Original Contribution

Plaque Inflammation and Unstable Morphology Are Associated With Early Stroke Recurrence in Symptomatic Carotid Stenosis

Michael Marnane, PhD; Susan Prendeville, MB; Ciaran McDonnell, MD; Imelda Noone, ANP; Mary Barry, MCh; Morgan Crowe, MB; Niall Mulligan, MB; Peter J. Kelly, MD

Background and Purpose—Although symptomatic carotid stenosis is associated with 3-fold increased risk of early stroke recurrence, the pathophysiologic mechanisms of high early stroke risk have not been established. We aimed to investigate the relationship between early stroke recurrence after initial symptoms and histological features of plaque inflammation and instability in resected carotid plaque.

Methods—Carotid endarterectomy tissue from consecutive patients with ipsilateral stenosis ≥50% and recent symptoms were analyzed using a validated histopathologic algorithm (Oxford Plaque Study [OPS] system). Nonprocedural stroke recurrence before carotid endarterectomy was ascertained at 7, 28, and 90 days after initial symptoms.

Results—Among 44 patients meeting eligibility criteria, 27.3% (12/44) had stroke recurrence after initial stroke/transient ischemic attack but before carotid endarterectomy. Compared with patients without recurrence, stroke recurrence was associated with dense macrophage infiltration (OPS grade ≥3; 91.7% versus 37.5%; \( P = 0.002 \)), extensive (>25%) fibrous cap disruption (90.9% versus 37%; \( P = 0.004 \)), neovascularization (OPS grade ≥ 2; 83.3% versus 43.8%; \( P = 0.04 \)), and low plaque fibrous content (OPS grade <2; 50% versus 6.3%; \( P = 0.003 \)). Early recurrence rates were 82.3% (confidence interval, 49.2%–98.8%) in patients with extensive plaque macrophage infiltration (OPS grade ≥ 3) compared with 22.2% (confidence interval, 3.5%–83.4%) in those with OPS grade <3 (log-rank \( P = 0.009 \)). On multivariable Cox regression, including OPS macrophage grade (≥3 or <3), age, and severity of stenosis (50%–69% or ≥70%), plaque inflammation was the only variable independently predicting stroke recurrence (adjusted hazard ratio, 9; confidence interval, 1.1–70.6; \( P = 0.04 \)).

Conclusions—Plaque inflammation and other vulnerability features were associated with highest risk of stroke recurrence and may represent therapeutic targets for future stroke prevention trials. (Stroke. 2014;45:00-00.)

Key Words: atherosclerosis • carotid stenosis • inflammation • pathology • stroke

In population studies, recently symptomatic carotid stenosis is associated with a 3-fold increase in stroke recurrence risk within 90 days of first stroke or transient ischemic attack (TIA) and a 5-fold increase in risk within 2 weeks of first symptoms, compared with other stroke mechanisms.1,2 Supporting these studies, the benefit of carotid endarterectomy (CEA) is most apparent when performed within 2 weeks of symptom onset in randomized trials.3 However, the underlying mechanisms of this high-risk state are not well established.

Studies of inflammatory biomarkers and plaque uptake of 18-fluorodeoxyglucose on positron emission tomography in patients with carotid disease suggest that inflammation may be an important contributor to recurrent cerebral thromboembolism independently of the severity of lumen stenosis.4 Almost no data exist from longitudinal studies that have directly investigated the association between early stroke recurrence and carotid plaque inflammation and instability.5 We hypothesized that histological features of carotid plaque inflammation and instability (such as fibrous cap disruption, neovascularization, and low fibrous content) in resected plaque would be associated with greater risk of early recurrent stroke in the interval between recent initial symptoms and CEA. We investigated this hypothesis in a prospective cohort study of patients with recently symptomatic carotid stenosis.

