Intraobserver and interobserver variability and spatial differences in histologic examination of carotid endarterectomy specimens

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Introduction: **Studies using histologic examination and protein analysis of atherosclerotic plaques are increasingly being performed, but reproducibility of plaque histology and variation of plaque composition among different parts of the plaque, which are key to reliability of these studies, are relatively unexplored. Therefore, this study investigated the intraobserver and interobserver variability of plaque histology and spatial variability in plaque composition.**

Methods: **Atherosclerotic plaques (n = 100) obtained during carotid endarterectomy were divided into 0.5-cm segments. Paraffin sections were stained and semiquantitatively analyzed (four categories: no, minor, moderate, and heavy) for fat, macrophages, smooth muscle cells, collagen, calcification, thrombus, and overall phenotype. First, to determine the intraobserver and interobserver reproducibility, two independent observers independently analyzed the plaques. Second, to investigate spatial variability in plaque composition, histologic appearances of the culprit lesions (0-segment) were compared with the histologic appearances of adjacent (+5 mm) and more distant (+10 mm) plaque segments of 30 specimens.**

Results: **The κ values for intraobserver variability of fat, macrophages, smooth muscle cells, collagen, calcifications, thrombus, and overall phenotype were 0.83, 0.85, 0.71, 0.63, 0.81, 0.80, and 0.86, respectively, and κ values for interobserver variability were 0.68, 0.74, 0.54, 0.59, 0.82, 0.75, and 0.71, respectively. Comparison of the histologic scorings of adjacent segments revealed a mean κ of 0.40 (range, 0.33 to 0.60). When the culprit segment was compared with the more distant segment, the mean κ was 0.24; however, in 91% of cases, the difference between the culprit segment and the distal segment was one category or less.**

Conclusion: **Semiquantitative analysis of carotid atherosclerotic plaque histology was well reproducible, both intraobserver and interobserver. Although variation between different plaque segments in histologic appearance was observed, differences were small in almost all cases. Variability in histologic examination needs to be taken into account in studies comparing plaque imaging with histopathology and plaque research studies. (J Vasc Surg 2007;46:1147-54.)**

The percentage carotid artery stenosis has traditionally been considered the main determinant of stroke risk related to carotid artery disease. It has recently become evident that composition of the atherosclerotic lesion is also an important determinant of clinical presentation. Three large studies have convincingly shown that symptomatic presentation of carotid artery disease is associated with specific histologic features of the carotid plaque such as a large lipid core, rupture of the fibrous cap, and increased inflammatory activity.[1-3] This is comparable with earlier findings in the coronary tree, where the same characteristics have been linked with unstable clinical presentation such as myocardial infarction and sudden cardiac death.[4-8]

The increasing knowledge on plaque pathophysiology has the potential to be clinically applied; for example, noninvasive imaging of these plaque characteristics could, in the future, aid in selection of the most appropriate treatment. Furthermore, it is currently being investigated if the plaque histology itself holds prognostic information.[9] When histologic characteristics of endarterectomy specimens could be identified that are related to a highly increased risk of restenosis, patients whose plaque histology showed these characteristics could undergo a more aggressive duplex surveillance scheme. For all of these applications, it is important to investigate the reliability of the histologic examination.

Present data on reproducibility of atherosclerotic plaque histology are scarce. To our knowledge, one study has been published that systematically investigated the reproducibility of carotid plaque histology.[10] This study showed good reproducibility of plaque histology between observers, and the authors concluded that examination of a single culprit segment of the plaque was reasonably representative for the plaque as a whole.[3] Hematoxylin and eosin was the only histologic staining used, however, and the reproducibility of scoring of immunohistochemical stainings, for example to assess macrophage infiltration, was not investigated.

