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Title: Carotid plaque neovascularization detected with Superb Microvascular Imaging Ultrasound without using contrast media

Cover title: Detection of carotid plaque neovascularization

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Abstract

Background and purpose: A significant proportion of ischemic strokes are caused by emboli from unstable carotid artery plaques with intraplaque neovascularization (IPN) as a key feature of plaque instability. IPN is not detectable with conventional Doppler ultrasound. Contrast-enhanced ultrasound (CEUS) can visualize IPN, but it`s use is limited in clinical practice because it requires an intravenous injection of contrast. Superb microvascular imaging (SMI) without contrast uses an algorithm to remove clutter and motion wall artefacts whilst preserving low velocity blood flow signals, enabling visualization of IPN. Our aim was to assess the feasibility of SMI for the detection of IPN.

Methods: Thirty-one patients with >50% carotid stenosis were included; twenty-two patients were symptomatic and 9 asymptomatic. All patients underwent conventional carotid ultrasound, CEUS, SMI and blood tests. CEUS and SMI findings were compared and correlated to histological plaque assessments after endarterectomy.

Results: There was significant positive correlation between a IPN visual 5-level classification of SMI and a semi-quantitative analysis of CEUS, (p<0.001, r=0.911). Plaques with higher SMI grades had higher numbers of neo-vessels quantified at histology, (p=0.041, r=0.460). Hypoechoic plaques had higher grades of IPN on both CEUS and SMI, (p<0.001). Higher visual IPN counts on SMI were associated with (i) Increased areas of inflammation (p=0.043, r=0,457), (ii) Combined rank scores of granulation tissue, inflammation and lipids (p=0.02, r=0.494) at histology, and (iii) Higher peak-intensity values on quantitative CEUS (p=0.042, r=0.514).

Conclusion: Superb Microvascular imaging ultrasound can detect neovascularization with accuracy comparable to CEUS, suggesting SMI to be a promising non-invasive alternative to CEUS for the assessment of carotid plaque stability.

Introduction

Ischemic strokes caused by thromboembolism from an unstable plaque in the carotid artery can be effectively prevented by carotid endarterectomy (CEA)[1, 2]. In current clinical practice the selection of patients for CEA is based on the degree of carotid artery stenosis and presence or absence of cerebral ischemic symptoms. It has in recent years, however, become increasingly clear that the degree of carotid artery stenosis alone is not the best predictor of stroke risk. This has led to the concept of the "unstable plaque" for the description of carotid plaques that carry high risk of stroke irrespective of the degree of artery stenosis. Research has therefore increasingly focused on identifying factors other than degree of artery stenosis that are important for plaque destabilization. One of these factors is intraplaque neovascularization (IPN) which has been documented to play a key role in plaque instability[3-7]. With increasing degrees of vascularization, plaques become more prone to hemorrhage and rupture, making them unstable and increasing the risk of thromboembolic stroke.

Ultrasound visualization of the vasa vasorum (VV) and quantification of IPN has a potential important role in cerebrovascular risk assessment and may be a potential surrogate marker of carotid plaque instability and stroke risk.

Conventional Doppler based ultrasound, however, applies a filter to remove wall motion artefacts and clutter which also excludes the detection of low velocity blood flow signals from IPN[8, 9]. Both contrast enhanced ultrasound (CEUS) and Superb Microvascular Imaging (SMI) have shown promise in visualizing this neovascularization[10-16]. CEUS applies high spatial and temporal resolution which combined with the intravascular traceability of contrast

microbubbles make CEUS a valuable tool in detection of IPN[10, 12, 17, 18]. Indeed, several studies have found that intraplaque neovascularization, assessed with CEUS, correlates well with micro-vessel density on histological assessments of excised plaques[10, 12, 14]. CEUS requires, however, an intra-venous injection of a contrast agent with an associated risk which limits its use in clinical practice.

SMI is a new ultrasound imaging technique, developed to overcome the limitations of conventional Doppler ultrasound which enables the visualization of neo-vessels without the use of intravenous contrast. This technique applies an exclusive algorithm to differentiate true microvascular flow signals from wall motion artefacts and clutter, thereby allowing the visualization of intraplaque microvascular flow signals (IMVF).

Recent studies comparing the detection of IPN using CEUS and comparing results with SMI demonstrated a good agreement between the two methods[16, 19, 20]. To secure validity of these results, IPN detection using SMI ultrasound should also be verified by histological assessments which were not carried out in most previous reports. The aims of this study were therefore to assess the ability of SMI to detect carotid plaque IPN, to compare the level of agreement between SMI and CEUS assessment of plaque IPN and to correlate findings with histological analyses.

