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New Quantitative Digital Image Analysis Method of Histological Features of Carotid Atherosclerotic Plaques

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WHAT THIS PAPER ADDS

Assessment of atherosclerotic plaque stability is considered to be important for both clinical and fundamental applications. Gold standard semi-quantitative classifications characterise important histological features of the plaque that help determine plaque stability, but they are based solely on visual estimation and can be limited by subjectivity and variability between observers. A new quantitative method for assessment of plaque features has been developed and internally validated, which provided greater accuracy and precision than pathological visual scoring of plaques. This quantitative method can be useful in the research setting for the identification of genetic and molecular signatures, as well as novel biomarkers that reflect histological features of stability. Moreover, it may be used indirectly in the clinical setting to help achieve precision medicine in the management of carotid atherosclerotic disease. For example, it may prove useful for validation of non-invasive imaging techniques to grade quantitatively vulnerable plaque features for association with future cerebrovascular events.

Objective: Atherosclerosis and its thrombotic complications are major causes of morbidity and mortality worldwide. Plaque stability assessment is considered to be important for both clinical and fundamental applications. The current gold standard method to investigate plaque stability is performed by histological assessment of plaque features using semi-quantitative classifications. However, these assessments can be limited by subjectivity and variability. Thus, the aim was to develop a new digital image analysis method to measure quantitatively individual plaque features that is more precise than existing semi-quantitative methods.
Methods: A quantitative method was developed using Image Pro Primer software. Carotid plaque specimens were obtained from patients who underwent carotid endarterectomy and categorised according to stability (definitely stable, probably stable, probably unstable, definitely unstable) based on the gold standard semi-quantitative method that assesses 10 histological plaque features. Using the new quantitative method, plaque features ($n = 15$) from each stability grade were then analysed by two independent raters. For the semi-quantitative analysis, quadratic weighted Cohen's kappa was used to test intra- and inter-rater reliability, while for the quantitative analysis, intraclass correlation coefficients (ICCs) were assessed.
Results: Intra-rater reliability demonstrated almost perfect agreement between both methods (Cohen's kappa range 0.831–0.969, ICC range 0.848–1.000). However, inter-rater reliability demonstrated mainly fair to moderate agreement (Cohen's kappa range 0.341–0.778) for the semi-quantitative analysis, while the digital image analysis method performed most optimally regarding reproducibility, yielding high ICCs close to 1 (ICC range 0.816–0.999). Using quantitative measurements, a statistically significant proportion of the individual plaque features ($p < .05$) were re-classified from one grade to another (shift by one) under the semi-quantitative classification.
Conclusion: A new quantitative digital image analysis was developed for the accurate assessment of histological plaque features, which demonstrated higher precision than the gold standard semi-quantitative methods, as measured by between and within rater analysis. Moreover, quantitative image analysis of histological plaque features provided more detailed insight into plaque morphology and composition.

Keywords: Atherosclerosis, Image analysis, Plaque instability, Quantitative method, Semi-quantitative method
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INTRODUCTION

Atherosclerosis, a chronic inflammatory arterial disease, and its thrombotic complications are major causes of morbidity and mortality worldwide.[1,2] Atherosclerotic

plaque instability and rupture are important causative factors of these complications.[3,4] Plaques have complex morphology and composition, consisting of an accumulation of inflammatory cells, smooth muscle cells, fibrous tissue, lipids, cholesterol crystals, and calcification.[5,6] Owing to their heterogeneity, plaques can be classified either as stable or unstable, based on the presence and severity of certain histological features. Unstable plaques are characterised specifically by a large lipid rich core, a thin fibrous cap, a chronic inflammatory state, thrombosis, and intraplaque haemorrhage, and are highly prone to rupture.[6,7] Plaque vulnerability is highly related to symptom status as the rupture of these plaques causes local thrombosis, leading to partial or total occlusion of the affected artery, as well as embolism to distal arteries, often resulting in clinical manifestations, including ischaemic stroke, transient ischaemic attack, and amaurosis fugax.[7]

To estimate plaque instability, Lovett *et al*. proposed a histological classification that ranges from definitely stable to definitely unstable plaques, based on the semi-quantitative assessment of 10 histological plaque features.[8–10] This semi-quantitative scale has been adopted as one of the "gold standards" used to categorise plaques as stable or unstable. On the one hand, this semi-quantitative standard was established based solely on visual estimation of histological features without quantitative measurements and thus can be limited by subjectivity and variability between raters. On the other hand, classifications based on quantitative image analysis are promising methods for the categorisation of plaque instability, which may provide a more precise and accurate assessment of histological plaque characteristics. Thus, the aim of this study was to develop a quantitative image analysis method for more accurate assessment of individual histological plaque features than traditional semi-quantitative methods.

PATIENTS AND METHODS

Patient recruitment and sample collection

Carotid plaque specimens were obtained immediately after surgical resection from patients who underwent a carotid endarterectomy (CEA) to treat high grade carotid artery stenosis, as described previously.[11,12] Ethics approval for this study (A12-M145-09B) was granted by the McGill University's Institutional Ethics Review Board; the study protocol conformed to the ethical guidelines of the Declaration of Helsinki. All recruited patients provided written informed consent prior to study participation.

Detailed demographic and clinical information was obtained from each patient and cross matched through various sources: (i) patient interview; (ii) questionnaire; and (iii) hospital medical records. As per guidelines, carotid stenosis was determined by carotid Doppler ultrasound imaging, according to the North American Carotid Endarterectomy Trial criteria.[13,14]

As a result of continuous patient recruitment, there was access to a biobank of 400 carotid plaque specimens. Plaques were categorised into four stability groups (grade 1–4), based on the gold standard histological semi-quantitative scale of plaque instability of Lovett *et al*.,[8] as detailed below. For the purpose of this study, a total of 40 plaque specimens (10% of the total plaque specimens), i.e., 10 representative plaque specimens from each stability grade (grade 1–4) were selected randomly (within each stability grade) by the last author (S.S.D.) for the quantitative assessment of plaque features (Fig. 1). S.S.D. did not participate in the semi-quantitative or quantitative measurements of the plaques.

