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学　位　論　文

Clinicopathological assessment of plaque instability in human carotid atherosclerosis
(ヒト頚動脈における粥状動脈硬化不安定性の臨床病理学的検証)

２０１９年３月

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Lists of paper and presentations

This study was published in the following journal.

1. Takao Konishi, Naohiro Funayama, Tadashi Yamamoto, Tohru Morita, Daisuke Hotta, Ryota Nomura, Yusuke Nakagaki, Takeo Murahashi, Kenji Kamiyama, Tetsuyuki Yoshimoto, Takeshi Aoki, Hiroshi Nishihara, Shinya Tanaka
Pathological quantification of carotid artery plaque instability in patients undergoing carotid endarterectomy Circulation Journal; 82: 258-266, (2017)

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Pathological quantitative assessment of plaque instability in patients undergoing carotid endarterectomy
AHA (American Heart Association) ATVB|PVD (Arteriosclerosis, Thrombosis and Vascular Biology | Peripheral Vascular Disease) 2017 Scientific Sessions, May 5, 2017. Minneapolis.

2. Takao Konishi, Naohiro Funayama, Tadashi Yamamoto, Tohru Morita, Daisuke Hotta, Ryota Nomura, Yusuke Nakagaki, Takeo Murahashi, Kenji Kamiyama, Tetsuyuki Yoshimoto, Takeshi Aoki, Hiroshi Nishihara, Shinya Tanaka
Clinicopathological assessment of plaque instability in human carotid atherosclerosis.
The 25th Annual Meeting of Japanese Vascular Biology and Medicine Organization, December 10, 2017. Osaka.

Abstract

Background: Ischemic strokes and transient ischemic attacks (TIA) are usually caused by unstable carotid lesions, which result in thrombus formation and occlusion of the artery. The histological characteristics of unstable carotid plaques have been described in several studies. In studies of coronary arteries, unstable plaques have been identified as ruptured, inflammatory plaques with thin fibrous caps (TFC) that were often the cause of acute coronary syndromes rather than stable angina. In the cerebral circulation, however, most ischemic strokes are caused by distal embolization originating from atherosclerotic plaques, or by an acute, instead of chronic, occlusion of a carotid artery. The interest in the morphology and functional characteristics of carotid plaques, using various imaging techniques and biochemical markers, has been growing. The progression of carotid plaques accelerated by hemorrhages developing inside the plaque has been observed in several imaging studies, and has been associated with predictors of future ischemic cerebrovascular events (CVE). In previously asymptomatic patients presenting with 50-79% carotid artery stenoses, thin or ruptured fibrous caps, hemorrhages within a plaque, and large, lipid-rich and necrotic cores (NC) have been associated with the development of adverse CVE. However, there is no comprehensive, pathological measure of carotid plaque instability that can be used to predict the risk of ischemic CVE. We performed this study in patients who underwent carotid endarterectomy (CEA) to describe in detail the pathology of carotid atherosclerosis and develop a diagnostic pathological scoring of unstable plaques. **Methods:** We analyzed data from 74 men and 8 women aged >30 years who, between May 2015 and August 2016, consecutively underwent CEA in the departments of neurosurgery of Nakamura Memorial Hospital, Kashiwaba Neurosurgical Hospital and Hokkaido Neurosurgical Memorial Hospital in Japan. The indications for surgery were >70% asymptomatic or symptomatic carotid artery stenosis. Patients were considered symptomatic if they had experienced an ischemic stroke or a TIA. Atherosclerotic plaques associated with symptoms were referred to as symptomatic or unstable plaques. The mean age of the 67 symptomatic patients was 73.2±6.9 years and that of the 15 asymptomatic patients was 71.3±7.6 years (NS). CEA was performed using standard surgical techniques with minimal handling of the specimens. The plaques were removed en bloc, fixed in 10% buffered formalin, transected transversely in 5-mm specimens, and embedded in paraffin. After hematoxylin-eosin and elastica-Masson (which stains elastin black and collagen and proteoglycans green) staining, 3-4 sections per specimen were examined. The sections with ulcerated plaques or thrombi, the most stenotic segments, or both, were retained for

further analysis. The sections were examined by 2 independent observers, 1 of whom was an experienced histopathologist unaware of the clinical status and identity of the patient. The sections were probed with anti-CD31, anti-CD34 and PDGFRβ antibodies, which recognize endothelial cells, to confirm the presence of microvessels in the plaque. Because one of the characteristics of an unstable plaque is the presence of ≥25 macrophages per high-power (0.3-mm diameter) field (HPF), and because immunohistochemical staining has revealed that most of the inflammatory cells at the site of plaque rupture are macrophages, we used ≥25 inflammatory cells/HPF as a threshold for plaque instability. Furthermore, because some studies have shown that (1) matrix metalloproteinase-12 (MMP-12) promotes atherosclerosis, (2) the main source of MMP-12 is a foamy cap macrophage, and (3) in patients undergoing CEA, the proportion of MMP-12 macrophages is approximately 10% of all macrophages, we used ≥3 foamy or hemosiderin-laden macrophages/HPF as a threshold for plaque instability. Cellular infiltration (i.e., ≥25 inflammatory cells/HPF) was scored semiquantitatively as absent (=0), limited to the fibrous cap or the shoulder of the cap (=1), or extending to the fibrous cap and the shoulder of the cap (=2) in >1 cross-sectional image of the lesion. Similarly, infiltration by ≥3 foamy or hemosiderin-laden macrophages/HPF was scored semiquantitatively as absent (=0), limited to the fibrous cap or the shoulder of the cap (=1), or abundant (=2) in the fibrous cap and the shoulder of the cap in ≥1 cross-sectional image of the lesion. The distribution of calcification was scored semiquantitatively as absent (0), 1-90° (1), 91-180° (2), or >180° (3) in ≥1 cross-sectional image of the lesion. Intraplaque hemorrhages were scored semiquantitatively as ≤1 (0), 2 (1), or ≥3 (2) cross-sections of the lesion. **Results:** The 82 patients enrolled in the study comprised 67 symptomatic and 15 asymptomatic patients. Plaques were resected from all patients and examined histopathologically. The clinical characteristics of the 67 symptomatic and 15 asymptomatic patients and the pathological characteristics of the lesions analyzed in each group are compared. By single-variable analysis, several pathological characteristics were significantly associated with symptomatic manifestations: plaque rupture, minimum TFC overlying a NC, microcalcifications in the fibrous cap, intraplaque hemorrhage, intraplaque microvessels, hemosiderin-laden macrophages within the cap and an extensive NC. The prevalence of plaque rupture was highest (73%) in patients presenting with stroke, significantly higher than in patients presenting with TIA (33%) or no symptoms (13%). In contrast, the prevalence of a calcified nodule was significantly higher in patients presenting with a TIA (25%) than in patients presenting with stroke (2%) or no symptom (0%). By multiple-variable logistic regression analysis, the presence of plaque rupture, minimum TFC, the presence of