Patients and methods

Thirty-one consecutive patients with internal carotid stenosis ≥50% were included in the study. Nine included patients were asymptomatic. Twenty-two patients were scheduled for CEA, twenty symptomatic and 2 asymptomatic. Plaques were defined as symptomatic when associated with ipsilateral cerebral ischemia (minor strokes, transitory ischemic attacks or amaurosis fugax) within 30 days prior to study inclusion. The decision to select the two

asymptomatic patients for CEA was based on the plaque characteristics on ultrasound (echolucent and irregular surface). Exclusion criteria were contra-indications for the use of ultrasound contrast agent such as right to left cardiac shunts, severe pulmonary hypertension (pulmonary artery pressure >90 mmHg), uncontrolled systemic hypertension, adult respiratory distress syndrome and contrast agent allergy. Patients underwent conventional Doppler ultrasound, SMI and CEUS of the carotid arteries prior to carotid endarterectomy or at a routine outpatient ultrasonographic control for the asymptomatic patients. The study protocol conformed with ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (ID REC 2014/1468), and written informed consent was obtained from all patients prior to study inclusion. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ultrasonography

Imaging was performed with a Canon ultrasound system Aplio 500 (Canon Medical Systems, Otawara, Japan) using a 7.5 MHz linear probe on both carotid arteries for standard Doppler ultrasound, CEUS and SMI. Common carotid artery, carotid bifurcation and internal carotid arteries were examined in longitudinal and transverse planes in standard ultrasound. The degree of carotid artery stenosis was determined based on peak systolic and end diastolic velocities according to consensus criteria of the society of radiologists in ultrasound[21]. Plaque echogenicity was classified visually in high-resolution B-mode grey-scale pictures according to the modified version of the classification proposed by Gray–Weale classification as follows: 1. Uniformly Hypoechoic, 2. Predominantly hypoechoic (hypoechoic with small hyperechoic regions), 3. Predominantly hyperechoic (hyperechoic with small <25% hypoechoic regions) or 4. Uniformly Hyperechoic[22, 23].

Superb Microvascular imaging (SMI)

An ultrasound examination was first performed as follows: following the assessment of plaque echogenicity and the degree of carotid stenosis the setting on the ultrasound scanner was switched to the monochrome SMI (mSMI) mode that showed a twin-view display of the plaque in B mode and mSMI side by side. After choosing the inbuilt mSMI software, the SMI specific region of interest (ROI) box was positioned around the entire plaque. Other SMI settings were optimized as follows: mechanical index of 1.5, frame rate of 50-60 frame per seconds (fps), dynamic range of 55-65 dB and SMI velocity 0.8-2.0 cm/s. Plaques were first observed in the transverse plane and then in the longitudinal plane for 2 minutes and the video images were stored in the ultrasound scanners hard drive. Static enhancements were excluded and moving enhancements were classified as intraplaque microvascular flow (IMVF). IMVF signals were firstly categorized on a visual scale as follows: Grade 0: no IMVF within the plaque or IMVF confined to the adjacent adventitia, Grade 1: moving IMVF confined to the adventitial side, Grade 2: moving IMVF at the plaque shoulder, Grade3: IMVF moving to the plaque core (Figure 1) and Grade 4: extensive IMVF. Secondly, a visual count of the IMVF signals was carried out and the number of neo-vessels observed in a two-minute SMI video clip was counted. Ten plaques were assessed on SMI from grade 0 to grade 4 IMVF by an experienced Neurologist with 10 years' experience in carotid ultrasonography who was blinded for research physician's findings to obtain interrater variability using Kappa statistics.

Contrast-enhanced ultrasound (CEUS)

CEUS was performed using a contrast-specific imaging mode with a low mechanical index (MI -0.12) to prevent destruction of contrast microbubbles (sized 1-11 μm). Patients received an intravenous bolus injection of 2.5 ml contrast microbubbles (Sono Vue; Bracco,Milan, Italy), in 5 ml 0.9 % saline followed by 5 ml physiological saline injection. Digital recordings