Histological and immunohistochemical staining

A segment of each carotid plaque specimen from the site of maximum stenosis was obtained for histological studies, as this is the largest plaque segment and typically contains the most vulnerable features.[8,10] The dissected segments were fixed routinely in 10% formalin, decalcified, then embedded in paraffin, and cut into 4 μm sections. For histological analyses, plaque sections were stained with haematoxylin and eosin. Immunohistochemical staining was performed on serial sections to stain for macrophage/foam cells and lymphocytes using a CD68 and CD3 antibody, respectively (abcam, 1:50). The EnVision+ System-HRP (DAB) kit (K4010; Dako-Agilent, Mississauga, ON, Canada) was used according to the manufacturer's instructions.

Semi-quantitative assessment of plaque features and instability

Semi-quantitative scorings of carotid plaques were performed by vascular pathologists (J.P.V. and C.L.: "rater 1" [assessment performed in collaboration]) and H.Z. ("rater 2"), according to the gold standard semi-quantitative scale by Lovett JK *et al*., as previously described (Table S1).[8,9,11,12] The raters were unaware of each other's scoring, and of patient characteristics and clinical symptomatology. Furthermore, H.Z. performed scorings at two time points with a three month interval. At the second observation, H.Z. was unaware of the previous scoring. Briefly, the semi-quantitative method assesses ten histological plaque features from a single haematoxylin and eosin stained section obtained from the site of maximum plaque stenosis, and defines each feature on a three or four grade scale according to their presence and severity.[8,9] The ten features include intraplaque haemorrhage; thrombus; foam cells; lipid core size; proportion of fibrous tissue; new vessel formation; calcification; cap rupture; overall inflammation; and infiltration of the fibrous cap with inflammatory cells (inflammation graded according to the number of macrophages and lymphocytes present). Based on the presence of a combination of these features, each plaque was classified as being definitely stable (grade 1); probably stable (grade 2); probably unstable (grade 3); or definitely unstable (grade 4).

Quantitative image analysis of plaque features

Quantitative image analysis of carotid plaques ($n = 40$) was performed using the Zeiss Axio Imager M2 imaging system (Carl Zeiss Canada, Toronto, ON, Canada). While the semi-quantitative scale of Lovett JK *et al*. assessed ten

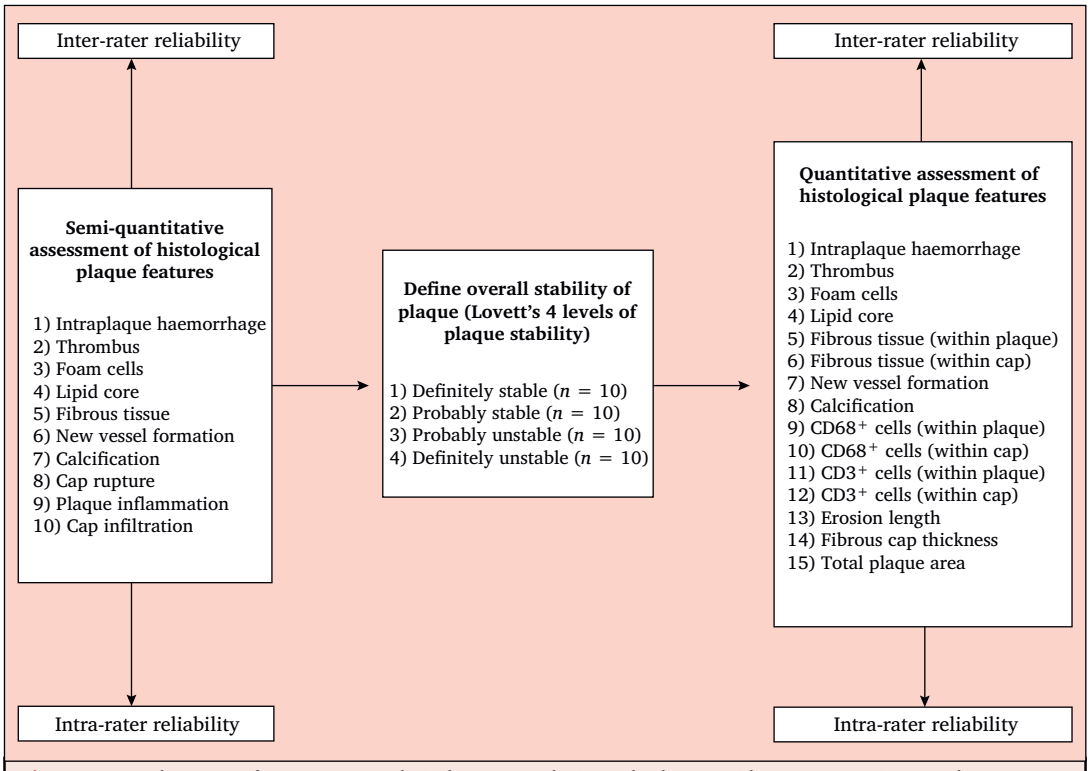

Figure 1. Development of a quantitative digital image analysis method to provide greater precision and accuracy in measuring histological plaque features, than the traditional semi-quantitative method. Collected plaques were assessed histologically using the gold standard semi-quantitative method of Lovett *et al.*,[8] which assesses 10 plaque features. Based on the semi-quantitative assessment of these 10 features, the plaques were classified into four stability categories: definitely stable; probably stable; probably unstable; or definitely unstable. A total of 40 plaque specimens, i.e., 10 representative plaque specimens from each stability grade (grade 1–4) were selected randomly (within each stability grade) and assessed quantitatively using the newly developed digital image analysis method, which measures 15 histological plaque features on a continuous scale. CD = cluster of differentiation.

