Peer Review File

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Review Comments:

Reviewer A:

This prospective study investigated an interesting topic of plaque characteristics as neovascularization and stiffness in carotid arteriosclerotic stenosis known to be associated with plaque vulnerability. The researchers used a new imaging modality of superb microvascular imaging (SMI) and shear wave elastography (SWE) to assess neovascularization with SMI and stiffness with SWE of the carotid plaque in 123 patients with symptomatic (n=65) and asymptomatic (n=58) carotid artery stenosis >50%. Intraplaque neovascularization on SMI was graded visually as grade 0 (no intraplaque blood flow), grade 1 (blood flow adventitial side), grade 2 (blood flow plaque shoulder), grade 3 (blood flow plaque core), grade 4 (extensive blood flow). SWE were measured directly after CEUS imaging within the plaque (Young's module = pc2).

Grade of intraplaque neovascularization on SMI was higher in symptomatic carotid stenosis compared to asymptomatic carotid stenosis. Plaque stiffness was lower in asymptomatic carotid stenosis compared with symptomatic carotid stenosis. In a multivariate analysis plaque stiffness and intraplaque neovascularization were associated with symptomatic carotid stenosis. The accuracy to differentiate asymptomatic from symptomatic carotid stenosis on a ROC increases using both plaque characteristics compared to the ROC curve for each characteristic alone.

The authors concluded that these advanced ultrasound technique of SMI and SWE are able to identify plaque characteristics of plaque vulnerability. The characteristics are associated with neurological symptoms of the patients.

In sum, this manuscript focuses on interesting topic of non-invasive atherosclerosis imaging in symptomatic and asymptomatic carotid stenosis using a new imaging method to assess intraplaque blood flow and plaque stiffness. It extended previous promising results using SMI and SWE in carotid lesions in order to assess intraplaque and plaque stiffness.

However, I suggest some additional discussion of the following comments:

Title:

The title is misleading as the researcher included only patients with symptomatic and asymptomatic carotid stenosis >50%. Therefore, I would suggest to change the title to:

Advance ultrasound techniques for the assessment of plaque vulnerability in symptomatic and asymptomatic carotid stenosis: a multimodal ultrasound study

Reply 1: Thank you for your careful review. We have modified the title of our manuscript according to your friendly advice.

Changes in the text: Page 1, Line 3-4

Introduction:

1. Are there any evidence that SWE provide information on the presence of lager lipid core and

intraplaque hemorrhage as indicator of plaque vulnerability as mentioned in the introduction? Please give any further information regarding correlation of plaque stiffness and plaque composition.

Reply 1: Thank you for your kind advice. Naim C et al. showed that ultrasound elastography detects a lipid core with high sensitivity and moderate specificity, and the findings were validated by MRI (Naim C, et al. Eur Radiol 2013). Garrard JW et al. revealed that the presence of intra-plaque hemorrhage, thrombus and increasing numbers of foam cells on histology were associated with a significantly lower Young' s Modulus (Garrard JW et al. Ultraschall Med 2015). We have added the references in our manuscript.
Changes in the text: Page 6, Line 2-4

2. Please also cite a further similar publication using SMI in carotid plaque (Oura K et al. Cerebrovasc Dis 2018, Chen X et al. Vasc Med 2020, Zhu YC J Stroke Cerebrovasc Dis 2019) and also publication with prospective date that predict future stroke: Yang DB et al. Value of superb micro-vascular imaging in predicting ischemic stroke in patients with carotid atherosclerotic plaques. World J Clin Case 2019

Reply 1: Thank you for your well-meaning suggestion. We have added the publications in our text according to your recommendation.
Changes in the text: Page 21, Line 16-18; Page 23, Line 7-15

Methods/Results:
3. Plaque echogenicity was graded as Type 1 to Type 4. However, Type 5 (calcified) is not mentioned. In the result, however, over 50% of plaques were categorized as calcified plaque. Calcification with acoustic shadowing have important limitation on ultrasound including also SMI and SWE. How the researchers handled this problem. How plaque assessment was possible in over 50% of plaques with calcification? As acoustic shadowing is a found very often in these patients it would be important to know if these plaques were excluded.