were started in longitudinal plane on the arrival of contrast microbubbles at the carotid artery bifurcation and continued for up to 7 minutes. Native raw data was stored in the scanners hard drive for later off-line assessments. IPN was assessed by two methods. Firstly semi-quantitatively, where the intraplaque contrast enhancement was categorized on a visual scale as follows; Grade 0: No bubbles within the plaque or bubbles confined to adjacent adventitia. Grade 1: Moving bubbles confined to the adventitial side. Grade 2: Moving bubbles at the plaque shoulder. Grade 3: Bubbles moving to the plaque core (Figure 1). Grade 4: Extensive intraplaque enhancement[10, 12, 24, 25]. Secondly using a quantitative assessment of plaque contrast enhancement of the off-line native uncompressed data. This is important because the scanner has the ability to store CEUS data as both video data (compression applied to the original signals in order to display them on video monitors) and RAW data (uncompressed data). The massive compression applied to original signals causes loss of important information which is vital for the quantification of enhancement intensity[26]. A time-intensity curve (TIC) was plotted using built-in quantification software (Canon Medical Systems, Otawara, Japan). A ROI was manually drawn around the plaque and a second circular ROI was placed in the lumen of the artery as reference. Curve fitting was applied to the TIC and TIC derived peak intensity (PI) values were obtained. PI is the maximum intensity of the TIC compared to that for local blood flow in the ROI in the artery. This gives a quantitative measure of plaque enhancement.

Tissue processing and histological analysis

Carotid plaques removed en bloc (intact) at endarterectomy were fixed in 4% formaldehyde, decalcified in ethylene diamine tetraacetic acid (EDTA) or 17% formic acid and cut in 2-3 mm slices. After dehydration and paraffin embedding, histological sections were cut at 5 micrometers (μm) and stained with hematoxylin and eosin. The plaques were assessed by (MZ) and an experienced pathologist (HS) blinded for the clinical and ultrasound findings. In

each section the plaque area was calculated based on measurements of length and average width of the plaque with a microscope equipped with an ocular lens with a micrometer scale. Areas with inflammation, granulation tissue, lipid, fibrosis and calcification were then scored using a semi-quantitative eyeballing system, modified from the scoring of kidney biopsies[27] and given as percentages of the section area. The percentage of a component in the plaque was calculated as the sum of these component areas in all the sections from the plaque divided by the total plaque area[28]. In each plaque section the number and diameter of vessels with a lumen diameter of 0.01 mm or greater were measured.

The plaques were ranked ascendingly based on the size of the measured areas of: 1. Granulation tissue, 2. lipid 3. Inflammation and then given a total rank score by combining all three components.

Histological assessments were carried out in 22 slices from 9 plaques on two occasions which were more than 2 months apart to assess the reproducibility of the findings. For this analysis the percentages of all components (granulation tissue, lipids, inflammation, calcium, and fibrosis) for each slice were classified by the two observers in 5% levels from 0-5% up to 95-100%. The results were assessed using Kappa statistics.

Blood samples were taken from all patients for the measurements of white blood cells, c-reactive protein (CRP), sedimentation rate (ESR), glucose, HbA1c, total cholesterol, high density lipoproteins (HDL), low density lipoprotein (LDL), triglycerides, glucose and glycated hemoglobin (HbA1c).

The IPN findings assessed using SMI and CEUS were compared and correlated to histological findings.

Statistical assessments

SPSS for Windows statistical software (version 25.0) was used for data analyses. Chi-square test was used to test the relationship between the categorical variables and Mann-Whitney U test was used to compare the non-parametric categorical variables with continuous variables. Coefficients of correlation were calculated by the Spearman rho correlation. All statistical results were considered significant when $p<0.05$. For the determination of interrater variability, Cohen's kappa was used to measure agreement between the two different ultrasound examiners using the established grading of agreement[29]: <0 (no agreement), 0 to 0.2 (poor), 0.21 to 0.4 (fair), 0.41 to 0.61 (moderate), 0.61-0.80 (substantial), 0.81 to 1.0 (nearly perfect). The receiver operating characteristic (ROC) curves was used to evaluate the accuracy of SMI-count and quantitative CEUS in predicting microvessels ≥50μm at histology.

Results

Clinical characteristics

Thirty-one patients participated in this study. There were 20 men (69 ± 5 years) and 11 women (72 ± 9 years). Twenty-two patients were symptomatic and 9 patients asymptomatic. Twenty-two plaques were removed at endarterectomy. Two symptomatic patients' plaques were not delivered for histological analysis. The first carotid plaque was not available for histological analysis because of logistical miscommunication where the researcher was not notified in time to collect the plaque after surgical removal, and the plaque therefore was not stored in formalin. The second carotid plaque was extensively calcified and despite multiple decalcification steps, it could not be sliced into sections to undergo histological analysis. One symptomatic patient had a contraindication for the use of contrast agent and did not have a CEUS examination. Baseline variables of patients and plaques are shown in table 1 and 2.