histological plaque features, the new digital image analysis method enabled the quantification of 15 histological plaque features (Fig. 1). The majority of these features are the same as those included in the semi-quantitative method, such as intraplaque haemorrhage size, thrombus size, foam cell count, lipid core size, fibrous tissue, new vessel formation count, amount of calcification, amount of inflammation in the plaque, and amount of inflammation in the cap. In contrast to the semi-quantitative method, the amount of fibrous tissue within the plaque was measured, separately from the amount of fibrous tissue within the cap. Moreover, the number of macrophages ($CD68^+$ cells) present in the plaque/cap was measured, separately from the number of lymphocytes ($CD3^+$ cells) present in the plaque/cap, while the method of Lovett *et al.* does not distinguish between the two types of cells. It was important to assess the presence of macrophages and lymphocytes separately, as each cell type plays a crucial and independent role in orchestrating the inflammatory response in plaques. Additional features included erosion length, fibrous cap thickness, and total plaque area, which were not assessed in the semi-quantitative method.

Quantitative measurements of the carotid plaque specimens were performed randomly and independently by two raters (H.Z. and K.G.). At the time of measurement both raters were unaware of the Lovett's stability—instability grading of each plaque, and they were unaware of the clinical symptom status of these patients. Both raters underwent similar training to use the image analysis software for the quantitative assessment of plaque features. H.Z. performed measurements at two time points with a three month interval. At the second observation, H.Z. was unaware of his previous scoring.

Whole plaque images of the haematoxylin and eosin stained plaque sections were obtained using the Zeiss Axio Imager M2 imaging system (Carl Zeiss Canada) at a magnification of 100 ×. The quantitative analyses were performed on the same haematoxylin and eosin stained section used for the semi-quantitative assessments, while for macrophage and lymphocyte quantification two additional serial consecutive sections were stained for CD68 or CD3, respectively.

The area of various pathological features (total plaque area, fibrous cap, fibrosis excluding cap tissue, lipid core, calcification, haemorrhage, and thrombus) was carefully traced and measured at 100 × using ImagePro Primer software (Media Cybernetics, Warrendale, PA, USA), combined with the help of high power (400 ×) microscopy to clearly identify the margins of each feature. The total plaque area includes the plaque and occasionally a tiny rim of media removed during the CEA, as a standard surgical procedure. Fig. S1 — S4 show how the areas of the different

features were marked and quantitatively measured in whole plaque images (one per grade of instability). A feature was accounted as zero when it did not exist in the plaque. Cap thickness and erosion length were measured linearly with the software. The average of 10 linear measurements per plaque with an equal distance from each other represented average cap thickness. Minimum cap thickness was measured by considering the thinnest area of the cap (one measurement per plaque). For inflammatory cell measurement, ten representative areas (at 400 ×) were systematically captured: five from the cap tissue (each image was captured at a distance equal to two images from each other), and five from the shoulder region and the area surrounding the necrotic core. The number of $CD68^+$ and $CD3^+$ cells (macrophages and lymphocytes, respectively) in each image was measured using the above image analysis software (see Fig. S5). Foam cells and new vessels in each plaque were counted under the microscope at a magnification of 400 × and 200 ×, respectively. Prior to quantitative analyses, Image-Pro Premier software was calibrated by adding a micron scale bar to the images.

The total plaque area can vary greatly in size from patient to patient, independently of the stability of the plaque or of the histological features it is composed of. Therefore, the measured area or total number of each histological feature (fibrous cap, fibrosis excluding cap tissue, lipid core, calcification, haemorrhage, thrombus, foam cells, and new vessels) was calculated and normalised based on the total area measured for each plaque. The number of $CD68^+$ or $CD3^+$ cells per area was calculated based on the total area of the 400 × images captured.

Statistical analysis

Chi-square analyses were performed to assess the differences in semi-quantitative plaque features between the different groups of plaque instability. Parametric (analysis of variance [ANOVA]) and non-parametric tests (Kruskal—Wallis) were used, as appropriate, to analyse differences in quantitative measurements of histological plaque features between plaques with the four different grades of stability (grade 1: definitely stable plaques; grade 2: probably stable plaques; grade 3: probably unstable plaques; grade 4: definitely unstable plaques). As the grouping variable has more than two levels (i.e., four herein), post-hoc analyses were performed following significance with the ANOVA or Kruskal—Wallis test to ascertain which pairs of groups differed significantly from one another. Partial Spearman or Pearson rank correlation coefficients were used, as appropriate, to assess correlations between the quantitative measurements of the histological plaque features.

Inter- and intra-rater reliability of semi-quantitative scorings (categorical scale) were analysed by percentage absolute agreement and Cohen's kappa (κ). Given the nature of the semi-quantitative categorisation, κ is presented with quadratic weightings, as the importance of the disagreement between categories (i.e., from category 1 to category 2 *vs.* from category 2 to category 3) is not equal.