Reply 1: Thank you for your careful review. Plaque echogenicity was classified visually in B-mode gray-scale ultrasound according to the modified classification proposed by Gray-Weale 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 (Arnold J, et al. Stroke 1999; Gray-Weale A, et al. J Cardiovasc Surg (Torino) 1988). Calcified plaque was defined by presence of any acoustic shadowing associated with carotid plaque, producing a reduction in echo amplitude due to intervening structures with high attenuation (Prabhakaran S, et al. Atherosclerosis 2007). We agreed with the reviewer that calcification with acoustic shadowing have important limitation on SMI and SWE. In our study, 5 severe calcified (> 75% of the entire plaque) plaque with broad acoustic shadowing were excluded from the analysis. On SMI imaging, fixed echogenic signals (considered to be tissue acoustic reflector) were excluded, whereas the presence of moving enhancements within the plaque or on the adventitia were defined as evidence of plaque neovascularization signals.
Changes in the text: Page 7, Line 14-15; Page 9, Line 9-10

4. Assessment of SMI was made on longitudinal and cross-section view. However, on with specific

section in the longitudinal and cross-sectional view quantification was made. There is no information on section view of SWE (longitudinal or cross-section?).

Reply 1: Thank you for your careful review. Assessment of SWE was made on longitudinal view. We have added that in our text.
Changes in the text: Page 10, Line 5-6

5. The assessment of SWE was performed immediately after SMI examination. Therefore, examinator was not blinded to the SMI result. Could there be any bias by calculating plaque stiffness based on known plaque echogenicity and SMI?

Reply 1: Thank you for your careful review. SMI is a technique to evaluate plaque neovascularization, while SWE is a technique for evaluating plaque stiffness. The B-mode images, SMI and SWE frames and videos were collected and stored on the device hard disk, and plaque characteristics were assessed within 2 hours after images collection. In addition, all images were independently assessed by two certified vascular sonographers with five years' experience, the intra- and interobserver variability tests of SMI and SWE examination showed good agreement between the 2 operators. So we think there were little bias in the assessment of plaque characteristics.

6. High-grade stenosis >70% were significantly associated with symptomatic stenosis (74% in symptomatic patients compared with 33% in asymptomatic patients, P<0.001). Therefore, the presence of stenosis >70% should also be included in the multivariate analysis (Table 2) and ROC-Curve. Does the additional information of SMI and SWE increase ROC-Curve of grade of stenosis alone significantly? Does the combination of SMI and SWE increases ROC-Curve significantly using only one of these characteristics? Maybe a calculation of moderate (n=56) and high-grade (N=67) carotid stenosis separately. Does the plaque characteristics (SMI and SWE) also distinguish symptomatic and asymptomatic stenosis within a specific stenosis category?

Reply 1: Thank you for your meaningful advice. We have added carotid stenosis degree in the multivariate analysis (Table 2) and ROC-Curve. The area under the curve (AUC) of carotid stenosis degree, IPN level, plaque stiffness and the combination of IPN level and plaque stiffness were 0.71, 0.80, 0.81, and 0.89, respectively. Delong test showed that the combination of IPN level and plaque stiffness model was significantly superior to that of the other three factors alone (all $p < 0.05$). According to your kind advice, to explore the relationship of the SMI and SWE with symptomatic plaques in patients where additional information to carotid stenosis severity might be most useful for clinical management, we added our analyses of patients with different degrees of carotid stenosis.
Changes in the text: Page 27, Table 2; Page 33, Figure 3; Page 13, Line 18-20; Page 14, Line 1-12

Discussion:
The following main general comments and limitations of the study should be mentioned in more detail in the discussion section:
7. The main concern is, if this new technology of can reliably depict microvessel within the plaque. These microvessels are known to be less then 100micron and may even have the dimension of a capillary. Maybe only very large vessels can be visualized by this method. The reviewer, therefore, has

some doubt of the plausibility of the results and conclusion of this study. Are there any other reports on SWE and plaque composition? Are there any correlation with histology?

Reply 1: Thank you for your careful review. IPN plays a key role in plaque instability (Moreno PR, et al. Circulation 2004; McCarthy MJ, et al. J Vasc Surg 1999). Contrast enhanced ultrasound (CEUS) is a well establish technique for visualization of IPN (Saito K, et al. Stroke 2014; Partovi S, et al. AJR Am J Roentgenol 2012). By using contrast agent, CEUS can allow the identification of small diameter (20-30 μm) micro-vessels (Giannoni MF, et al. Eur J Vasc Endovasc Surg. 2009). SMI is a emerging ultrasound technique, which enables the identification of small diameter microvessels without using intravenous contrast agent. SMI uses an exclusive algorithm to discriminate true micro-vessel flow signals from wall motion artifacts and clutter, thereby allowing the visualization of IPN signals. Recent studies demonstrated that CEUS and SMI had a good consistency in the detection of IPN (Oura K, et al. J Stroke Cerebrovasc 2018; Zhang H, et al. Exp Ther Med 2017). Zamani M, et al. reported that plaques with higher SMI grades had higher numbers of neovessels quantified at histology. Higher visual IPN counts on SMI were associated with (1) increased areas of inflammation, and (2) combined rank scores of granulation tissue, inflammation and lipids at histology (Zamani M, et al. Stroke 2019). Plaque stiffness is correlated with plaque composition. Histologically, plaques characterized by a large lipid core, thinning and rupture of the fibrous cap, and intraplaque hemorrhage have a high risk of becoming the embolic source of ischemic stroke (Redgrave JN, et al. Circulation 2006). Naim C et al. showed that ultrasound elastography detects a lipid core with high sensitivity and moderate specificity, and the findings were validated by MRI (Naim C, et al. Eur Radiol 2013). Garrard JW et al. revealed that the presence of intra-plaque hemorrhage, thrombus and increasing numbers of foam cells on histology were associated with a significantly lower Young' s Modulus (Garrard JW et al. Ultraschall Med 2015).

