Neovascularization from the Carotid Artery Lumen into the Carotid Plaque Confirmed by Contrast-Enhanced Ultrasound and Histology

Yuto Uchihara, MD,[*,†] Kozue Saito, PhD,[†,‡] Rie Motoyama, MD,[ʃʃ] Hatsue Ishibashi-Ueda, PhD,[§] Eriko Yamaguchi, MD,[‡] Kinta Hatakeyama, PhD,[§] Akito Tanaka, MD,[*,†] Hiroharu Kataoka, PhD,[‖] Koji Iihara, PhD,[‖] Kazuma Sugie, PhD,[†] Masatoshi Koga, PhD,[*] Kazunori Toyoda, PhD,[*] Kazuyuki Nagatsuka, PhD,[*] Masafumi Ihara, PhD[‡]

[*] Departments of Cerebrovascular Medicine, National Cerebral and Cardiovascular Center, Osaka, Japan; [†] Department of Neurology, Nara Medical University, Nara, Japan; [‡] Departments of Neurology, National Cerebral and Cardiovascular Center, Osaka, Japan; [ʃʃ] Departments of Neurology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan; [§] Departments of Pathology, National Cerebral and Cardiovascular Center, Osaka, Japan; [‖] Departments of Neurosurgery, National Cerebral and Cardiovascular Center, Osaka, Japan;

Corresponding author:

Yuto Uchihara, MD,

National Cerebral and Cardiovascular Center,

6-1 Kishibeshinmachi, Suita, Osaka 564-8565, Japan

Tel. no.: +81-6-6170-1070

E-mail: yuchihara.729@outlook.com

Abstract

Background:

This study aimed to assess intraplaque neovessels focusing on neovascularization from the vascular luminal side using contrast-enhanced ultrasound (CEUS) and demonstrate that this contrast effect indicates that the neovessel is connected to the vessel lumen histopathologically. We also investigated whether plaque vulnerability can be assessed more accurately.

Methods:

We enrolled consecutive patients with internal carotid artery stenosis who underwent carotid endarterectomy (CEA) and preoperatively examined CEUS with perflubutane of the carotid arteries. We graded the contrast effect semi-quantitatively from the vascular luminal and adventitial sides. We compared the contrast effect with the pathological findings, especially the neovascularization of the CEA specimens.

Results:

In total, 68 carotid arterial atheromatous plaques (47 symptomatic) were analyzed. Symptomatic plaques were significantly correlated with stronger contrast effects from the luminal side than those from the adventitial side ($p=0.0095$). Microbubbles from the luminal

side appeared to flow mainly into the plaque shoulder. The contrast effect value for the plaque shoulder and neovessel density were significantly correlated (ρ=0.35, p=0.031). Neovessel density was significantly higher in symptomatic than in asymptomatic plaques (56.2±43.7/mm^2 and 18.1±15.2/mm^2, respectively; p<0.0001). Serial histological sections of CEA specimens in a symptomatic plaque with a strong contrast effect from the luminal side showed multiple neovessels fenestrated to the vessel lumen with endothelial cells, consistent with the CEUS findings.

Conclusion:

CEUS can evaluate neovessels originating from the luminal side, histopathologically confirmed in serial sections. Symptomatic vulnerable plaque is correlated more significantly with intraplaque neovascularization from the luminal side than with neovascularization from the adventitia.

Key words: Atherosclerosis, Neovascularization, Contrast-enhanced ultrasound

INTRODUCTION

Carotid artery plaques can cause artery-to-artery embolism and are important risk factors for cerebral infarction.[1] Plaques with a high risk of causing stroke are vulnerable plaques characterized histopathologically by a large lipid/necrotic core, thin fibrous cap, marked inflammation, and intraplaque hemorrhage.[2,3] Tissue hypoxia and chemical mediators released from inflammatory cells recruited into the plaques induce neovessel formation and proliferation in the plaques.[4] Neovessels are fragile, easily collapse, and are densely distributed, particularly in the portion at both edges of a plaque, which is called the plaque shoulder.[5] Shear stress is high at the plaque shoulder, which is susceptible to plaque rupture.[6] Intraplaque hemorrhage is associated with a rapid increase in plaque volume and fibrous cap rupture and is recognized as a factor for plaque vulnerability.[7] Intraplaque hemorrhage is a parameter for the qualitative diagnosis of plaques, and plaque imaging, mainly by T1-weighted images such as Magnetization Prepared Rapid Acquisition Gradient Echo, is widely used.[8] However, in some cases involving plaques that are mainly composed of a necrotic core without intraplaque hemorrhage, plaque vulnerability may be difficult to evaluate.[9]