Table 1. Population demographic and clinical characteristics of patients studied for carotid atherosclerotic plaque stability (n = 40)

Population characteristic	All patients (n = 40)	Patients with definitely stable plaques (n = 10)	Patients with probably stable plaques (n = 10)	Patients with probably unstable plaques (n = 10)	Patients with definitely unstable plaques (n = 10)	p value*
Age — y	71.6 ± 9.1	70.2 ± 11.0	67.9 ± 7.4	71.0 ± 9.2	77.1 ± 6.6	.13
Sex — % men	29 (73)	7 (70)	6 (60)	9 (90)	7 (70)	.50
BMI — kg/m^2	27.1 ± 4.0	27.3 ± 2.6	26.8 ± 4.4	27.5 ± 3.8	26.8 ± 5.1	.98
Ever smoker — %	32 (80)	9 (90)	8 (80)	8 (80)	7 (70)	.74
Carotid artery stenosis — 50—79%/80—99%	9 (23)/31 (78)	2 (20)/8 (80)	2 (20)/8 (80)	2 (20)/8 (80)	3 (30)/7 (70)	.93
Neurologically symptomatic — %	25 (63)	7 (70)	3 (30)	6 (60)	9 (90)	.046
AF/TIA/stroke — %	3 (12)/11 (44)/11 (44)	1 (14)/2 (29)/4 (57)	1 (33)/2 (67)/0 (0)	0 (0)/2 (33)/4 (67)	1 (11)/5 (56)/3 (33)	.47
CAD — %	16 (40)	6 (60)	3 (30)	3 (30)	4 (40)	.48
SBP — mmHg	136 ± 16	142 ± 19	135 ± 17	132 ± 12	137 ± 16	.66
DBP — mmHg	70 ± 11	73 ± 9	67 ± 7	68 ± 11	73 ± 14	.46
Hypertension — %	34 (85)	9 (90)	7 (70)	9 (90)	9 (90)	.50
Antihypertensive medication — %	33 (97)	9 (100)	6 (86)	9 (100)	9 (100)	.26
T2DM — %	10 (26)	3 (30)	3 (30)	2 (20)	2 (20)	.87
Antihyperglycaemic medication, — %	9 (90)	2 (67)	3 (100)	2 (100)	2 (100)	.46
Hypercholesterolaemia — %	30 (75)	6 (60)	7 (70)	8 (80)	9 (90)	.45
Statin use — %	28 (70)	7 (70)	6 (60)	7 (70)	8 (80)	.81

Continuous data are presented as mean ± standard deviation; categorical data presented as n (%). BMI = body mass index; AF = amaurosis fugax; TIA = transient ischaemic attack; CAD = coronary artery disease; SBP = systolic blood pressure; DBP = diastolic blood pressure; T2DM = type 2 diabetes mellitus.*p value indicates comparison among the four instability groups: definitely stable; probably stable; probably unstable; and definitely unstable (analysis was done by chi-square or analysis of variance, as appropriate).

Values of κ between 0 and 0.2 indicated poor agreement, 0.21—0.40 fair, 0.41—0.60 moderate, 0.61—0.80 good, and 0.81—1.00 almost perfect agreement. Inter- and intra-rater reliability of quantitative scorings (continuous scale) were analysed by calculating intraclass correlation coefficients (ICCs), by a two way mixed, single measures model. Interpretation was performed accorded to Koo and Li's guidelines: < 0.050 (poor); 0.50—0.75 (fair); 0.75—0.90 (good); and 0.90—1.00 as excellent.[15] In addition to ICCs, Bland—Altman plots were used to analyse the agreement between the quantitative scorings. Quadratic weighted κ for ordinal scales is considered to be identical to a two way, mixed, single measures ICC for continuous scales.[16]

Statistical analyses were performed using SPSS version 20 (IBM, Armonk, NY, USA). A p value of $< .05$ (two tailed) was considered to be statistically significant. The degree of significance for the correlation matrix was adjusted using Bonferroni correction to $p \leq .003$.

RESULTS

Patient characteristics

The demographic and clinical parameters of the 40 patients who underwent CEA are presented in Table 1. All patients were divided into four groups according to the stability of their plaques, as classified by the vascular pathologists. Among all parameters, only cerebrovascular symptomatology

Table 2. Prevalence of histological features of the carotid atherosclerotic plaque according to the semi-quantitative scale of plaque instability ($n = 40$)

Histological feature	Definitely stable plaques ($n = 10$)	Probably stable plaques ($n = 10$)	Probably unstable plaques ($n = 10$)	Definitely unstable plaques ($n = 10$)	p value*
Haemorrhage — %					.19
None	9 (90)	8 (80)	8 (80)	4 (40)	
Small	1 (10)	1 (10)	2 (20)	4 (40)	
Large	0 (0)	1 (10)	0 (0)	2 (20)	
Thrombus — %					<.001
None	10 (100)	10 (100)	10 (100)	2 (20)	
Small	0 (0)	0 (0)	0 (0)	7 (70)	
Large	0 (0)	0 (0)	0 (0)	1 (10)	
Lipid core — %					<.001
None	4 (40)	5 (50)	0 (0)	0 (0)	
Small	6 (60)	5 (50)	0 (0)	1 (10)	
Large	0 (0)	0 (0)	10 (100)	9 (90)	
Fibrous tissue — %					<.001
Very little	0 (0)	1 (10)	1 (10)	4 (40)	
~50%	0 (0)	0 (0)	9 (90)	6 (60)	
Predominantly fibrous	10 (100)	9 (90)	0 (0)	0 (0)	
Foam cells — %					.02
None	9 (90)	8 (80)	5 (50)	2 (20)	
<50	1 (10)	1 (10)	3 (30)	2 (20)	
At least 50	0 (0)	1 (10)	2 (20)	6 (60)	
New vessels — %					.41
None	3 (30)	0 (0)	1 (10)	1 (10)	
<10 per section	4 (40)	2 (20)	4 (40)	4 (40)	
At least 10 per section	3 (30)	8 (80)	5 (50)	5 (50)	
Calcification — %					.49
None	1 (10)	1 (10)	2 (20)	1 (10)	
Stippling only	2 (20)	0 (0)	2 (20)	0 (0)	
Calcified nodules	7 (70)	9 (90)	6 (60)	9 (90)	
Inflammatory cells — %					.003
None	5 (50)	1 (10)	0 (0)	0 (0)	
Occasional	5 (50)	4 (40)	4 (40)	2 (20)	
2–5 groups of >50 cells	0 (0)	5 (50)	6 (60)	8 (80)	
>5 groups of >50 cells	0 (0)	0 (0)	0 (0)	0 (0)	
Cap infiltration — %					<.001
None	10 (100)	6 (60)	0 (0)	1 (10)	
<10 cells	0 (0)	1 (10)	2 (20)	0 (0)	
10–50 cells	0 (0)	3 (30)	6 (60)	5 (50)	
>50 cells	0 (0)	0 (0)	2 (20)	4 (40)	
Cap rupture — %					<.001
Intact cap	10 (100)	10 (100)	10 (100)	0 (0)	
Probably intact	0 (0)	0 (0)	0 (0)	2 (20)	
Probably ruptured	0 (0)	0 (0)	0 (0)	3 (30)	
Definitely ruptured	0 (0)	0 (0)	0 (0)	5 (50)	