8. Please provide more technical information on the SMI technique as this technique seems to be new and specific for the Canon ultrasound system. Previous non-ultrasound contrast based microflow imaging technique (e.g. B-flow from GE, MFI form Philips, etc…) were not able to depict these very low flow microvessels (intraplaque neovascularization). Please declare in the limitation that SMI in contrast to CEUS only can be assessed with a specific ultrasound system of Canon Aplio.

Reply 1: Thank you for your kind suggestion. We have provided more information on the SMI and SWE techniques in our text. According to your kind advice, we have added that SMI in contrast to CEUS only can be assessed with a specific ultrasound system of Canon Aplio in the limitation section.
Changes in the text: Page 18, Line 5-7

9. The prospective study results are supported by previous published results on correlation of SMI in order to quantify intraplaque neovascularization on carotid plaque (Oura K et al. Evaluation of intraplaque neovascularization using SMI and CEUS. Cerebrovasc Dis 2018, Chen X et al. Neovascularization in carotid atherosclerotic plaque con be effectively evaluated by SMI: Initial experience. Vasc Med 2020, Zhu YC et al. Evaluation the efficacy of atorvastatin on patients with carotid plaque by an innovative ultrasonography. J Stroke Cerebrovasc Dis 2019, Yang DB et al. Value of superb micro-vascular imaging in predicting ischemic stroke in patients with carotid atherosclerotic plaques. World J Clin Case 2019). However, in fact not really new evidences are presented by this

study. Please discuss in more detail what would be the clinical impact of this new method and what kind of further studies are needed (prospective randomized trial?).

Reply 1: Thank you for your meaningful suggestion. Previous studies are mainly focused on the feasibility and reproducibility of SMI and SWE to identify unstable carotid plaques, and the sample size of these studies are relatively small. No study has systematically investigated the association of IPN level or plaque stiffness with symptomatic plaques in such large sample size, however. Our findings suggested that the addition of advanced ultrasound techniques with conventional ultrasound improves diagnostic confidence and performance for the detection of high-risk carotid plaque in patients with asymptomatic carotid stenosis. We have added more information about the clinical impact of our study in the discussion section. Large-scale, multicenter studies with long-term follow-up are needed to validate that criteria observed with advanced ultrasound techniques are predictive of ischemic events and that advanced ultrasound techniques allow better patient selection for revascularization therapies. We have added that in the Conclusion section.
Changes in the text: Page 14, Line 13-20; Page 15, Line 6-8; Page 18, Line 20-22

Reviewer B:
Advanced carotid ultrasound techniques such as superb microvascular imaging (SMI) and shear wave elastography (SWE) with features such as plaque stiffness and intraplaque neovascularization can be used to compare symptomatic and asymptomatic patients and can identify plaque characteristics that are associated with ischemic events and hence can improve individual risk stratification.

My Comments:
1. In both Abstract and text, the author had used “asymptomatic” and “symptomatic” for both ‘patient’ and ‘plaque’. Please choose a different word for ‘patient’ and ‘plaque’.

Reply 2: Thank you for your meaningful suggestion. We have modified that in our manuscript.

2. The study had chosen patients with > 50% internal carotid stenosis. Please provide a reason for this. Does the analysis fail for patients with < 50% internal carotid stenosis?