microcalcifications in the fibrous cap and intraplaque microvessels were independent correlates of symptomatic plaques. We used receiver-operating characteristic statistics to examine the diagnostic performance of these indices of plaque instability. Among all correlates of symptomatic cerebral ischemic event, minimum TFC had the highest diagnostic performance (area under the curve [AUC] 0.78, optimal cutoff value 70.2 μm, sensitivity 90%, specificity 60%, PPV 91%, NPV 56%, diagnostic accuracy 84%). However, combining the 4 independent correlates into a single score, using the equation derived from the multiple-variable logistic regression model (Logit(Score)=0.179+2.277* (insert 1 if plaque rupture present; otherwise 0)-0.355* (insert minimum TFC in multiples of 10 μm)+2.057* (insert 1 if microcalcification in the fibrous cap present; otherwise 0)+0.646* (insert intraplaque microvessels/mm^2), increased the diagnostic performance to an AUC of 0.92 (95% CI 0.85-0.99; optimal cutoff value 0.814; sensitivity 90%; specificity 87%; PPV 97%; NPV 65%; diagnostic accuracy 89%).**Discussion:** This pathological study is the first to show that the presence of microcalcifications in the fibrous cap is an important sign of symptomatic carotid plaque. Microcalcifications typically 10 μm in diameter within a TFC promote plaque rupture by increasing the local stress and causing interfacial debonding. Therefore, microcalcifications in the fibrous cap represent an additional factor of risk of carotid plaque instability. The individual and combined power of these plaque characteristics to measure plaque instability is incompletely understood. In this study, we combined these previously described individual characteristics of plaque instability into a single score. From our comprehensive pathological study and multiple-variable analysis, we identified the factors correlating with plaque instability. Among all the pathological indices, plaque rupture, minimum TFC, presence of microcalcifications and intraplaque microvessels were the only independent correlates of symptomatic plaque in this sample of patients with carotid artery stenosis. **Conclusions:** This pathological analysis of carotid artery plaques suggested that our diagnostic scoring may facilitate understanding of plaque instability. Pathological quantification by this scoring simplified the assessment of carotid artery plaques, instead of requiring the wide variety of pathological characteristics reflecting plaque instability.

Abbreviations

CEA	carotid endarterectomy
CI	confidence interval
CVE	cerebrovascular events
HPF	high power field
NC	necrotic cores
NPV	negative predictive value
NS	not significant
PPV	positive predictive value
TFC	thin fibrous cap
TIA	transient ischemic attacks

Introduction

Ischemic strokes and transient ischemic attacks (TIA) are usually caused by unstable carotid lesions, which lead to thrombus formation and occlusion of the artery. The histological characteristics of unstable carotid plaques have been described in several studies.(Carr et al., 1996; Carr et al., 1997; Howard et al., 2015; Jeziorska and Woolley, 1999) In studies of coronary arteries, unstable plaques were identified as ruptured, inflammatory plaques with thin fibrous caps (TFC) were often the cause of acute coronary syndromes instead of stable angina.(Hansson, 2005; Virmani et al., 1998) In the cerebral circulation, however, most ischemic strokes are caused by distal embolization originating from atherosclerotic plaques, or by an acute, instead of chronic, occlusion of a carotid artery.(Golledge et al., 2000) The interest in the morphology and functional characteristics of carotid plaques, using various imaging techniques and biochemical markers, has been growing.(Chai et al., 2016; Gaemperli et al., 2012; Hetterich et al., 2016; Liu et al., 2011; Saito et al., 2014; Wallis de Vries et al., 2009) The progression of carotid plaques accelerated by hemorrhages developing inside the plaque has been observed in several imaging studies, and has been associated with predictors of future cerebrovascular ischemic events.(Altaf et al., 2014; Hosseini et al., 2013) In previously asymptomatic patients presenting with 50 to 79% carotid artery stenoses, thin or ruptured fibrous caps, hemorrhages within a plaque, and large, lipid-rich and necrotic cores have been associated with the development of adverse cerebrovascular events.(Takaya et al., 2006) However, there is no comprehensive, pathological measure of carotid plaque instability, which can be used to predict the risk of ischemic cerebrovascular event. We performed this study to describe in detail the pathology of carotid atherosclerosis and develop a diagnostic pathological scoring of unstable plaques in patients who underwent carotid endarterectomy (CEA).

Methods

Sample population

We analyzed the data from 74 men and 8 women >30 years of age who, between May 2015 and August 2016, consecutively underwent CEA in the departments of neurosurgery of Nakamura Memorial Hospital, Kashiwaba Neurosurgical Hospital and Hokkaido Neurosurgical Memorial Hospital, in Japan. The indications for surgery were >70%, asymptomatic or symptomatic carotid artery stenosis. Patients were considered symptomatic if they had experienced an ischemic stroke or a TIA. Atherosclerotic plaques associated with symptoms were

referred to as *symptomatic* or *unstable plaques*. The mean age of the 67 symptomatic patients was 73.2±6.9 years and that of the 15 asymptomatic patients was 71.3±7.6 years (ns). Patients whose excised CEA specimen criteria were severely damaged were excluded from this analysis. This study, approved by the Ethics Committee of each participating medical institution, is in compliance with the Declaration of Helsinki on ethical principles for medical research involving human subjects, and all patients granted their written, informed consent to participate.

Histologic examinations

CEA was performed, using standard surgical techniques with minimal handling of the specimens. The plaques were removed *en bloc*, fixed in 10% buffered formalin, transected transversely in 5-mm specimens, and embedded in paraffin. After hematoxylin-eosin and elastica-Masson (which stains elastin black and collagen and proteoglycans green) staining, 3 to 4 sections per specimen were examined. The sections with ulcerated plaques or thrombi, the most stenotic segments, or both, were retained for further analysis. The sections were examined by two independent observers, one of whom was an experienced histopathologist unaware of the clinical status and identity of the patient. The sections were probed with anti-CD31, anti-CD34 and PDGFRβ antibodies, which recognize endothelial cells, to confirm the presence of microvessels in the plaque. The factors we chose as potential predictors of unstable carotid plaque are listed in the appendix.

Definitions

Plaque rupture is an area of fibrous cap disruption, where the overlying thrombus is in continuity with the underlying necrotic core.(Virmani et al., 2000) *Plaque erosion* is a luminal thrombosis without communication between thrombus and necrotic core.(Virmani et al., 2000) A *thrombus* consists of laminated platelets or fibrin, with or without interspersed red and white blood cells. A *calcified nodule* is a plaque with luminal thrombi containing a calcific nodule protruding into the lumen through a disrupted TFC.(Virmani et al., 2006) An *intraplaque hemorrhage* is a microscopically visible blood and thrombus inside the plaque. *Intraplaque microvessels* indicate the presence of neovascularization in a carotid plaque. A *foamy macrophage* within cap is a foam cell in the superficial intima. *Immature fibrillization* is a plaque stained in light green by elastica-Masson staining. A *necrotic core* is an

acellular lipid pool within the intima, near the media where smooth muscle cells are scarce and proteoglycans and lipid deposition are abundant.(Virmani et al., 2000) *Microcalcifications* are 0.5-15 μm in diameter calcifications.(Otsuka et al., 2014; Otsuka et al., 2016) *Fragmented or sheet calcification* is a ≥15 μm in diameter aggregation of microcalcifications.(Otsuka et al., 2014; Otsuka et al., 2016) *Nodular calcifications* are breaks in calcified plates with fragments of calcium separated by fibrin.(Kolodgie et al., 2007; Otsuka et al., 2014)

Semiquantification of pathological observations

Since a characteristic of an unstable plaque is the presence of ≥25 macrophages per high-power (0.3-mm-diameter) field (HPF),(Burke et al., 1997) and since immunohistochemical staining revealed that most inflammatory cells at the site of rupture are macrophages,(Kolodgie et al., 2000) we used ≥25 inflammatory cells/HPF as a threshold for plaque instability. Furthermore, since some studies have shown that 1) matrix metalloproteinase-12 (MMP-12) promotes atherosclerosis,(Liang et al., 2006; Yamada et al., 2008) 2) the main source of MMP-12 is a foamy cap macrophage,(Matsumoto et al., 1998; Scholtes et al., 2012) and 3) in patients undergoing CEA, the proportion of MMP-12 macrophages is approximately 10% of all macrophages,(Kangavari et al., 2004; Scholtes et al., 2012) we used ≥3 foamy or hemosiderin-laden macrophages/HPF as a threshold for plaque instability.