Methods

Subjects

The Dublin Carotid Atherosclerosis Stroke Study is a prospective cohort study of multimodal imaging and blood biomarkers in recently symptomatic carotid stenosis, as previously described in detail.1 Briefly, we recruited consecutive patients with TIA, mild-to-moderate stroke (modified Rankin score ≤3), or retinal artery embolism and ipsilateral nonocclusive internal carotid artery stenosis (≥50% luminal narrowing by The North American...
Symptomatic Carotid Endarterectomy Trial [NASCET] criteria). We excluded those who were aged <50 years, pregnant, had active malignancy, prior neck irradiation, prior ipsilateral CEA/stenting, or carotid occlusion.

At recruitment, patients were assessed by a trained stroke physician, who was independent of patient care and blinded to histological data. All episodes of TIA, stroke, or retinal embolism within the 28 days before the stroke or TIA that prompted presentation to medical attention were recorded. The first such event within 28 days of medical assessment was defined as the index episode, with subsequent events defined as recurrent. If no earlier stroke or TIA occurred before the event for which the patient sought medical attention, then this stroke/TIA was coded as the index event. Patients were followed up in person or by telephone interview by an independent study physician to ascertain recurrent stroke at 72 hours, 7, 28, and 90 days after the index event or before CEA (if CEA was performed within 90 days). Clinical details were confirmed with proxies where appropriate (including in 3 patients with persistent moderate aphasia). Patients were censored at the time of CEA. Therefore, recurrent events that occurred during or after surgery were not included in the analysis.

Clinical stroke recurrence was defined as a new, sudden-onset focal neurological deficit lasting >24 hours in the same carotid territory in a patient whose symptoms had resolved or whose examination findings were initially stable for ≥24 hours, confirmed by a trained stroke physician. Cases in which new neurological deficits or deterioration in existing deficits could reasonably be attributable to stroke-in-evolution, cerebral edema, hemorrhagic transformation, or comorbid illness were excluded. Only recurrent stroke in the same carotid territory as the index (qualifying) event was included in the analysis. All patients with potential recurrences were confirmed by in-person physician assessment and review of initial and repeat brain imaging.

Ethics committee approval was obtained from all participating institutions, and all participants provided informed consent.

Table 1. Semiquantitative Grading Scales for Plaque Histological Analysis

<table>
<thead>
<tr>
<th>Histological Feature</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque macrophage infiltration*</td>
<td>None</td>
<td>Occasional or 1 group &gt;50</td>
<td>2–5 groups of &gt;50</td>
<td>&gt;5 groups of &gt;50 or 1 group &gt;500</td>
</tr>
<tr>
<td>Plaque lymphocyte infiltration†</td>
<td>None</td>
<td>Occasional or 1 group &gt;20</td>
<td>2–5 groups of &gt;20</td>
<td>&gt;5 groups of &gt;20 or 1 group &gt;100</td>
</tr>
<tr>
<td>Fibrous cap rupture</td>
<td>Intact cap</td>
<td>Probably intact, eg, artifactual break in cap from surgical incision</td>
<td>Probably ruptured, eg, site of rupture not clear, but thrombus seen adherent to lipid in lumen</td>
<td>Definitely ruptured</td>
</tr>
<tr>
<td>Extent of fibrous cap disruption</td>
<td>None</td>
<td>&lt;25%</td>
<td>&gt;25%</td>
<td>N/A</td>
</tr>
<tr>
<td>Luminal thrombus</td>
<td>None</td>
<td>Small</td>
<td>Large</td>
<td>N/A</td>
</tr>
<tr>
<td>Intraplaque hemorrhage</td>
<td>None</td>
<td>Present but less than grade 3</td>
<td>&gt;2 mm in circumferential length and &gt;0.5 mm in maximum width</td>
<td>N/A</td>
</tr>
<tr>
<td>Lipid-rich necrotic core</td>
<td>None</td>
<td>Present but less than grade 3</td>
<td>&gt;50% of plaque thickness or &gt;25% of cross section</td>
<td>N/A</td>
</tr>
<tr>
<td>Calcification</td>
<td>None</td>
<td>Stipping only</td>
<td>Calcified nodules</td>
<td>N/A</td>
</tr>
<tr>
<td>Fibrous tissue</td>
<td>Very little fibrous tissue</td>
<td>≥50% fibrous</td>
<td>Predominantly fibrous</td>
<td>N/A</td>
</tr>
<tr>
<td>Neovascularization</td>
<td>No microvessels present</td>
<td>≤10 microvessels per section</td>
<td>&gt;10 microvessels per section</td>
<td>N/A</td>
</tr>
<tr>
<td>Foam cells</td>
<td>None</td>
<td>&lt;50</td>
<td>≥50</td>
<td>N/A</td>
</tr>
<tr>
<td>Brown histiocytes</td>
<td>None</td>
<td>Present</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Oxford Plaque Study composite assessment</td>
<td>Stable—predominantly fibrous plaque with thick, intact cap</td>
<td>Predominantly stable—some features of instability, eg, inflammation, but thick, intact cap</td>
<td>Unstable with intact cap—thin cap, large lipid core, but no definite rupture or surface thrombus</td>
<td>Unstable with ruptured cap—rupture or thrombus present</td>
</tr>
</tbody>
</table>