Carotid plaque neovascularization detected using superb microvascular imaging ultrasound (SMI)

Ultrasound examinations of plaques using SMI visualized IMVF signals in 21 of the 31 plaques. Ten (32.3%) patients had no IMVF signals (grade 0), six (19.4%) patients had signals confined to the adventitial side of the plaque (grade 1), in one (3.2%) patient signals were seen at the plaque shoulder (grade 2), and fourteen (45.2%) patients had signal moving to the plaque core (grade 3). No patients were found to have extensive IMVF signals (grade 4) on SMI. CEUS examinations showed that ten (32.3%) patients had no contrast enhancement (grade 0), four (12.9%) patients had moving micro-bubbles confined to the adventitia (grade 1), four (12.9%) patients had moving micro-bubbles to the plaque shoulder (grade 2), eleven (35.5%) patients had micro-bubbles moving to the plaque core (grade 3), and one (3.2%) patient had extensive plaque enhancement (grade 4), Table 2. A comparison of the 5-level visual classifications of IMVF using SMI with semi-quantitative intraplaque enhancement on CEUS showed that patients with higher degrees of IMVF on SMI also had higher degrees of enhancement on CEUS (grade 3) (p<0.001, r=0.911) 95% CI (0.782, 0.992), (Figure 1). Patients with high neo-vessel counts on SMI also had higher grading of plaque enhancement on semi-quantitative CEUS (p<0.001, r=0.869), 95% CI (0.688, 0.961) and higher peak intensity values on quantitative CEUS (p=0.042, r=0.514), 95% CI (-0.072, 0.876). There was no statistically significant difference between symptomatic and asymptomatic patients with regards to the different grades of IMVF or neo-vessels counts using SMI. Plasma levels of CRP, total cholesterol, LDL, HDL, glucose and HbA1c were not significantly different across the different grades of IMVF assessments using SMI and in the two groups of patients (symptomatic vs asymptomatic patients).

Inter-rater variability for assessment of IPN using the 5-level classification with SMI was found to have Kappa=0.650, CEUS= 0.722, SMI-count= 0.756 and B-mode ultrasound 0.856 between two independent observers.

Carotid plaque enhancement and correlation with echogenicity on ultrasound

Plaques which were hypoechoic on B mode ultrasound had significantly higher grades of IMVF signals on SMI (p<0.001, r=-0.600), 95% CI (-0.816, -0.9) and enhancement on CEUS (p=0.0002, r= -0.625), 95% CI (-0.822, -0.329). Hypoechoic plaques on B mode ultrasound had also higher neo-vessel counts on SMI (p=<0.001, r= -0.747), 95% CI (-0.866, -0.553).

Intraplaque neovascularization SMI correlated with histology

Higher grades of IMVF on SMI were positively correlated to increasing number of observed neo-vessels on histological assessments (p=0.041, r=0.460), 95% CI (-0.007, 0.817). Increasing number of neo-vessels counted on SMI were also positively correlated to increased area of inflammation (p=0.043, r=0.457), 95% CI (0.009, 0.829), area of granulation tissue added to the area of inflammation (p=0.038, r=0.467), 95% CI (0.026, 0.767) and the total plaque rank score (combined rank scores of granulation tissue, inflammation and lipids) (p=0.02, r=0.494), 95% CI (0.058, 0.795) assessed on histology. When comparing the number of neo-vessels observed at histology in groups of patients with grade 0 and grades 1-3 IMVF signals on SMI, the latter had higher numbers of neo-vessels on histology. None of the patients had grade 4 IMVF signals on SMI (Figure 2).

Neo-vessels could be seen in granulation tissue and in fibrotic areas, a few also in the necrotic core. The neo-vessels in granulation tissue were surrounded by extravasated erythrocytes (Figure 3). The neo-vessels in fibrotic tissue were not surrounded by erythrocytes and had an intact endothelial layer (Figure 4).

When the histological assessments were repeated on two occasions more than two-months apart we found that percentages of the different components were in the same 5% levels as follows: Granulation tissue area (21 of 22 slices, Kappa =0.95), Lipid (17 of 22 slices, Kappa

=0.77), inflammation (18 of 22 slices Kappa =0.82), calcium (19 of 22 slices, Kappa =0.86) and fibrosis (15 of 22 slices, Kappa =0.68).

Diagnostic value of SMI and CEUS

According to Receiver operating characteristic curve analysis the area under the curve for SMI-count predicting plaques containing neo-vessels with diameter ≥50 μm was 0.742 and AUC for CEUS-peak intensity was 0.854. The optimal diagnostic point with the best combined sensitivity and specificity was found to be for SMI-count ≥4 (Table I, suppl) and for CEUS-peak intensity ≥4.9 ($\times 10^{-5}$ arbitrary units [AU]) (Table II, suppl).

