manually outlined. Whenever the adventitia could not be identified, a straight line between the beginning and end of the trace was delineated. All plaques were measured during the R-to-R interval identified on the ECG trace corresponding to the selected image loop. The area was thus delineated manually and expressed in mm. The mean value of three different measurements was reported as "plaque area".

## Statistical analysis

Inter-operator and inter-system reproducibility was analysed by the intraclass correlation coefficient (ICC), calculated from a one-way random model with subjects as random factor, where  $ICC > 0.75$  was used as an indicator of excellent agreement.

In **study I**, the inter-system variability was also assessed by coefficient of variation (CV) calculated as the variance of differences divided by the overall mean cIMT and using the root mean square approach (CVRMS) to compare it with previous studies. Differences were characterized by Bland-Altman analysis, and evaluated using the paired t-test. The mean differences in cIMT measurements were tested using the “two one-sided t-test” (TOST) approach. Statistical significance was defined as  $P < 0.05$ .

In **study II** inter- and intraoperator reproducibility was analysed by ICC and by the CV calculated as described above. Differences were characterized by Bland-Altman plots, showing the mean of the two measurements made by the expert and the novices (whole group) on each patient on the x-axis, and the difference between the readings by the expert and the novices was plotted on the y-axis. The non-parametric Wilcoxon test was used to test if the average difference was different from zero. A P value  $< 0.05$  was considered as significant. Weekly averages of CV were compared using the non-parametric Friedman test.

In **study III** continuous data were reported as mean  $\pm$  standard deviations. Nominal variables were reported as counts and percentages. Multivariate analysis was performed using stepwise logistic regression. All variables with a p-value  $< 0.15$  were included in the model. The model was calibrated by the Hosmer-Lemeshow goodness-of-fit test, as well as residual diagnostics (deviance and df of  $\beta$ ). Model discrimination was evaluated by using the area under the receiver operating characteristic (ROC) curve. All tests were two-sided with the  $\alpha$  level set at 0.05 for statistical significance.

In **study IV** inter-method and interobserver reproducibilities was analysed using the intraclass correlation coefficient (ICC). Differences were characterized by a modified Bland Altman analysis. Discrepancies between all acquired measurements were assessed using linear effects mixed modelling, where results were presented as estimated marginal means and 95% confidence intervals of plaque area.

# Results

## Study I

### *Feasibility*

cIMT measurements were successfully made in all 50 patients. The acquisition time of the automated ultrasonograph was significantly shorter than that of the conventional system ( $P < 0.01$ ) (Table 1).

**Table 1. Acquisition time (seconds) for conventional CIMT measurements.**

	SG A, left (Conv)	SG A, left (Aut)	SG B, left (Conv)	SG B, left (Aut)	SG A, right (Conv)	SG A, right (Aut)	SG B, left (Conv)	SG B, left (Aut)
Mean (s)	130	76*	111	55*	102	71*	139*	78*
SD	64	33	52	21	54	36	56	37

\*P value <0,01 between methods (conventional versus automated system).

Abbreviations: Aut, automated system; Left, left common carotid artery; Conv, conventional system; Right, right common carotid artery; SG A, sonographer A; SG B, sonographer B.

### *Intersystem variability*

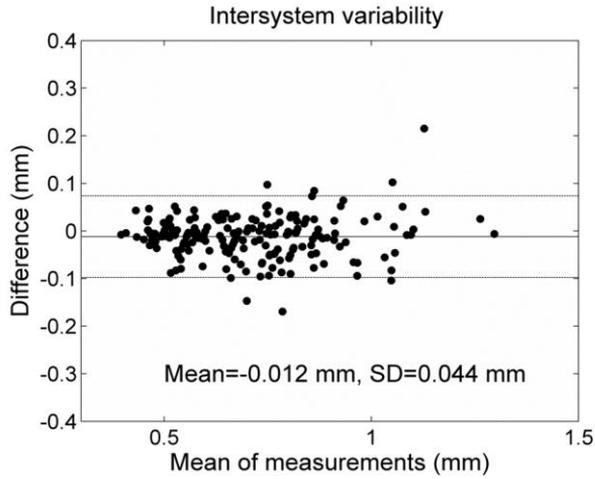
It was analysed based on measurements from both operators and both carotid arteries ( $n = 200$ , paired observations). The ICC was 0.98 ( $P < 0.001$ ) and CV of 4.6%. The mean difference in the measurements from the two systems was  $-0.012 \pm 0.04$  mm ( $P < 0.001$ ). Please see Figure 4.

