Early enhancement with contrast-enhanced ultrasonography relates to the number of small-diameter neovessels in the carotid plaque

Sho Takeshita MD[1], Toshiyasu Ogata MD, PhD[1], Noriko Uesugi PhD[2], Kazuki Nabeshima PhD[2], Hirofumi Shimada MT[3], Hisatomi Arima PhD[4], Tooru Inoue MD, PhD[5], Yoshio Tsuboi MD, PhD[1]

[1] Department of Neurology, Fukuoka University, Fukuoka, Japan

[2] Department of Pathology, Fukuoka University, Fukuoka, Japan

[3] Department of Laboratory Medicine, Fukuoka University, Fukuoka, Japan

[4] Department of Preventive Medicine and Public Health

[5] Department of Neurosurgery, Fukuoka University, Fukuoka, Japan

Corresponding author and reprint requests to: Yoshio Tsuboi MD, PhD

Department of Neurology, Faculty of Medicine, Fukuoka University

7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

TEL: +81-92-801-1011; FAX: +81-92-865-7900

E-mail: tsuboi@cis.fukuoka-u.ac.jp

Word Count: 3559 words

Total number of tables and figures: Tables 3; Figures 2; Supple table 2; Supple figure 1

Running title: Small diameter neovessels in the carotid plaque

Keywords: CEUS; intraplaque neovessels; intraplaque hemorrhage; plaque vulnerability

ABSTRACT

Background and Purpose: Intraplaque neovessels (INVs) has been recognized as a major cause of intraplaque hemorrhage and subsequent vulnerability of the carotid plaque. However, the exact mechanisms by which INVs cause intra-plaque hemorrhage remain unclear. Various size of INVs are coexist in carotid plaques in pathologically, and we hypothesized that the size of INVs would be associated with carotid plaque histology, particularly in terms of intraplaque hemorrhage. Detection method of INV is important when determining whether carotid plaques are vulnerable and contrast-enhanced ultrasonography (CEUS) is one of the most useful methods to detect them. The purpose of this study was to examine the relationship between findings from CEUS and vascular pathology obtained by carotid endarterectomy (CEA). We focused on associations between small and large intraplaque neovessels (INVs) evaluated by CEUS and histologically defined intraplaque hemorrhage.

Methods: Participants comprised 115 patients (mean age, 73.0±7.2 years; 96 men) who underwent preoperative CEUS and underwent CEA. CEUS findings were evaluated as vascular grade at 0 minutes (Vas-G0) and 10 minutes (Vas-G10) after contrast injection. Plaques were histologically evaluated quantitatively for the total area of intraplaque hemorrhage, cholesterol and calcification, and the thinnest fibrous cap. Immunohistochemical studies were conducted using anti-CD-34 antibody as a marker for endothelial cells. INVs were divided into two groups depending on diameter: small INVs, <50 μm; and large INVs, ≥50 μm. Numbers of small and large blood vessels in the plaque were quantified histologically. Associations of small and large INVs with CEUS, plaque histology, and clinical findings were assessed by uni- and multivariable analyses.

Results: Multivariable analyses indicated that CEUS Vas-G0 was associated with the 4th

quartile of the number of small INVs compared with other quartiles and Vas-G10 was associated with the 4th quartile of the number of large INVs. Histologically, the presence and area of intraplaque hemorrhage were associated with the number of small INVs, while the increased number of large INVs was associated with infrequent plaque disruption and thicker fibrous cap.

Conclusions: Our study showed that early-phase enhancement in the CEUS can help identify plaque vulnerability by predicting a larger number of small INVs. This information can also help determine treatment strategies for carotid plaque.

INTRODUCTION

Carotid artery stenosis is a major risk factor for ischemic stroke, but the occurrence of ischemic stroke greatly depends on the characteristics of the carotid plaque, as well as the degree of stenosis. Assessing the histological characteristics of a plaque is therefore important for subsequent treatment decisions. Intraplaque hemorrhage is one of the histological features of vulnerable plaque significantly associated with the increase of the risk of cerebral infarction. Among the histological features, intraplaque hemorrhage, which can be identified on magnetic resonance imaging (MRI), has been reported to increase the risk of ischemic stroke by 5–7 fold.[1, 2, 3] Because the rupture of INVs in the carotid plaque has been considered to underlie intraplaque hemorrhage, development of intraplaque neovessels (INVs) has been recognized as a major cause of intraplaque hemorrhage and subsequent vulnerability of the carotid plaque.[4, 5] Dynamic contrast-enhanced MRI have been shown to correlate well with INVs,[6] and contrast-enhanced ultrasonography (CEUS) was reported to correlate positively with a greater density of INVs.[7, 8, 9] Enrichment of INVs evaluated by CEUS was also suggested to be associated

with strong inflammation and fibrous cap disruption in carotid plaque, which might lead to symptomatic ischemic stroke.[4, 10] Although the diversity of INV diameters has been pointed out, no studies have focused on associations between small and large INVs and intraplaque hemorrhage. Small and large INVs may coexist in carotid plaques, but their respective contributions to plaque vulnerability remain unclear.

This study therefore focused on the role of INV size in plaque vulnerability. We also examined whether CEUS could predict the size of INVs by comparing CEUS findings with plaque histology.