Data presented as n (%). Inflammation was graded according to the number of macrophages and lymphocytes present.*p value indicates comparison among the four instability groups: definitely stable; probably stable; probably unstable; and definitely unstable (chi-square analysis).

was statistically significantly different across the patient groups ($p = .046$). To demonstrate that the selected patients for the present study ($n = 40$) were representative of the larger pool of patients in the biobank, the clinical parameters of all 400 patients based on their plaque stability are included in Table S2.

Semi-quantitative assessment of plaque features

The semi-quantitative results of the histological features of 40 plaques based on the plaque stability/instability scoring of Lovett *et al*. are given in Table 2. All histological features, except for intraplaque haemorrhage, neovascularisation, and calcification, were statistically significantly different between definitely stable, probably stable, probably unstable, and definitely unstable plaques. Fibrous tissue content was greater in definitely stable and probably stable plaques than in unstable plaques ($p < .001$). In contrast, lipid core size was larger in definitely unstable and probably unstable plaques than in stable plaques ($p < .001$). Plaque and cap inflammation increased with greater plaque instability. Furthermore, a greater number of foam cells was also present in unstable *vs.* stable plaques ($p = .015$). Presence of a thrombus as well as cap rupture, were key histological features of definitely unstable plaques ($p < .001$). The distribution of histological characteristics of the 40 plaques across the different stages of stability was representative of those in the 400 patient cohort (see Table S3).

Inter- and intra-rater reliability of semi-quantitative analysis

The inter- and intra-rater reliability for the semi-quantitative scoring of all histological plaque features are presented in Table 3. The inter-rater reliability was good for only half of the graded plaque features, while the remaining characteristics had fair to moderate agreement. Intra-rater reliability demonstrated almost perfect agreement among all plaque features. Inter- and intra-rater reliability for plaque stability demonstrated almost perfect agreement ($\kappa = 0.918$ and $\kappa = 0.969$, respectively).

Quantitative image analysis of plaque features

The quantitative image analysis results of the histological features of the 40 plaques based on the plaque stability/instability scoring of Lovett *et al*. are given in Table 4, while a correlation matrix of the quantitative measurements of the histological plaque features is presented in Table S4. Similar to the semi-quantitative analysis, fibrous tissue content within the plaque, as well as within the cap, was statistically significantly greater in stable plaques (grades 1 and 2) than in unstable plaques (grades 3 and 4), while lipid core size was statistically significantly smaller (Fig. S6A). Intraplaque haemorrhage was also statistically significantly larger in definitely unstable plaques compared with stability grades 1 to 3 ($p < .001$, $p = .05$, and $p = .025$, respectively).

In addition to the 10 features scored by the semi-quantitative method, using the digital image analysis method other important plaque features were measured, including erosion length, fibrous cap thickness, and $CD68^+$ (macrophages/foam cells) and $CD3^+$ (lymphocytes) cells in the fibrous cap and plaque tissue, separately. Vascular thrombi and plaque surface erosion were extensively present in definitely unstable plaques, and statistically significant differences were observed compared with the other stability grades 1–3 (thrombus: $p < .01$; erosion: $p = .01$). Average cap thickness was greatest among stable plaques (grades 1 and 2) *vs.* definitely unstable plaques (Fig. S6B). Moreover, plaque and cap CD68 and $CD3^+$ cells were highly and significantly present in unstable *vs.* stable plaques (Figs. S6C and D).

The aim was to determine whether using the quantitative measurements of plaque features obtained from the newly developed image analysis method, would result in plaque features being reclassified as a different grade under the semi-quantitative classification of Lovett *et al*. (see Table S1). As a result, a significant proportion of plaque features (fibrous tissue content, lipid core size, number of foam cells, neovascularisation, and amount of inflammation and cap infiltration) were reclassified as a different grade under the semi-quantitative classification (Table 5). However, in the majority

Table 3. Inter- and intra-rater reliability of semi-quantitative scoring of histological carotid atherosclerotic plaque features and stability