### *Interoperator variability automated system*

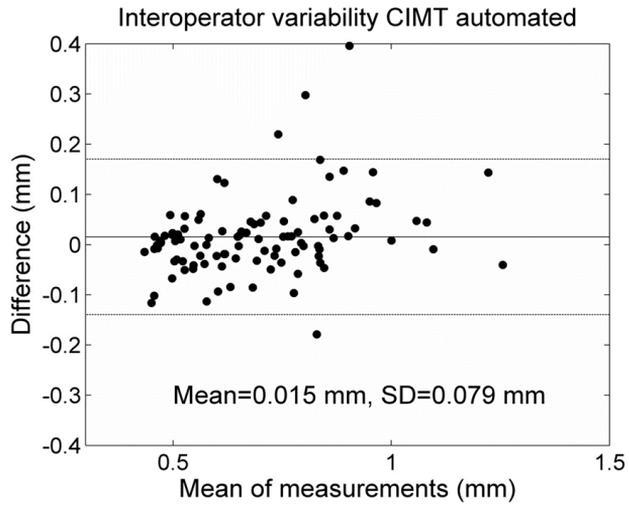
The overall mean values of the automated measurements made by both sonographers were  $0.70 \pm 0.19$  mm and  $0.68 \pm 0.17$  mm respectively calculated as pooled data from both sides,  $n=100$  paired observations. The mean difference was  $-0.01 \pm 0.08$  mm ( $P = 0.05$ ). The agreement between the two sonographers was express as ICC and it was 0.95 with  $P < 0.001$ . The CV of the two sonographers was 8.2%. Please see Figure 5.

### *Intraoperator variability automated system*

The intraoperator variability of the automated measurements was analysed using the first three scans made by each sonographer on the left side. For operator A, the CV was 8.4% with an ICC of 0.91 ( $P < 0.001$ ) and for operator B the CV was 6.5% with a ICC = 0.94 ( $P < 0.001$ ).



**Figure 4. Intersystem variability.**



**Figure 5. Interoperator variability CIMT automated.**

## Study II

### *Feasibility*

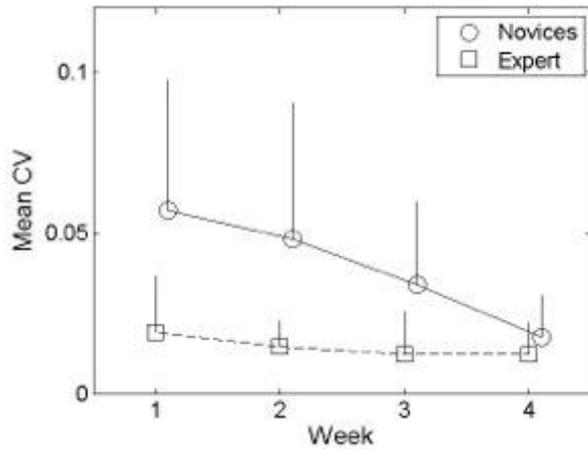
During the first week the feasibility was low for novice 1 and 4. Novice 1 was unable to achieve the cIMT measurement in 2 of 6 subjects (33%). Novice 4 could not achieve the cIMT measurement in 1 of 6 (17%). During the second week novice 2 was unable to achieve the cIMT measurement in 3 of 6 subjects (50%). During the same week novices 3 and 4 were unable to achieve cIMT measurement in 1 subject (17%) each. During the fourth week, the feasibility was 100% for all novices. For the expert the feasibility was 83% in the first week and 100% in the fourth week.