Reply 2: Thank you for your careful review. Carotid atherosclerosis plays a fundamental part in the occurence of ischaemic stroke. To data, guidelines for prevention of stroke in patients with carotid plaques are based on quantification of carotid lumen narrowing due to the atherosclerotic process to select the best therapeutic approach (Kernan WN, et al. Stroke 2014; Naylor AR, et al. Eur J Vasc Endovasc Surg 2018). In general, carotid revascularization is indicated in symptomatic patients with moderate to severe stenosis (>50%). However, the management of asymptomatic carotid stenosis is controversial at present. Risk stratification of asymptomatic carotid stenosis allow better patient selection for revascularization therapies. In addition, carotid revascularization is not recommended for patients with stenosis less than 50% because the risk for stroke is low in these patients (North American Symptomatic Carotid Endarterectomy Trial investigators. Stroke 1991). So the inclusion criteria was patients with > 50% internal carotid stenosis. However, a growing number of evidence shows that vulnerable plaques are highly likely to cause ischemic stroke, independent of the degree of carotid stenosis (Millon A, et al. J Vasc Surg 2013; Yamada K, et al. Cerebrovasc Dis 2016). In the

future study, we will investigate the discrimination performance of SMI and SWE in patients with mild and moderate carotid stenosis.

3. In conclusion, the author talks about “Plaque stability”, but the reviewer can’t see the potential contribution of current work towards this topic. Conclusion should be based on results obtained in the research. Please do modifications accordingly.

Reply 2: Thank you for your careful review. Histologically, carotid plaques measured by a large lipid core, macrophage infiltration, thinning and rupture of the fibrous cap, and intraplaque hemorrhage have a high risk of becoming the embolic source of neurological events (Redgrave JN, et al. Circulation 2006; Davies MJ, et al. Br Heart J 1993). Naghavi M, et al. reported that vulnerable plaque including: (a) Rupture-prone plaque with large lipid core and thin fibrous cap infiltrated by macrophages. (b) Ruptured plaque with subocclusive thrombus and early organization. (c) Erosion-prone plaque with proteoglycan matrix in a smooth muscle cell-rich plaque. (d) Eroded plaque with subocclusive thrombus. (e) Intraplaque hemorrhage secondary to leaking vasa vasorum. (f) Calcific nodule protruding into the vessel lumen. (g) Chronically stenotic plaque with severe calcification, old thrombus, and eccentric lumen (Naghavi M, et al. Circulation. 2003). In our study, clinical neurological symptoms were regarded as a surrogate measure of vulnerable plaques. Our findings confirmed that advanced ultrasound techniques (including SMI and SWE) can identify plaque vulnerable characteristics (such as intraplaque neovascularization and plaque stiffness) that are associated with ischemic events and may be potentially indicative of plaque stability. We have modified the Conclusion section accordingly.
Changes in the text: Page 4, Line 2-3

4. Page 6, Introduction section. “However, contrast-enhanced ultrasound is a minimally invasive examination that requires an intravenous injection of a contrast agent with a related risk that limits its use in clinical practice. SMI is a novel ultrasound technique devised for overcoming the limitations of conventional Doppler ultrasound, which can successfully visualize the microvascular blood flow signals without the use of contrast agents.”
Please pad the above statements with relevant references.

Reply 2: Thank you for your meaningful advice. We have added relevant reference about that statements in the Introduction section.
Changes in the text: Page 23, Line 16-18

5. In the last paragraph of the Introduction section, the ‘aim’ can be replaced by ‘We hypothesize that …’.

Reply 2: Thank you for your kind suggestion. We have replaced the ‘aim’ by ‘We hypothesize that’ in the Introduction section according to your advice.
Changes in the text: Page 6, Line 14-19

6. Page 11, in the Result section, subsection ‘Plaque characteristics’ should come in Method section as ‘Patient Demographics’ under ‘Study population’ as this is not the result.

Reply 2: Thank you for your careful review. In the Method section as ‘Patient Demographics’, we have described the assessment methods of plaque characteristics in detail, including B-mode ultrasound, SMI, and SWE. In the Result section, in order to compare the different characteristics of plaques on ultrasound imaging between symptomatic and asymptomatic groups, we added the subsection of ‘plaque characteristics’. Actually, this subsection belongs to the 'Patient Demographics' of the included 'Study population'.

7. In the Discussion section, the reviewer feels that at many places the author after discussing the relevant works speaks about the observed results without providing a clear justification. The sole aim of the Discussion section is to justify the results. If the results are good then why?? If the results are not significant then why??

Reply 2: Thank you for your kind advice. In the Discussion section, we have discussed the similarities and differences about the observed results between the relevant works and our study. According to your advice, we have added more information about the reasonableness of the observed results.
Changes in the text: Page 15-16, Line 21-2; Page 16, Line 6-11; Page 17, Line 5-9; Page 17, Line 11-14

8. In the Discussion section, Benchmarking Table is missing. A Benchmarking Table shows the limitation of the previous studies and reveals the novelty of the proposed work.

Reply 2: Thank you for your meaningful suggestion. We have added the Benchmarking Table in the discussion section.
Changes in the text: Page 29-30, Table 3