N/A indicates not applicable.

*Counts of macrophages identified using CD68 antibody stain and confirmed on hematoxylin and eosin sections.
†Counts of lymphocytes identified using CD3 antibody stain.
Modified from Oxford plaque study methods.6

Tissue Processing and Histological Analysis

Endarterectomy tissue was removed en bloc and placed in formalin. After fixation, the tissue was sectioned transversely at 3-mm intervals and embedded in paraffin wax. Four adjacent 5- to 10-μm thick transverse sections were cut from each paraffin block and stained with hematoxylin and eosin, elastic Van Gieson (fibrous tissue), CD68 antibody (macrophages), and CD3 antibody (lymphocytes).

An experienced pathologist and trained research physician graded all cases blinded to clinical details. All sections were reviewed, and the 3 contiguous sections with the most advanced atherosclerotic disease were analyzed. The semiquantitative scales used to grade specific plaque characteristics (Table 1) were those of the Oxford Plaque study6 which have previously been shown to have good to excellent intra- and inter-rater reproducibility. In addition, we graded the extent of fibrous cap disruption. Further methods are provided in the online-only Data Supplement.

Statistical Analysis

Continuous and categorical variables were compared using the t test, Wilcoxon rank-sum test, χ2 test, and Fisher exact test as appropriate. Life-table analysis was used to compare recurrence-free survival time with censoring of patients at the time of CEA. Bivariate analyses of time to stroke recurrence associated with specific risk variables were performed using the log-rank test, and multivariable Cox regression analysis with adjustment for covariates was performed using STATA (version 9.0).

Results

Clinical Characteristics

Forty-four patients met prespecified inclusion criteria for the study. The index event (first symptoms within 28 days of study recruitment) was ischemic stroke in 29.5% (13/44), TIA in...
47.7% (21/44), and retinal artery embolism in 22.7% (10/44). Medical attention for the index event was sought by 61.4% (27/44), whereas 38.6% (17/44) presented to medical attention after a recurrent stroke (12/44) or recurrent TIA (5/44) within 28 days of the index event. No difference was observed in clinical characteristics, acute treatment, or time to CEA of patients with and without stroke recurrence (Table 2).

All patients had carotid duplex ultrasound and brain computed tomography or MRI at presentation. Of these patients, 20.5% (9/44) had 50% to 69% ipsilateral carotid stenosis, and 79.5% (35/44) had ≥70% ipsilateral carotid stenosis. Diffusion-weighted MRI was performed in 79.5% (35/44) patients, and acute ischemic lesions were seen in 74.3% (26/35) of these patients. All patients with clinical evidence of stroke recurrence had brain diffusion-weighted MRI confirming acute ischemic change. CEA was completed at a median interval of 10 (interquartile range, 7–17) days from onset of symptoms for which the patient sought medical attention. None were lost to follow-up at 90 days after the index event.