MATERIALS AND METHODS

We prospectively registered 140 patients with carotid stenosis who were evaluated by CEUS before undergoing carotid endarterectomy (CEA) between July 2012 and December 2017. Patient characteristics including age, sex, hypertension, diabetes mellitus, dyslipidemia, smoking and drinking habits, ischemic heart disease, peripheral arterial disease as well as the degree of carotid stenosis were obtained from medical records. Symptomatic carotid plaque was defined if the plaque was identified as a cause of ischemic stroke or transient ischemic attack within 120 days preoperatively. Pharmacotherapy with statins, angiotensin receptor blockers, and antiplatelet agents was also noted. CEA methods have been described elsewhere.[11] CEUS was performed within a month of CEA after obtaining written informed consent from the patient. This study was approved by the human subject ethics committee at Fukuoka University Hospital (approval no.: 18-9-04).

CEUS was performed by a specialist in carotid ultrasonography (H. S.) using a 7-MHz linear transducer (GE LOGIQ7; GE Healthcare, Milwaukee, WN, USA).[9] The

97 method of CEUS was described in our previous report.[12] CEUS findings were evaluated
98 according to vascular grade at 0 minutes (Vas-G0) and 10 minutes (Vas-G10) after
99 administration of perflubutane (Daiichi Sankyo, Tokyo, Japan).[13] INVs were
100 ultrasonographically defined as rapid movement of echogenic microbubbles within the
101 plaque, graded as: G0, no microbubbles visible within the plaque; G1, moderate
102 microbubbles confined to the shoulder and/or adventitial side of the plaque; or G2,
103 extensive microbubbles throughout the plaque. This definition was obtained from a
104 previous report.[13]

105 The preparation for histological evaluation of carotid plaque has been described
106 previously.[14] All sections of each plaque were stained with hematoxylin and eosin and
107 with Elastica van Gieson. Masson trichrome was used for the visualization of the fibrous
108 collagen matrix and hemorrhage. Plaque area was calculated by subtracting the area of
109 lumen from that of the entire blood vessel. Histological definitions were obtained from a
110 previous report.[15] We confirmed the presence or absence of fibrous cap disruption and
111 intraplaque hemorrhage. We measured the thickness of the thinnest site of fibrous cap in
112 all sections and decided on the thinnest value. Intraplaque hemorrhage was defined as the
113 area of erythrocyte and fibrin deposit with Masson trichrome stained inside the plaque.
114 The area of intraplaque hemorrhage was calculated by measuring the hemorrhage area of
115 all sections and adding all measured values together. Cholesterol, calcification and plaque
116 area were calculated using the same method applied for intraplaque hemorrhage. Newly
117 formed INVs were identified with staining for CD-34 (mouse monoclonal, NU-4A1;
118 Nichirei, Tokyo, Japan) and were determined by a vascular cavity surrounded by
119 endothelial cells. Small INVs were defined as those with short diameter <50 μm, while
120 large INVs were determined as ≥50 μm, then numbers were counted. The numbers of

small and large INVs were counted in all sections and added together, respectively. Plaques were then divided into two groups between the 4th and other quartiles (1st, 2nd and 3rd) of the number of small and large INVs, respectively. Density of neovessels was measured as the number of INVs divided by the plaque area in each section, and the average was calculated (/mm^2). Quantitative evaluators of histological findings were blinded to CEUS results.

Statistical analyses

First, patients were divided into two groups between the 4th and other quartiles of the number of small INVs. Age, sex, atherosclerotic risks, degree of stenosis, and Vas-G0 and Vas-G10 were compared between groups using uni- and multivariable logistic regression analyses to identify factors significantly associated with the 4th quartile of the number of small INVs. Histological findings of plaque area, plaque rupture, thinnest fibrous cap, density of neovessels, presence of intraplaque hemorrhage, intraplaque hemorrhage area, cholesterol area and calcification area were then compared between the two groups. Next, after patients were divided into two groups between the 4th and other quartiles of the number of large INVs, the same uni- and multivariable analyses were performed.

Data are expressed as mean value ± standard deviation, median and range (25th and 75th percentiles) or frequencies (%). Uni- and multivariable logistic regression analyses were conducted to identify patient characteristics and findings significantly associated with the 4th quartile. IBM SPSS version 28.0 (IBM, Armonk, NY, USA) was used for all statistical analyses. All values of $p<0.05$ were considered to denote a significant difference.

RESULTS

Among the 140 patients who underwent CEA, we excluded 8 patients who did not consent to participation in this study. We also excluded 9 patients who were difficult to evaluate ultrasonographically due to severe calcification, and 8 patients who were difficult to evaluate histologically due to destruction of plaque tissue during surgery. Finally, we included 115 patients in this study. Mean age at surgery was 73.0 ± 7.2 years and 96 patients (83%) were men. The median degree of stenosis as measured by CT angiography was 74%, and the median period from CEUS to CEA was 17 days. Patient characteristics and histological findings are shown in Table 1.

Table 2 shows uni- and multivariable logistic regression analyses significantly associated with the 4^{th} quartile of the number of small INVs. Univariable analyses indicated that the complication of diabetes mellitus and degree of carotid artery stenosis were associated with an increase in the number of small INVs, while the complication of hypertension and statin use showed an inverse correlation. CEUS findings indicated that both Vas-G0 and Vas-G10 correlated with the number of small INVs. Multiple logistic regression analysis confirmed that factors associated with the 4^{th} quartile of the number of small INVs were Vas-G0 ($p=0.012$) as well as complication of diabetes mellitus ($p=0.034$) and degree of carotid artery stenosis ($p=0.012$). Histological evaluation suggested that plaque area and density of neovessels were associated with the number of small INVs ($p=0.012$, <0.001, respectively) (Supplemental table 1). With respect to the intraplaque hemorrhage, frequency and area were significantly associated with the number of small INVs ($p=0.006$, 0.001, respectively: Figure 1).