Ultrasound contrast agents do not permeate through blood vessels and are superior for visualizing blood vessels as a vascular tracer.[10,11] In addition, over recent years, ultrasound contrast agents have shown good stability *in vivo* and can trace microbubbles in real-time without the collapse of the bubbles, even with a slow blood flow.[12,13] Accordingly, neovessels

78 in plaques can be visualized using contrast ultrasound.[14] We have previously reported that the
79 contrast effect is high at the plaque shoulder in symptomatic plaques and that neovascular
80 density is histopathologically high, which can be an indicator of plaque vulnerability.[15] Blood
81 flow into the plaques is thought to occur via vasa vasorum in the adventitial layer of blood
82 vessels.[16] However, when observing the microbubbles flowing into the plaques in real-time,
83 images of neovessels, delineated by a flow from the vessel lumen into the plaques, are
84 frequently found. Pathological neovascularization from the luminal side has been reported.[17]
85 Neovascularization and intraplaque microvascular flow from the luminal side have been
86 observed using a new ultrasonographic technique.[18,19]
87 This study aimed to investigate the intraplaque neovessels originating from the adventitial and
88 luminal sides using contrast-enhanced ultrasound (CEUS) and their association with
89 symptoms. Additionally, we aimed to demonstrate that this contrast effect from the luminal
90 side indicated pathological neovessel connected to the vessel lumen.
91

92 **MATERIALS AND METHODS**

93 *Study population*

94 We enrolled consecutive patients with internal carotid artery stenosis who underwent carotid
95 endarterectomy (CEA) and preoperative CEUS of the carotid arteries at the National Cerebral
96 and Cardiovascular Center between July 2010 and June 2014. This study was approved by the

97 Ethics Committee of the National Cerebral and Cardiovascular Center (M22-019). Written
98 informed consent was obtained from all patients prior to enrollment. The exclusion criterion
99 was a previous allergic reaction to the contrast medium, perflubutane (Sonazoid; GE
100 Healthcare, Tokyo, Japan), or eggs because the lipid-stabilized suspension of Sonazoid
101 contains egg yolk.
102
103 *Patient characteristics*
104 Data on vascular risk, stenosis severity, and symptoms associated with previous ischemic
105 events on the ipsilateral side were collected. Symptomatic events were classified as transient
106 ischemic attack (TIA), amaurosis fugax, or cerebral infarction. TIA was defined as a sudden
107 focal neurological deficit that lasted <24 h. Amaurosis fugax was defined as a sudden,
108 temporary loss of vision in the ipsilateral eye. Cerebral infarction was defined as a sudden
109 focal neurological deficit that lasted for ≥24 h. Vascular risk factors were defined as follows:
110 hypertension was defined as blood pressure ≥140/90 mmHg and/or antihypertensive drug use;
111 diabetes mellitus was confirmed according to established guidelines and/or the use of
112 medication for diabetes mellitus; dyslipidemia was defined as a low-density lipoprotein level
113 of >3.6 mmol/L, high-density lipoprotein level of <1.0 mmol/L, triglyceride level of >3.8
114 mmol/L, and/or statin use; stenosis severity was assessed according to the North American
115 Symptomatic Carotid Endarterectomy Trial with computed tomographic angiography or

magnetic resonance (MR) angiography; symptomatic plaques were defined as plaques associated with a history of TIA, cerebral infarction, or both on the ipsilateral side.