Histological feature	Category	Inter-rater agreement		Intra-rater agreement	
		Absolute agreement, %	Quadratic weighted Cohen's kappa (95% CI)	Absolute agreement, %	Quadratic weighted Cohen's kappa (95% CI)
Intraplaque haemorrhage	3	55.0	0.341 (0.048–0.634)	85	0.831 (0.610–1.0)
Thrombus	3	87.5	0.498 (0–1.0)	97.5	0.925 (0.528–1.0)
Lipid core	3	77.5	0.778 (0.594–0.961)	95.0	0.936 (0.765–1.0)
Fibrous tissue	3	82.5	0.781 (0.520–1.0)	95.0	0.909 (0.597–1)
Foam cells	3	42.5	0.383 (0.168–0.595)	90.0	0.863 (0.605–1.0)
New vessels	3	65.0	0.654 (0.412–0.896)	87.5	0.897 (0.791–1.0)
Calcification	3	57.5	0.536 (0.322–0.750)	80.0	0.845 (0.774–0.916)
Inflammatory cells	4	53.7	0.434 (0.063–0.805)	92.5	0.903 (0.522–1.0)
Cap infiltration	4	52.5	0.633 (0.407–0.858)	85.0	0.892 (0.742–1.0)
Cap rupture	4	60.0	0.727 (0.626–0.828)	85.0	0.930 (0.760–1.0)
Plaque stability	4	87.5	0.918 (0.817–1.0)	92.5	0.969 (0.853–1.0)

CI = confidence interval.

Table 4. Quantitative image analysis of histological features of the carotid atherosclerotic plaque (n = 40)

Histological feature	Definitely stable plaques (n = 10)	Probably stable plaques (n = 10)	Probably unstable plaques (n = 10)	Definitely unstable plaques (n = 10)	p value*
Fibrous cap – % area	22.57 ± 6.55	23.68 ± 6.57	12.74 ± 3.10	9.34 ± 4.27	<.001
Fibrosis (minus cap tissue) – % area	38.33 ± 9.74	38.70 ± 6.94	12.75 ± 8.15	13.55 ± 5.70	<.001
Lipid core – % area	13.00 ± 4.09	13.74 ± 7.71	49.15 ± 12.23	46.02 ± 12.87	<.001
Calcification – % area	3.18 ± 4.79	5.88 ± 9.57	1.66 ± 2.87	4.38 ± 4.00	.16
Average cap thickness – μm	716.40 (590.62–914.82)	707.39 (562.10–826.34)	455.20 (408.00–735.28)	120.50 (103.83–591.93)	.005
Minimum cap thickness – μm	411.45 (237.78–620.21)	413.31 (312.37–612.38)	250.15 (217.27–529.65)	19.68 (6.06–267.86)	.004
Haemorrhage – % area	0.00 (0.00–0.00)	0.14 (0.00–0.49)	0.001 (0.00–0.54)	1.38 (0.58–2.49)	<.001
Thrombus – % area	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	1.21 (0.0–1.81)	<.001
Erosion – μm	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.01 (0.00–124.94)	0.00 (0.00–1370.26)	.029
Foam cells – no. per 10 mm^2	7.00 (0.00–14.50)	2.00 (0.00–25.00)	19.00 (4.50–33.25)	15.00 (6.25–22.75)	.19
New vessels – no. per 10 mm^2	6.00 (2.75–14.50)	8.00 (6.75–12.50)	6.00 (3.75–9.50)	6.50 (4.00–13.00)	.48
Plaque CD68 – no. per 1 mm^2	83.50 (65.00–106.50)	96.50 (56.25–185.25)	235.00 (169.75–339.50)	231.50 (152.00–619.75)	<.001
Cap CD68 – no. per 1 mm^2	44.00 (32.00–67.25)	79.00 (68.00–159.00)	297.50 (188.00–389.25)	157.00 (84.25–334.25)	<.001
Plaque CD3 – no. per 1 mm^2	56.00 (37.75–92.00)	108.50 (61.00–145.50)	156.50 (100.25–190.50)	205.50 (166.00–282.25)	<.001
Cap CD3 – no. per 1 mm^2	28.50 (18.75–42.75)	32.00 (20.25–75.75)	69.00 (45.50–103.00)	63.50 (22.75–149.25)	.029

Normally distributed data are presented as mean ± standard deviation; non-normally distributed data are presented as median (interquartile range). *p value indicates significance among the four instability groups: definitely stable; probably unstable; probably unstable; and definitely unstable (analysis was performed by analysis of variance or Kruskal–Wallis test, as appropriate). CD = cluster of differentiation.

of cases only a shift by one grade (i.e., grade 1 to grade 2 or grade 3 to grade 4) was observed.

Inter- and intra-rater reliability of quantitative analysis

Inter-rater ICCs were found to be excellent for the majority of histological features graded, ranging from 0.931 to 0.999 (Table 6). The rest of the features had good agreement, with the exception of calcification and thrombus, which had fair agreement. The intra-rater reliability demonstrated similar ICC values as the inter-rater reliability (Table 6). Bland–Altman plots demonstrated that the variability in inter- and intra-rater measurements remained consistent across the graph as the average increased (see Figs. S7 and S8, respectively).

DISCUSSION

To the authors' knowledge, this is the first study to develop a novel and comprehensive method that uses quantitative image analysis to assess histological features of carotid atherosclerotic plaques. Unlike the semi-quantitative method, which assesses plaque histological features on a grade scale of 1–3, or 1–4, based solely on visual estimation, digital image analysis provides exact measurements (on a continuous scale) for each histological feature, thus minimising the bias and inter-individual variability often associated with semi-quantification. Herein, it is reported that the novel quantitative image analysis technique achieved better reproducibility than the traditional semi-quantitative analysis. Both methods achieved high intra-rater agreement; this was expected given that repeated analysis was performed by the same experienced pathologist/rater in each case. However, for inter-rater agreement, which relies more on objective criteria, the quantitative method demonstrated superiority over the semi-quantitative method. For the vast majority of histological features quantified using the new image analysis method, inter-rater ICCs ranged from 0.816 to 0.999, with narrow 95% confidence intervals. By contrast, inter-rater Cohen's κ values for the histological features scored using the semi-quantitative method ranged between 0.341 and 0.778.