Table 3 shows uni- and multivariable logistic regression analyses associated with

the 4th quartile of the number of large INVs. The complication of hypertension and statin use were inversely correlated with the 4th quartile of the number of large INVs. Univariable analyses showed that Vas-G0 and Vas-G10 were associated with the 4th quartile of the number of large INVs (p=0.001, <0.001). On the other hand, multivariable logistic regression analysis confirmed Vas-G10 as the factor associated with the 4th quartile of the number of large INVs (p<0.001). Histological evaluation suggested that the frequency of plaque rupture correlated inversely with the number of large INVs (p=0.004), while thickness of the fibrous cap and vascular density increased according to the number of large INVs (p=0.001, <0.001, respectively) (Figure 2). With respect to intraplaque hemorrhage, frequency and area were not associated with the number of large INVs (Supplemental table 2).

DISCUSSION

The present results indicate: 1) CEUS Vas-G0 was associated with a number of small INVs; 2) CEUS Vas-G10 was strongly associated with a number of large INVs; 3) the number of small INVs was associated with the frequency and severity of intra-plaque hemorrhage; and 4) the number of large INVs was associated with fibrous cap thickening and less frequent fibrous cap destruction.

We have previously reported that the enhancement inside a carotid plaque on CEUS fluctuates over time from the injection of contrast.[16] The results of the present study showed that carotid plaques rich in small INVs showed an enhancing effect immediately after injection of contrast medium, whereas plaques rich in large INVs showed a delayed enhancement effect of several minutes. One possibility is that the migration and proliferation of vascular endothelial cells forms small INVs in the plaque,

which, as shown in the supplementary figure, have direct contact with the vascular lumen, resulting in an early enhancement effect for CEUS. Although small INVs are growing and sprouting in the plaque, the vascular networks are underdeveloped, leading to disappearance earlier on CEUS. In contrast, large INVs are formed by the aggregation and enlargement of INVs, and their vascular networks may be well developed.[17] This may lead to late-stage enhancing effects. Our results also show that each carotid plaque usually has large and small INVs coexisting, and that the size of the INV is the predominant determining factor regarding the nature of the plaque. The results also show that observing CEUS findings over time is important to estimate their properties.

Although the rupture of INVs in the carotid plaque has been considered to underlie intraplaque hemorrhage, the exact mechanisms have yet to be identified. Our study revealed that an increased number of small INVs correlated with the presence and area of intraplaque hemorrhage. Small INVs are conceivably in the early stages of neovascularization and have an immature structure, so a reasonable interpretation seems to be that small INVs break easily and cause frequent intraplaque hemorrhage.[18] INVs may also serve as a pathway for inflammatory cells to infiltrate into the plaque,[19, 20] and can exert an important role in tissue remodeling and reactive proliferation after hemorrhage.[21]

On the other hand, large INVs were significantly associated with reduced frequency of fibrous cap disruption and thickening of the fibrous cap. This study indicated that large INVs may contribute to plaque stability, while small INVs may relate to plaque vulnerability. Histologically, small INVs were aggregating in the site of fibrous cap

disruption in vulnerable plaque (Supplemental figure). Unlike small INVs, the majority of large INVs include not only endothelial cells, but also difficult-to-rupture elastic fibers. A recent study concerning hypoxia-inducible factor 1-alpha and vascular endothelial growth factor indicated the association of plaque hypoxia with plaque vulnerability.[22] Because the maturation and enlargement of pre-existing INVs plays an important role in vascular remodeling and collateral growth,[17, 18, 23] increasing the size of INVs may contribute to the resolution of hypoxia in carotid plaque and plaque vulnerability. Such development is highly likely to represent the healing process of vulnerable plaque.

Although the density of INVs detected by CEUS was already reported to be related to intraplaque hemorrhage and plaque rupture[24], it could not show the deterioration and improvement of intraplaque hemorrhage and plaque vulnerability. However, this study provides a possible perspective the information of both the deterioration and improvement by evaluating the size of INVs. The plaque with the large number of SINVs may be unstable and aggressive surgical treatment should be performed even if mild stenosis, however the plaque with the large number of LINVs may be stable and medical treatment is considered even if severe stenosis. Evaluation of the size of INVs with CEUS can help identify plaque vulnerability and determine treatment strategies for carotid plaques.

This study had some limitations. First, this was a small, retrospective, single-center study. Second, we did not evaluate CEUS findings in cases with strong calcified plaque. Third, we did not evaluate inter-rater reliability for histological findings. Fourth, we divided neovessels size using a cutoff value of 50 μm, but this method is not established and its validity needs further investigation. Further studies may enable to elucidate the mechanism of plaque healing and the factor of plaque events.

SUMMARY

In conclusion, CEUS can predict the size and number of INVs. Histological analyses suggest that an increased number of small INVs leads to intraplaque hemorrhage, while an increased number of large INVs is significantly associated with plaque stability. Predicting the size of INVs on CEUS may help determine treatment strategies for carotid plaques.

STATEMENTS

Acknowledgment

The authors thank Ms. Miki Sashikata for her technical assistance.