### Table 2. Clinical Characteristics of Patients With and Without Pre-Endarterectomy Stroke Recurrence (Defined as Clinical, Ipsilateral, Nonprocedural Ischemic Stroke Occurring Between Qualifying Index Event and Endarterectomy)

<table>
<thead>
<tr>
<th></th>
<th>Stroke Recurrence (n=12)</th>
<th>No Stroke Recurrence (n=32)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y, mean (±SD)</strong></td>
<td>68 (9.7)</td>
<td>69 (9.7)</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Male sex, % (n)</strong></td>
<td>91.7 (11)</td>
<td>68.8 (22)</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Hypertension, % (n)</strong></td>
<td>41.7 (5)</td>
<td>62.5 (20)</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>Hyperlipidemia, % (n)</strong></td>
<td>66.7 (8)</td>
<td>68.8 (22)</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Diabetes mellitus, % (n)</strong></td>
<td>8.3 (1)</td>
<td>21.9 (7)</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Smoker, % (n)</strong></td>
<td>91.7 (11)</td>
<td>71.9 (23)</td>
<td>0.24</td>
</tr>
<tr>
<td><strong>Peripheral vascular disease, % (n)</strong></td>
<td>16.7 (2)</td>
<td>12.5 (4)</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>High-risk cardiac source, % (n)</strong></td>
<td>33.3 (4)</td>
<td>28.1 (9)</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>Carotid stenosis ≥70%, % (n)</strong></td>
<td>91.7 (11)</td>
<td>75 (24)</td>
<td>0.41</td>
</tr>
<tr>
<td><strong>Statin preindex event, % (n)</strong></td>
<td>50 (6)</td>
<td>40.6 (13)</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Antiplatelet preindex event, % (n)</strong></td>
<td>50 (6)</td>
<td>40.6 (13)</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Statin &lt;72 h of medical presentation, % (n)</strong></td>
<td>100 (12)</td>
<td>75 (24)</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Antiplatelet &lt;72 h of medical presentation, % (n)</strong></td>
<td>91.7 (11)</td>
<td>81.3 (26)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>NIHSS &lt;72 h of medical presentation, median (IQR)</strong></td>
<td>2 (0–3)</td>
<td>0 (0–2)</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Delay from presentation to carotid endarterectomy, d, median (IQR)</strong></td>
<td>9 (4–11)</td>
<td>10 (8–19)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

IQR indicates interquartile range; and NIHSS, National Institutes of Health Stroke Scale score, performed by a trained study physician.

Plaque Inflammation and Early Stroke Recurrence

After the index clinical event, 27.3% of the cohort (12/44) had a stroke recurrence before CEA. Stroke recurrence occurred within 48 hours of the index event in 16.7% (2/12), within 7 days in 33.3% (4/12), and within 14 days in 58.3% (7/12). In all 12 patients, early recurrent stroke occurred before presentation to medical attention. The index clinical event was stroke in 25% (3/12), TIA in 41.7% (5/12), and retinal artery embolism in 33.3% (4/12). Stroke recurrence was found in 14.7% (8/54) of those with hemispheric stroke or TIA as their index event, whereas 40% (4/10) of those with retinal embolism as their index event had stroke recurrence. The median interval between the index event and stroke recurrence was 16 days (interquartile range, 2–20) and between stroke recurrence and CEA was 9 days (interquartile range, 4–11). An additional 20.5% (9/44) who had not had a stroke recurrence had a TIA recurrence before CEA.