Cellular infiltration, i.e. ≥25 inflammatory cells/HPF, was scored semiquantitatively as absent (= 0), limited to the fibrous cap or the shoulder of the cap (= 1), or extending to the fibrous cap and the shoulder of the cap (= 2) in >1 cross-sectional image of the lesion. Similarly, infiltration by ≥3 foamy or hemosiderin-laden macrophages/HPF was scored semiquantitatively as absent (= 0), limited to the fibrous cap or the shoulder of the cap (= 1), or abundant (= 2) in the fibrous cap and the shoulder of the cap in ≥1 cross-sectional image of the lesion. The distribution of calcification was scored semiquantitatively as absent (0), 1 to 90° (1), 91 to 180° (2), or >180° (3) in ≥1 cross-sectional image of the lesion. Intraplaque hemorrhages were scored semiquantitatively as ≤1 (0), 2 (1), or ≥3 (2) cross-sections of the lesion.

Statistical analysis

Continuous variables are reported as means ± standard deviations (SD) and categorical variables as counts and percentages. Between-group differences were analyzed using Pearson's chi-square or Fisher's exact test for categorical variables and Student's *t*-test or Mann-Whitney *U*-test for continuous variables, as appropriate. The diagnostic value of the morphologic characteristics of the plaques as predictors of symptomatic adverse cerebral events was examined by single variable logistic regression analysis. Characteristics which emerged with p values (Wald statistics) <0.05 in the single variable analysis were entered in a multiple variable regression analysis. Characteristics not defined for non-lipid plaques, such as TFC were assigned a score of 0.

The score was derived from the multiple regression equation, to represent the probability of a correlation between a specific morphologic characteristic of the plaque and a symptomatic lesion. The results are reported as p values, odd ratios (OR) and 95% confidence intervals (CI). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), diagnostic accuracy and optimal cut-off values were calculated from receiver operating characteristic curves. Values with the highest Youden-index (sensitivity + specificity - 1) were identified as optimal cut-off-values. A p value <0.05 was considered to indicate statistical significance. The data were analyzed with the SPSS 22.0 statistical system software (IBM Corporation, Armonk, NY).

Results

Clinical and pathological characteristics of symptomatic and asymptomatic patients presenting with unstable plaques

The 82 patients enrolled in the study were divided into 67 symptomatic and 15 asymptomatic patients. Plaques were resected from all patients and examined histopathologically. The clinical characteristics of 67 symptomatic and 15 asymptomatic patients and the pathological characteristics of the lesions analyzed in each group are compared in Table 1. By single variable analysis, several pathological characteristics were significantly associated with symptomatic manifestations, including plaque rupture, minimum TFC overlying a necrotic core, microcalcifications in the fibrous cap, intraplaque hemorrhage, intraplaque microvessels, hemosiderin-laden macrophages within the caps and an extensive necrotic core.

Table 1. Clinical and pathological characteristics of symptomatic and asymptomatic study groups

	Symptomatic (n=67)	Asymptomatic (n=15)	Odd ratio (95%CI)	p
Clinical characteristics				
Age, y	73.2±6.9	71.3±7.6	1.88 (0.38-9.25)	0.667
Men	61 (91)	13 (87)	1.56 (0.28-8.64)	0.972
Diabetes mellitus	25 (37)	5 (33)	1.19 (0.37-3.88)	0.994
Hypertension	53 (79)	12 (80)	0.95 (0.23-3.82)	0.783
Dyslipidemia	57 (85)	11 (73)	2.07 (0.55-7.81)	0.476
Chronic kidney disease	17 (25)	8 (53)	0.30 (0.09-0.94)	0.069
Current smoker	22 (33)	5 (33)	0.98 (0.30-3.21)	0.790
History of				
TIA or cerebral infarction	15 (22)	3 (20)	1.15 (0.29-4.63)	0.886
Coronary artery disease	9 (13)	3 (20)	0.62 (0.15-2.64)	0.805
Peripheral artery disease	4 (6)	2 (13)	0.41 (0.07-2.50)	0.659
Prior drug therapy				
Statin	9 (13)	5 (33)	0.31 (0.09-1.12)	0.141
Aspirin	6 (9)	4 (27)	0.27 (0.07-1.12)	0.145
Clopidogrel	3 (4)	2 (13)	0.30 (0.05-2.01)	0.485
Cilostazol	1 (1)	1 (7)	0.22 (0.01-3.60)	0.804
Days between onset of disease manifestations and operation	52±50	-		-
Admission laboratory measurements				
Glycemia, mg/dl	136±51	128±55	2.06 (0.66-6.44)	0.343
Cholesterol, mg/dl				
Low-density	118±32	101±25	4.33 (1.34-14.0)	0.025

High-density	52±12	54±17	0.18 (0.05-0.64)	0.015
LDL-C to HDL-C ratio	2.4±0.8	2.0±0.8	4.99 (1.48-16.8)	0.017
Triglyceride, mg/dl	156±76	191±113	0.31 (0.09-1.12)	0.141
Pathological characteristics				
Plaque area, mm^2	41.3±19.4	34.9±20.4	3.07 (0.97-9.71)	0.096
Cross sectional area luminal narrowing, %	84.1±11.0	83.6±11.2	4.39 (0.54-36.1)	0.257
Plaque rupture	44 (66)	2 (13)	12.4 (2.58-59.9)	<0.001
Plaque erosion	9 (13)	4 (27)	0.43 (0.11-1.63)	0.380
Calcified nodule	4 (6)	0 (0)		0.759
Luminal thrombi	57 (85)	6 (40)	8.55 (2.49-29.3)	<0.001
Thinnest fibrous cap, μm	50.4±19.2	78.3±33.6	0.10 (0.03-0.36)	<0.001
Calcification	62 (93)	14 (93)	0.89 (0.10-8.19)	0.659
Extent of calcification	1.5±0.9	1.7±0.9	0.57 (0.18-1.79)	0.499
<15 μm microcalcification in the fibrous cap	53 (79)	7 (47)	4.33 (1.34-14.0)	0.025
Nodular calcification	26 (39)	2 (13)	4.12 (0.86-19.8)	0.114
Fragmented or sheet calcification	62 (93)	14 (93)	0.89 (0.10-8.19)	0.659
Maximum thickness of calcification, μm	679±552	799±746	0.35 (0.09-1.35)	0.201
Intraplaque hemorrhage	63 (94)	12 (80)	3.94 (1.04-19.5)	0.213
Intraplaque hemorrhage, sections	1.7±0.6	1.2±0.9	4.33 (1.34-14.0)	0.025
Intraplaque microvessels, $/mm^2$	2.5±1.9	1.6±1.1	5.94 (1.24-28.4)	0.031
Inflammatory cells within cap	1.8±0.5	1.3±0.9	3.80 (1.11-13.0)	0.064
Foamy macrophages within cap	1.6±0.6	1.1±0.8	3.07 (0.97-9.71)	0.096
Hemosiderin-laden macrophages within cap	1.5±0.6	0.9±0.7	4.38 (1.13-16.9)	0.048
Immature fibrillization	66 (99)	13 (87)	10.2 (0.86-120)	0.148

Myxoid change in media	61 (91)	13 (87)	1.56 (0.28-8.64)	0.972
Necrotic core	63 (94)	13 (87)	2.42 (0.40-14.6)	0.659
>120° necrotic core	58 (87)	9 (60)	4.30 (1.23-15.0)	0.042
Eccentric shape	58 (87)	13 (87)	0.99 (0.19-5.14)	0.683

Values are means ± SD or number (%) of observations. TIA, transient ischemic attack.