Author Contributions

S. T. drafted the manuscript, revised the manuscript for content, contributed to the conception of the study, and acquired data. T. O. drafted the manuscript, revised the manuscript for content, contributed to the design of the study, performed statistical analysis, supervised the study, and obtained funding. N. U. and K. N. assessed the histopathological findings. H. S. implemented the ultrasonographic examinations and acquired data. H. A. contributed to the statistical analysis. T. I. performed the surgery and critically revised the manuscript for content. Y. T. supervised the study.

Grant support

This work was partly supported by the Taiju Life Social Welfare Foundation and the Japan Society for the Promotion of Science (KAKENHI, grant no. 20K10544).

Disclosure Statement

The authors have no conflicts of interest to disclose.

REFERENCES

1) Saam T, Hetterich H, Hoffmann V, Yuan C, Bamberg F, et al. Meta-analysis and Systematic Review of the Predictive Value of Carotid Plaque Hemorrhage on Cerebrovascular Events by Magnetic Resonance Imaging. *J Am Coll Cardiol* 2013;62:1081–1091.

2) Azuma M, Maekawa K, Yamashita A, Yokogami K, Hirai T, et al. Characterization of Carotid Plaque Components by Quantitative Susceptibility Mapping *AJNR Am J Neuroradiol*. 2020;41:310–317.

3) Bitar R, Moody AR, Leung G, Symons S, Maggisano R, et al. In Vivo 3D High-spatial-resolution MR Imaging of Intraplaque Hemorrhage. *Radiology* 2008;249:259–267.

4) Kashiwazaki D, Koh M, Uchino H, Akioka N, Kuroda S, et al. Hypoxia Accelerates Intraplaque Neovascularization Derived from Endothelial Progenitor Cells in Carotid Stenosis. *J Neurosurg* 2018;131:884–891.

5) Horie N, Morofuji Y, Morikawa M, Tateishi Y, Nagata I, et al. Communication of Inwardly Projecting Neovessels with the Lumen Contributes to Symptomatic Intraplaque Hemorrhage in Carotid Artery Stenosis. *J Neurosurg* 2015;123:1125–1132.

6) Kerwin W, Hooker A, Spilker M, Vicini P, Yuan C, et al. Quantitative Magnetic Resonance Imaging Analysis of Neovasculature Volume in Carotid Atherosclerotic Plaque. *Circulation* 2003;107:851–856.

7) Ten Kate GL, van den Oord SC, Sijbrands EJ, van der Lugt A, Schinkel AF, et al. Current Status and Future Developments of Contrast-enhanced Ultrasound of Carotid Atherosclerosis. *J Vasc Surg* 2013;57:539–546.

8) Giannoni MF, Vicenzini E, Citone M, Ricciardi MC, Mauriello A, et al. Contrast Carotid Ultrasound for the Detection of Unstable Plaques with Neoangiogenesis: A Pilot Study. *Eur J Vasc Endovasc Surg* 2009;39:722–727.

9) Hoogi A, Adam D, Hoffman A, Kerner H, Gaitini D, et al. Carotid Plaque Vulnerability: Quantification of Neovascularization on Contrast-enhanced Ultrasound with Histopathologic Correlation. *AJR Am J Roentgenol* 2011;196:431–436.

10) Owen DR, Shalhoub J, Miller S, Gauthier T, Leen EL, et al. Inflammation within Carotid Atherosclerotic Plaque: Assessment with Late-phase Contrast-enhanced US. *Radiology* 2010;255:638–644.

11) Ogata T, Inoue T, Okada Y. Outcome of 312 Japanese Patients with Carotid Endarterectomy and Factors Associated with Cardiovascular Events--A Single-center Study in Japan. *J Stroke Cerebrovasc Dis* 2014;23:529–533.

12) Amamoto T, Sakata N, Ogata T, Shimada H, Inoue T. Intra-plaque Vessels on Contrast-enhanced Ultrasound Sonography Predict Plaque Histology. *Cerebrovasc Dis* 2018;46:265–269.

13) Deyama J, Nakamura T, Takishima I, Fujioka D, Mishina H, et al. Contrast-enhanced Ultrasound Imaging of Carotid Plaque Neovascularization is Useful for Identifying High-risk Patients with Coronary Artery Disease. *Circ J* 2013;77:1499–1507.

14) Hamada O, Sakata N, Ogata T, Shimada H, Inoue T. Contrast-enhanced Ultrasonography for Detecting Histological Carotid Plaque Rupture: Quantitative Analysis of Ulcer. *Int J Stroke* 2016;11:791–798.

15) Konishi T, Funayama N, Yamamoto T, Morita T, Yoshimoto T, et al. Pathological Quantification of Carotid Artery Plaque Instability in Patients Undergoing Carotid

Endarterectomy. *Circ J* 2017;82:258–266.

16) Shimada H, Ogata T, Takano K, Abe H, Inoue T, et al. Evaluation of the Time Dependent Changes and the Vulnerability of Carotid Plaques Using Contrast Enhanced Carotid Ultrasonography. *J Stroke Cerebrovasc Dis* 2018;27:321–325.

17) Carmeliet P. Mechanisms of Angiogenesis and Arteriogenesis. *Nat Med* 2000;6:389–395.

18) Parma L, Baganha F, Quax PHA, de Vries MR, et al. Plaque Angiogenesis and Intraplaque Hemorrhage in Atherosclerosis. *Eur J Pharmacol* 2017;816:107–115.

19) Lindstedt KA, Kovanen PT. Proteolysis of Pericellular Matrix: A Process Linking Inflammation to Plaque Destabilization and Rupture. *Arterioscler Thromb Vasc Biol* 2004;24:2205–2206.