The current study therefore investigated the reproducibility of the plaque histology. For this purpose, two sub-

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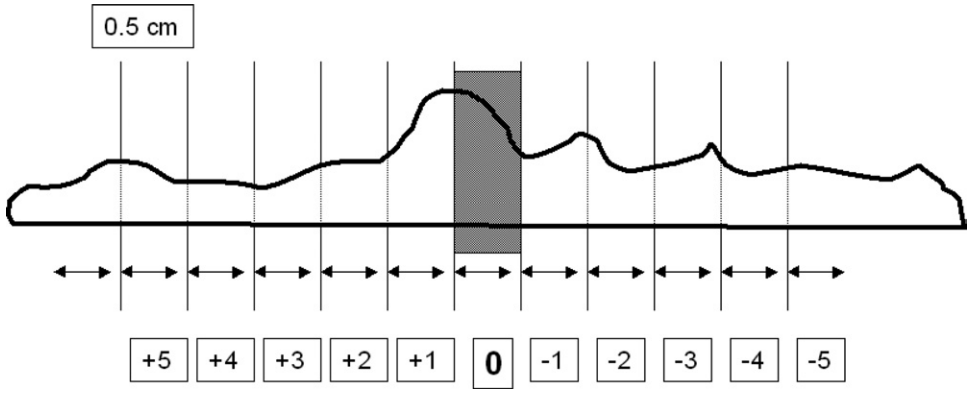

Fig 1. In this schematic representation of plaque segmentation, each transverse segment measures approximately 0.5 cm. The 0 segment, the reference segment that is routinely used for assessment of plaque histology in the Athero-Express study, is defined as the segment with greatest plaque burden. The adjacent segments at both sides are called +1, +2 etc, at one side and −1, −2, etc, at the other side.

studies were performed. First, sections were repeatedly examined to determine intraobserver and interobserver variability, and semiquantitative ratings based on visual estimation were compared with computer-aided analyses. Second, the variability of scorings along different segments of the plaque was examined.

METHODS

Patients. A random subset of 100 carotid endarterectomy specimens from the Athero-Express study was included.[9] The study has been approved by the Institutional Review Boards of the two participating hospitals, and written informed consent was obtained from all patients. The patients had significant carotid artery stenosis as assessed by preoperative duplex examination: 3% had 50% to 69% stenosis and 97% had 70% to 99% stenosis. Most patients (85%) were symptomatic and had symptoms of amaurosis fugax, transient ischemic attack (TIA), or stroke.

Plaque processing. All carotid endarterectomies were performed by an experienced vascular surgeon or by a trainee under specialist supervision. Before the carotid artery was cross-clamped, 5000 U of heparin was administered intravenously. The plaques were carefully dissected in toto and directly transferred to the laboratory after excision. The plaques were dissected by a dedicated technician into 5-mm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden as determined by visual assessment of plaque macroscopy was called the zero segment (Fig 1). The rationale is that the segment of a plaque with largest plaque burden is generally the plaque segment where most vulnerable characteristics, such as large lipid core and inflammation, are present.[11] This was not necessarily the middle segment of the endarterectomy specimen.

Adjacent segments (5-mm thickness) were numbered sequentially: +1, +2, etc, at one side and –1, –2, etc, at the other side. Segments used for the analysis were fixed in formalin, quickly decalcified in ethylenediaminetetraacetic acid, and embedded in paraffin. Cross-sections of 5-μm thickness were cut on a microtome and used for staining with hematoxylin and eosin (H&E), elastin von Gieson (EvG), picrosirius red, and immmunohistochemical stainings for α-actin and CD68 (Fig 2).

Semiquantitative assessment. Table I gives an overview of all semiquantitative assessments and the stainings used for each item. The definitions of each staining category have been published previously.[9] Briefly, all scorings are based on visual estimates and are rated on ordinal scales. Overall phenotype is established by overall appearance: the typical atheromatous plaque has a large lipid core (high fat content, defined as >40% of plaque area) and high macrophage infiltration with low smooth muscle cell and collagen content, whereas the typical fibrous plaque has a small (<10% of plaque area) or absent lipid core, low macrophage infiltration, and high smooth muscle cell and collagen content. The fibrous-atheromatous phenotype is an intermediate between the two other phenotypes.