CEUS image analysis

Carotid ultrasound examination was performed using a LOGIQ E9 ultrasound system (GE Healthcare, Milwaukee, WI, USA) with a linear probe (4–9 MHz phased array transducer). CEUS examinations were performed using phase-inversion mode to delineate the neovessels. The mechanical index was 0.2–0.3. The image depth was adjusted to 4–5 cm, and the focus position was 3–4 cm. Sonazoid (0.01 mL/kg body weight), a lipid-stabilized suspension of perflubutane gas microbubbles, was injected as an intravenous bolus, followed by a 10-mL saline flush through an antecubital vein. It was necessary to discriminate the true contrast effect from artifacts, which appeared as bright echoes and moving microbubbles. We initiated observation before the injection of the contrast agent and traced the microbubbles moving into the plaques from the vascular luminal or adventitial side to eliminate artifacts. The appearance of microbubbles was observed within 10–20 s following injection, and we observed the plaques and recorded images as cine clips in the short and long axes. Intraplaque neovessels were identified by the movement of echogenic reflectors of microbubbles within the plaque. Images for evaluation were acquired for at least 5 min after injection of each bolus.

We recorded images using the amplitude modulation mode for further offline analysis and the phase inversion mode for neovessel delineation.

Amounts of microbubbles flowing into plaques from both the vascular luminal and adventitial sides were classified as semi-quantitative. The contrast effects were classified semi-quantitatively on a scale from 0 to 3, where 0 = absent, 1 = small, 2 = large, and 3 = extensive. Plaques were defined as follows: grade 0, plaques with no visible microbubbles; grade 1, plaques with a small number of microbubbles; grade 3, plaques with several microbubbles that were constantly seen, and grade 2, plaques with microbubbles between grades 1 and 3.[20] Two observers (R.M. and K.S.) independently graded the cine clips offline at different time points with no prior knowledge of the patient's clinical information. Disagreements were resolved by consensus.

We measured the contrast grade from the luminal side (G_L) and that from the adventitial side (G_A) in each plaque. The grade difference (G_D) was defined as G_L minus G_A, and we compared G_D in symptomatic and asymptomatic plaques to assess which had a stronger correlation with symptoms.

To quantitatively evaluate microbubbles flowing into the plaque shoulder as a contrast effect, we recorded images using the amplitude modulation mode of the short axis of the narrowest point of the stenosis before and after injection. We defined the four circled regions as those of interest in the plaque core, plaque shoulders as lateral edges of the plaques, and the vessel

153 lumen. The size of ROIs was set to 2 mm (see Supplementary Figure 1A). Subsequently, a
154 time-intensity curve was generated, and enhanced intensity (EI) was calculated by subtracting
155 the baseline from peak intensities in the core (EI_C), the plaque shoulders (EI_S), and the vessel
156 lumen (EI_L) (see Supplementary Figure 1B). For further analysis, we used the larger EI_S of
157 the two shoulders[15] (Supplementary Figure1).
158
159 *Histological analysis*
160 For histological analysis, obtained carotid specimens by CEA were immersed immediately
161 in the fixative solution (HistoChoice; Amresco, Solon, OH, USA) for 24–48 h. After
162 decalcification in EDTA for 1 week, CEA specimens were cut into blocks by a 3-mm-thick
163 interval and embedded in paraffin. Thin slices, 5-μm-thick, were stained with hematoxylin-
164 eosin, Masson trichrome stains, and immunostaining of von Willebrand factor. Plaque
165 morphology was evaluated according to the American Heart Association (AHA) classification
166 of atherosclerotic plaques.[21]
167 AHA classification is defined as follows: I to III, an early to moderate degree of
168 atherosclerosis; IV, atheroma with thick fibrous cap; V, calcified plaque; VI, atheroma with
169 large necrotic core and thin fibrous cap.
170 Histological examinations were performed by an experienced pathologist (H.I-U.) who was
171 blinded to the CEUS findings. Immunohistochemistry was performed using a monoclonal

antibody (diluted 1:50) against the endothelial cell marker von Willebrand factor (DAKO, Japan) to stain neovessels in the plaque. Neovessel density (per square millimeter) was counted in the shoulder of the narrowest point of the stenosis. We compared histological neovessel density to the contrast effect, defined as EI_S, to validate the correlation of the contrast effect. We prepared serial sections of a symptomatic plaque showing a high contrast effect (grade 3: G_L 3, G_A 2, G_D +1) to demonstrate the fenestration of neovessels connected to the blood vessel lumen.