### *Intraobserver variability*

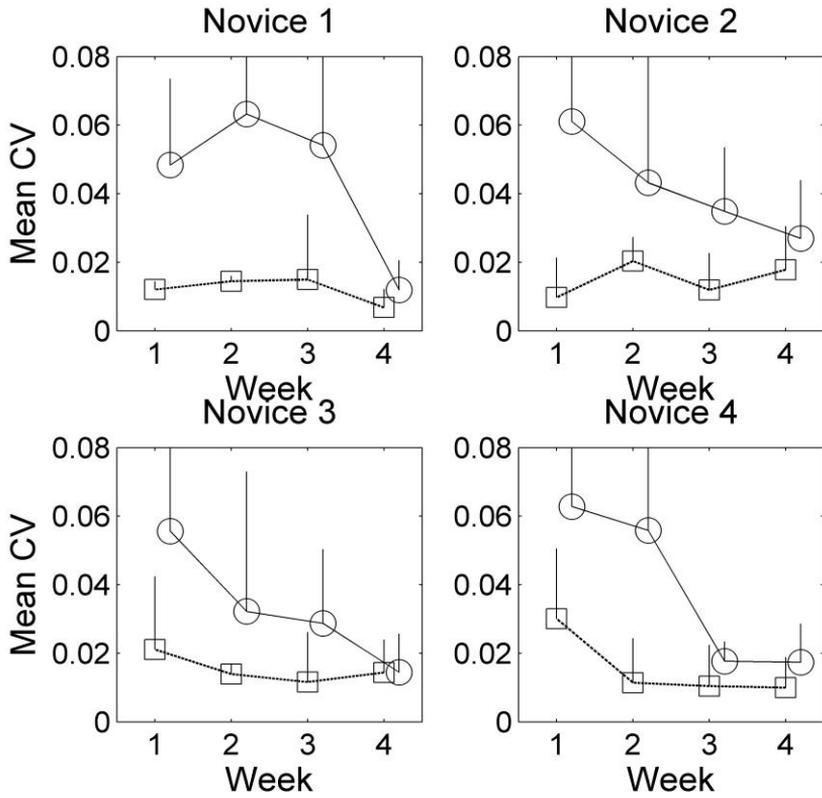
The mean CV of the novices' measurements of the 24 patients decreased over the course of four weeks: 0.06, 0.05, 0.03 and 0.02, respectively ( $P = 0.03$ ) (figure 6). The corresponding mean CV for the expert was 0.02, 0.02, 0.03 and 0.02, respectively ( $P = 0.68$ ). The pattern of reduced weekly mean CV was seen in all four novices. The ICC expressed the agreement between novice measurements: 0.97 ( $P < 0.001$ ) in the first week, increasing to 0.99 ( $P < 0.001$ ) in the 4<sup>th</sup> week. For the expert, the ICC in the first week was 0.99 ( $P < 0.001$ ) and remained unchanged (0.99,  $P < 0.001$ ) at the 4<sup>th</sup> week.

### *Interobserver variability*

The ICC expressed the interobserver variability between novice and expert, showing good correlation already at the end of the first week (0.98,  $P < 0.001$ ), and the agreement remained throughout the four weeks (0.95,  $P < 0.001$  at the end of the fourth week). As shown in Figure 7, there was no obvious relationship between the difference and the mean of the measurements made by the novices and the expert, in the 4<sup>th</sup> week.



**Figure 6. Weekly means of CV:s of all novices compared with those from an expert operator. Data shown are weekly averages of CV (symbols) and SD:s of CV:s (lines).**



**Figure 7. Weekly changes in the agreement between the measurements made by the different novices and the expert. Data shown are weekly averages of CV (symbols) and SD:s of CV:s (lines).**

## Study III

Of the 162 enrolled patients, 29 (17.9%) were excluded because of poor visualization of the plaques due to shadowing or poor quality of the B-mode frame. A total of 327 carotid plaques images in 133 patients were analysed. Of the 133 patients, 34 (25.5%) experienced a recurrent ipsilateral CVE (stroke or TIA, including retinal events) before carotid surgery. There were no hemorrhagic events. Patients affected by recurrent CVE (group II) had higher levels of total cholesterol ( $5.2 \pm 1.2$  vs  $4.7 \pm 1.1$ ) and HDL ( $1.3 \pm 0.4$  versus  $1.2 \pm 0.4$ ) compared to those who did not experience a recurrent CVE (group I), see Table 2.