Quantitative assessment. The sections were visualized under a microscope at ×40 magnification and digitally captured on a computer workstation with a 3-megapixel camera. Three representative fields were identified in each section. These fields contained only intima; lumen, and fragments of media were avoided. The percentage of plaque area occupied by the specific staining (CD68 for macrophages and α-actin for smooth muscle cells) was measured automatically in each of these fields, based on color thresholds (AnalySIS 3.2, Soft Imaging Systems GmbH, Münster, Germany). The mean of the three fields was calculated and used as the representation of percentage of positive staining in the section.

Reproducibility. To determine interobserver reproducibility, two independent observers assessed the zero segments of the plaques. The observers did not have access to scorings of the other observer. All cross-sections were blinded so that patient characteristics were not available to the observ-

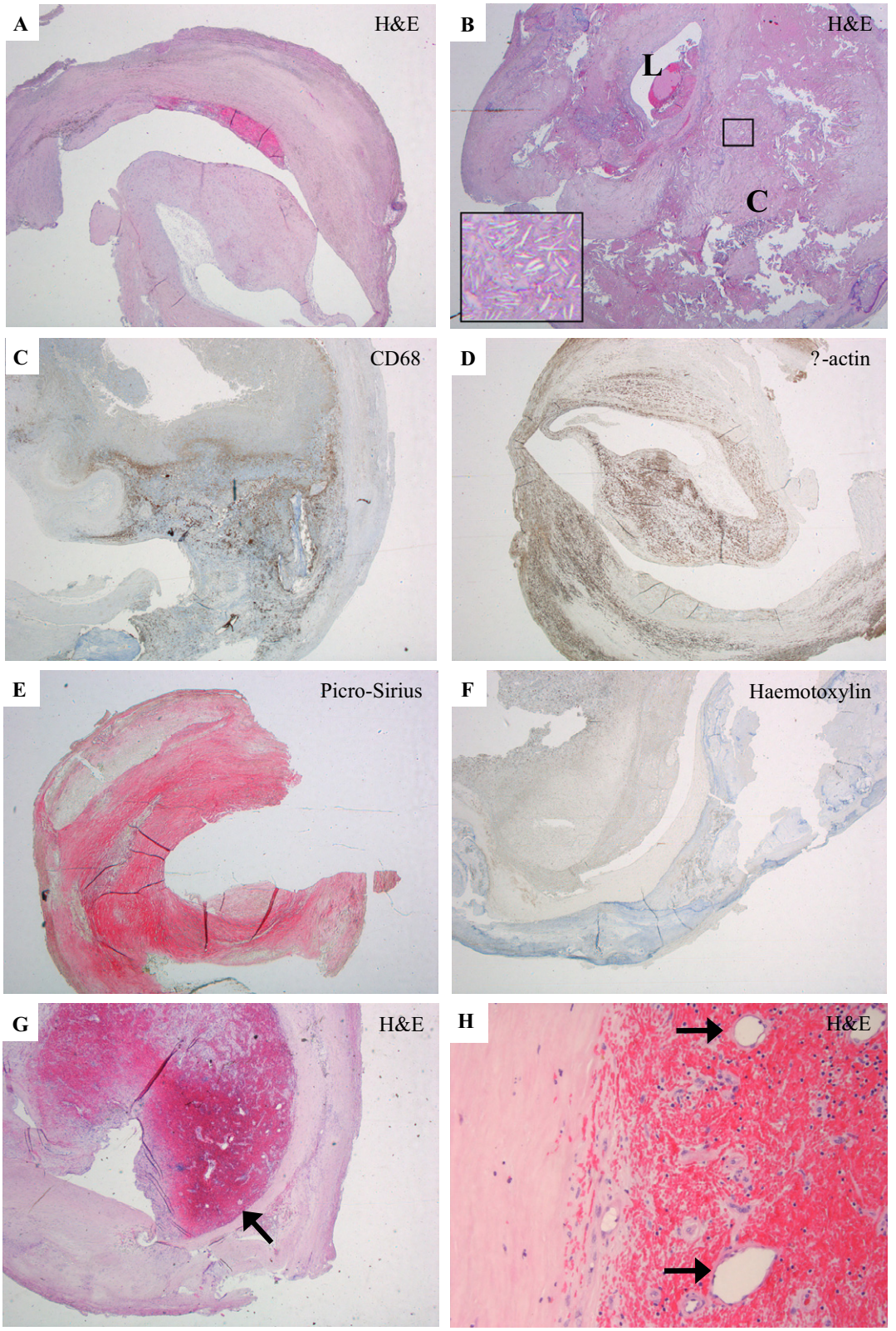

Fig 2. Representative carotid plaque histology. Images A-G were photographed at ×20 original magnification. **A,** Hematoxylin and eosin (H&E) staining shows a plaque with no lipid core. **B,** H&E staining, large lipid core (*L,* lumen; *C,* core). The inset shows cholesterol crystals, which were clearly visible at higher magnification. **C,** Immunohistochemistry analysis with CD68 shows heavy macrophage infiltration. **D,** Immunohistochemistry analysis with α-actin shows heavy smooth muscle cell infiltration. **E,** Picrosirius red shows heavy collagen staining. **F,** Hematoxylin stains heavy calcification (blue). **G,** H&E, thrombus *(arrows)*. **H,** At higher magnification (H&E, ×200 original magnification), signs of organization of the thrombus can be appreciated: angiomatosis *(arrows)* and influx of inflammatory cells.

Table I. Semiquantitative assessment of plaque histology

Histologic characteristic	*Staining*	*Categories*			
		1	*2*	*3*	*4*
Fat (lipid core)	H&E, Picrosirius	<10%	10%-40%	>40%	
Macrophages	CD68	No	Minor	Moderate	Heavy
SMC	α-Actin	No	Minor	Moderate	Heavy
Collagen	Picrosirius	No	Minor	Moderate	Heavy
Calcifications	H&E, hematoxylin*	No	Minor	Moderate	Heavy
Thrombus	H&E, EvG	No	Minor	Moderate	Heavy