20) Marnane M, Prendeville S, McDonnell C, Noone I, Kelly PJ, et al. Plaque Inflammation and Unstable Morphology are Association with Early Stroke Recurrence in Symptomatic Carotid Stenosis. *Stroke* 2014;45:801–806.

21) Bauer SM, Bauer RJ, Velazquez OC. Angiogenesis, Vasculogenesis, and Induction of Healing in Chronic Wounds. *Vasc Endovascular Surg* 2005;39:293–306.

22) Ogata A, Kawashima M, Wakamiya T, Nishihara M, 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.

23) Hosaka A, Koyama H, Kushibiki T, Tabata Y, Nagawa K, et al. Gelatin Hydrogel Microspheres Enable Pinpoint Delivery of Basic Fibroblast Growth Factor for the Development of Functional Collaterals. *Circulation* 2004;110:3322–3328.

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

Figure legends

Figure 1: Ultrasonographic and histological findings of the vulnerable carotid plaque with intraplaque hemorrhage. A) Contrast-enhanced ultrasonography (CEUS) findings at 0 minutes. Extensive microbubbles throughout the plaque (white arrows). Vascular grade at 0 minutes (Vas-G0) is 2. B) CEUS findings at 10 minutes. Vas-G10 is 0. C) Histological findings with Masson trichrome stain. An area of erythrocytes and fibrin deposits is recognized, indicating intraplaque hemorrhage. D) Finding of intraplaque neovessels (INVs) with CD-34 stain. Many small INVs aggregate inside the plaque. E) The fibrous cape with Masson trichrome stain. This shows the thin fibrous cap (45.2 μm).

Figure 2: Ultrasonographic and histological findings of stable plaque. A) CEUS findings at 0 minutes. Vas-G0 is 0. B) CEUS findings at 10 minutes. Extensive microbubbles throughout the plaque (white arrows). Vas-G10 is 2. C) Histological findings with Masson trichrome stain. No area of intraplaque hemorrhage is observed. Rich staining for collagen fibers is seen as blue stain around INVs. This shows fibrous plaque. D) Findings of INVs with CD-34 stain. Some large INVs are apparent. E) Findings for INVs with Elastica van Gieson. Large INVs are surrounded by collagen and elastic fibers.

Table 1: Baseline characteristics and ultrasonographic and histological findings of patients

Baseline characteristics	All N=115
Age	73 ± 7.2
Sex, male, n (%)	96 (83.5)
Symptomatic, n (%)	53 (46.1)
Hypertension, n (%)	86 (74.8)
Diabetes mellitus, n (%)	52 (45.2)
Dyslipidemia, n (%)	75 (65.2)
Smoking, n (%)	77 (67)
Drinking, n (%)	80 (70)
Ischemic heart disease, n (%)	28 (24.3)
Peripheral arterial disease, n (%)	8 (7)
Statins, n (%)	73 (63.5)
Angiotensin receptor blocker, n (%)	48 (41.7)
Antiplatelet drugs, n (%)	98 (85.2)
Degree of stenosis (%)	74.2±18.1
Ultrasonographic findings	
Vas-G0; 0, 1, 2, n (%)	67 (58), 39 (34), 9 (8)
Vas-G10; 0, 1, 2, n (%)	60 (52), 40 (35), 15 (13)
Histological findings	
Plaque area, mm^2	237±113
Plaque rupture	48 (41.7)
Thinnest fibrous cap, μm	177 ± 239
Density of neovessels	1.45 ± 0.89
Presence of intraplaque hemorrhage	96 (83.5)
Intraplaque hemorrhage area, mm^2	7.8 ± 12.3
Cholesterol area, mm^2	28.1 ± 30.9
Calcification area, mm^2	9.6 ± 14.7
Number of small INVs (0-49μm)	305 ± 214

Number of large INVs (50-μm)	10 ± 15

Data are expressed as mean ± standard deviation or number (percentage) of subjects.

Table 2: Uni- and multivariable logistic regression analyses of associations between the 4th quartile of the number of small intraplaque neovessels (INVs) and baseline characteristics and ultrasonographic findings

	Univariable			Multivariable adjusted		
	OR	95%CI	p value	OR	95%CI	p value
Age	0.95	0.89-1.00	0.067			
Sex, male, n (%)	0.88	0.29-2.71	0.83			
Symptomatic, n (%)	1.49	0.63-3.50	0.36			
Hypertension	0.26	0.10-0.65	0.004	0.45	0.14-1.48	0.19
Diabetes mellitus	3.46	1.40-8.53	0.007	3.19	1.09-9.32	0.034
Dyslipidemia	0.77	0.32-1.86	0.57			
Smoking	2.13	0.78-5.81	0.14			
Drinking	0.9	0.36-2.25	0.82			
Ischemic heart disease	0.61	0.21-1.78	0.36			
Peripheral arterial disease	1.04	0.20-5.46	0.96			
Medications						
Statins	0.39	0.16-0.93	0.034	0.62	0.21-1.83	0.39
Angiotensin receptor blocker	0.58	0.24-1.43	0.24			
Antiplatelet drugs	0.74	0.24-2.31	0.6			
Degree of stenosis	1.07	1.03-1.11	<0.001	1.05	1.01-1.09	0.012
Ultrasonographic findings						
Vas-G0	3.94	1.93-8.01	<0.001	3.38	1.31-8.68	0.012
Vas-G10	1.88	1.04-3.39	0.036	0.91	0.40-2.07	0.82

Vas-G0: vascular grade at 0 minutes; Vas-G10: vascular grade at 10 minutes.