Extensive plaque macrophage infiltration (Oxford Plaque Study [OPS] plaque macrophage grade ≥3) was more common among patients with stroke recurrence compared with those without recurrence (91.7% [11/12] versus 37.5% [12/32]; P=0.002). On life-table analysis, stroke recurrence rates before CEA were 82.3% (confidence interval [CI], 49.2%–98.8%) in patients with high plaque macrophage counts (OPS grade ≥3) compared with 22.2% (CI, 3.5%–83.4%) in those with low or absent macrophages (OPS grade 1 and 2; log-rank P=0.009; Figure 1). The hazard ratio (HR) of stroke recurrence associated with high plaque macrophage counts was 9.7 (CI, 1.2–72.1; P=0.03). In a multivariable Cox regression model including plaque macrophage count (OPS grade <3 and ≥3), age, and carotid stenosis severity (50%–69% or ≥70%), macrophage count was the only independent predictor of early stroke recurrence (adjusted HR, 9; CI, 1.1–70.6; P=0.04).

Extensive plaque lymphocyte infiltration (OPS plaque lymphocyte grade ≥3) was also more common in patients with stroke recurrence compared with those without (100% [12/12] versus 59.4% [19/32]; P=0.009). On multivariable Cox regression including plaque macrophage count (OPS grade <3 and ≥3), age, and carotid stenosis (50%–69% or ≥70%), low plaque fibrous tissue content was 6.2 (CI, 1.9–20.3; P=0.004). The unadjusted HR for stroke recurrence associated with low plaque fibrous tissue content was 6.2 (CI, 1.9–20.3; P=0.004). On multivariable Cox regression including plaque fibrous tissue (categorized as OPS grade 1 or ≥2), age, and carotid stenosis (50%–69% or ≥70%), low plaque fibrous tissue content was the only independent predictor of early stroke recurrence before CEA (adjusted HR, 5.8; CI, 1.6–21.6; P=0.03). On life-table analysis, stroke recurrence rates were 100% (CI, 100%–100%) in those with low plaque fibrous tissue counts (OPS grade ≥3) versus 0% (CI, 0%–0%) in those with low counts (log-rank P=0.06; Figure 2). The HR for early stroke recurrence associated with high plaque lymphocyte infiltration could not be calculated because all stroke recurrences occurred in patients in the high category.

Plaque Fibrous Content and Early Stroke Recurrence

Plaques with low plaque fibrous tissue content (OPS fibrous tissue grade 1) were more common in patients with early stroke recurrence than in those without recurrence (50% [6/12] versus 6.3% [2/32]; P=0.003). The unadjusted HR for stroke recurrence associated with low plaque fibrous tissue content was 6.2 (CI, 1.9–20.3; P=0.004). On multivariable Cox regression including plaque fibrous tissue (categorized as OPS grade 1 or ≥2), age, and carotid stenosis (50%–69% or ≥70%), low plaque fibrous tissue content was the only independent predictor of early stroke recurrence before CEA (adjusted HR, 5.8; CI, 1.6–21.6; P=0.03). On life-table analysis, stroke recurrence rates were 100% (CI, 100%–100%) in the low plaque fibrous content group (grade 1) versus 47.6% (CI, 20.5%–83.8%) in the high (OPS grade ≥2) plaque fibrous content group (log-rank P=0.0006).

Plaque Fibrous Cap Disruption, Neovascularization, and Other Instability Features

The extent of luminal cap disruption could not be reliably quantified in 6 patients because of loss of plaque surface...
integrity during CEA or tissue processing. In the remaining 38 patients, extensive fibrous cap disruption (>25% of lumen exposed to lipid core) was more common among those with stroke recurrence than those without (90.9% [10/11] versus 37% [10/27]; P=0.004). On life-table analysis, early stroke recurrence rates were 81.7% (CI, 48.1%–98.8%) for those with extensive cap disruption and 5.6% (CI, 0.8%–33.4%) for those without (log-rank P=0.08). The unadjusted HR for stroke recurrence before CEA associated with extensive fibrous cap disruption was 5.2 (CI, 0.6–41.1; P=0.12).