Figure 1 illustrates the main characteristics of plaque instability.

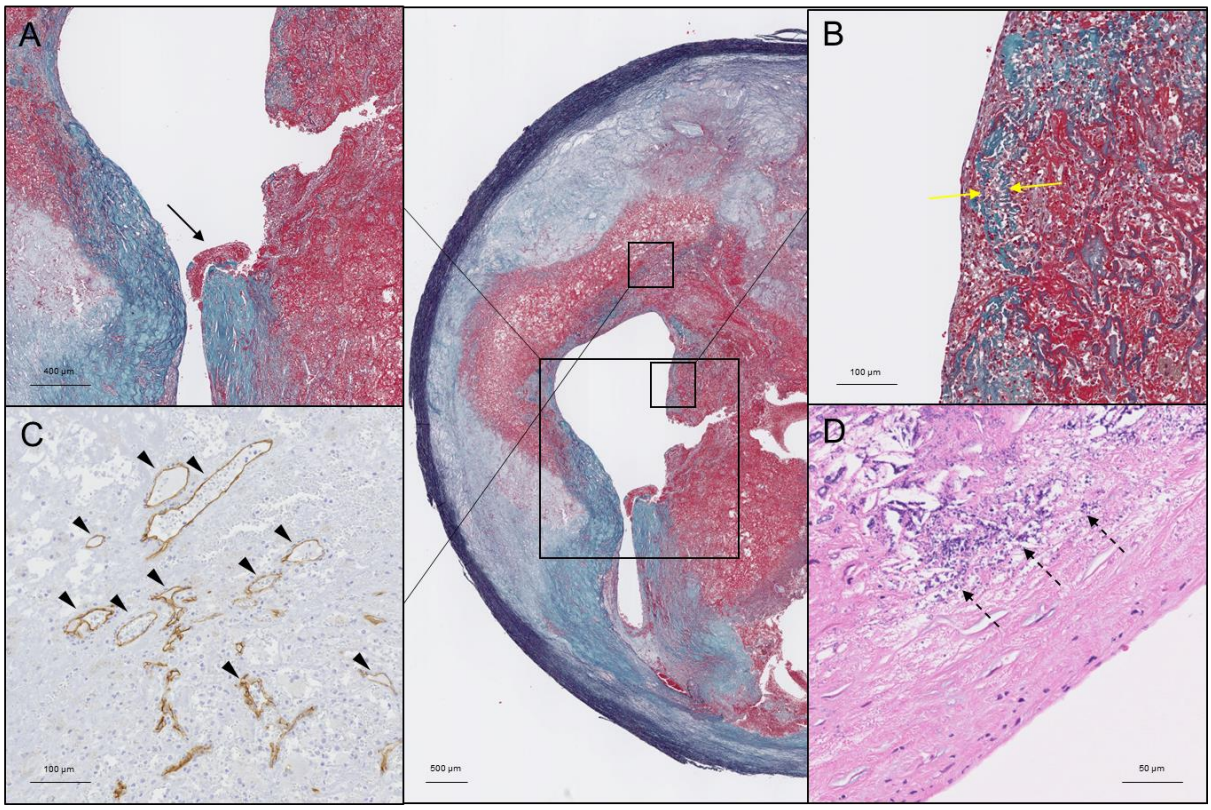

Figure 1. Representative microscopic plaque characteristics.

A. Ruptured plaque with a luminal thrombus (black arrows) stained in elastica-Masson.

B. The fibrous cap thickness measures 49.4 µm (yellow arrows).

C. Intraplaque microvessels stained in CD34 (arrowheads).

D. Another section shows microcalcifications in the fibrous cap in Hematoxylin-Eosin staining (dashed arrows).

Table 2 shows the prevalence of plaque ruptures, erosions and calcified nodules among patients presenting with strokes, versus TIA, versus no symptom. The prevalence of plaque ruptures was highest (73%) in patients presenting with strokes, significantly higher than in patients presenting with TIA (33%) or no symptom (13%). In contrast, the prevalence of calcified nodules was significantly higher in patients presenting with TIA (25%) than in patients presenting with strokes (2%) or no symptoms (0%).

Table 2. Rates of plaque ruptures, erosions and calcified nodules among the various study subgroups

	Number of plaques (%)			p		
				Stroke versus		TIA versus
	Major ipsilateral stroke (n=55)	TIA (n=12)	Asymptomatic (n=15)	TIA	Asymptomatic	Asymptomatic
Plaque rupture	40 (73)	4 (33)	2 (13)	0.023	<0.001	0.076
Plaque erosion	7 (13)	2 (17)	4 (27)	0.917	0.360	0.535
Calcified nodule	1 (2)	3 (25)	0	0.002	0.599	0.040

Values are means ± SD or numbers (%) of observations. TIA, transient ischemic attack.

By multiple variable logistic regression analysis, the presence of plaque rupture, minimum TFC, the presence of microcalcifications in the fibrous cap and intraplaque microvessels were independent correlates of symptomatic plaques (Table 3).

Table 3. Pathological lesion characteristics independently associated with symptomatic cerebral ischemic events

Variable	OR (95%CI)	p

Presence of plaque rupture	9.75 (1.62-58.6)	0.013
Minimum thin fibrous cap (10 μm)	0.70 (0.51-0.96)	0.025
Presence of microcalcification in the fibrous cap	7.82 (1.35-45.4)	0.022
Intraplaque microvessels (/mm^2)	1.91 (1.02-3.57)	0.043

Diagnostic performance of predictors of plaque instability

We used receiver operating characteristics statistics to examine the diagnostic performance of these indices of plaque instability (Figure 2; Table 4).