Table 3: Uni- and multivariable logistic regression analyses of associations between the 4th quartile of the number of large INVs and baseline characteristics and ultrasonographic findings

	Univariable			Multivariable adjusted		
	OR	95%CI	p value	OR	95%CI	p value
Age	1.01	0.95-1.07	0.72			
Sex, male, n (%)	1.25	0.38-4.13	0.72			
Symptomatic, n (%)	0.46	0.19-1.14	0.093			
Hypertension	0.32	0.13-0.81	0.016	0.44	0.14-1.36	0.16
Diabetes mellitus	0.88	0.37-2.08	0.77			
Dyslipidemia	0.43	0.18-1.02	0.055			
Smoking	2.81	0.97-8.11	0.056			
Drinking	1.83	0.67-5.02	0.24			
Ischemic heart disease	0.81	0.29-2.24	0.68			
Peripheral arterial disease	1.97	0.44-8.82	0.38			
Medications						
Statins	0.39	0.16-0.93	0.034	0.52	0.19-3.36	0.21
Angiotensin receptor blocker	0.47	0.19-1.18	0.11			
Antiplatelet drugs	0.39	0.13-1.15	0.087			
Degree of stenosis	1.03	0.99-1.06	0.1			
Ultrasonographic findings						
Vas-G0	3.07	1.56-6.03	0.001	1.43	0.63-3.36	0.38
Vas-G10	5.41	2.62-11.18	<0.001	4.36	1.99-9.60	<0.001

Supplemental table 1: Histological findings according to the number of small INVs

Number of small INVs	SINVs Q1 Q1≤165 n=29	SINVs Q2 166≤Q2≤254 n=29	SINVs Q3 255≤Q3≤374 n=29	SINVs Q4 375≤Q4 n=28	P value
Histological findings					
Plaque area, mm^{2*}	191±87	223±120	249±91	285±132	0.012
Plaque rupture	10 (34.5%)	16 (55.2%)	12 (41.4%)	10 (35.7%)	0.36
Thinnest fibrous cap, μm†	91 (31-364)	68 (30-219)	83 (19-212)	83 (28-315)	0.79
Density of neovessels*	0.74±0.53	1.18±0.49	1.52±0.68	2.39±0.87	<0.001
Presence of intraplaque hemorrhage	23 (79.3%)	19 (65.5%)	28 (96.6%)	26 (92.9%)	0.006
Intraplaque hemorrhage area, mm^{2*}	3.4±3.1	4.9±5.7	7.7±5.8	15.3±21.7	0.001
Cholesterol area, $mm^{2†}$	17.8 (2.1-33.5)	21.6 (1.6-48.5)	22.8 (9.1-60.2)	16.7 (1.9-30.3)	0.22
Calcification area, $mm^{2†}$	3.7 (0-16.7)	2.1 (0-8.8)	1.6 (0-10.9)	10.2 (0.8-27.1)	0.067

SINVs, small intraplaque neovessels; LINVs, large intraplaque neovessels; Q, quartile; Vas-G0, vascular grade at 0 minutes; Vas-G10, vascular grade at 10 minutes. Data are expressed as mean ± standard deviation, median (1st and 3rd quartiles) or number (percentage) of subjects.

*: Analysis of variance; †: Kruskal-Wallis test.

Supplemental table 2: Histological findings according to number of large INVs

Number of large INVs	LINVs Q1 Q1≤2 n=37	LINVs Q2 3≤Q2≤5 n=25	LINVs Q3 6≤Q3≤12 n=25	LINVs Q4 13≤Q4 n=28	P value
Histological findings					
Plaque area, mm^{2*}	212±83	217±116	252±115	271±135	0.14
Plaque rupture	20 (54.1%)	10 (40.0%)	14 (56.0%)	4 (14.3%)	0.004
Thinnest fibrous cap, μm†	65 (18-219)	51 (26-134)	79 (17-133)	277 (78-512)	0.001
Density of neovessels*	1.03±0.68	1.34±0.93	1.57±0.75	2.01±0.92	<0.001
Presence of intraplaque hemorrhage	30 (81.1%)	22 (88.0%)	21 (84.0%)	23 (82.1%)	0.9
Intraplaque hemorrhage area, mm^{2*}	5.3±5.4	5.5±5.9	12.3±20.7	9.1±12.3	0.11
Cholesterol area, $mm^{2†}$	22.8 (8.1-48.9)	18.4 (3.4-38.3)	23.4 (1.6-51.0)	13.6 (1.2-21.8)	0.16
Calcification area, $mm^{2†}$	5.8±8.1	11.1±11.2	6.7±10.8	15.7±23.2	0.12

Supplemental table 3: Univariate analyses of baseline characteristics and ultrasonographic findings according to the number of small INVs