Statistical analysis

JMP 14.4.3 software (SAS Institute, Cary, NC, USA) was used for statistical analysis. Descriptive characteristics of all variables are expressed as mean±standard deviation for continuous variables and as percentages for categorical variables. Statistical analysis was performed using the Wilcoxon rank sum test, $\chi 2$ test, or Fisher's exact test. Correlation analysis between EI and neovessel density was performed using Spearman's rho correlation. A value of $p<0.05$ indicated statistical significance. The intra-rater agreement between the two observers for the CEUS grade was calculated using the kappa statistic.

RESULTS

Patients' characteristics

A total of 71 patients were enrolled; however, three patients were later excluded for the following reasons: pathological tissue sample error (n=1) and difficulty in quantifying the contrast effect (n=2). Finally, data on 68 patients were analyzed. The demographic data of the study group are presented in Table 1.

Symptomatic plaques were found in 47 patients, and symptoms were classified as TIA (n=8; 17%), amaurosis fugax (n=8; 17%), and cerebral infarction (n=31; 66%).

Correlation between contrast effect and symptomatic findings

The vessel lumen was clearly visualized using CEUS in all patients, and the plaques, particularly the shoulders of the plaques, were enhanced by microbubbles filling the plaques via neovessels mainly from the vessel lumen and/or via vasa vasorum from the adventitial side, while the cores of plaques were minimally enhanced.

For measuring the agreement between the observers in the determination of the contrast grade, the kappa statistic was obtained (kappa=0.77 for the luminal side; kappa=0.50 for the adventitial side).

Contrast grades from both the adventitial and luminal sides were higher in symptomatic than in asymptomatic plaques ($p=0.03$ and $p<0.0001$, respectively). G_D was greater in symptomatic than in asymptomatic plaques ($p=0.0095$), which indicated that contrast G_L was higher than G_A in symptomatic plaques (Figure 1).

Comparison with pathological images

According to the AHA classification of plaques, there were three cases of type V and 44 cases of type VI plaques in the symptomatic patients, and three cases of type V and 18 cases of type VI plaques in the asymptomatic patients. Neovessel density was significantly higher in symptomatic than in asymptomatic plaques (56.2±43.7/mm^2 and 18.1±15.2/mm^2, respectively; $p<0.0001$) (Table1). There were no type IV lesions.

The contrast effect value for the plaque shoulder, defined as EI_S, and neovessel density were significantly correlated ($\rho=0.35$, $p=0.031$) (Figure 2).

Pathological evidence of fenestrated neovessels to the arterial lumen

In the prepared serial sections of the symptomatic plaques showing a high contrast effect (grade 3) mainly from the vessel lumen (G_D +1: G_L 3, G_A 2), many neovessels were observed in the fibrous cap in vulnerable plaque shoulders showing a large necrotic core or plaque hemorrhage. Multiple neovessels approximately 50 to 100 μm in diameter were found, which fenestrated to the lumen of blood vessels in serial sections and continued to the inside of the plaque accompanied by endothelial cells from the arterial lumen. The sites matched the neovessel images depicted linearly by CEUS, demonstrating that the CEUS findings and histopathological images were consistent (Figure 3).

DISCUSSION

We reported that neovessels from the luminal side were more significantly contrasted than the vasa vasorum from the adventitial side in symptomatic plaques and that multiple neovessels fenestrated to the arterial lumen in serial sections. Furthermore, we demonstrated matching between the contrast image and histopathological image. A previous report showed that neovessels sprouting from the endothelium contributed to symptomatic plaque hemorrhage.[17,22] However, this has not been previously demonstrated using both pathological examination and CEUS *in vivo*.

Arteries are originally supplied and nourished by the vasa vasorum in the adventitia. Nonetheless, as the intima thickens with the progression of arteriosclerosis, hypoxia induces neovessels through mediators such as Hypoxia Inducible Factor (HIF)-1a and Vascular Endothelial Growth Factor (VEGF),[23] and some of these mediators have been histopathologically shown to flow from the luminal side into coronary plaques.[24] The induced neovessels are vulnerable and tend to collapse, resulting in intraplaque hemorrhage.[25] Intraoperative evaluation of carotid plaques using indocyanine green video angiography revealed that luminal neovessel sprouting and communication with the lumen were correlated with artery-to-artery embolism, contributing to intraplaque hemorrhage more specifically than the vasa vasorum.[22] Three-dimensional reconstruction of microvessels using CEA specimens

showed the microvasculature of carotid plaques with intraplaque hemorrhage, some of which fenestrated to the arterial lumen. In this study, the neovessel connected to the lumen was also covered with smooth muscle actin-positive cells (data not shown), indicating that they were exposed to high internal pressure due to the blood flow from the arterial lumen.[17] Contrary to the vasa vasorum, neovessels induced from the luminal side were directly affected by the blood flow through the carotid artery.

