ORIGINAL ARTICLE

In vivo measurement of plaque neovascularisation and thermal heterogeneity in intermediate lesions of human carotid arteries

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ABSTRACT

Objectives Both neoangiogenesis and inflammation contribute in atherosclerosis progression. Contrast-enhanced ultrasound (CEUS) provides visualisation of plaque neovascularisation. Microwave radiometry (MR) allows in vivo non-invasive measurement of temperature of tissues, reflecting inflammatory activation. We assessed the association of carotid plaque temperature, measured by MR, with plaque neovascularisation assessed by CEUS in intermediate lesions.

Methods Consecutive patients with coronary artery disease and carotid atherosclerosis underwent carotid ultrasound imaging, CEUS and MR. Plaque texture, plaque surface and plaque echogenicity were analysed. Contrast enhancement (CE) by CEUS was defined as the % percentage of signal intensity difference, prior and post contrast infusion. Thermal heterogeneity (ΔT) was assigned as maximal temperature along the carotid artery minus minimum.

Results Eighty-six carotid arteries of 48 patients were included. Fatty plaques had higher CE% and ΔT compared with mixed and calcified ($p<0.01$ for all comparisons). Heterogeneous plaques had higher CE% and ΔT compared with homogenous ($p<0.01$ for all comparisons). Plaques with irregular surface had higher CE% and ΔT compared with plaques with regular ($p<0.01$ for all comparisons). There was a good correlation between ΔT and CE ($R=0.60$, $p<0.001$).

Conclusions Carotid plaque neovascularisation on CEUS examination is associated with increased thermal heterogeneity and ultrasound characteristics of plaque vulnerability in intermediate lesions.

INTRODUCTION

Carotid artery atherosclerosis is the most important cause of ischaemic stroke.[1] Although the degree of carotid artery stenosis is presently the sole determinant for performing carotid intervention, it is a relatively poor predictor for future stroke.[2] This limitation has been mainly attributed to the inability of ultrasound to detect other important characteristics of atheromatous plaques.

Thus, atheromatous plaques with inflammatory activation and neovascularisation are correlated with higher probability for stroke. A potential positive feedback loop between plaque neovascularisation and inflammatory activation has emerged.[3] Several non-invasive methods have been developed for the identification of neovascularisation and plaque inflammation.[4] Contrast-enhanced carotid ultrasound (CEUS) is a non-invasive imaging modality that provides direct visualisation of plaque neovascularisation, using contrast microspheres as intravascular tracers.[5–7] Moreover, microwave radiometry (MR) detects plaque inflammation by measuring atheromatous plaque temperature.[8 9] Recently, we described an association of in vivo human carotid plaque temperature measured by MR with increased inflammatory cell and neovessel density in significant lesions.[10] However, there are no data regarding a possible association between noninvasive CEUS imaging of neovascularisation with in vivo carotid plaque temperature in intermediate lesions. The aim of this prospective study was to investigate in human carotid arteries whether local inflammatory activation assessed by MR was associated with neovascularisation assessed by CEUS in intermediate lesions.

METHODS

Study population

Consecutive patients undergoing coronary angiography for chest pain evaluation suggestive of ischaemic heart disease were prospectively enrolled in the study. Patients with coronary artery disease as documented by coronary angiography (≥50% stenosis in one major epicardial vessel), were evaluated by standard carotid ultrasound imaging. Patients with carotid artery atherosclerosis defined as a focal thickening of intima-media thickness (IMT) ≥1.2 mm in one or both carotid arteries were finally included in the study.[11] As a control group 15 healthy volunteers were screened. All participants provided an informed consent and the study was approved by our institution ethical committee.

Patients with known cerebrovascular disease were excluded from the study. The main exclusion criteria for CEUS study were known hypersensitivity to the contrast agent (SonoVue); severe pulmonary hypertension; previous carotid surgery or angioplasty; and poor quality of the ultrasound imaging study. Exclusion criteria for MR were vasculitis, non-atherosclerotic carotid artery disease

and intermittent inflammatory, infectious or neoplastic conditions.

Conventional risk factors for coronary artery disease and current medical therapy were recorded. All participants underwent standard carotid ultrasound examination followed by CEUS and MR measurement, blindly by different specialists.