**Table 2. Clinical data in patients with and without recurrent cerebrovascular events.**

Variable	All patients	No recurrent CVE	Recurrent CVE	P
		Group I , n = 99	Group II, n = 34	
<b>Demographics</b>				
Age, yrs	70.0 $\pm$ 7.8	69.8 $\pm$ 7.9	70.8 $\pm$ 7.7	0.496
Female, %	48 (36.1)	33 (33.3)	15 (44.1)	0.259
<b>Baseline characteristics</b>				
Diabetes, %	33 (24.8)	28 (28.3)	5 (14.7)	0.167
Hypertension, %	124 (93.2)	92 (92.9)	32 (94.1)	0.812
Claudicatio, %	23 (17.3)	21 (21.2)	2 (5.9)	0.063
Prior AMI, %	30 (22.6)	25 (25.3)	5 (14.7)	0.242
Prior stroke, %	20 (15.0)	17 (17.2)	3 (8.8)	0.518
Heart failure, %	4 (3.0)	3 (3.0)	1 (2.9)	0.999
Angina, %	20 (15.0)	14 (14.1)	6 (17.6)	0.622
History of AF, %	14 (10.9)	12 (12.8)	2 (5.9)	0.351
Smoking, %	28 (21.9)	21 (21.9)	7 (21.9)	0.999
Antihypertensive drugs, %	133 (100)			
Statin, %	121 (91.0)	91 (91.9)	30 (88.2)	0.518
Antiplatelets/Anticoagula	133 (100)			
SAP, mmHg	145 $\pm$ 24	145 $\pm$ 22	146 $\pm$ 29	0.660
DAP, mmHG	78 $\pm$ 11	78 $\pm$ 10	78 $\pm$ 13	0.747
Total Chol, mmol/l	4.8 $\pm$ 1.2	4.7 $\pm$ 1.1	5.2 $\pm$ 1.2	0.024
HDL, mm/l	1.2 $\pm$ 0.4	1.2 $\pm$ 0.4	1.3 $\pm$ 0.4	0.039
LDL, mm/l	2.8 $\pm$ 1.1	2.8 $\pm$ 1.1	3.1 $\pm$ 1.1	0.082

Data are expressed as mean  $\pm$  SD, absolute number (percentage)

Abbreviations: SAP, systolic arterial pressure; DAP, diastolic arterial pressure; AF, atrial fibrillation; AMI, acute myocardial infarction; HDL, high density lipoprotein; LDL, low density lipoprotein

### *Ultrasound based texture feature*

At univariate and multivariate analyses (Table 3 and 4) no texture variables were related to recurrent CVE.

**Table 3. Ultrasound based plaque texture characteristics.**

Variable	All patients	No recurrent CVE		Recurrent CVE		P
		Group I, n = 99	Group II, n = 34	Group I, n = 99	Group II, n = 34	
DWA presence, (%)	111 (77.6)	86 (86.9)	25 (73.5)		0.071	
JBA presence, (%)	62 (43.4)	45 (45.5)	17 (50.0)		0.647	
GSM	44.10 ± 15.54	44.86 ± 16.65	45.40 ± 17.53		0.841	
Area, mm <sup>2</sup>	42.95 ± 21.41	42.62 ± 20.61	40.52 ± 20.53		0.506	
Entropy	3.53 ± 0.28	3.51 ± 0.26	3.58 ± 0.34		0.438	
Coarseness	14.13 ± 3.11	14.22 ± 3.22	14.39 ± 3.17		0.646	
JBA GSM	8.71 ± 13.39	7.58 ± 11.41	10.68 ± 24.05		0.880	
N. Plaques	2.16 ± 1.57	2.41 ± 1.35	2.59 ± 1.28		0.355	

Data are expressed as mean ± SD, absolute number (percentage). DWA, discrete white area; JBA juxtaluminal black area; GSM, grey scale median averaged; N. Plaques, numbers of plaques