The new image analysis method is more accurate and reproducible in categorising histological plaque features, as its measurements do not rely on visual estimation. Based on the criteria of Lovett *et al*. to classify plaque features on a grade scale of 1–3 or 1–4, the image analysis method led to a more precise classification of the severity of plaque features causing, in the majority of cases, a shift by one grade when compared with the semi-quantitative classification.

Table 5. Re-classification of semi-quantitative grading of various histological features of carotid atherosclerotic plaque using the quantitative method

Histological feature	Method	Grade 1	Grade 2	Grade 3	Grade 4	*p* value*
Fibrous tissue	Criteria	<45%	45–55%	>55%	–	<.001
	SQ – %	10	40	50	–	
	Q – %	52.5	5	42.5	–	
Lipid core	Criteria	0%	≤25%	>25%	–	<.001
	SQ – %	20	30	50	–	
	Q – %	5	42.5	52.5	–	
Foam cells	Criteria	0	<50 cells	≥50 cells	–	.024
	SQ – %	62.5	12.5	25	–	
	Q – %	22.5	42.5	35	–	
New vessels	Criteria	0	<10 vessels	≥10 vessels	–	.013
	SQ – %	12.5	37.5	50	–	
	Q – %	0	40	60	–	
Inflammation	Criteria	0	≤100 cells	101–250 cells	>250 cells	.003
	SQ – %	12.5	37.5	50	0	
	Q – %	2.5	42.5	45	10	
Cap infiltration	Criteria	0	<10 cells	10–50 cells	>50 cells	.003
	SQ – %	42.5	7.5	30	20	
	Q – %	0	0	25	75	

SQ = semi-quantitative method; Q = quantitative method. *p value indicates comparison between semi-quantitative and quantitative scorings (chi-square analysis).

Table 6. Inter- and intra-rater reliability of quantitative scoring of histological carotid atherosclerotic plaque features

Histological feature	Inter-observer agreement	Intra-observer agreement
	ICC (95% CI)	ICC (95% CI)
Fibrous cap – % area	0.853 (0.746–0.964)	0.943 (0.847–0.980)
Fibrosis (minus cap tissue) – % area	0.849 (0.616–0.945)	0.944 (0.844–0.980)
Lipid core – % area	0.976 (0.934–0.991)	0.985 (0.955–0.995)
Calcification – % area	0.567 (0.124–0.823)	0.650 (0.234–0.863)
Average cap thickness – μm	0.932 (0.820–0.975)	0.848 (0.617–0.945)
Minimum cap thickness – μm	0.816 (0.553–0.931)	0.895 (0.724–0.962)
Haemorrhage – % area	0.817 (0.561–0.931)	0.871 (0.676–0.953)
Thrombus – % area	0.745 (0.422–0.902)	0.740 (0.413–0.900)
Erosion – μm	0.978 (0.940–0.992)	1.000 (0.999–1.000)
Foam cells – no. per 10 mm^2	0.999 (0.998–1.000)	0.999 (0.997–1.000)
New vessels – no. 1 10 mm^2	0.950 (0.864–0.982)	0.996 (0.988–0.999)
Plaque CD68 – no. per 1 mm^2	0.951 (0.867–0.983)	0.984 (0.952–0.994)
Cap CD68 – no. per 1 mm^2	0.937 (0.831–0.978)	0.848 (0.620–0.944)
Plaque CD3 – no. per 1 mm^2	0.931 (0.813–0.975)	0.996 (0.988–0.999)
Cap CD3 – no. per 1 mm^2	0.890 (0.252–0.972)	0.895 (0.704–0.963)

ICC = intraclass correlation coefficient; CI = confidence interval; CD = cluster of differentiation.

Moreover, the quantitative method provided a more comprehensive insight into plaque morphology and composition. The results confirmed the findings from several other studies that the amount of fibrous tissue, lipid core size, cap thickness, inflammatory cell infiltration, and intraplaque haemorrhage size are major determining factors of plaque instability. In addition to the features used by the semi-quantitative method, other important features were also measured, including cap thickness, erosion length, and $CD68^+$ and $CD3^+$ cells in the fibrous cap and plaque tissue, separately, allowing for more detailed characterisation of each plaque. As a result of the normalisation of the quantitative data by total plaque area, the new method provides greater precision and accuracy in measuring histological plaque features than the semi-quantitative method, which does not account for the inter-individual variability in plaque size. Additionally, the new method confirmed known concepts of histological plaque features (e.g., large lipid core, thin fibrous cap, increased inflammation in unstable plaques, etc.), which internally validates the results (see Appendix A for a detailed discussion on histological plaque features).

Over the past decade, dramatic improvements in computerised image analysis algorithms have promoted the development of powerful quantitative approaches for pathological diagnosis and prognosis.[17] Quantitative pathology has become more prevalent in several fields owing to its clear advantages in reducing pathological interpretation bias and improving the accuracy of disease prognosis/diagnosis and severity grading. For example, the application of computerised image analysis in breast cancer has improved the ability for reliable prognosis,[18,19] and has allowed more accurate monitoring of fibrotic changes in different stages of liver disease.[20,21] However, there are limited quantitative pathology studies in the field of atherosclerosis. Thus, there is a need for quantitative image based assessment of atherosclerotic plaque features, as

semi-quantitative methods remain the gold standard with which to assess these features. Similar to the present results, Vrijenhoek *et al.*[22] also demonstrated that a semi-automated method for digital quantification of atherosclerotic plaque features provides greater precision and agreement in repeated measurements compared with semi-quantitative analyses. While their method is certainly promising for future research applications, quantitative analysis was only provided to measure macrophage and smooth muscle cell infiltration. By contrast, a more comprehensive quantitative analysis of a total of 15 plaque features is present herein, which are available for more accurate identification of plaque instability.