Plaque neovascularization (OPS plaque microvessel grade ≥2) was more common in patients with early stroke recurrence than in those without recurrence (83.3% [10/12] versus 43.8% [14/32]; P=0.04). Actuarial stroke recurrence rates were 74.1% (CI, 44.8%–95.3%) in patients with neovascularization (OPS grade ≥2) compared with 17.7% (CI, 4.1%–59.4%) in those without.

Table 1. Kaplan–Meier curve showing stroke recurrence–free survival for those with (-----) and without (──) extensive plaque macrophage infiltration (ie, Oxford Plaque Study [OPS] plaque macrophage grade ≥3). *Numbers at risk of stroke recurrence after censoring patients at time of carotid endarterectomy (CEA) or recurrent stroke pre-CEA. No patients died or were lost to follow-up.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>OPS plaque macrophage grade &lt; 3</th>
<th>OPS plaque macrophage grade ≥ 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Kaplan–Meier curve showing stroke recurrence–free survival for those with (-----) and without (──) extensive plaque lymphocyte infiltration (ie, Oxford Plaque Study [OPS] plaque lymphocyte grade ≥3). *Numbers at risk of stroke recurrence after censoring patients at time of carotid endarterectomy (CEA) or recurrent stroke pre-CEA. No patients died or were lost to follow-up.

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<thead>
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<th>Time (days)</th>
<th>OPS plaque lymphocyte grade &lt; 3</th>
<th>OPS plaque lymphocyte grade ≥ 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
without neovascularization (OPS grade 1; log-rank \( P=0.11 \)). The unadjusted HR for early stroke recurrence pre-CEA for patients with neovascularization (OPS grade \( \geq 2 \)) was 3.2 (CI, 0.7–14.8; \( P=0.09 \)). On multivariable Cox regression, after adjusting for age and severity of carotid stenosis, neither fibrous cap disruption (HR, 4.67; \( P=0.19 \)) or neovascularization (HR, 2.96; \( P=0.21 \)) was independently associated with early recurrent stroke.

No association was observed between early stroke recurrence and other plaque instability features (lipid-rich core, luminal thrombus, intraplaque hemorrhage, calcification, foam cells, brown histiocytes) or overall plaque instability assessments according to the OPS, American Heart Association or modified American Heart Association classification systems (online-only Data Supplement).6,8

**Discussion**

Similar to other hospital-based studies of symptomatic carotid stenosis, we observed high rates of early stroke recurrence, with 27.3% of the cohort having stroke recurrence before CEA.9 An important therapeutic opportunity exists to prevent early recurrent stroke in patients with symptomatic stenosis of carotid, vertebral, and intracranial arteries, particularly those with carotid stenosis awaiting revascularization and noncarotid patients for whom stenting or endarterectomy are currently unproven. To guide selection of agents for future randomized trials, longitudinal studies of the pathophysiology of early recurrent stroke in symptomatic cranio cervical atherosclerosis are needed. We observed a 9-fold increase in the risk of stroke recurrence before CEA associated with abundant plaque macrophage content. High plaque macrophage content independently predicted the risk of early stroke recurrence after adjustment for age and severity of carotid lumen stenosis. Supporting these findings, high plaque lymphocyte content was also associated with early stroke recurrence. The rate of recurrent stroke before CEA was 82% in patients with high plaque macrophage content despite early CEA and high rates of treatment with statins and antiplatelet agents at the time of the index event and after medical presentation.

Experimental studies have identified a key role of inflammation in atherosclerotic plaque destabilization and rupture, partly mediated by expression of matrix metalloproteinases leading to fibrous cap erosion and rupture.11,12 Cross-sectional studies have reported higher prevalence of carotid plaque inflammation in symptomatic patients13,14 and in those with cerebral compared with ocular clinical events.15 Longitudinal data from the Athero-Express study have established an association between a subpopulation of macrophages expressing matrix metalloproteinase-12 and late occurrence of major cardiovascular events and stroke16 and between macrophage-expressed proteins, such as matrix metalloproteinase-8 and adipocyte fatty acid-binding protein, and late major cardiovascular events (but not stroke alone).17,18 Longitudinal studies using 18-fluorodeoxyglucose positron emission tomography to image plaque inflammation have reported improved risk stratification for late vascular events by addition of plaque 18-fluorodeoxyglucose uptake data to the Framingham risk score.19 In a longitudinal positron emission tomography study, we recently observed that inflammation-related 18-fluorodeoxyglucose uptake predicted early stroke recurrence independently of the severity of carotid stenosis.4