Overall phenotype	H&E, Picrosirius	Fibrous	Fibro-atheromatous	Atheromatous	

SMC, Smooth muscle cell; *H&E,* Hematoxylin and eosin; *EvG,* elastin von Gieson.
*Hematoxylin was used as counterstaining in CD68 and α-actin stainings.

ers. To determine intraobserver reproducibility, the second observer reassessed the sections in a random order about 2 months after the first assessment. The initial ratings of the second observer were compared with the repeated assessment of the same observer. The observer was blinded for earlier scorings of the plaques. Macrophage and smooth muscle cell infiltration were also measured by computer-aided analysis in the sections from the zero segment and compared with the semiquantitative ratings.

Intersegment variation. In a subset of 30 plaques, the zero segment, the directly adjacent segment, and the next segment underwent histologic examination by the second observer. Per plaque, either segments 0, +1, +2, or segments 0, −1, −2 were examined. The examination of these segments was blinded and was performed in a random order. Comparison was made between adjacent segments (0 vs +1 and +1 vs +2) and between nonadjacent segments (0 vs +2).

Data analysis. Weighted kappa (κ) statistics were used as the measure of agreement between different observations. Two different weightings were employed: First, linear weights are presented, in which the weights are calculated as $[1 - abs(i-j)/(m-1)]$ and second, quadratic weights are presented, in which the weights are calculated as $1 - abs(i-j)^2/(m-1)^2]$, with $abs(i-j)$, the number of categories difference between observers; m, the number of categories, and $m-1$, maximum difference between two observations.[12] When four semiquantitative categories are used, linear weightings for disagreement (no difference between observers), and difference of 0, 1, 2, or 3 categories between observers are 0, 1/3, 2/3, and 1, respectively. With quadratic weighting, these numbers are 0, 1/9, 4/9, and 1, respectively. This clarifies that small disagreements affect the quadratic-weighted κ less than linear-weighted κ. Given κ values are quadratic-weighted unless otherwise specified. In addition, the percentages of sections in which both observations were exactly equal are given (percentage agreement).