Discussion

The main findings of this study were: Firstly, with increasing degrees of IMVF on SMI ultrasound, the degree of enhancement on CEUS also increased. Secondly, increasing IMVF grades on SMI were significantly correlated to increasing number of neo-vessels on histology providing the first histological validation on comparison of these two methods. These findings demonstrate that SMI can detect neovascularization with accuracy comparable to CEUS suggesting SMI as a promising non-invasive alternative to CEUS.

The finding in the current study of good correlation between SMI and CEUS is in keeping with three previous published reports which documented good consistency between SMI and CEUS in the evaluation of IPN[16, 19, 20]. Oura et al[16] reported that the sensitivity and specificity of SMI for predicting IPN visualized on CEUS were 100% and 63% respectively. Zhu et al[20] reported a good consistency between SMI and CEUS in visualizing IPN (p<0.001) in a randomized controlled study evaluating the efficiency of atorvastatin in patients with carotid plaques compared to placebo treatment. Zhang et al[19] also reported good consistency between CEUS and SMI in detection of IPN (p< 0.05) finding sensitivity, specificity and accuracy of SMI to be 100, 65.0 and 68.8%, respectively. These previous studies used

different methods to assess the degree of IPN with SMI and CEUS, making the comparison of the results difficult. Furthermore, histological analysis of neovascularization which is important to validate the finding of IPN was only included in one previous study[19].

The only previous study which combined histological assessments of IPN with SMI was carried out by Zhang et al[19]. They investigated micro-vessel density assessed by immunohistochemistry in plaques with different SMI grading. They graded SMI assessment of IPN based on the location of IMVF signals in the plaque. They found significant differences in micro-vessel density in different SMI grade (p<0.001)[19]. In this study the time from the ultrasound examinations and histological assessments was not given, and therefore a change in the degree of IPN between the two assessments cannot be excluded. To our knowledge, our study is the first which has performed SMI and CEUS ultrasound assessments of carotid plaques within 24 hours of endarterectomy.

In this current study, we applied the 5-level visual classification of IPN used by Chaolun Li[12], for the semi-quantitative CEUS assessments to our SMI analysis, as this represents in our view the simplest and most intuitive comparison between the two methods. For quantitative assessment of SMI the number of observed neo-vessels within the plaque was counted from a 2-minute SMI video clip. This count was then compared with CEUS quantitative assessments (peak intensity values) to which it was significantly, positively correlated. This current study is, to our knowledge, the first to carry out a comparison of both visual and quantitative findings with SMI and CEUS. This provides a more robust comparison of the two methods.

Previous studies on excised carotid plaques have reported a good correlation between microvessel density on histology and degree of enhancement on CEUS[10, 18]. The majority of

these studies used immunological markers of vascularization such as CD31 staining[10, 14]. In this current study microvessels were defined histologically by direct observations of the vessel endothelial cells allowing for the number of micro-vessels in each plaque to be counted. With this assessment we found that plaques with higher IMVF SMI counts had higher numbers of neo-vessels and also larger areas of inflammation. This is in agreement with previous studies[14 19], where areas of inflammation on histology were defined as the direct observation of macrophages. Higher percentages of macrophage infiltration were found in plaques from patients with higher grades of IPN on CEUS[14]. Inflammation plays a key role in atherosclerosis with macrophages and T-lymphocytes recruited during the early stages of the disease. Macrophages ingest oxidized lipoproteins becoming foam cells. The release of growth factors and cytokines recruits more inflammatory cells to the plaque. Smooth muscle cells (SMCs) migrate into the intima, promoting the formation of a collagenous fibrous cap. Death of foam cells and SMCs contributes to the formation of a necrotic core. At the same time surviving SMCs attenuate inflammation by triggering fibrosis which stabilizes the plaque[30]. This fibrosis starts with formation of granulation tissue which is rich in microvessels that are prone to leakage and rupture. We observed this phenomenon on histological assessments as accumulation of red blood cells around microvessels in granulation tissue and a lack of surrounding erythrocytes around microvessels in fibrotic tissue area. We interpreted the observation of extravasated erythrocytes around microvessels in granulation tissue as intraplaque hemorrhage (IPH) secondary to the vessel disruption due to loss of endothelial integrity. The absence of such erythrocytes around microvessels which were found in fibrotic tissue was interpreted as representative of more maturely developed and stable vessels. In the current study, plaques were therefore histologically assessed using a total plaque rank score which was based on these three tissue components (inflammation, granulation tissue and lipids) known to play a key role in IPN formation. We found that plaques with high total rank

scores had higher neo-vessel counts on SMI and higher peak intensity enhancements on quantitative CEUS. This is in keeping with increasing scientific evidence supporting a key role for inflammation in plaque destabilization and thromboembolic stroke risk.