A pathophysiological study on CEA specimens revealed that intimal capillaries were most numerous in the plaque shoulder, and this was more prevalent in unstable plaques.[26] We showed that neovessels were mainly localized in the plaque shoulder from the luminal side *in vivo* with real-time observation using CEUS, and this localization was more prevalent in symptomatic plaques. The plaque shoulder is subjected to strong wall shear stress, and neovessels from the luminal side, in particular, are at high risk for plaque rupture and are involved in plaque vulnerability.[27] With respect to the blood flow direction, plaque rupture is often observed in the upstream shoulder with increased neovascularization and hemorrhage, whereas endothelial erosion more frequently occurs downstream. The specific geometry of plaques ruptured upstream increased the shear stress and pressure drop between the upstream and downstream plaque shoulders.[28] Over recent years, the vector flow mapping method, which analyzes flow dynamics using ultrasound, has become available for use in blood vessels, and wall shear stress is calculated to evaluate not only the blood flow velocity but

267 also the force applied to the blood vessels by the blood flow itself.[29] Analysis of flow
268 dynamics may reveal the progression of arteriosclerosis in the future by assessing how
269 neovessels contribute to plaque vulnerability.
270 Ultrasound contrast agents are superior for visualizing blood vessels as vascular tracers, and
271 tracing microbubbles can delineate even very slow blood flow, visualizing neovessels and
272 small ulcers corresponding to fibrous cap rupture. Plaque ulceration is visible as a concavity
273 or column-like sea creek, and neovessels are delineated as a linear shape by the trace of the
274 microbubbles. However, in some plaques, swirling microbubbles that flow from the artery
275 lumen into the plaque as a line were documented histopathologically as plaque ruptures,[27]
276 which may complicate their differentiation. Although both plaque rupture and neovessels may
277 show plaque vulnerability, they are histopathologically different. The former shows a gap
278 between the ruptured edges of the fibrous cap, and the latter appears as a lumen lined with
279 endothelium that connects to the inside of the plaque. We characterized thin-line delineation
280 from the arterial lumen as neovessels. However, pathological evidence of the presence of
281 neovessels is challenging because it is difficult to identify the opening to the arterial lumen
282 when examining endothelial cells in CEA specimens. Horie et al. confirmed luminal
283 neovessel sprouting and communication with the lumen intraoperatively using indocyanine
284 green video angiography; however, how luminal neovessels communicate with the vessel
285 lumen remains unclear.[22]

Serial sections enable the precise assessment of the microvasculature. Moreover, three-dimensional reconstruction using CEA specimens showed a complex network of microvasculature, with fenestration of some neovessels to the arterial lumen.[17] To the best of our knowledge, this is the first report showing that serial sections identified the thin-line delineations of the CEUS image as multiple neovessels that fenestrated to the vessel lumen, accompanied by endothelial cell lining, and extended into the plaque shoulder.

This study has several limitations. First, the participants were patients with internal carotid artery stenosis who underwent CEA, and there was a selection bias, including several high-risk patients. However, this study aimed to perform pathological verification, and we considered it necessary to demonstrate in serial sections that neovessels originated from the luminal side; thus, CEA cases were included.

Second, in this study, the contrast effect from the adventitial and intimal sides was qualitatively evaluated. While the inter-rater agreement was applied, it was difficult to verify whether neovascularization from the adventitial and luminal sides could be compared. In this study, pathological verification was performed on the CEA specimens. It was difficult to assess the vasa vasorum histopathologically from the adventitial side because they included the intima and a part of the media but not the adventitia.