Ultrasound imaging

Extracranial (common, internal, external) carotid arteries were examined with a high resolution B-mode ultrasound unit (Philips iEE33 ultrasound machine, Philips, Bothell, Washington), using the 7.5-MHz linear probe L9-3. For signal detection the second harmonic technique was used. All data were collected and interpreted by two experienced ultrasonographers (CA and IF). In case of disagreement, the difference was resolved by a third sonographer.

The carotid arteries were scanned from their point of origin, throughout their whole length. Both the internal and external carotid arteries were interrogated in transverse and longitudinal sections. The vertebral arteries were also studied with colour and power Doppler ultrasound. B-mode ultrasound was used to depict and evaluate atheromatous plaque morphology and plaque consistency as well as to measure the IMT.

Measurements of IMT were performed in three segments of 200 mm along each carotid artery. The middle segment was the region of common carotid bifurcation (bifurcation segment), which was defined as the last cross section encompassing a single common carotid artery lumen and used as a marker. The two other segments were defined as the regions 200 mm proximal and 200 mm distal to the bifurcation region. The proximal was referred as common carotid artery segment and the distal as internal carotid artery segment. Maximum IMT for each segment was calculated from three preselected images. The segment of each carotid artery with the highest IMT value was designated as 'the segment under investigation'.

Plaques were identified as focal echogenic thickenings with a minimal intimal plus medial thickness ≥1.2 mm.[12] Plaques were evaluated for the following main parameters: echogenicity, texture and surface contour.

To assess plaque echogenicity the Gray-Weale's classification was used as previously described (type I–V).[13 14] Thus, there are five descriptions of plaque morphology: type I, dominantly echolucent plaque, with a thin echogenic cap; type II, predominantly echolucent lesions with <50% echogenic areas; type III, predominantly echogenic lesions with <50% echolucent areas; type IV, uniformly echogenic lesions; and type V, plaques with heavy calcification and acoustic shadows.[13] We considered types I and II as fatty plaques, types III and IV as mixed plaques and type V as calcified plaques. For the analysis of plaque echogenicity, which was based on the adjacent adventitia echogenicity, we used the classification previously described by considering fatty plaque as heterogeneous, and mixed and calcified as homogeneous.[13 15]

The plaque surface was considered regular when it was smooth and irregular if a variation ≥0.3mm was observed on the surface of the plaque with a depth of 1 mm.[16]

Contrast enhanced ultrasound imaging (CEUS)

For each patient enrolled, CEUS examination was performed at the 'segment under investigation'. All CEUS examinations were performed with the commercially available contrast agent, SonoVue (Bracco Imaging, Milan). The contrast agent was infused intravenously via an infusion pump at a rate of 0.8 ml/min.[17–22] Basic ultrasound imaging and the corresponding CEUS examination of a representative case is presented in online supplementary videos S1 and S2.

Image settings were not changed during contrast infusion and a low mechanical index was used. In all cases a low MI was implemented ranging between 0.07–0.09. The MI was constant during each patient study. Machine gain settings were adjusted at 75% and optimised during baseline echo study.

For CEUS the machine was adjusted for low mechanical index imaging. The studies were then digitally stored for subsequent analysis. Using the advanced quantification software QLAB (V.8.1), which enables echo intensity measurement, contrast enhancement (CE) of the identified lesions was quantified.

CE was defined as the percentage of signal intensity difference, prior and post contrast infusion. Standard and contrast-enhanced images were reviewed separately offline by two experienced readers, and inter- and intra-observer agreement were calculated.

Microwave radiometry measurements

The MR measurements were performed with the RTM 01 RES microwave computer based system (Bolton, UK) that measures temperature from internal tissues at microwave frequencies.[9] MR measurements were obtained at least 10 min after ultrasound and CEUS examination in order to avoid any influence on temperature from palpation or the ultrasound study. The basic principles of MR have been previously described.[8 10] In brief, the sensor of the antenna measures with an accuracy of 0.20°C the 'volume under investigation' as a rectangular area of 3 cm in width, 2 cm in length, and 3–7 cm in depth depending on the water content of the body.