This current study is, to the best of our knowledge, the first to demonstrate that hypoechoic plaques on B-mode ultrasound have both higher degrees of IMVF on SMI and higher degrees of enhancement on CEUS. This suggests the finding of a hypoechoic plaque on ultrasound should prompt clinicians to consider assessment of neovascularization when evaluating carotid plaque stability. Low echogenicity on B-mode ultrasound is documented to be correlated with both histopathological features of plaque instability[10] as well as an increased risk of stroke. A recent study done by Zhang et al[19] reported an increase in CEUS enhancement levels in low echogenic plaques.

Yang et al[31] in a recent study reported that SMI and CEUS peak intensity could predict ischemic stroke. In our study, we could not replicate this finding with no correlation between SMI findings and clinical symptoms or plasma level of lipoproteins (HDL, LDL, total cholesterol nor triglycerides). This lack of association in our study may be due to the relatively small study sample size.

The main limitation of this study is the relatively small sample size and further studies including higher numbers of patients are therefore needed to offer a definitive conclusion on the potential role of SMI evaluation of IPN in stroke risk assessment.

Future direction

Currently, monochrome SMI with black and white images is the only available mode for the examination of carotid plaques and in some images it can be challenging to distinguish

plaque microvessels from intra-plaque calcification signals. The development of Colour SMI mode which can distinguish pulsatile microvessels from static calcification signals will improve the specificity of the examination. Furthermore, a quantitative method for the assessment of neovascularization with SMI such as TIC analysis, currently available for CEUS, would enable a more accurate comparison between the two methods.

Conclusion

This study has demonstrated that SMI can detect neovascularization with accuracy comparable to CEUS, suggesting SMI to be a promising non-invasive alternative to CEUS for the assessment of carotid plaque stability.

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All authors contributed to the design and implementation of the research, collection of data, analysis and interpretation of results and to the writing of the manuscript. M.Z had the main responsibility for collecting the data, performing the research and wrote the manuscript with input from all authors. All authors have read and approved the final manuscript.

Disclosures

None

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Table 1. Baseline variables in patients (n=31)

Age, years	70 ± 6.3
Male, sex	20 (64.5)
Symptoms	22 (71)
Hypertension	21 (67.7)
Diabetes mellitus	3 (3.9)
Dyslipidemia	13 (67.7)
Family history of stroke	4 (12.9)
Coronary artery disease	13 (41.9)
Peripheral artery disease	3 (9.7)
Atrial fibrillation	3 (9.7)
Chronic obstructive pulmunary disease	4 (12.9)
Current or former smoker	17 (54.8)
Alcohol intake >10 unit/week	10 (33.4)
Systolic blood pressure, mm Hg	144 ±19.7
Diastolic blood pressure, mm Hg	76 ± 12.5
Cholesterol, mmol/l	4.2 ± 0.9
Non-HDL cholestrol, mmol/l	2.8 ± 1.04
HDL, mmol/l	2.4 ± 0.6
LDL, mmol/l	2.4 ± 0.9
Triglycerides, mmol/l	1.2 ± 0.5
Glucose, mmol/l	6 ± 1.7
ESR, MM	10.6 ± 9.5
CRP, mg/l	3.9 ± 6.3
HbA1c, %	5.5 ± 1.1

BMI, kg/m^2	25.4 ± 3.7
Statin treatment	29 (93.5)
Antihypertensive treatment	18 (58.1)
Aspirin treatment	23 (74.2)
Clopidogrel treatment	24 (77.4)

Numbers given as number (percentage) or mean ± SD

CRP; high sensitivity C-reactive protein. LDL; low density lipoprotein. HDL; high density lipoprotein.