The Spearman correlation was used to test the relation between semiquantitative and quantitative measurements. For comparison of different plaque segments, quadratic-weighted κ were used. The κ values for comparison of different segments were also expressed with correction for the intraobserver κ, because perfect alikeness of two adjacent segments could theoretically not result in a κ value higher than the intraobserver κ. Applying generally accepted definitions, κ values ≤0 indicate no agreement; 0 to 0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, good agreement; and 0.8 to 1, excellent agreement.[13]

RESULTS

The semiquantitative analysis of plaque histology was well reproducible, with a mean κ of 0.78 (Table II). Intraobserver reproducibility showed highest κ values for macrophage infiltration (0.85) and overall phenotype (0.86). Percentage exact agreement was 68% to 83%. Reproducibility between observers was slightly lower than intraobserver reproducibility: on average, κ was 0.69. The mean percentage interobserver agreement was 70% compared with 77% intraobserver agreement. Most of the disagreements between the observers were minor (1 category): in the intraobserver assessments, disagreements of two categories were observed in 0.8% of cases and disagreements of 3 categories in 0.1% of cases. This observation was comparable in interobserver analysis: difference of two categories was observed in 0.9% of cases and disagreements of three categories in 0.2% of cases. Moreover, ratings for macrophages and smooth muscle cells did not show any differences greater than one category in either intraobserver or interobserver analyses.

The reliability of the scorings of macrophage and smooth muscle cell presence was confirmed by comparison with quantitative analyses. For macrophages, semiquantitative and quantitative measurements were very well correlated ($R = 0.77$; $P < .0005$), and there was no overlap between the 25th and 75th percentiles of subsequent scoring categories (Fig 3, *A*). The cutoff values between semiquantitative scoring categories were approximately 0.03% (no–minor), 0.3% (minor–moderate), and 1.4% (moderate–heavy) of plaque area. With increasing semiquantitative ratings, the quantitative measurements increased more than linear.

For smooth muscle cells, the same relations were found. Although the quantitative measurements of subsequent semiquantitative rating categories overlapped a little more, the

Table II. Intraobserver and interobserver variability of semiquantitative plaque examination

		Intraobserver variability			*Interobserver variability*		
		κ *(95% CI)*	κ *(95% CI)*		κ *(95% CI)*	κ *(95% CI)*	
Histologic characteristic	*Categories**	*Quadratic weighted*	*Linear weighted*	*Agreement*	*Quadratic weighted*	*Linear weighted*	*Agreement*
Fat	3	0.83 (0.69-0.96)	0.77 (0.65-0.89)	83%	0.68 (0.49-0.87)	0.60 (0.48-0.73)	71%
Macrophages	4	0.85 (0.59-1.00)	0.78 (0.67-0.89)	83%	0.74 (0.46-1.00)	0.63 (0.50-0.75)	73%
SMC	4	0.71 (0.24-1.00)	0.63 (0.48-0.79)	78%	0.54 (0.12-0.95)	0.42 (0.25-0.57)	66%
Collagen	4	0.63 (0.19-1.00)	0.54 (0.40-0.68)	68%	0.59 (0.33-0.85)	0.51 (0.38-0.67)	69%
Calcification	4	0.81 (0.65-0.98)	0.75 (0.64-0.85)	76%	0.82 (0.74-0.90)	0.71 (0.61-0.81)	70%
Thrombus	4	0.80 (0.64-0.95)	0.68 (0.55-0.80)	71%	0.75 (0.52-0.98)	0.65 (0.53-0.76)	71%
Overall phenotype	3	0.86 (0.78-0.94)	0.79 (0.68-0.89)	82%	0.71 (0.55-0.87)	0.62 (0.49-0.74)	71%

CI, Confidence interval; *SMC,* smooth muscle cell.
*Categories are specified Table I.