Figure 2A

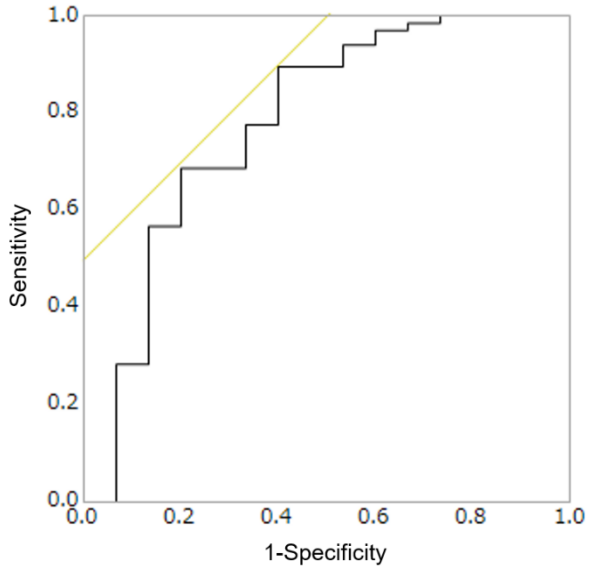

Figure 2B

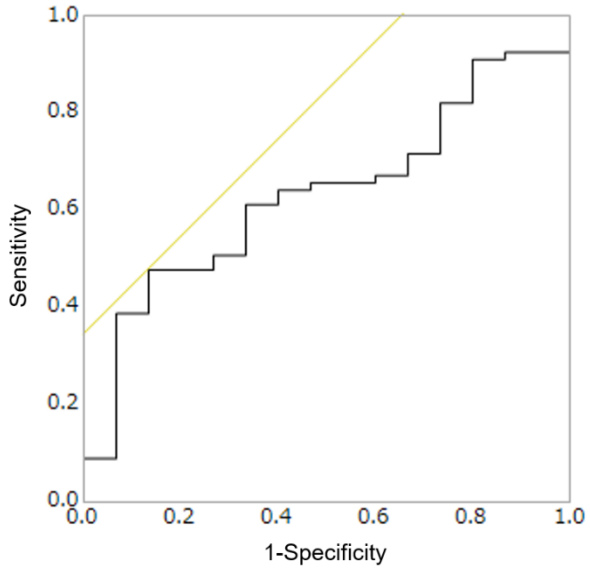

Figure 2C

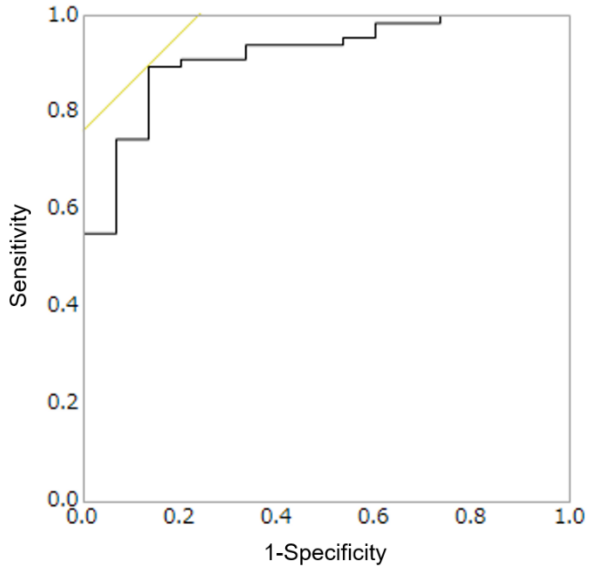

Figure 2. Pathological characteristics identify symptomatic plaques individually as well as when combined in a score.

Receiver-operating characteristic curves for **A)** minimum fibrous cap thickness, **B)** intraplaque microvessels to identify symptomatic plaque individually and **C)** combined into a score. The score, which includes the presence of plaque rupture and microcalcifications in the fibrous cap, was calculated as described in the text.

Table 4. Outcome of receiver-operator-characteristics analysis of pathological variables

	Area under the curve (95% CI)	Cut-off	Sensitivity	Specificity	Predictive value		Diagnostic accuracy
					Positive	negative	
Plaque rupture		Present	65.7	86.7	95.7	36.1	69.5
Minimum thin fibrous cap	0.78 (0.62-0.94)	70.2 μm	89.6	60.0	90.9	56.3	84.1
Fibrous cap microcalcifications		Present	79.1	53.3	88.3	36.4	74.4
Intraplaque microvessels	0.63 (0.49-0.77)	2.20/mm^2	47.8	86.7	94.1	27.1	54.9
Score	0.92 (0.85-0.99)	0.814	89.6	86.7	96.8	65.0	89.0

Among all correlates of symptomatic cerebral ischemic event, minimum TFC had the highest diagnostic performance (area under the curve 0.78, optimal cut-off value 70.2 μm, sensitivity 90%, specificity 60%, PPV 91%, NPV 56%, diagnostic accuracy 84%). However, combining the four independent correlates into a single score, using the equation derived from the multiple variable logistic regression model (Logit(Score) = 0.179 + 2.277* (insert 1 if plaque rupture present; otherwise 0) - 0.355* (insert minimum TFC in multiples of 10 μm) + 2.057* (insert 1 if microcalcification in the fibrous cap present; otherwise 0) + 0.646* (insert intraplaque microvessels /mm^2), increased the diagnostic performance to an area under the curve of 0.92 (95% CI 0.85-0.99; optimal cut-off value 0.814; sensitivity 90%; specificity 87%; PPV 97%; NPV 65%; diagnostic accuracy 89%). The application of this score to grade the instability of two separate lesions is illustrated in Figure 3.

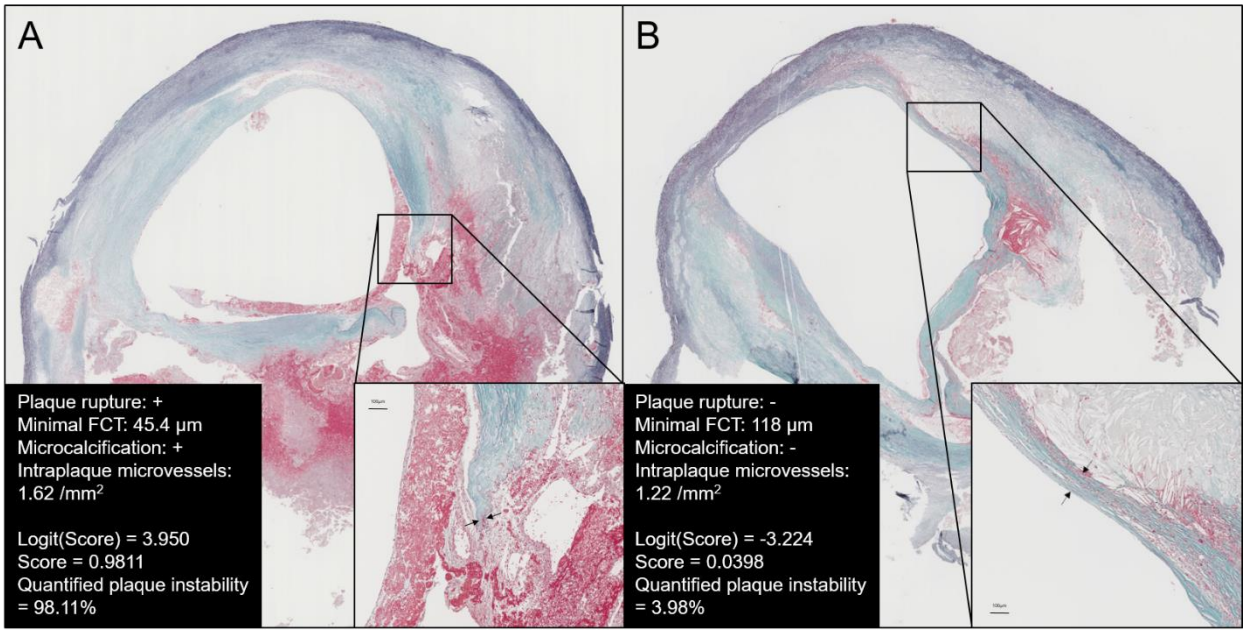

Figure 3. Examples of measurement of lesion instability.