Table 2. Plaque characteristics using standard Doppler ultrasound, IMVF grading using SMI (n=31) and contrast enhancement using CEUS (n=30)

Degree of stenosis %	
• 50-69%, ICA PSV≅125-230 cm/s	5 (16.1)
• >70%, ICA PSV >230	26 (83.9)
Plaque echogenicity	
• Uniformly Hypoechoic	3 (9.7)
• Predominantly Hypoechoic	11 (35.5)
• Predominantly Hyperechoic	10 (32.3)
• Uniformly Hyperechoic	7 (22.6)
Intraplaque Microvascular flow signals on SMI	
• Grade 0 No IMVF signals within the plaque or IPN signals confined to adjacent adventitia	10 (32.3)
• Grade 1 Moving IMVF signals confined to the adventitial side	6 (19.4)
• Grade 2 Moving IMVF signals at the plaque shoulder	1 (3.2)
• Grade 3 IMVF signals moving to the plaque core	14 (45.2)
• Grade 4 Extensive IMVF signals	0
Contrast enhancement on CEUS	
• Grade 0 No bubbles within the plaque or bubbles confined to adjacent adventitia.	10 (32.3)
• Grade 1 Moving bubbles confined to the adventitial side.	4 (12.9)
• Grade 2 Moving bubbles at the plaque shoulder.	4 (12.9)
• Grade 3 Bubbles moving to the plaque core.	11 (35.5)
• Grade 4 Extensive intraplaque enhancement.	1 (3.2)

Numbers are shown as No (percentages)

Figure 1. Contrast-enhanced ultrasound (CEUS) (A), Superb Microvascular Imaging (SMI) (B) and histological slice (C). >70% carotid stenosis, predominantly hypoechoic plaque located at near wall of left internal carotid artery in a symptomatic patient. Panel (A): Left, CEUS examination of the plaque. Contrast material influx is yellow in color, arrow points at contrast microbubble enhancement moving toward the plaque core and lumen, Grade 3. Panel (B): Middle, SMI examination of the plaque. The SMI ROI is positioned to show the entire plaque, arrow points at IMVF signals moving towards the plaque core and lumen, Grade 3. Panel (C): Histological slice of the plaque showing an intraplaque neo-vessel (arrow) in the granulation tissue.

Figure 2. Box plot showing the distribution of the median number of neo-vessels assessed on histology in patients with respectively no IMVF signals and those with IMVF signal (grade 1-3) on SMI. The bottom and top of the boxes represents the first and third quartile. Horizontal lines in boxes represent the median values (second quartile) and the whiskers the range limits. The median number of neo-vessels was 12 (range 2-58) in plaques with IMVF signal grade 0 compared with 27 (range 5-146) in plaques with IMVF grade 1-3.

Figure 3. Histological slice of an excised predominantly hypoechoic plaque on ultrasound which caused a >70% stenosis. Arrow points at a neo-vessel in the granulation tissue area surrounded by extravasated red blood cells. Arrow head points at erythrocytes outside the neo-vessel extravasated from it.

Figure 4. Histological slice of another predominantly hypoechoic plaque on ultrasound which caused a >70% stenosis. Arrow head points at a neo-vessel in the fibrotic area. Arrow points at a calcium area in the plaque. Note the lack of extravasated erythrocytes surrounding the neo-vessel.