Number of small INVs	SINVs Q1 Q1≤165 N=29	SINVs Q2 166≤Q2≤254 N=29	SINVs Q3 255≤Q3≤374 N=29	SINVs Q4 375≤Q4 N=28	P value
Age*	74.2±6.6	74.6±7.1	72.2±6.6	70.8±8.2	0.14
Sex, male, n (%)	24 (82.8%)	25 (86.2%)	24 (82.8%)	23 (82.1%)	0.98
Symptomatic, n (%)	9 (31.0%)	14 (48.3%)	15 (51.7%)	15 (53.6%)	0.3
Hypertension, n (%)	24 (82.8%)	21 (72.4%)	26 (89.7%)	15 (53.6%)	0.011
Diabetes mellitus, n (%)	11 (37.9%)	12 (41.4%)	10 (34.5%)	19 (67.9%)	0.047
Dyslipidemia, n (%)	17 (58.6%)	22 (75.9%)	19 (65.5%)	17 (60.7%)	0.52
Smoking, n (%)	13 (44.8%)	21 (72.4%)	21 (72.4%)	22 (78.6%)	0.031
Drinking, n (%)	21 (72.4%)	19 (65.5%)	21 (72.4%)	19 (67.9%)	0.92
Ischemic heart disease, n (%)	5 (17.2%)	9 (31.0%)	9 (31.0%)	5 (17.9%)	0.42
Peripheral arterial disease, n (%)	2 (6.9%)	3 (10.3%)	1 (3.4%)	2 (7.1%)	0.79
Statins, n (%)	19 (65.5%)	21 (72.4%)	20 (69.0%)	13 (46.4%)	0.18
Angiotensin receptor blocker, n (%)	14 (48.3%)	10 (34.5%)	15 (51.7%)	9 (32.1%)	0.34
antiplatelet drugs, n (%)	23 (79.3%)	28 (96.6%)	24 (82.8%)	23 (82.1%)	0.25
Degree of stenosis*	70.0±16.7	73.4±15.7	69.6±17.2	84.0±12.6	0.002

Supplemental table 4: Univariate analyses of baseline characteristics and ultrasonographic findings according to the number of large INVs

Number of large INVs	LINVs Q1 Q1≤2 N=37	LINVs Q2 3≤Q2≤5 N=25	LINVs Q3 6≤Q3≤12 N=25	LINVs Q4 13≤Q4 N=28	P value
Age*	73.9±7.8	74.4±5.9	69.7±8.3	73.4±5.9	0.079
Sex, male, n (%)	31 (83.8%)	18 (72.0%)	23 (92.0%)	24 (85.7%)	0.28
Symptomatic, n (%)	21 (56.8%)	11 (44. 0%)	12 (48.0%)	9 (32.1%)	0.27
Hypertension, n (%)	30 (81.1%)	21 (84.0%)	19 (76.0%)	16 (57.1%)	0.088
Diabetes mellitus, n (%)	12 (32.4%)	15 (60.0%)	13 (52.0%)	12 (42.9%)	0.16
Dyslipidemia, n (%)	29 (78.4%)	15 (60.0%)	17 (68.0%)	14 (50.0%)	0.11
Smoking, n (%)	26 (70.3%)	12 (48.0%)	16 (64.0%)	23 (82.1%)	0.064
Drinking, n (%)	25 (67.6%)	16 (64.0%)	17 (68.0%)	22 (78.6%)	0.67
Ischemic heart disease, n (%)	10 (27.0%)	4 (16.0%)	8 (32.0%)	6 (21.4%)	0.57
Peripheral arterial disease, n (%)	3 (8.1%)	1 (4.0%)	1 (4.0%)	3 (10.7%)	0.71
Statins, n (%)	26 (70.3%)	17 (68.0%)	17 (68.0%)	13 (46.4%)	0.2
Angiotensin receptor blocker, n (%)	16 (43.2%)	11 (44. 0%)	13 (52.0%)	8 (28.6%)	0.37
antiplatelet drugs, n (%)	33 (89.2%)	21 (84.0%)	23 (92.0%)	21 (75.0%)	0.29
Degree of stenosis*	71.9±14.6	75.7±15.8	71.0±16.7	78.7±18.9	0.28