We identified other plaque morphological characteristics that were associated with stroke recurrence risk. Low fibrous tissue content was independently associated with a 6-fold increased risk of early stroke recurrence, after adjustment for age and degree of stenosis. This finding is supported by other studies, which have reported associations between lower plaque fibrous content and greater likelihood of clinical symptoms and of cerebral compared with ocular events.14,15 In our study, early stroke recurrence was also associated with fibrous cap disruption on bivariate but not multivariate analysis. This is consistent with previous cross-sectional studies, which found that thrombosis and cap rupture were highly associated with symptomatic (but not asymptomatic) plaque,13 and with angiographic data, in which surface irregularity and ulceration were associated with cap rupture and late stroke recurrence at all grades of stenosis severity.7 We also observed greater frequency of early recurrence among patients with plaque neovascularization, supporting recent data from the Athero-Express study which found higher risk of late cardiovascular events in patients with abundant neovascularization at CEA.20 In contrast to some recent reports, intraplaque hemorrhage was not associated with stroke recurrence in our study. This may reflect differing definitions in other studies.20,21

Strengths of our study include its longitudinal design, prospective follow-up, and emphasis on early ipsilateral recurrent stroke, which is the most relevant clinical consequence of acute carotid plaque instability. Because patients with TIA and recurrent stroke frequently present to medical attention following stroke recurrence rather than for the initial TIA, we also carefully ascertained all new ipsilateral TIA and stroke events within 28 days before medical presentation to avoid undercounting clinically relevant stroke recurrences. The importance of detecting these early outcome events was underlined in our study because all recurrent strokes occurred before patients sought medical attention.22 Two of these patients went on to have a second stroke recurrence while awaiting CEA. We minimized the potential for recall bias by applying standard definitions for all TIA and stroke events, which were confirmed by a trained stroke physician blinded to the histological data. By performing CEA soon after presentation (median, 10 days), it is likely that plaque histology in our study was representative of that at the time of clinical symptoms, thus minimizing confounding by plaque remodeling and plaque stabilizing medications which may have limited earlier studies with longer intervals between symptoms and plaque retrieval.

The main limitation of our study is the small sample size, which is likely to have reduced statistical power to detect modest associations of some variables on recurrence risk. However, we observed significant associations between early recurrent stroke, inflammation, and other unstable features despite our relatively small sample, indicating a large effect of these variables on stroke outcome. It is also possible that some patients who may have gone on to have stroke recurrence were included in the nonrecurrence group because of early CEA. We think that this is an unavoidable consequence of observational studies of real-world clinical practice and reflects current treatment according to guidelines recommending
CEA within 14 days. Our study excluded patients with more severe stroke (modified Rankin score $>3$) at presentation, and therefore, our results may not be extrapolated to this patient subgroup. No patients were excluded from the study or CEA because of disabling stroke recurrence.

In our study, plaque inflammation, low fibrous content, neovascularization, and cap rupture were associated with high risk of early stroke recurrence in patients with recently symptomatic carotid stenosis. Our results require confirmation, but provide initial data that may guide development of emerging imaging techniques to noninvasively identify symptomatic patients at highest recurrence risk for urgent treatment and new therapeutic strategies to prevent recurrent stroke.

**Acknowledgments**

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**Disclosures**

None.