Temperature measurements were performed at each carotid artery at the 'segment under investigation'. To ensure that matching cross sections were compared between ultrasound, CEUS and MR the measurements were performed at each segment, as previously defined in ultrasound imaging, starting from the proximal common carotid artery and moving distally, based on skin markers located under the guidance of ultrasound. We analysed segments of 20 mm in length, as MR measured temperature in a length of 2 cm, as previously described.[10] The antenna was held at this position for 10sec, required for the receiver to integrate the microwave emission and the conversion of the measured signal to temperature by a microprocessor. The temperature variables used in the statistical analyses were the temperature of the 'segment under investigation' and the minimal temperature across the carotid artery. For the analysis we used the temperature difference (ΔT), assigned as the temperature of the segment under investigation minus the minimal temperature of each carotid (reference temperature).

Statistical analysis

Statistical analysis was performed with the commercially available software (Statview, SPSS Inc., Chicago, Illinois, USA). Quantitative data are presented as rates or mean value±SD. Probability values are two-sided from the Student t-test for continuous variables were compared and Analysis of variance (ANOVA) test for more than 2 categories. Non-continuous values were compared by χ^2 test. A value of $p<0.05$ was considered significant. The optimal cutoff values of CE and ΔT for the identification of plaques with ≥1 characteristic of vulnerable plaque (fatty, heterogeneous or irregular) by ultrasound criteria were determined by receiver operator characteristic (ROC) curve analysis. To evaluate a value of 0.50 of the effect

size measure (correlation coefficient) between ΔT and CE, a sample size of n=50 was a-posteriori considered adequate to achieve statistical power equal to 0.95% at 5% type I error rate.

RESULTS

Baseline demographic and clinical characteristics

Seventy-four patients were included in the screening process for carotid atherosclerosis. Nineteen patients (25.6%) with IMT≤1.2 mm in both carotid arteries, two patients (3%) with acute hypersensitivity reaction to contrast agent and five (6%) with poor ultrasound imaging quality, were excluded from the study. Forty-eight patients (64.8%) finally met the inclusion criteria and 86 carotid arteries were analysed.' The mean IMT of carotid arteries was 2.68±1.08 mm. There were no patients with severe stenosis (>80%). Patient demographics and clinical data are summarised in table 1.

Contrast enhanced ultrasound analysis (CEUS)

During CEUS analysis inter- and intra-observer agreement were calculated at 92% and 95% respectively. Fatty plaques had higher CE% compared with mixed and calcified plaques (figure 1A). Heterogeneous plaques had higher CE% compared with homogenous (figure 1B). Plaques with irregular surface had higher CE% compared with plaques with regular surface (figure 1C) (table 2).

We categorised plaques into two groups based on ultrasound criteria: vulnerable plaques with ≥1 ultrasound characteristic, and plaques without vulnerable plaque characteristics.

By ROC curve analysis the optimal cutoff point of CE for predicting plaques with ≥1 vulnerable plaque characteristics was determined as ≥15.0% with a sensitivity of 77.2% and a specificity of 86.5% (AUC:0.782, 95% CI 0.683 to 0.880). Forty-nine plaques had high CE and 37 plaques low CE. Based on ultrasound criteria, all fatty plaques had high CE (n=9) and none was found having low CE. All calcified plaques had low CE (n=22) and none was found having high CE (p<0.01). The majority of plaques with irregular surface had high CE (n=27) compared with low CE (n=5) (84.4% vs 15.6%). The majority of plaques with regular surface had low CE (n=32) compared with high CE (n=22) (59.3% vs 40.7%). CE did not exceed 22% even in calcified plaques.

Table 1 Demographic characteristics

Number of patients (n)	48
Clinical variables	
Age (Years)	66.45±10.13
Male gender(%)	32 (66.6%)
Hypertension	33 (68.7%)
Family history	29 (60.4%)
Dyslipidaemia	40 (83.3%)
Diabetes	17 (35.4%)
Smoking	26 (54.2%)
Previous medication	
ASA	48 (100%)
ACE	13 (27.1%)
b-blockers	20 (41.6%)
Statins	48 (100%)
Previous cardiac history	
CABG	9 (18.7%)
PCI	27 (56.2%)
Multivessel CAD (≥2 vessel disease)	12 (24.5%)

ACE, Angiotensin-converting-enzyme inhibitor; ASA, Acetylsalicylic acid; CABG, Coronary artery bypass graft; CAD, Coronary artery disease; PCI, percutaneous coronary intervention.
All values are expressed as mean±SD or n (%).