Figure 1

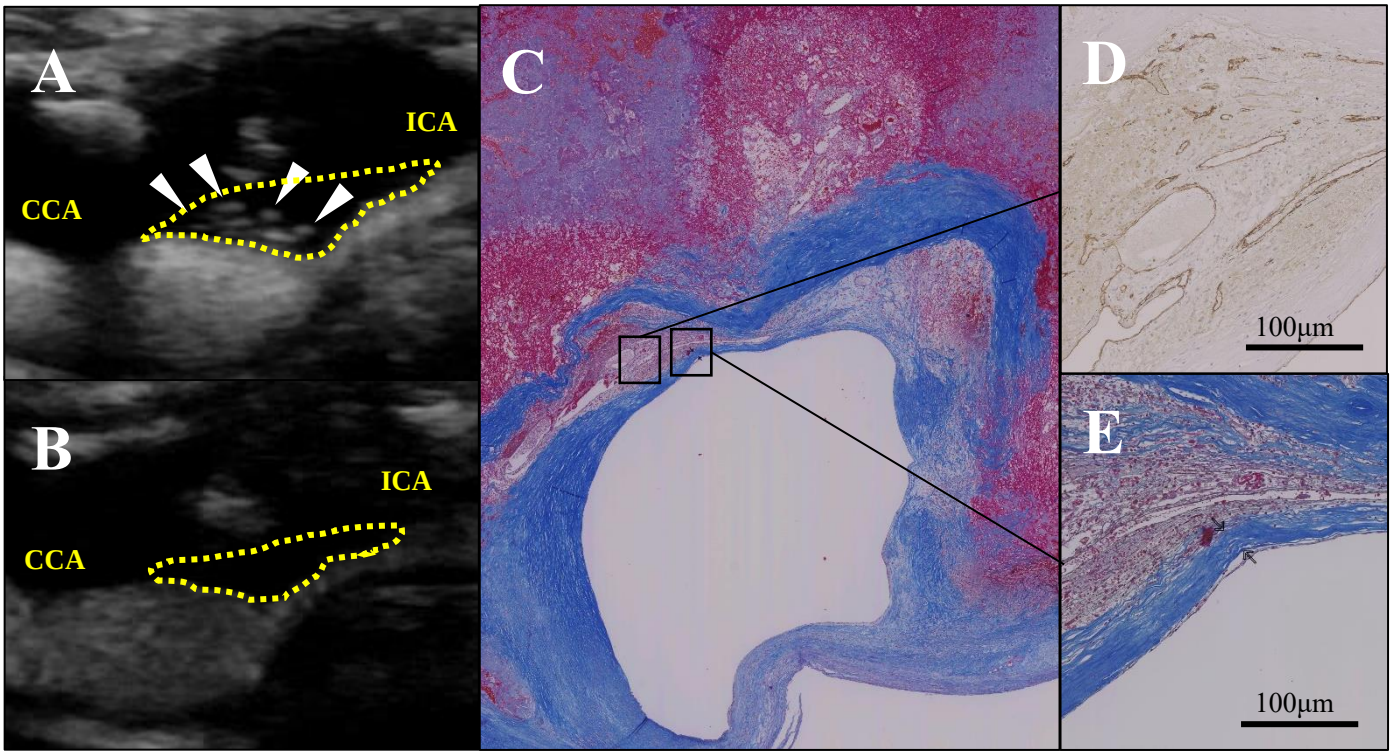
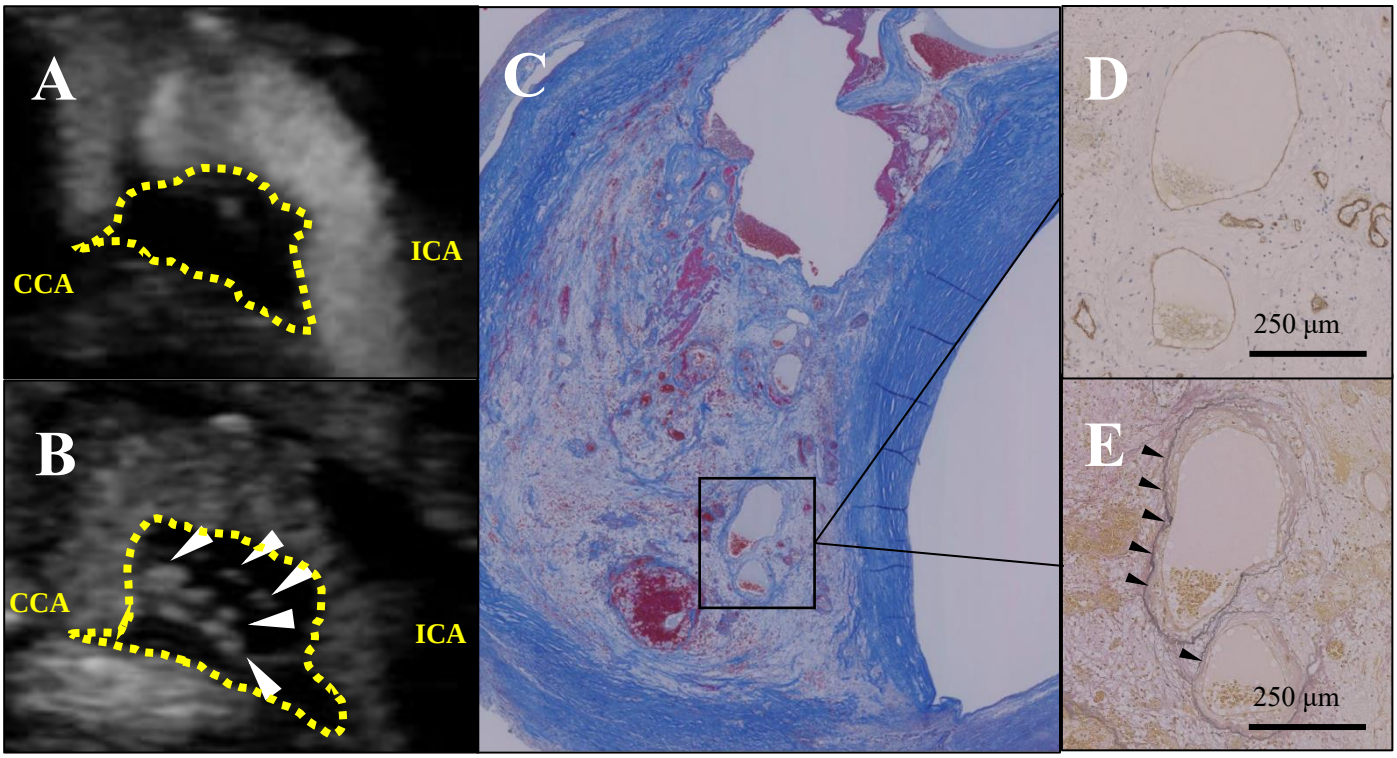


Figure 2



Supplemental figure: Pathological findings of unstable carotid plaque. A) Histological findings with Masson trichrome stain. Intraplaque hemorrhage and fibrous cap disruption are apparent. B) Finding of INVs with CD-34 stain. Small INVs are identified around the site of fibrous cap disruption, and some have contact with the vascular lumen through the site of fibrous cap disruption.