**Table 4. Multivariate analysis with risk factors for recurrent ipsilateral cerebrovascular events and ultrasound variables.**

Variable	Wald X <sup>2</sup>	p Value	OR	95% CI
Age	0.58	0.810	1.01	0.95-1.07
Prior CEA/CAS	1.36	0.244	0.45	0.12-1.72
Tot Chol	0.01	0.956	1.16	0.26-3.60
HDL	2.82	0.093	4.66	0.77-28.06
LDL	0.31	0.576	1.45	0.39-5.37
Claudication	2.19	0.139	3.31	0.68-16.12
DWA presence	1.51	0.219	2.13	0.64-7.10

CEA, carotid endoarterectomy; CAS, carotid artery stenting; OR, odds ratio; CI, confidence interval  
Tot Chol, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein;  
DWA, discrete white area

## Study IV

### *Feasibility*

Forty-two plaques were detected and analysed in 33 subjects (18 men, age mean  $59.0 \pm 2.9$  years). The analysis of B-mode images was feasible in 37 plaques (4 plaques were excluded due to shadowing artefacts, 1 plaque was echo lucent and went undetected by B-mode).

With the CEUS technique, at time point  $T_5$  analysis of 35 plaques was possible: ultrasound artefacts overshadowed 4 plaques, 3 plaques were unable to be analysed due to motion artefacts. Thirty-four and 35 plaques were analysed at time point  $T_{10}$  and  $T_{15}$ , respectively (4 plaques were covered by “shadows” at both  $T_{10}$  and  $T_{15}$ , motion artefacts were present in image loops of 4 plaques at time point  $T_{10}$  and in 3 at time point  $T_{15}$ ). For the resulting ultrasound measurements see Table 5. These measurements show no significant differences between plaque area values obtained from B-mode or CEUS image loops.

**Table 5. Plaque area measurements obtained with CEUS and B-mode based images. P-values refer to comparisons with measurements from B- mode that was used as the baseline reference in the models. CEUS, contrast-enhanced ultrasound; CI confidence interval.  $T_5$ ,  $T_{10}$ , and  $T_{15}$ , refer to time points.**

Method	Estimated Marginal Means ( $\text{mm}^2$ )	$p$ Value	CI ( $\text{mm}^2$ )
B-mode	24.75	0.810	20.20-29.30
<b>CEUS</b>			
$T_5$	24.67	0.58	19.83-29.51
$T_{10}$	24.70	0.72	19.86-29.54
$T_{15}$	24.81	0.70	19.97-29.65

*Inter-methods variability*

In study IV a strong agreement was found between B-mode and CEUS measurements at time points  $T_5$  (ICC=0.998),  $T_{10}$  (ICC=0.999) and  $T_{15}$  (ICC=0.998), respectively.

*Interobserver variability*

The ICC for the inter-observer variability was 0.96 for B-mode measurements, 0.98, 0.98 and 0.99 respectively for CEUS measurements at  $T_5$ ,  $T_{10}$  and  $T_{15}$ .

*Intraobserver variability for CEUS*

The ICC for the intra-observer variability was 0.99 for operator A and 0.98 for operator B.

# Discussion

The 2004 European Society of Hypertension/European Society of Cardiology (ESH - ESC) guidelines proposed a new risk stratification scheme for estimating absolute risk for CVD (3). Compared to the previous 1999 World Health Organization–International Society of Hypertension (WHO/ISH) guidelines, the new criteria include additional risk factors such as obesity and abnormal high density (HDL) or low density lipoprotein (LDL), and define a slight increase in creatinine and microalbuminuria as signs of target organ damage. New methods, in combination, have been suggested and developed to classify future potential individual risk for CVD (73). “Vascular age” (73) can be calculated using several measured risk markers and determines the age a person would have based on the measured parameters. If the “vascular age” calculated is greater than the actual age of the patient then a patient is diagnosed as having an increased risk of developing CVDs (73). However, there are some issues to resolve with this method, including the following;

What are the cut-off values dictating when to recommend intensive pharmacological prevention?

Are the methods used to assess the early atherosclerotic lesion accurate enough to detect small changes in the vascular wall?