Representative images of unstable (A) and stable (B) carotid plaques quantified by a pathological score, with values indicated for the presence of plaque rupture, minimum thin fibrous cap (TFC), presence of microcalcifications in the cap and intraplaque microvessels. The score was calculated in these two examples and the absolute probability of symptomatic plaque is presented for each lesion.

Discussion

The main observations made in this study were 1) while several pathological characteristics were associated with symptomatic plaques, plaque rupture, minimum TFC, microcalcifications present in the fibrous cap and intraplaque microvessels were independent correlates, and 2) when combined into a single score, the performance of these four independent correlates in the diagnosis of pathologically unstable plaques in patients with carotid artery stenoses was very high. To the best of our knowledge, this study is the first to a) compare the pathological indices of unstable plaque by multiple variable analysis, and b) quantify the instability of carotid artery plaques in patients undergoing CEA.

Comparison with previous studies

Our pathological observations are concordant with those reported in previous publications.(Carr et al., 1996; Golledge et al., 2000; Redgrave et al., 2006; Spagnoli et al., 2004) In this study, the proportion of symptomatic carotid plaques associated with plaque ruptures was higher than that of asymptomatic plaques, and the prevalence of plaque rupture in patients presenting with strokes was higher than that of patients with presenting with TIA or of asymptomatic patients. Previous studies found that symptomatic carotid artery disease is more often associated with plaque ruptures than asymptomatic disease.(Carr et al., 1996; Golledge et al., 2000; Redgrave et al., 2006; Spagnoli et al., 2004) The prevalence of plaque ruptures in stroke victims (67%) was significantly higher than in patients presenting with TIA (23%) or in asymptomatic patients (13%).(Spagnoli et al., 2004)

Earlier studies have observed certain lesion morphologies more often in symptomatic than in asymptomatic patients undergoing CEA.(Carr et al., 1996; de Rotte et al., 2015; Redgrave et al., 2010) Histopathological studies have identified plaque ruptures,(Bentzon et al., 2014; Redgrave et al., 2010) very thin fibrous caps overlying lipid cores,(Carr et al., 1996; Kedhi et al., 2016) intimal foamy macrophages,(Childs et al., 2016; Redgrave et al., 2006) intraplaque hemorrhages (Kolodgie et al., 2003; Redgrave et al., 2006) and intraplaque microvessels (McCarthy et al., 1999; Willems et al., 2013) as major characteristics of atherosclerotic plaque instability. Our own observations are concordant with these studies and add specific information, including the demonstration of a) a higher prevalence of plaque ruptures, b) thinner fibrous caps, c) the presence of microcalcifications in the fibrous cap, and d) a greater number of microvessels inside the plaques in symptomatic than asymptomatic patients presenting with carotid artery stenoses.

Microcalcifications in the fibrous cap

This pathological study is the first to show that the presence of microcalcifications in the fibrous cap is an important sign of symptomatic carotid plaque. An increase in plaque structural stress increases the instability of atherosclerotic plaques.(Imoto et al., 2005) Microcalcifications approximately 5 to 10 μm in diameter accumulate in the fibrous cap inside apoptotic smooth muscle cells or macrophages,(Vengrenyuk et al., 2006) and superficial calcifications around or inside the fibrous cap

increase the stress by nearly 50%.(Imoto et al., 2005) When microcalcifications are clustered along the tensile axis of the cap in coronary arteries, they may increase the local structural stress over fivefold.(Kelly-Arnold et al., 2013) In addition, microcalcifications typically 10 μm in diameter within a thin fibrous cap promote the plaque rupture by increasing the local stress and causing interfacial debonding.(Vengrenyuk et al., 2006) Therefore, microcalcifications in the fibrous cap represent an additional factor of risk of carotid plaque instability.

Measuring the plaque vulnerability

The individual and combined power of these plaque characteristics to measure the plaque instability is incompletely understood. In this study, we combined these previously described, individual characteristics of plaque instability into a single score. By our comprehensive pathological study and multiple variable analysis, we identified the factors correlated with plaque instability. Among all the pathological indices, plaque rupture, minimum TFC, presence of microcalcifications and intraplaque microvessels were the only independent correlates of symptomatic plaques in this sample of patients with carotid artery stenosis. Among all the pathological factors we examined, minimum TFC was the most accurate to quantify plaque instability. Furthermore, when combined into a score, these risk factors were highly accurate to quantify plaque instability and identify unstable plaques in patients with carotid artery stenoses. Although plaque rupture has been described as one of the major features of unstable plaque in histological studies,(Carr et al., 1996; Howard et al., 2015; Redgrave et al., 2006) the plaque rupture is not the only characteristics of unstable atherosclerotic lesion. A representative example of unstable plaque without plaque rupture is shown in Figure S1.

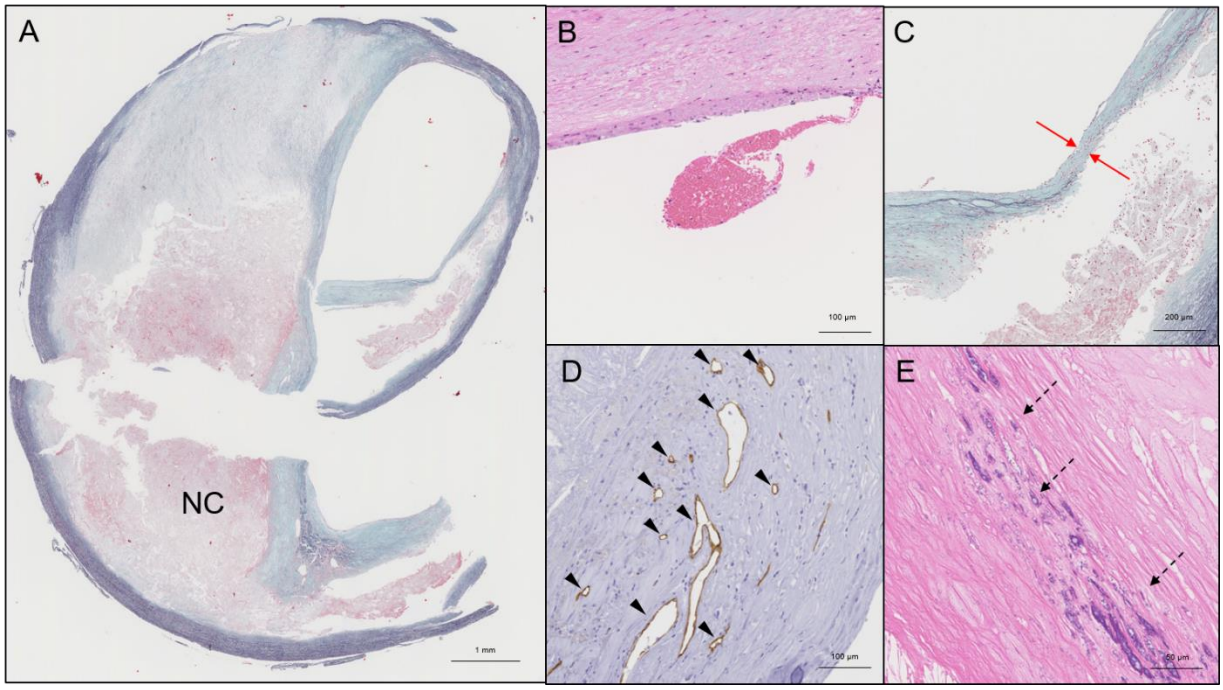

Figure S1. A representative example of unstable plaque without plaque rupture.