**References**

Plaque Inflammation and Unstable Morphology Are Associated With Early Stroke Recurrence in Symptomatic Carotid Stenosis
Michael Marnane, Susan Prendeville, Ciaran McDonnell, Imelda Noone, Mary Barry, Morgan Crowe, Niall Mulligan and Peter J. Kelly

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SUPPLEMENTAL MATERIAL

TITLE PAGE:

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Plaque inflammation and unstable morphology are associated with early stroke recurrence in symptomatic carotid stenosis

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SUPPLEMENTAL MATERIAL

Supplemental Methods:

Stroke was defined according to the World Health Organisation definition, with neuroimaging confirmation. The Oxfordshire clinical definition of TIA was applied (acute loss of focal cerebral or ocular function, less than 24 hours duration, presumed after investigation due to thrombotic or embolic vascular disease), regardless of sub-clinical acute ischaemic injury on brain imaging.

Carotid stenosis was identified on duplex ultrasound by standardised methods combining peak systolic flow velocity thresholds with visual estimation of stenosis on B-mode imaging. As in the NASCET study, stenosis was graded by comparison of the lumen diameter at the maximum site of stenosis to that of normal-appearing carotid artery distal to the stenosis.

Fibrous cap rupture was defined as presence of any communication of luminal thrombus with lipid-rich necrotic core, not related to surgery or plaque processing. The extent of fibrous cap disruption was defined as the proportion of luminal surface with exposed lipid-rich necrotic core. We used this grading to explore whether the presence of extensive cap disruption (defined as >25% of luminal surface) might be associated with increased risk of stroke recurrence. Luminal thrombus was defined as an organised collection of fibrin and red blood cells at the plaque surface. Areas of macrophage infiltration were identified on CD68 antibody staining. We also performed macrophage counts on contiguous H&E stained sections to exclude false positive results from CD68 background staining. We found that the identification of macrophages by the two methods demonstrated excellent correlation (kappa [κ] =0.91). Lymphocyte counts were performed on CD3 antibody stained sections. The fibrous content of each plaque was estimated using an elastic van gieson stain which identifies collagen and other connective tissues. Plaques were assigned a composite assessment designation according to the Oxford Plaque Study (OPS) grading system, and the American Heart Association (AHA) and modified AHA classification systems. The OPS grading system codes plaques as (1) stable, (2) predominantly stable, (3) unstable or (4) unstable with rupture, based on fibrous cap morphology, inflammatory cell infiltrate, lipid-rich necrotic core size, luminal thrombus and the presence of plaque rupture. The AHA system grades lesions as type 4 (atheroma), type 5a (fibroatheroma), type 5b (calcified), type 5c (fibrous) and type 6 (complicated lesion). The modified AHA system uses the following lesion descriptors: pathological intimal thickening (with or without erosion), fibrous cap atheroma (with or without erosion), thin fibrous cap atheroma (with or without rupture), calcified nodule and fibrocalcific plaque.

Supplemental Results:

Clinical characteristics:

A co-existing cardioembolic source was identified in 29.5% (13/44), this was dilated ischaemic cardiomyopathy in 2 cases, sick sinus syndrome in 1 case, a bioprosthetic aortic valve in 1 case and atrial fibrillation in the remaining 9 cases.

Pre-operative care was at the discretion of treating physicians. In most patients this consisted of a single antiplatelet agent (aspirin 75mg-300mg once daily) and statin therapy
(atorvastatin, pravastatin, simvastatin or rosuvastatin). No patients were treated with dual antiplatelet therapy or ‘high dose’ statin therapy pre-operatively.

Stroke recurrence and plaque classification:

We also investigated 3 atherosclerotic plaque classification systems (OPS, modified AHA and AHA) with regard to stroke recurrence. Both “OPS- unstable plaque with ruptured cap” (91.7% (11/12) versus 65.6% (21/32), p=0.13) and “modified AHA- thin fibrous cap atheroma with plaque rupture” (91.7% (11/12) versus 59.4% (19/32), p=0.07) morphologic subtypes were more common in those with stroke recurrence compared to those without, although neither association met statistical significance. There was no association between other OPS and modified AHA or any AHA morphologic plaque classification and stroke recurrence.
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