Mean IMT was higher in carotid plaques with high CE compared with carotid plaques with low CE (3.05±1.12 vs 2.18 ±0.80%, p<0.001). There was a positive correlation between CE and IMT (R=0.47, p<0.001).

Microwave radiometry analysis (MR)

Measurements obtained at each carotid artery sample were constant at each site varying by 0.10°C (0–0.20°C). ΔT was higher in the atheromatous arteries (n=86) compared with control group (n=30) (0.86±0.38 vs 0.23±0.01°C, p<0.01). Fatty plaques had higher ΔT compared with mixed and calcified plaques (figure 2A). Heterogeneous plaques had higher ΔT compared with homogenous (figure 2B). Plaques with irregular surface had higher ΔT compared with plaques with regular surface (figure 2C) (table 2).

By ROC curve analysis the optimal cutoff point of ΔT for predicting vulnerable plaques was ≥0.90°C with a sensitivity of 70.3% and a specificity of 75.5% (AUC:0.786, 95% CI 0.691 to 0.881, p<0.001). Thirty-eight plaques had high ΔT and 48 plaques low ΔT. The majority of fatty plaques had high ΔT (n=7) compared with low ΔT (n=2) (77.7% vs 22.2%). The majority of calcified plaques had low ΔT (n=20) and only two were found having high ΔT (90.9% vs 9.1%). The majority of plaques with irregular surface had high ΔT (n=23) compared with low ΔT (n=9) (71.9% vs 28.1%).

Mean IMT was higher in carotid arteries with high ΔT (n=54) compared with carotid arteries with low ΔT (n=32) (3.18±1.10 vs 2.28±0.88°C, p<0.001). Mean ΔT was higher in carotid plaques with high CE compared with carotid plaques with low CE (1.03±0.36 vs 0.64±0.25°C, p<0.01) (figure 3A). Mean CE was higher in carotid arteries with high ΔT compared with arteries with low ΔT (18.71±5.84 vs 12.73±4.97%, p<0.01) (figure 3B). There was a positive correlation between mean ΔT and CE (R=0.60, p<0.001) (figure 3C).

We further categorised plaques according to CE and ΔT in three groups: plaques with high CE and high ΔT (high risk), plaques with low CE and ΔT (low risk), and plaques with high CE and low ΔT or low CE and high ΔT (intermediate risk; table 3). The majority of fatty plaques were found in the high risk group, whereas only two were found in the intermediate risk group (77.7% vs 22.2%, p<0.01). The majority of plaques with irregular surface were in the high risk group compared with intermediate and low risk groups (62.5% vs 31.2% vs 6.2%, respectively p<0.01). The majority of heterogeneous plaques were in the high risk group (77.7%) and all plaques from the low risk group were homogenous. Interestingly the majority of the plaques had concordance of high CE and high ΔT or low CE and low ΔT compared with plaques with high or low in CE and ΔT (68.6% vs 31.4%, p<0.01).

By multivariate analysis, ΔT was found to be an independent predictor for CE variation (based on a cut-off value of 0.15) after adjustment for other potential co-factors (OR=0.18, p=0.002).

By logistic regression analysis, ΔT and CEUS were found to be independent predictors for the type of plaque (fatty, mixed, calcified) after adjustment for other potential co-factors (ΔT: OR=0.16, p=0.01, CEUS: OR=0.30, p=0.01).

DISCUSSION

In the present study, we demonstrated in carotid atheromatous plaques of patients with coronary artery disease that plaque neoangiogenesis as assessed by CEUS, correlated with plaque temperature as measured by MR, and ultrasound plaque characteristics. Thus, neovascularisation and inflammation coincided