Finally, because CEUS is observed with low acoustic power, it is difficult to evaluate lesions with calcification or deep lesions, and CEUS could not be assessed in some cases.

CONCLUSION

CEUS allowed for the evaluation of neovessels originating from the luminal side, which were confirmed histopathologically in serial sections of one symptomatic plaque. Moreover, symptomatic plaques were more significantly correlated with neovessels originating from the luminal side than those from the adventitia, reflected in higher G_L than G_A in symptomatic plaques. It is important to evaluate plaque vulnerability using CEUS, considering not only the intensity of the contrast effect but also the origins of the neovessels.

CONFLICT OF INTEREST STATEMENT

Dr. Saito reports the lecture's fee from GE Healthcare LLC, Takeda, Sumitomo Pharma, Daiichi Sankyo, Otsuka Pharmaceutical, and Bayer outside the submitted work. Dr. Kataoka reports the research funding and lecture's fee from Eizai, Daiichi Sankyo, Otsuka Pharmaceutical, CSL Behling, Abbott, Astellas, Bayer, JB, Kaneka, and Carl Zeiss outside the submitted work. Dr. Iihara reports grant support from Chugai Pharmaceutical Company Limited, Eisai Co., KM Biologics Company Limited, Nihon Medi-Physics, Nippon Zoki Pharmaceutical, Otsuka Pharmaceutical Company Limited, and Idorsia Pharmaceuticals Japan Limited outside the submitted work. Dr. Koga reports honoraria from Daiichi-Sankyo and research support from Daiichi-Sankyo, Nippon Boehringer Ingelheim, and Shionogi, all of

which are outside of the submitted work. Dr. Toyoda reports the lecturer's fees from Daiichi Sankyo, Otsuka, Novartis, Bayer, Bristol-Myers Squibb, and Abbott Medical outside the submitted work. Dr. Ihara reports the lecturer's fees from Daiichi Sankyo and Eisai and grant supports from Panasonic, GE Healthcare LLC, Bristol-Myers Squibb, and Shimadzu Corporation outside the submitted work. The other authors report no conflicts of interest.

Acknowledgments

We would like to thank Amy Kawai for the English language editing.

Sources of Funding

This study was supported by the Intramural Research Fund (22-6-10) for Cardiovascular Diseases of the National Cerebral and Cardiovascular Center.

Data Availability Statement

Data will be made available on request.

REFERENCES

1. U-King-Im JM, Young V, Gillard JH. Carotid-artery imaging in the diagnosis and management of patients at risk of stroke. *Lancet Neurol*. 2009;8:569–580.

2. Molloy KJ, Thompson MM, Jones JL, Schwalbe EC, Bell PRF, Naylor AR, Loftus IM. Unstable carotid plaques exhibit raised matrix metalloproteinase-8 activity. *Circulation*. 2004;110:337–343.

3. Nighoghossian N, Derex L, Douek P. The vulnerable carotid artery plaque: current imaging methods and new perspectives. *Stroke*. 2005;36:2764–2772.

4. Sluimer JC, Gasc JM, van Wanroij JL, Kisters N, Groeneweg M, Sollewijn Gelpke MD, Cleutjens JP, van den Akker LH, Corvol P, Wouters BG, et al. Hypoxia, Hypoxia-Inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. *J. Am. Coll. Cardiol.* 2008;51:1258–1265.

5. Jeziorska M, Woolley DE. Neovascularization in early atherosclerotic lesions of human carotid arteries: Its potential contribution to plaque development. *Hum. Pathol.* 1999;30:919–925.

6. Yamada H, Yuri K, Sakata N. Correlation between stress/strain and the retention of lipoproteins and rupture in atheromatous plaque of the human carotid artery: A finite element study. *J. Biomech. Sci. Eng.* 2010;5:291–302.

7. Michel JB, Virmani R, Arbustini E, Pasterkamp G. Intraplaque haemorrhages as the trigger of plaque vulnerability. *Eur. Heart J.* 2011;32:1977–1985.

8. Liu J, Balu N, Hippe DS, Ferguson MS, Martinez-Malo V, Demarco JK, Zhu DC, Ota H, Sun J, Xu D, et al. Semi-automatic carotid intraplaque hemorrhage detection and

quantification on Magnetization-Prepared Rapid Acquisition Gradient-Echo (MP-RAGE) with optimized threshold selection. *J. Cardiovasc. Magn. Reson.* 2016;18:1–9.