Insignificant variables by multiple variable analysis

Intraplaque hemorrhages, hemosiderin-laden macrophages and extensive necrotic cores, though related with unstable plaques by single variable analysis, were no longer statistically significant by multiple variable analysis. While plaques removed ≤60 days after the most recent event are more unstable after a stroke than after a TIA, the instability persists after a TIA, and plaques removed >180 days after a recent event are less unstable after a stroke than after a TIA.(Redgrave et al., 2006) Since over 80% of symptomatic patients presented with major ipsilateral strokes in this study, and the average timing of CEA was 52±50 days after the onset of symptoms, intraplaque hemorrhages, inflammatory cells and extensive necrotic cores might no longer be significant factors due to the long interval between the onset of symptoms and CEA. In contrast, since the statistical significance between plaque instability and intraplaque microvessels is not related to the time since onset of stroke,(Redgrave et al., 2006) it might remain an independent predictor of unstable plaque by multiple variable analysis. Furthermore, intraplaque hemorrhages have been correlated with plaque ruptures in symptomatic carotid artery stenosis.(Carr et al., 1996) It is noteworthy that plaque rupture and intraplaque hemorrhages were correlated in this study ($r = 0.362$; $r^2 = 0.131$;

$p < 0.001$). Therefore, it might be a confounding factor in the multiple variable analysis.

The higher prevalence of calcified nodule in TIAs than strokes

Calcified nodule might be associated with more stable atherosclerotic lesions than plaque rupture in symptomatic patients. Several matrix metalloproteinases produced by activated macrophages digest fibrillar collagen, thus reducing the mechanical plaque stability.(Newby et al., 2009) The comparisons of plaques with plaque rupture and calcified nodule showed significantly less severe infiltration of inflammatory cells (1.9±0.4 vs. 1.3±0.9, p=0.015) and foamy macrophages (1.7±0.5 vs. 1.0±0.8, p=0.037) for those with calcified nodule (Table S1).

Table S1. Comparisons of plaque rupture and calcified nodule groups

	Plaque rupture (n=46)	Calcified nodule (n=4)	p
Age, y	73.5±6.3	75.5±6.8	0.556
Male	45 (98)	3 (75)	0.025
Diabetes mellitus	19 (41)	1 (25)	0.523
Hypertension	40 (87)	2 (50)	0.053
Dyslipidemia	39 (85)	3 (75)	0.609
Chronic kidney disease	11 (24)	0 (0)	0.268
Current smoker	12 (26)	1 (25)	0.962
History of			
TIA or cerebral infarction	11 (24)	0 (0)	0.268
Coronary artery disease	7 (15)	0 (0)	0.400
Peripheral artery disease	4 (9)	0 (0)	0.539
Prior drug therapy			
Statin	7 (15)	0 (0)	0.400
Aspirin	5 (11)	0 (0)	0.487
Clopidogrel	3 (7)	0 (0)	0.598
Cilostazol	0 (0)	0 (0)	
Days between onset of disease manifestations and operation	47±44	64±101	0.752
Glycemia, mg/dl	145±58	113±30	0.124

Cholesterol, mg/dl			
Low-density	116±30	115±41	0.955
High-density	50±13	55±19	0.647
LDL-C to HDL-C ratio	2.4±0.9	2.2±0.6	0.535
Triglyceride, mg/dl	169±82	97±49	0.087
Plaque area, mm^2	42.5±20.2	52.4±34.7	0.610
Cross sectional area luminal narrowing, %	84.5±10.6	78.4±15.0	0.485
Thinnest fibrous cap, μm	50.0±18.9	31.3±17.6	0.063
Calcification	43 (93)	4 (100)	0.598
Extent of calcification	1.4±0.8	2.5±0.6	0.013
<15 μm microcalcification in the fibrous cap	35 (76)	4 (100)	0.268
Nodular calcification	14 (30)	4 (100)	0.005
Fragmented or sheet calcification	41 (89)	4 (100)	0.487
Maximum thickness of calcification, μm	599±488	1,674±790	<0.001
Intraplaque hemorrhage, sections	1.8±0.4	1.3±1.0	0.061
Intraplaque microvessels, $/mm^2$	2.5±2.1	1.3±1.5	0.204
Inflammatory cells within cap	1.9±0.4	1.3±0.9	0.015
Foamy macrophages within cap	1.7±0.5	1.0±0.8	0.037
Haemosiderin laden macrophages within cap	1.6±0.5	1.0±1.2	0.252
Immature fibrillization	45 (98)	4 (100)	0.766
Myxoid change in media	42 (91)	4 (100)	0.539
>120° necrotic core	43 (93)	2 (50)	0.005
Eccentric shape	40 (87)	3 (75)	0.509

Values are means ± SD or number (%) of observations. TIA, transient ischemic attack.

Therefore, calcified nodule might be more frequently observed in patients with TIA, compared to those with stroke. As for coronary artery disease, Jia et al showed that the prevalence of calcified nodule was higher in patients with non-ST segment elevation myocardial infarction (NSTEMI) compared to those with ST segment elevation myocardial infarction (STEMI) in OCT analysis (15% vs. 0%, p=0.004).(Jia et al., 2013)

Clinical perspectives

First, using the indices of plaque instability applied in this study, new techniques, such as 3-dimensional or contrast-enhanced ultrasonography,(Kumar et al., 2016; Saito et al., 2014) computed tomography,(Gupta et al., 2015) magnetic resonance imaging (MRI),(Kumar et al., 2016; Selwaness et al., 2016) and positron-emission tomography (PET),(Hyafil et al., 2016) which can be used to measure these indices, may supplement conventional plaque imaging and improve our prediction of adverse cerebrovascular events. High-resolution MRI is the best means of imaging carotid plaques and is effective to visualize plaque ruptures, intraplaque hemorrhages and TFC. Seven-tesla MRI might enable a further detailed visualization of the structure of carotid plaques.(Erturk et al., 2017) Contrast-enhanced ultrasound allows a quantification of intraplaque neovascularization as a feature of instability in the carotid plaque, and has been closely correlated with histopathologic observations.(Hoogi et al., 2011) 1-μm resolution optical coherence tomography is capable of revealing microcalcifications as the accumulation of small, punctate high-density signals within the fibrous caps.(Liu et al., 2011) These microcalcifications in the atherosclerotic plaque can also be identified with ^{18}sodium fluoride PET or ^{18}sodium fluoride PET/computed tomography.(Irkle et al., 2015) Thus, further technological developments are needed, including of spatial resolution, to detect unstable plaques more precisely. Second, we can verify the consistency between the preoperative diagnosis made by these new techniques and the plaque instability quantified by this pathological score. In our study, the plaque instability diagnosed by preoperative examinations including ultrasonography, MRI and CT was consistent with pathological analysis of plaque instability in 72 out of 82 patients (88%). Third, the plaque instability score might be a predictor of cerebrovascular event (CVE). During the follow-up of six months, CVE occurred in two of 55 patients presenting with major ipsilateral stroke. The mean instability score was 97.8±2.3% in patients with CVE compared with 90.2±17.1% in those without CVE (p=0.329, Table S2).