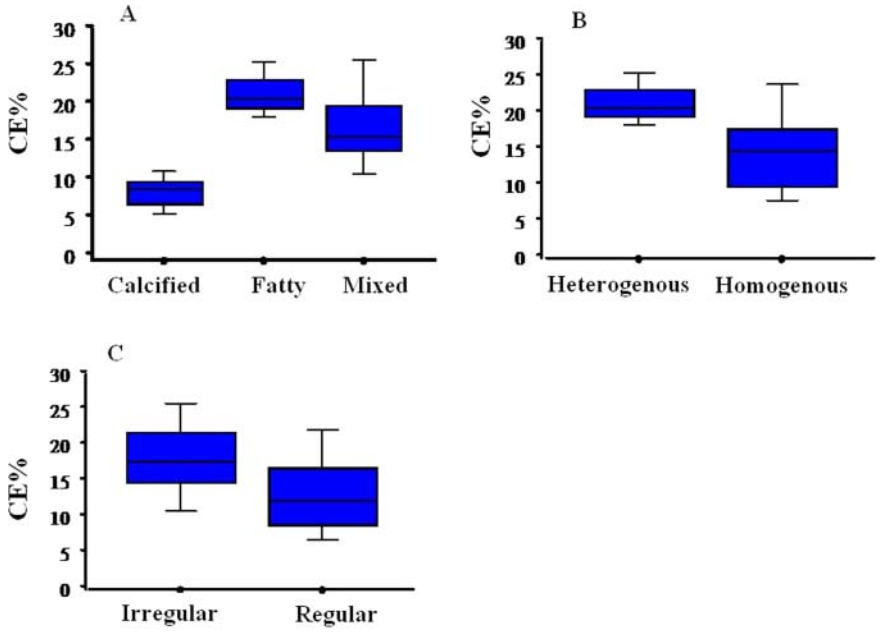

Figure 1 Percentage of contrast enhancement ultrasound (CE%) in carotid artery plaques stratified by ultrasound plaque characteristics. Fatty (A), irregular (B), and heterogeneous (C) plaques had higher CE% ($p<0.01$ for all comparisons). This figure is only reproduced in colour in the online version.

in carotid atheromatous plaques, as indicated by two non-invasive methods.

During the last decades there is growing interest on imaging modalities aimed to reveal these biological activities of atherosclerotic lesions.[3 4 23 24] Despite the high sensitivity of these methods, several limitations, including radioactive probes, partial volume effect and poor spatial resolution, have not yet permitted the wide application in humans.

Neovascularisation was measured by CEUS, a new imaging modality for the detection of vasa vasorum.[20 25 26] CEUS provided direct visualisation of the adventitial vasa vasorum and intraplaque neovascularisation.[5 20 25 27] In a recent study, neovascularisation as quantified by CEUS was directly associated with established cardiovascular disease, supporting the concept that neovascularisation is associated with plaque vulnerability.[21]

Inflammatory activation within carotid plaques has been previously shown to produce heat, which was correlated with local inflammatory activation.[28] In this study, inflammation was assessed by MR, which provides accurate temperature measurements and is associated with histological inflammatory infiltration.[8]

These two important features, neovascularisation and inflammation, were found to be pronounced in plaques with high-risk characteristics determined by ultrasound.3 Previous studies have shown that fatty, heterogeneous plaques with irregular surface have a dynamic inflammatory process and more neovascularisation in both adventitia and plaque.[25] Indeed, the use of CEUS and MR in patients with IMT≥1.2 mm revealed this association with ultrasound characteristics. However, an interesting finding was that in 31.4% of the plaques (27 out of 86) a discrepancy between neovascularisation and inflammation was found. This observation needs further investigation to clarify the potential other pathophysiological mechanisms influencing the development of atheromatosis.

Thus, a prospective study evaluating both features is justified in order to evaluate the individual and additional prognostic value of CEUS and MR for predicting cerebrovascular events.

The current study included patients with documented coronary artery disease and asymptomatic non-occlusive carotid artery disease. In this specific population a weak, but statistical significant correlation between IMT with CE was observed, consistent with previous observations and has been attributed to the association of neoangiogenesis with atherosclerosis.[22]

This study demonstrated the association of temperature and neovascularisation in vivo in patients without significant lesions in carotid arteries. We showed previously that neovascularisation is very well correlated with inflammatory activation in significant lesions requiring surgical endarterectomy.[10] In the current study we demonstrated this correlation in intermediate lesions of patients without cerebrovascular events. Thus, a clinical study with serial CEUS and MR measurements is justified in patients with intermediate lesions, and the combination of these new non-invasive imaging modalities may prove a tool for primary and secondary prevention of cerebrovascular and/or coronary events. The potential additional predictive value of CEUS and/or MR measurements on plaque risk stratification needs to be investigated. A prospective study is required to prove this hypothesis.