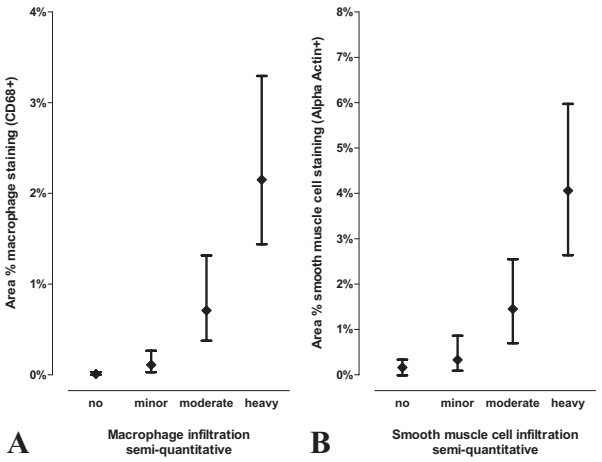

Fig 3. Semiquantitative vs computer-aided measurements. Comparison of semiquantitative assessments, expressed as "no," "minor," "moderate," and "heavy" (x-axis) and the computer-aided measurements, expressed as the percentage of plaque area occupied by the specific immunohistochemical staining in three representative fields (y-axis). **A,** Macrophage infiltration (CD68+). **B,** Smooth muscle cell infiltration (α-actin+).

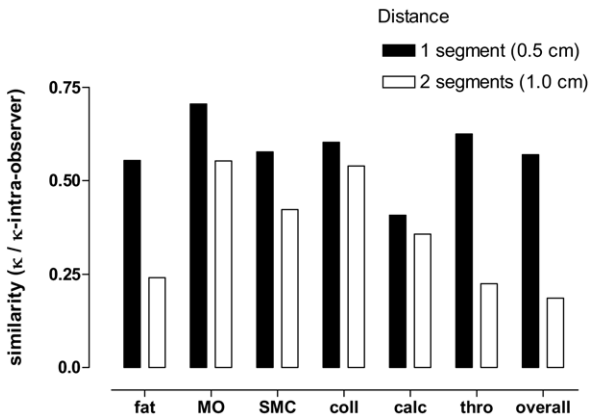

Fig 4. Intersegment variability of histologic examination of the plaque. The similarity between different segments expressed as percentage of the κ value of the intraobserver repeatability, which is the theoretic maximum when two adjacent segments are completely equal: 100% indicates perfect similarity, 0% indicates no similarity, and negative values (not observed) indicate inverse associations. The similarity is compared between adjacent segments (intersegment distance "1") and between the reference segment and a nonadjacent segment (intersegment distance "2"). *MO,* Macrophage infiltration; *SMC,* smooth muscle cell infiltration; *coll,* collagen; *calc,* calcifications; *thro,* thrombus; *overall,* overall phenotype.

semiquantitative ratings compared very well with the quantitative ratings overall ($R = 0.67$; $P < .0005$; Fig 3, *B*). In general, the area occupied by smooth muscle cells amounted to more than twofold the area occupied by macrophages (mean, 2.3% vs 0.89%). The cutoff points between semiquantitative scoring categories for smooth muscle cells were therefore higher: 0.2% (no–minor), 0.7% (minor–moderate), and 2.5% (moderate–heavy) of plaque area.