Can we really measure these small changes with accuracy?

Thickening of the carotid intima media (cIMT) has been established as an early sign of organ damage. A study conducted in over 580 subjects revealed a favourable cost/benefit ratio for ultrasound as a tool for early organ damage (cIMT) assessment. A study involving 1288 subjects, demonstrated the role of carotid ultrasound as a tool for assessment of cardiovascular risk; the presence of any structural changes in the common carotid arteries or carotid bulbs was associated with a 3.29-fold risk of acute myocardial infarction compared with men free of any structural changes in the carotid artery wall at baseline. The presence of intimal-medial thickening was associated with a 2.17-fold risk of acute myocardial infarction and even greater risk was observed for small carotid plaques (4.15-fold) and large "stenotic" plaques (6.71-fold) (74). A meta-analysis of cIMT data concluded that with each 1-SD increment of cIMT, the relative risk of coronary heart disease and stroke increase with an OR of 1.26 and 1.32, respectively (75, 76). cIMT over the 75th percentile for sex and age was shown to be associated with future risk of CVD, independent of traditional risk factors (77). In addition to cIMT, the presence of carotid arterial plaques has proven to be superior in predicting coronary artery disease (1).

A complementary strategy involves coronary calcium scoring (CAC) using computer tomography (CT). This method was shown to provide accurate prognostic information (2). However, CT exposes the patient to low doses of radiation, it is expensive and not suited for low density populations with long distance to hospitals with CT

In fact direct assessment of carotid arteries of individuals for detection of carotid plaque and measurement of cIMT resulted in a reclassification of individuals risk as either low or high (78).

Morphological studies using ultrasound have identified echographic plaque characteristics, beyond the severity of stenosis, that reflect a higher risk of vulnerability, thus defining the “unstable” plaque (27, 68, 79-82). Adventitial vasa vasorum and plaque neovascularization have been well established and confirmed in histological studies as predictors of unstable atherosclerotic lesions in cerebro- and cardiovascular patients (43, 44, 83-86). CT, magnetic resonance imaging (MRI), digital angiography and even positron emission tomography (PET) have been recently applied to determine the biological functional pathways of carotid plaques (3-5). CT and MRI offer the best anatomical resolution; however, none of these techniques can be employed as screening tools because of the prohibitive cost, and for CT, the radiation delivered to subjects.

PET has been used to assess the metabolic activity of plaque’s angiogenesis and inflammation (6). Naturally, such a sophisticated tool will open new frontiers for the study of the complex pathophysiological mechanisms of tissue (plaques) inflammation; however, PET currently cannot be systematically and efficiently used for clinical routine or for large population studies.

Compared to CT, MRI and PET, ultrasound is considerably more suited for large population studies and routine clinical screening. Ultrasound is an efficient tool for collecting a large amount of data in a relatively short amount of time with a high cost/benefits ratio. Ultrasound is transportable, harmless for the patient and can be decentralized under guidance from specialized laboratories to other health care providers. However, the question is how to use ultrasound in the most reliable way?

#### Study I.

Assessment of early signs of atherosclerosis such as increased cIMT could be challenging because of the lack of standardization of protocols and the reproducibility of results. Reproducibility of cIMT measurements was the main objective of study I.

The major findings of this study were that the automated ultrasonograph provides values for cIMT comparable to those obtained by the conventionally

used manual system. Measurements were highly reproducible between systems, irrespective of the operator acquiring the images. Moreover, the acquisition time of the automated ultrasonograph was significantly shorter than that of the conventional system. The study was conducted following a test re-test model to replicate a clinical “environment”. The intersystem variability was low with an excellent ICC (0.98,  $P < 0.01$ ). Moreover, the interoperator variability (ICC 0.95) and the intraoperator variability (ICC 0.91 and 0.94 for operator A and B respectively) for the automated system had shown a good reproducibility of measurements. The automated system achieved cIMT measurements faster than a conventional manual system making it an ideal tool for survey studies. A major limitation of this study was to have compared an automatic system to a manual method instead of a semi-automatic one. In this study we aimed to compare measurements achieved by different methods in an environment as similar as possible to a clinical one. All available semi-automatic systems at that time were able to perform only time consuming off-line analyses, making them not useful in a clinical setting.