Figs 1-2

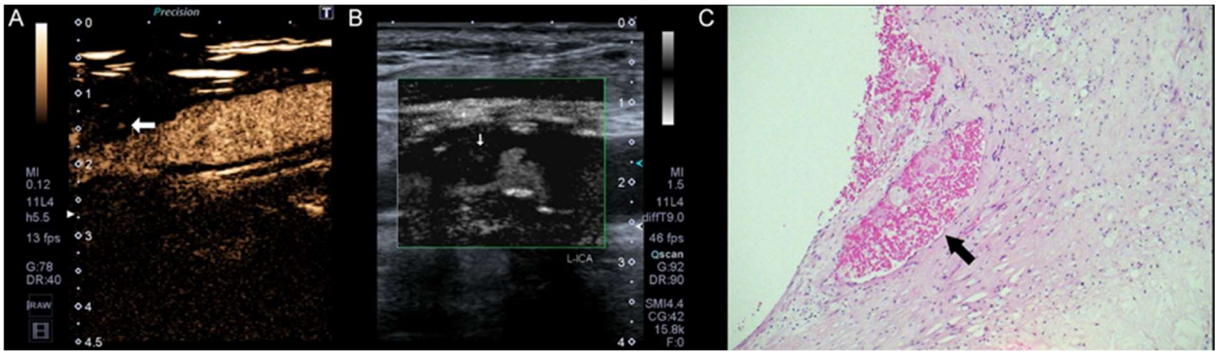


Fig 3

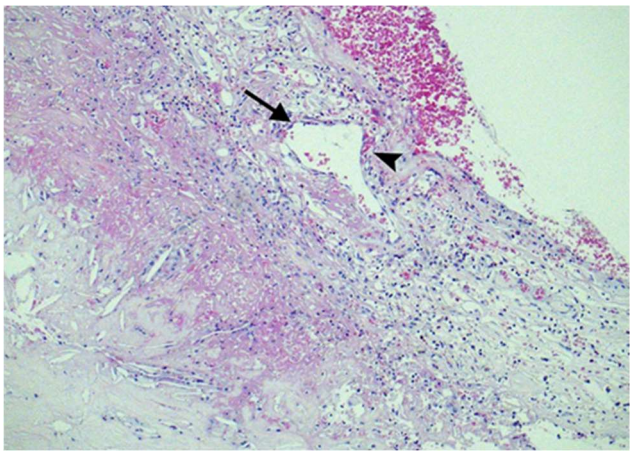
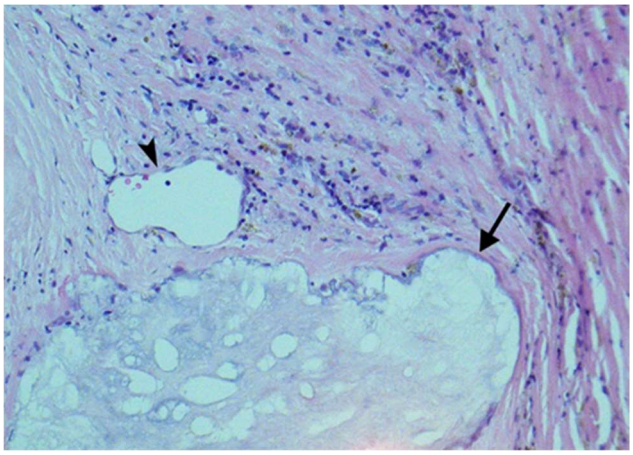


Fig 4



SUPPLEMENTAL MATERIAL

Title: Carotid plaque neovascularization detected with Superb Microvascular Imaging Ultrasound without using contrast media

Cover title: Detection of carotid plaque neovascularization

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Key words: Unstable Carotid Plaque, Atherosclerosis, Neovascularization, Carotid stenosis, Ultrasound, Ischemic stroke, Prevention.

Table I. ROC curve statistics for SMI-count vs Histology

Criterion	Sensitivity	Specificity	LR+	LR-	PPV	NPV	Diagnostic odd	Yuden index
≥0	100 %	0 %	1,00		65.0			0
≥1	76.92 %	57.14 %	1.79	0.40	76.9	57.1	4.44	0.34
≥4*	61.54 %	71.43 %	2.15	0.54	80.0	50.0	3.99	0.33
≥5	46.15 %	85.71 %	3.23	0.63	85.7	46.2	5.14	0.32
≥6	38.46 %	100 %		0.62	100	46.7	0	0.38
≥7	30.77 %	100 %		0.69	100	43.7	0	0.30
≥10	15.38 %	100 %		0.85	100	38.9	0	0.15
≥11	7.69 %	100 %		0.92	100	36.8	0	0.07

Area under the ROC curve (AUC): 0,742, 95% Confidence interval (0.500-0.908)

*Optimal diagnostic point

Table II. ROC curve statistics for CEUS (Peak intensity) vs Histology

Criterion	Sensitivity	Specificity	LR+	LR-	PPV	NPV	Diagnostic odd	Yuden index
≥0.5	100%	0%	1.00	0.00	57.1			0
≥0.6	100%	16.67%	1.20	0.00	61.5	100.0		0.17
≥1.6	100%	33.33%	1.50	0.00	66.7	100.0		0.33
≥1.9	87.50%	33.33%	1.31	0.37	63.6	66.7	3.50	0.37
≥3.3	87.50%	50%	1.75	0.25	70.0	75.0	7.00	0.37
≥3.5	87.50%	66.67%	2.62	0.19	77.8	80.0	14	0.54
≥4.2	75%	66.67%	2.25	0.37	75.0	66.7	6.00	0.42
≥4.9*	75%	83.33%	2.25	0.30	85.7	71.4	7.50	0.58
≥5.3	62.50%	83.33%	4.50	0.45	83.3	62.5	10	0.46
≥5.4	62%	100%	3.70	0.37	100.0	66.7	9.86	0.62
≥6.5	50%	100%		0.50	100.0	60.0	0	0.50
≥7.6	37.50%	100%		0.62	100.0	54.5	0	0.37
≥12.1	25%	100%		0.75	100.0	50.0	0	0.25
≥14	12.50%	100%		0.87	100.0	46.2	0	0.12

Area under the ROC curve (AUC): 0.854, 95% Confidence interval (0.568-0.981)

*Optimal diagnostic point