Table 2 Comparative evaluation of ultrasound plaque characteristics with percentage of contrast enhancement ultrasound (CE%)

Ultrasound characteristics	N of carotid plaques=86	CE%	p Values for CE%	ΔT (°C)	p Values for ΔT(°C)
Plaque texture					
Calcified	22 (25.6%)	8.55±2.42	<0.01	0.53±0.26	<0.01
Fatty	9 (10.5%)	21.44±2.70		1.13±0.27	
Mixed	55 (63.9%)	17.11±5.23		0.95±0.34	
Plaque surface					
Regular	54 (62.7%)	13.64±6.06	<0.01	0.75±0.32	<0.01
Irregular	32 (37.2%)	18.29±5.09		1.05±0.32	
Plaque echogenicity					
Homogenous	77 (89.5%)	14.66±6.02	<0.01	0.83±0.37	<0.01
Heterogeneous	9 (10.4%)	21.44±2.7		1.13±0.27	

All values are expressed as mean±SD or n (%).

Limitations

We included patients with IMT≥1.2 mm and selected the segment with the highest IMT for the analysis in order to avoid the interference of adjacent plaques exhibiting thermal heterogeneity. However, in case of diffuse arterial wall carotid

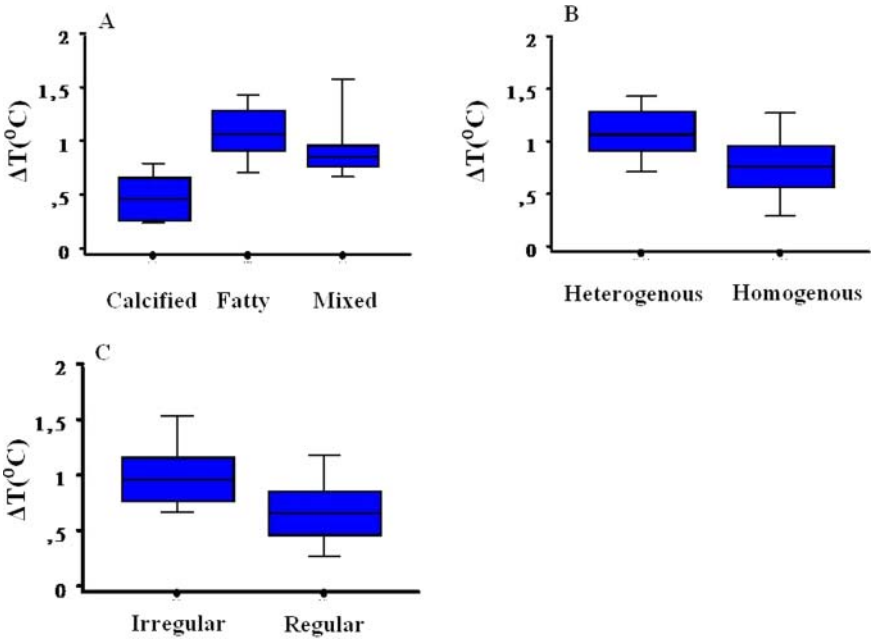

Figure 2 Temperature differences (ΔT) in carotid artery plaques stratified by ultrasound plaque characteristics. Fatty (A), irregular (B), and heterogeneous (C) plaques had higher ΔT ($p<0.01$ for all comparisons). This figure is only reproduced in colour in the online version.

artery inflammatory activation our results may have been underestimated, as the temperature of the segment selected for the analysis with the minimal temperature could be increased. Moreover, the possible impact of other adjacent tissues on the measurements of temperature seems limited, as the control group had a trivial thermal heterogeneity and our results were in accordance with previously published data.[10] Moreover, we categorised the plaques as high-, intermediate and low risk based on the sensitivity and specificity for predicting at least one vulnerable characteristic. Whether the characterisation of a plaque, based on these criteria, is correlated with the clinical outcome needs to be further investigated.

Although this method detects accurately the plaque temperature, information concerning other plaque characteristics is not provided. Thus the additional morphological information of other non-invasive imaging modalities, such as computed angiography, is still critical.[29 30] Finally, CEUS and MR are two modalities targeting different characteristics of vulnerable plaque: neovascularisation and inflammation. Although, a good correlation was found between these two characteristics, there is no 'gold standard' method for the in vivo quantification of neovascularisation and/or inflammation. Thus, the impact of the resolutions of these two methods on the final results cannot be measured.