9. Saito A, Sasaki M, Ogasawara K, Kobayashi M, Hitomi J, Narumi S, Ohba H, Yamaguchi M, Kudo K, Terayama Y. Carotid plaque signal differences among four kinds of T1-weighted magnetic resonance imaging techniques: A histopathological correlation study. *Neuroradiology*. 2012;54:1187–1194.

10. Vicenzini E, Giannoni MF, Puccinelli F, Ricciardi MC, Altieri M, Di Piero V, Gossetti B, Valentini FB, Lenzi GL. Detection of carotid adventitial vasa vasorum and plaque vascularization with ultrasound cadence contrast pulse sequencing technique and echo-contrast agent. *Stroke*. 2007;38:2841–2843.

11. Schinkel AFL, Kaspar M, Staub D. Contrast-enhanced ultrasound: clinical applications in patients with atherosclerosis. *Int. J. Cardiovasc. Imaging*. 2016;32:35–48.

12. Unnikrishnan S, Klibanov AL. Microbubbles as ultrasound contrast agents for molecular imaging: Preparation and application. *Am. J. Roentgenol.* 2012;199:292–299.

13. Ignee A, Atkinson NSS, Schuessler G, Dietrich CF. Ultrasound contrast agents. *Endosc. Ultrasound*. 2016;5:355–362.

14. Shah F, Balan P, Weinberg M, Reddy V, Neems R, Feinstein M, Dainauskas J, Meyer P, Goldin M, Feinstein SB. Contrast-enhanced ultrasound imaging of atherosclerotic

carotid plaque neovascularization: a new surrogate marker of atherosclerosis? *Vasc. Med.* 2007;12:291–7.

15. Saito K, Nagatsuka K, Ishibashi-Ueda H, Watanabe A, Kannki H, Iihara K. Contrast-enhanced ultrasound for the evaluation of neovascularization in atherosclerotic carotid artery plaques. *Stroke*. 2014;45:3073–3075.

16. Fleiner M, Kummer M, Mirlacher M, Sauter G, Cathomas G, Krapf R, Biedermann BC. Arterial neovascularization and inflammation in vulnerable patients: Early and late signs of symptomatic atherosclerosis. *Circulation*. 2004;110:2843–2850.

17. Kurata M, Nose M, Shimazu Y, Aoba T, Kohada Y, Yorioka S, Suehiro S, Fukuoka E, Matsumoto S, Watanabe H, et al. Microvasculature of carotid atheromatous plaques: hemorrhagic plaques have dense microvessels with fenestrations to the arterial lumen. *J. Stroke Cerebrovasc. Dis.* 2014;23:1440–6.

18. Hoshino M, Shimizu T, Ogura H, Hagiwara Y, Takao N, Soga K, Usuki N, Moriya J, Nakamura H, Hasegawa Y. Intraplaque microvascular flow signal in superb microvascular imaging and magnetic resonance imaging carotid plaque imaging in patients with atheromatous carotid artery stenosis. *J. Stroke Cerebrovasc. Dis.* 2018;27:3529–3534.

19. Zamani M, Skagen K, Scott H, Lindberg B, Russell D, Skjelland M. Carotid plaque neovascularization detected with superb microvascular imaging ultrasound without using contrast media. *Stroke*. 2019;50:3121–3127.

20. Motoyama R, Saito K, Tonomura S, Ishibashi-Ueda H, Yamagami H, Kataoka H, Morita Y, Uchihara Y, Iihara K, Takahashi JC, et al. Utility of complementary magnetic resonance plaque imaging and contrast-enhanced ultrasound to detect carotid vulnerable plaques. *J. Am. Heart Assoc.* 2019;8:1–8.

21. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: A report from the Committee on Vascular Lesions of the council on arteriosclerosis, American heart association. *Circulation*. 1995;92:1355–1374.

22. Horie N, Morofuji Y, Morikawa M, Tateishi Y, Izumo T, Hayashi K, Tsujino A, Nagata I. Communication of inwardly projecting neovessels with the lumen contributes to symptomatic intraplaque hemorrhage in carotid artery stenosis. *J. Neurosurg.* 2015;123:1125–1132.