Table S2. Comparisons of CVE and no CVE groups

	CVE within six months after CEA		
	Absent (n=53)	Present (n=2)	p
Age, y	72.7±7.2	75.5±6.4	0.597
Male	50 (94)	1 (50)	0.018

Diabetes mellitus	20 (38)	1 (50)	0.726
Hypertension	43 (81)	2 (100)	0.497
Dyslipidemia	47 (89)	1 (50)	0.107
Chronic kidney disease	12 (23)	1 (50)	0.371
Current smoker	17 (32)	0 (0)	0.335
History of			
TIA or cerebral infarction	11 (21)	0 (0)	0.471
Coronary artery disease	6 (11)	0 (0)	0.614
Peripheral artery disease	3 (6)	0 (0)	0.729
Drug therapy during follow-up			
Statin	34 (64)	1 (50)	0.683
Aspirin	12 (23)	1 (50)	0.371
Clopidogrel	38 (72)	1 (50)	0.507
Cilostazol	9 (17)	0 (0)	0.524
Days between onset of disease manifestations and operation	43±35	18±7	0.024
Plaque area, mm^2	42.4±19.8	52.1±5.1	0.496
Cross sectional area luminal narrowing, %	83.9±11.0	78.2±1.7	0.024
Plaque rupture	38 (72)	2 (100)	0.378
Plaque erosion	7 (13)	0 (0)	0.582
Calcified nodule	1 (2)	0 (0)	0.845
Thinnest fibrous cap, μm	51.3±19.1	52.1±4.2	0.851
Calcification	49 (92)	2 (100)	0.687
Extent of calcification	1.5±0.8	1.5±1.5	0.981
<15 μm microcalcification in the fibrous cap	40 (75)	2 (100)	0.423
Nodular calcification	19 (36)	0 (0)	0.295
Fragmented or sheet calcification	48 (91)	1 (50)	0.071
Maximum thickness of calcification, μm	631±491	395±384	0.508
Intraplaque hemorrhage, sections	1.7±0.6	2.0±0.0	0.472
Intraplaque microvessels, /mm^2	2.6±2.0	2.4±1.9	0.857
Inflammatory cells within cap	1.9±0.4	1.5±0.7	0.129
Foamy macrophages within cap	1.6±0.6	2.0±0.0	0.380
Haemosiderin laden macrophages	1.4±0.6	2.0±0.0	0.161

within cap			
Immature fibrillization	52 (98)	2 (100)	0.845
Myxoid change in media	48 (91)	2 (100)	0.649
>120° necrotic core	46 (87)	2 (100)	0.582
Eccentric shape	47 (89)	2 (100)	0.614
Plaque instability score, %	90.2±17.1	97.8±2.3	0.028

Values are means ± SD or number (%) of observations. CVE included any stroke or restenosis of carotid artery. During the follow-up of six months, CVE occurred in two of 55 patients presenting with major ipsilateral stroke. One had the second stroke and the other had restenosis. CVE, cerebrovascular event; TIA, transient ischemic attack.

Fourth, carotid artery plaques with certain morphological features might be better target for carotid artery stenting (CAS). Previous studies have shown that some morphological plaque features are associated with cerebrovascular complications pertaining to CAS. Tsutsumi et al showed that severe calcification of the carotid artery might be a cause of incomplete stent expansion despite aggressive dilatation.(Tsutsumi et al., 2008) Bicknell et al reported that lipid-rich plaque was one of the independent predictors of distal embolization during the procedure.(Bicknell and Cheshire, 2003) Furthermore, Kolodgie et al described extensive inflammation as an important risk factor of restenosis after CAS.(Kolodgie et al., 2007) In our study, age and the thickness of calcification were positively correlated (Figure S2).

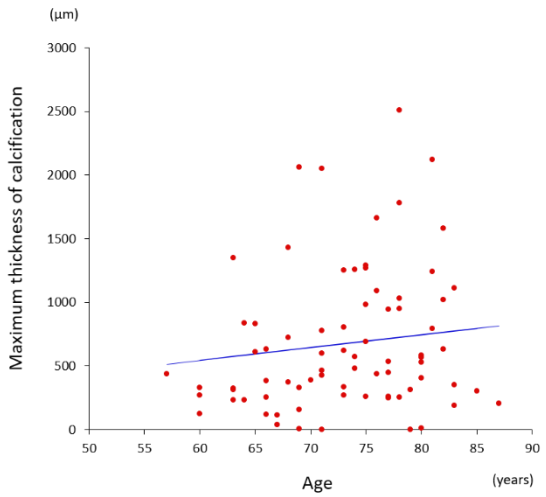

Figure S2. Correlation between age and maximum thickness of calcification.

Infiltration of inflammatory cells as well as foamy and hemosiderin-laden macrophages were less observed in patients with necrotic core <120 ° than in those with necrotic core ≥120 ° (Table S3).

Table S3. Comparisons of NC <=120° and NC >120° groups

	NC <=120° (n=15)	NC >120° (n=67)	p
Inflammatory cells	0.6±0.7	1.5±0.5	<0.001
Foamy macrophages	0.7±0.8	1.7±0.5	<0.001
Hemosiderin-laden macrophages	0.6±0.7	1.5±0.5	<0.001

Values are means ± SD of observations. NC, necrotic core.

These observations suggest that non-elderly patients without severe calcification and extensive necrotic core would be likely to preferentially benefit from CAS. Fifth, several studies have shown that the mechanisms of carotid plaque instability are similar to those found in the coronary circulation,(Redgrave et al., 2006; Spagnoli et al., 2004) although, unlike the myocardial circulation, the carotid vascular bed is exposed to a high blood flow. Therefore, this study should provide useful information, including in the identification of unstable plaques in patients presenting with coronary artery disease.

Limitations of our study

The sample size of this retrospective, observational study, conducted at three medical centers, was small. Its results need to be confirmed by a study including a larger number of patients. Second, in our population, the median time between the last adverse clinical event and CEA was 52±50 days, while the current professional practice guidelines recommend that patients presenting with symptomatic carotid stenosis undergo CEA within 14 days of the last event. Therefore, in further histopathological analyses, observations such as infiltration by inflammatory cells could turn out to be strong predictors of unstable plaques in patients undergoing CEA. Third, instead of

being functional, this study was mainly a quantitative, morphologic analysis of excised specimen of carotid plaques, which did not include measurements of enzymatic concentrations derived from foamy macrophages, such as matrix metalloproteinase.(Childs et al., 2016) Fourth, while observations that are not derived from atherosclerotic plaques, for instance wall shear stress, have also been found to predict plaque instability,(Samady et al., 2011) this study was limited to pathological indices to quantify plaque instability.

Conclusion

This pathological analysis of carotid artery plaques suggests that our diagnostic scoring may facilitate the understanding of plaque instability. The pathological quantification by this scoring simplifies the assessment of carotid artery plaques, instead of requiring a wide variety of pathological characteristics reflecting plaque instability.

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Conflicts of interest

We have conflicts of interest regarding to this study.

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