CONCLUSIONS

Carotid plaque neovascularisation on CEUS examination is associated with increased thermal heterogeneity by MR and ultrasound characteristics of plaque vulnerability in intermediate lesions. In vivo non-invasive assessment of the functional

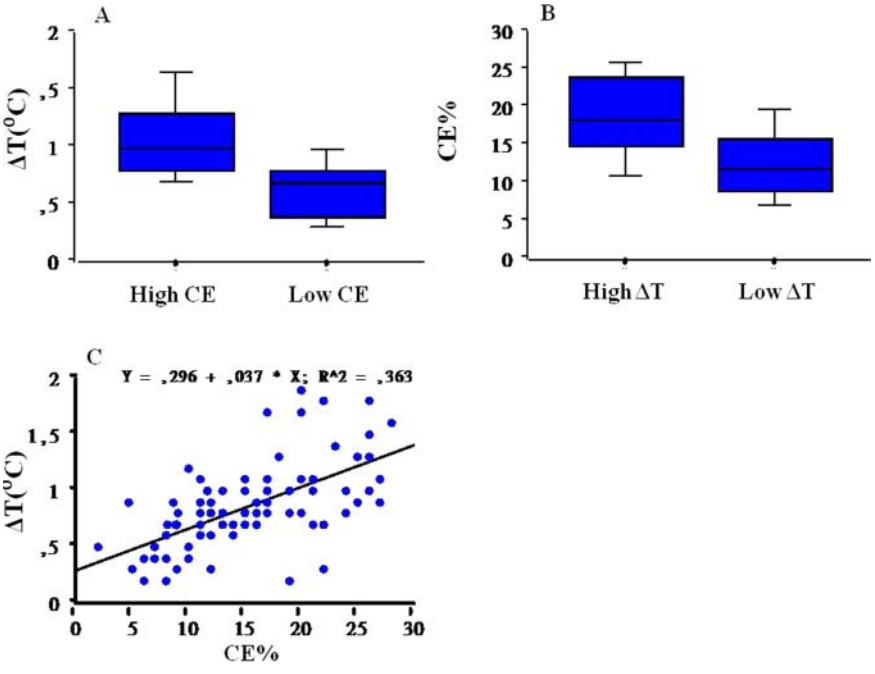

Figure 3 Association of temperature differences (ΔT) in carotid artery plaques with contrast enhancement percentages (CE%). A. ΔT of carotid arteries with high CE was higher compared with low CE% ($p<0.01$) B. CE% of carotid arteries with high ΔT was higher compared with low ΔT ($p<0.01$), C. There was a positive correlation of ΔT and CE ($R=0.60$, $p<0.001$). This figure is only reproduced in colour in the online version.

Table 3 Comparative evaluation of ultrasound plaque characteristics based on CE and ΔT

Ultrasound characteristics	High risk plaques (N=30)	Low risk plaques (N=29)	Intermediate risk plaques (N=27)	
Plaque texture				
Calcified	0 (0%)	20 (68.9%)	2 (7.4%)	<0.01
Fatty	7 (23.3%)	0 (0%)	2 (7.4%)	
Mixed	23 (76.6%)	9 (31.0%)	23 (85.2%)	
Plaque surface				
Regular	10 (33.3%)	27 (93.1%)	17 (62.9%)	<0.01
Irregular	20 (66.6%)	2 (6.9%)	10 (37.0%)	
Plaque echogenicity				
Homogenous	23 (76.6%)	29 (100%)	25 (92.6%)	<0.01
heterogeneous	7 (23.3%)	0 (0%)	2 (7.4%)	

All values are expressed as n (%).

and morphological characteristics of carotid artery atherosclerotic plaques may serve as an additional screening tool to identify 'high risk' patients.

Contributors KT: revising the manuscript for important intellectual content; MD: drafting of the manuscript, collection of data; CA: conception and design of the manuscript, performance of ultrasound imaging study; CN: drafting of the manuscript, collection of data; IF: drafting of the manuscript, performance of ultrasound imaging study; HG: conception and design of the manuscript; AS: revising the manuscript for important intellectual content; KS: drafting of the manuscript; AK: drafting of the manuscript; ET: conception and design of the manuscript; ES: conception and design of the manuscript; CS: revising the manuscript critically for important intellectual content and final approval of the manuscript submitted.

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Ethics approval Institutional ethical committee.

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