23. Ogata A, Kawashima M, Wakamiya T, Nishihara M, Masuoka J, Nakahara Y, Ebashi R, Inoue K, Takase Y, Irie H, et al. Carotid artery stenosis with a high-intensity signal

plaque on time-of-flight magnetic resonance angiography and association with evidence of intraplaque hypoxia. *J. Neurosurg.* 2017;126:1873–1878.

24. Kumamoto M, Nakashima Y. Intimal Neovascularization in Human Coronary Atherosclerosis : Its Origin and Pathophysiological Significance. *Hum Pathol*. 1995;26:450–456.

25. Shah BN, Gujral DM, Chahal NS, Harrington KJ, Nutting CM, Senior R. Plaque neovascularization is increased in human carotid atherosclerosis related to prior neck radiotherapy: A contrast-enhanced ultrasound study. *JACC Cardiovasc. Imaging*. 2016;9:668–675.

26. Mofidi R, Crotty TB, McCarthy P, Sheehan SJ, Mehigan D, Keaveny T V. Association between plaque instability, angiogenesis and symptomatic carotid occlusive disease. *Br. J. Surg.* 2001;88:945–950.

27. Lyu Q, Tian X, Ding Y, Yan Y, Huang Y, Zhou P, Hui P. Evaluation of carotid plaque rupture and neovascularization by contrast-enhanced ultrasound imaging: An exploratory study based on histopathology. *Transl. Stroke Res.* 2021;12:49–56.

28. Cicha I, Wörner A, Urschel K, Beronov K, Goppelt-Struebe M, Verhoeven E, Daniel WG, Garlichs CD. Carotid plaque vulnerability: A positive feedback between hemodynamic and biochemical mechanisms. *Stroke*. 2011;42:3502–3510.

29. Saito K, Abe S, Kumamoto M, Uchihara Y, Tanaka A, Sugie K, Ihara M, Koga M, Yamagami H. Blood flow visualization and wall shear stress measurement of carotid arteries using vascular vector flow mapping. *Ultrasound Med. Biol.* 2020;46:2692–2699.

Figure Legends

Figure 1

G_D, defined as G_L minus G_A, is shown for each patient divided into symptomatic and asymptomatic plaques. G_D was greater in symptomatic plaques than in asymptomatic ones ($p=0.0095$).

Figure 2

Correlation between EIs and neovascular density

The contrast effect value for the plaque shoulder, defined as EI_S, and neovessel density were significantly correlated ($\rho=0.35$, $p=0.031$).

Figure 3

Presentation of symptomatic plaque in the internal carotid artery with strong contrast enhancement (grade 3: G_D +1, G_L 3, G_A 2)

A. Previous plaque rupture with a large necrotic core and a little hemorrhage is shown by Masson's trichrome stain.

B. Multiple neovessels are stained using immunohistochemistry of the von Willebrand factor. (high-power field of Figure 3A enclosed in a square)

C. D. A serial immunostained section with von Willebrand factor shows an opening of neovessel that flowed into the accompanied by endothelial cell lining from the vessel lumen (*). Figure 3D is an enlarged figure of the square part of Figure 3C.

E. Contrast-enhanced ultrasound shows that many microbubbles flowed from the vessel lumen and were linearly delineated (arrows), which matched the neovessels that flowed from the vessel lumen around the plaque shoulder (yellow parts pointed by arrows in Figure 3F).

F. A serial immunostained section with von Willebrand factor shows a neovessel opening into the vessel lumen (arrowhead). Figure 3F corresponds to the square part of Figure 3E.

Supplementary Figure1

Contrast-enhanced ultrasound parameters.

A. Four regions of interest (ROIs) were set (red: plaque core, blue and green: plaque shoulder, yellow: vessel lumen) on the short axis of the narrowest point of the stenosis. The size of ROIs was set to 2mm.

B. A time-intensity curve was generated, and enhanced intensity (EI) was calculated by subtracting the baseline from peak intensities in the core (EI_C), plaque shoulder (EI_S), and vessel lumen (EI_L). We used a larger EI_S of the two plaque shoulders for further analysis.