Personnel involved in this study were sonographers who are highly trained and specialized in vascular ultrasound, thus explaining the excellent ICC achieved for the inter-system variability.

## Study II

In study II, the same automatic system used in study I was operated by two groups of operators. The first one represented an expert in carotid ultrasound and the second one constituted four novices with no experience in ultrasound. In this study, we aimed to answer a simple question: “could any operator produce reliable and stable cIMT measurements with the assistance of the technology available for ultrasound imaging, and thus make cIMT a truly useful tool in atherosclerosis assessment?”

It should not be forgotten that cIMT increases normally with age, from an average of 0,5 mm in early childhood to an average of 0,8 mm in healthy octogenarians (7), thereby accounting for an approximate annual increase of thickness between 0.03 and 0.15 mm (8, 9). Such variations in cIMT and technical bias connected to the scanning technique make consideration of absolute measurements as well as their change overtime potentially difficult to collocate in a clinical scenario. A number of factors contribute to the reproducibility of cIMT measurements reported in the literature. These include the type of ultrasound system used (manual, semi-automated or fully automated with direct on screen results) and the techniques applied (B-mode imaging based or RF data based) (10-15). A number of algorithms have been developed for the fully automatic segmentation of the carotid intima-

media (16-18). Among the systems currently available, fully automatic RF-data based software seems to have a comparable, or even higher, reproducibility than off-line semi-automatic programs or manual systems, with ICCs of 0.95, 0.91 and 0.73, respectively (10, 13, 19). From the 1980s a tendency toward automation of carotid intima-media segmentation (measurement) seems to have taken hold promising to improve the reproducibility of results and to shorten the learning curve of sonographers. In fact the experience of the operators using the ultrasound systems, above all if manual or semi-automatic, could multiply the potential variability of the measurements, which can compromise the clinical value of the cIMT. As a result, the use of cIMT measurements is still confined to cardiovascular centres where only well trained (46, 53) personnel undertake such examinations, particularly where a disease prevention service is established.

In study II we observed the variability of the cIMT measurements obtained by a group of novice ultrasound operators during a learning curve period of four weeks. The end point of the study for the group of novices was to approach, at the end of the learning curve, the intra-observer variability similar to that of an expert using a full automatic ultrasound scanner. The major finding of study II was that even novices who receive well structured and designed brief training, preceded by an intensive theoretical background on carotid anatomy, ultrasound physiology and optimum measurement acquisition succeed in achieving highly reproducible measurements not different from experts (ICC 0.97 at baseline increasing to ICC 0.99 at the end of the study). This result was in part obtained thanks to the segmentation algorithm that freezes the image on the CIMT once an excellent quality is obtained. Such consistency in the image acquisition must have contributed to the rapid improvement of the novices' measurement reproducibility. A major limitation of the study was the small number of patients enrolled: in a larger group, with more challenging patient from a technical point of view, perhaps the end point could be still achieved but in a longer period of time.

In studies I and II was evaluated the reproducibility of conventional ultrasound marker of early of atherosclerosis. In studies III and IV we studied ultrasound-based markers of atherosclerosis, other than cIMT, based on mounting evidence that the presence of plaque as well the plaque burden predicts better cerebrovascular events (CVE) than cIMT (1) and that echo lucent plaques are more often associated with acute cerebrovascular events than the echogenic ones (20-25).

### Study III

In study III, we hypothesised that grey scale median (GSM), juxtaluminal hypo echoic area (JBA), coarseness, entropy, and discrete white area (DWA) could be useful in predicting the short term (90 days) risk of recurrent ipsilateral ischemic stroke or TIA in patients with symptomatic carotid stenosis (70-99% stenosis) waiting for carotid surgery. The major findings in this study seem to contradict what was reported in the literature (23, 24, 26). At the multi- and univariate analyses there were no significant association between any of the 5 tested plaque ultrasound based characteristics. A possible explanation for the lack of association could be the high echogenicity (GSM > 40) of the plaques observed in the group that developed a second CVE as well in those who did not.