The variation in plaque histology between different plaque segments was investigated. Average κ between adjacent segments was 0.40, and the percentage of exact agreement between adjacent segments was 60% compared with 77% for intraobserver analysis of the same segment. Similarity between the adjacent segments was good for macrophage infiltration (0.60), moderate for fat (0.46), smooth muscle cell infiltration (0.41), collagen (0.46), and overall phenotype (0.50), and fair for thrombus (0.33) and calcifications (0.38). Because assessment of the similarity of segments is hampered by intraobserver variation, the similarity of segments was corrected for the intraobserver variability (Fig 4).

In the distant segments (two-segment distance from the reference segment), similarity was generally less compared with the reference segment, but most items still showed fair to moderate agreement (Fig 1; average κ, 0.24). For all histologic assessments, similarity decreased with increasing distance. Still, in the distal segments, most differences with the reference segment were minor (one category), and 91% showed exact agreement or at maximum one category difference.

DISCUSSION

The present study shows that histologic examination of carotid plaque histology is well reproducible. The current study did not achieve 100% reproducibility. However, even clinical standards such as angiography sometimes show considerable variation, but this does not mean that the diagnostic tool can not be used reliably.[14] Moreover, comparison of the semiquantitative analysis with quantitative measurements of smooth muscle cell and macrophage infiltration showed that each semiquantitative scoring category corresponded with a distinct range of quantitative measurements. We further found that differences in plaque characteristics between adjacent segments are relatively small. When the distance was bigger, however, the similarity consistently decreased.

The results of the current study are comparable with the previously published results from Lovett et al.[10] The current data have added value because macrophage infiltration, smooth muscle cell infiltration, and collagen are also included in the analysis and because our results are backed up by quantitative computer-based measurements. The previous study found good intraobserver and interobserver reproducibility for repeated assessment in the same sections of lipid core size, calcifications, and thrombus, which is comparable with our results. Findings on intersegment difference were also comparable. The current study adds the intersegment difference in macrophages, smooth muscle cells, and collagen. Compared with the other plaque characteristics, macrophages and smooth muscle cells showed low variability between segments.

Clinical implications. The interpretation of the extent of variability depends greatly on the underlying purpose. On the one hand, plaque imaging is an upcoming field, and validation of these imaging modalities requires comparison with the gold standard of plaque histology. On the other hand, histologic examination of atherosclerotic plaques is used by studies linking the plaque with clinical characteristics. For both purposes, it is very important to quantify the amount of variability in histologic assessment.

Implications for validation of plaque imaging studies. Preoperative noninvasive imaging of the plaque has the potential to help guide the choice of treatment modality. Supporting this is that the composition of symptomatic plaques is different from asymptomatic plaques. Symptomatic presentation is related to a large lipid core, infiltration of macrophages, plaque rupture, and thrombus.[1-3,15] Some studies have already shown that the size of the lipid core can be determined reliably with magnetic resonance imaging.[16-18] To be clinically applicable, the imaging technique needs to be validated against the gold standard of plaque histology.

The present study clarifies that the accuracy of plaque imaging may be underestimated because of variability in histologic assessment of plaques. The observed variability between imaging modalities and histology is the sum of (1) variability and imperfections of the imaging technique and (2) the variability in assessment of plaque histology. When the latter is not taken into account, the ability of imaging techniques to identify plaque characteristics noninvasively will be underestimated.

From our current results, we can make the following recommendations with regard to validation of plaque imaging against histology. First, sections along the whole endarterectomy specimen need to be taken, especially when thrombus or calcifications are studied, which show marked variablility within the plaque. Optimally, the site where histological sections are taken should be documented and matched to the images following a standardized protocol. Second, we recommend histological assessment of each section by two independent observers, in order to minimize variability. Studies investigating plaque imaging should report the variability in their histological assessments and consider this in their conclusions.