A limitation of the present study is that the quantitative assessment of plaque features is based on the analysis of a single 4 μm plaque section at the site of maximum stenosis, rather than a serial analysis of various sections throughout the plaque. However, this was done in order to analyse the same single 4 μm section that was used by the pathologists for the semi-quantitative analysis. Furthermore, quantitative image analysis of various features in the plaque is inevitably time consuming, as the process is not fully automated and still requires some manual evaluation.

To conclude, in this "methodology development" study a new method for the histological assessment of plaque features is presented, which provided better accuracy and precision than the gold standard semi-quantitative method. Moreover, it was demonstrated that quantitative image analysis of histological features of atherosclerotic plaques can provide more detailed insight into plaque morphology and composition. Based on these findings, future work will use advanced statistical analyses to identify (i) the relative weight/contribution of each plaque feature to plaque instability; and (ii) severity cut off values of the individual plaque features. The cut off values will be used to convert continuous values of the individual histopathological features of the quantitative method into valid ordinal categories. Using Rasch models or other item response theory models, fully quantitative diagnostic criteria for plaque stability classification will be established, which will then be externally validated in a larger plaque population. Thus, the method developed herein may serve as a stepping stone for future fundamental research, as well as for clinical application of accurate atherosclerotic plaque stability classification.

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CONFLICT OF INTEREST

None.

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APPENDIX A. SUPPLEMENTARY DATA

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COUP D'OEIL

A Rare Case of Arterial Tortuosity Syndrome

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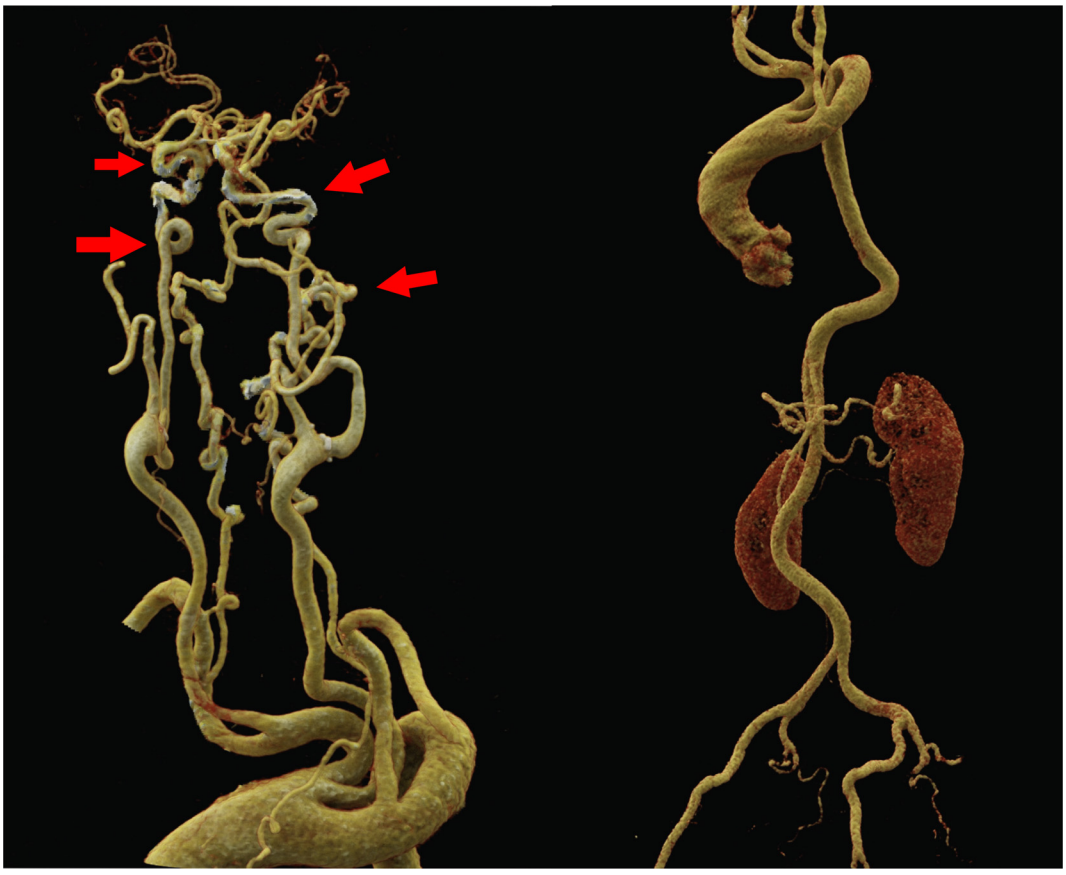

A 60 year old woman presented with widespread joint pain and ligament laxity. She had previously undergone lumbar hernia repair. A previous diagnosis of arterial tortuosity syndrome was confirmed by molecular genetic testing for a homozygotic mutation in *SLC2A10*. Duplex ultrasound showed multiple atherosclerotic carotid plaques without significant stenosis. Computed tomography angiography revealed unusual, widespread vessel tortuosity, particularly in the supra-aortic area, with multiple kinking/coiling (arrows). No surgical procedures were undertaken given the absence of symptoms and the diffuse nature of the vascular tortuosity. The patient remains under clinical and radiological surveillance, as these patients can also present with aortic aneurysms.

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