### Study IV

Beside the above mentioned texture characteristics, plaque burden has been reported to be associated with CVE (29-31). In the Tromsø study, the total carotid plaque area (TPA) was shown to be a predictor of CVE (32, 33). The larger TPA, the higher is the risk for CVE. Thus, quantification of carotid plaque has become an attractive tool to predict risk for cerebrovascular events. Evaluation of plaque area can be performed using conventional B-mode images and contrast enhanced ultrasound images (CEUS) (5). Measurements of plaque area with CEUS can be subjected to multiple technical biases. In study IV we hypothesize that plaque area measurements may change due to the effect of CEUS blooming artefacts at different time points, and we tested the agreement of plaque area measurements performed using CEUS and conventional B-mode ultrasound images. The major finding of the study was the strong ICC relationship between plaque areas measured from B-mode image loops and from CEUS image loops, at progressive time points suggesting that blooming artefacts may not affect the assessment of carotid plaque areas at any time. In the study, the interobserver variability between two expert operators measuring plaque areas from conventional B-mode image loops (ICC=0.96) and CEUS image loops (ICC > 0.97 at any time point) was low. This could suggest that CEUS does not improve the reproducibility of results for plaque areas between 7 and 75 mm<sup>2</sup>.

The results of studies III and IV lead to a reflection of the role that ultrasound could play in the future of imaging of carotid atherosclerosis. Although the advancing in technology could produce and support the use of several sophisticated makers of early atherosclerosis or markers of atherosclerotic progression, these technologies are not yet ready for clinical use.

## **Future directions**

The future role of ultrasound in assessment of carotid atherosclerosis is strictly dependent on the improvement of the current technology. The above-mentioned issues in this work are, de facto, limiting factors and any improvement in any of the recalled problems will eventually boost the role of ultrasound, perhaps not as a tool for primary diagnosis, but for monitoring the never ending changing of the atherosclerotic lesions. The current ultrasound technology can give us a reliable diagnosis of “plaque” or “thickened intima-media” but struggles to follow the evolution of these structures; in fact artefacts are the major limitations of ultrasound. Reverberation and refraction produce “false” echos that alter the signal/noise ratio and produce overestimation of the thickness of a given anatomical structure, and therefore reduce the precision of measurements. With more advance, post-processing algorithms should be possible to improve the signal/noise ratio and therefore assess with more precision the anatomy of the carotid vessels.

New subtraction algorithms may produce a better filtering of the over reflected signal coming from calcified plaque responsible for the shadowing artefact. Already available are second-harmonic based blood-signal enhance software capable of enhancing weak signals. These two latter improvements will give us the possibility to “read” the plaque under the “shadow”, and therefore assess structures that are “de facto” invisible today. Already available technical feature such auto-detecting probe-position with displayed angle of insonation will further reduce the inter- and intraobserver variability improving the reproducibility of measurement, and therefore in clinical terms, the possibility to detect small changes in the plaque anatomy.

Better tools for monitoring changes will produce a better understanding about what treatment “works” and what does not in a single patient; this is the ultimate goal still to be achieved.

# Conclusions

- In study I we have demonstrated the high feasibility and reproducibility of cIMT measurements obtained with an automated carotid ultrasonograph and with conventional, B-mode images based system.
- In study II we have shown that automatic system with high grade of automation can provide cIMT measurements with high reproducibility when used by novice operator after a short but well designed training program.
- In study III we have shown that measurements of new ultrasound based atherosclerotic markers can be performed with high reproducibility but those markers were not shown to be beneficial in predicting plaque vulnerability and recurrent ipsilateral ischemic cerebrovascular events in patients with symptomatic carotid stenosis waiting for carotid endarterectomy.
- Study IV has demonstrated that measurements of plaque burden, expressed as plaque area, can be performed with high feasibility and reproducibility with and without contrast enhanced ultrasound technique.

In summary medical ultrasound technology has proven to produce measurements of the tested markers with high reproducibility and low interobserver variability. Application of more sophisticated methods for assessment of plaque vulnerability in clinical practice at an individual level, needs further improvement, research and testing

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