Implications for plaque research and biobanking. The study of endarterectomy specimens without involvement of imaging is of interest for two reasons. First, the study of endarterectomy specimens may reveal insights into pathophysiology of the disease and underlying mechanisms. Second, the endarterectomy specimen may hide prognostic information that could predict risk of restenosis and risk of adverse vascular events during follow-up. The latter is currently under investigation in the Athero-Express study.[9]

The interpretation of the current results may be different for these biobanking studies then for validation of plaque imaging. For plaque research studies, a perfect segment-to-segment match is not needed. When one wants to investigate the difference in plaque composition between patient groups, it is sufficient to study a large cohort with less extensive sampling per plaque. This will minimize the probability of chance findings and also provide power to perform multivariable statistical analysis to correct for confounders. It is unlikely that with the currently observed degree of variation, clear differences between patient groups would be missed in large series of endarterectomy specimens. This is underlined by the strong differences we observed between plaque histology of men compared with women in 450 patients.[19] Furthermore, for biobanking studies, we recommend that histologic assessment needs to be done by two independent observers, that the sampling of the plaques is performed by following a standardized protocol, and that variability in histologic examination is reported.

When biobanking would succeed in identifying predictive markers in the atherosclerotic plaque, such as for restenosis, the possibility of comparable assessment at different laboratories would be warranted. Semiquantitative histologic assessment is well reproducible within one laboratory but may be difficult to reproduce by others. The semiquantitative methods are well suited for research studies, but quantitative techniques such as computer-based analysis or measurement of protein markers by enzyme-linked immunosorbent assay (eg, macrophage markers) will be more suitable for extrapolation of results to other centers.

Another issue for biobanking studies is the comparison of plaque histology with protein expression within the plaques. In the Athero-Express study, the segments adjacent to the zero segment are regularly used for protein extraction.[9] The protein extracts can be used for determination of different proteins, such as inflammatory markers (interleukin-6 and -8 are routinely determined) and matrix metalloproteinases (MMP-2, MMP-8, MMP-9). The current study shows that intersegment differences in histology are relatively small, especially for the smooth muscle cells and macrophages, the main cell types responsible for production of several substances (eg, cytokines and proteases) in atherosclerotic plaques. Therefore, the use of the plaque segment adjacent to the reference segment for comparisons within plaques (histology in the reference segment vs protein in the adjacent segment) is a valid approach. This is underlined by the fact that well-known associations, for example between macrophage infiltration and production of MMP-9,[20] could be readily confirmed when the histology in the zero segment was compared with protein analysis from the adjacent segment.[21] In distant segments, however, the differences in histology compared with the reference segment were larger. This indicates that when histopathology is compared with protein analyses within a plaque, this is best done in adjacent segments.

Strengths and limitations. This study contained a random sample of plaques taken from an ongoing consecutive series of carotid endarterectomies. Macroscopy was not used to select specimens, and therefore, this study provides a real-world comparison. To assess variability of different observations, the weighted κ statistic was used. Weightings are arbitrary, however, and thus we attempted to give optimal insight into our data by providing two different weightings.

A potential limitation of the current study is that plaque rupture was not assessed. In our experience, the plaque morphology is disrupted by surgical trauma in many cases, thus making assessment of cap integrity difficult. In line with these observations, plaque rupture had relatively low reproducibility in the study by Lovett et al.[10] We have not included cap rupture in our own plaque assessments for this reason. Assessments of plaque rupture are probably much more accurate in postmortem series where vessels can be examined with the plaque in situ.

CONCLUSION

Semiquantitative analysis of carotid atherosclerotic plaque histology is well reproducible. Although variation between different plaque segments in histologic appearance was observed, differences were small in almost all cases. Variability in histologic examination needs to be taken into account in studies that compare plaque imaging with histopathology and plaque research studies.

AUTHOR CONTRIBUTIONS

Conception and design: WH, GP, CS, DK, FM
Analysis and interpretation: WH, GP, AV, FM
Data collection: WH, AV, CS, EV, JD, FM
Writing the article: WH
Critical revision of the article: GP, AV, CS, JD, EV, DK, FM
Final approval of the article: GP, AV, CS, JD, EV, DK, FM
Statistical analysis: WH, GP, AV
Obtained funding: Not applicable
Overall responsibility: FM

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