Carotid Plaque Vulnerability
A Positive Feedback Between Hemodynamic and Biochemical Mechanisms

Iwona Cicha, PhD; Anja Wörner, MD; Katharina Urschel, MSc; Kamen Beronov, PhD; Margarete Goppelt-Stru¨be, PhD; Eric Verhoeven, MD; Werner G. Daniel, MD; Christoph D. Garlicks, MD

Background and Purpose—Rupture of atherosclerotic plaques is one of the main causes of ischemic strokes. The aim of this study was to investigate carotid plaque vulnerability markers in relation to blood flow direction and the mechanisms leading to plaque rupture at the upstream side of carotid stenoses.

Methods—Frequency and location of rupture, endothelial erosion, neovascularization, and hemorrhage were determined in longitudinal sections of 80 human carotid specimens. Plaques were immunohistochemically analyzed for markers of vulnerability. Plaque geometry was measured to reconstruct shape profiles of ruptured versus stable plaques and to perform computational fluid dynamics analyses.

Results—In 86% of ruptured plaques, rupture was observed upstream. In this region, neovascularization and hemorrhage were increased, along with increased immunoreactivity of vascular endothelial and connective tissue growth factor, whereas endothelial erosion was more frequent downstream. Proteolytic enzymes, mast cell chymase and cathepsin L, and the proapoptotic protein Bax showed significantly higher expression upstream as compared with the downstream shoulder of atherosclerotic lesions. Comparison of geometric profiles for ruptured and stable plaques showed increased longitudinal asymmetry of fibrous cap and lipid core thickness in ruptured plaques. The specific geometry of plaques ruptured upstream induced increased levels of shear stress and increased pressure drop between the upstream and the downstream plaque shoulders.

Conclusions—Vulnerability of the upstream plaque region is associated with enhanced neovascularization, hemorrhage, and cap thinning induced by proteolytic and proapoptotic mechanisms. These processes are reflected in structural plaque characteristics, analyses of which could improve the efficacy of vascular diagnostics and prevention. (Stroke, 2011;42:00-00.)

Key Words: atherosclerosis ■ carotid plaque rupture ■ vulnerability markers ■ hemodynamic forces ■ shear stress

Stroke, the leading cause of disability in adults and the third most common cause of death in the Western countries, is most often caused by atherosclerotic plaque rupture.1 Vulnerable lesions may block the blood flow to the brain by atherothrombosis of large cerebral arteries or as a result of cardioembolism. In advanced stages of atherosclerosis, the presence of lumen-narrowing lesions dramatically alters the local hemodynamic conditions in affected arteries. As reported by Thrysøe et al,2 peak mechanical stress levels are asymmetrically distributed along the carotid plaque, with 50% occurring proximal and 25% distal to the point of maximal stenosis, and correlate inversely with the fibrous cap (FC) thickness. These differences in mechanical load distribution can affect cellular composition and stability of the lesions. According to a large angiographic study by Lovett and Rothwell,1 vulnerable carotid plaques mostly rupture at the upstream side. Hemodynamically, this region is characterized by steeply increasing wall shear stress,4–6 which, nonetheless, even in case of a 75% stenotic artery, remains several orders of magnitude lower than tensile stress induced by the blood pressure pulse.7 In contrast, on the downstream (distal) side of stenosis, the abrupt increase in cross-sectional area of the vessel lumen causes a flow separation, leading to flow instabilities, formation of vortices, and oscillatory shear stress. Although elevated wall shear stress in the upstream region is unlikely to directly induce mechanical rupture, the distinct shear stress conditions along atherosclerotic plaque can certainly result in a differential activation of biological processes, such as inflammatory mechanisms. For example, higher numbers of macrophages8 and dendritic cells9 were observed at the upstream plaque shoulder, which may indirectly contribute to plaque destabilization by activation of inflammatory pathways in this region. In contrast, higher numbers of smooth muscle cells (SMCs) were detected...
downstream, corresponding with the distal plaque growth. The downstream region was also characterized by increased endothelial cell apoptosis.\textsuperscript{10}

Recent studies, including the Predictors of Response to CRT Trial,\textsuperscript{11} showed that characterization of plaque morphology by noninvasive or invasive imaging modalities allows the identification of lesions that are at especially high risk for future adverse cardiovascular events. Together with pathological studies,\textsuperscript{12,13} these findings clearly underscore the importance of morphometric analyses of the suspect plaques. However, whereas the existing studies have concentrated on plaque classification based on cross-sectional geometry of the affected vessels, reports are missing that considered the longitudinal heterogeneity within an atherosclerotic plaque. Although the degree of stenosis is currently accepted as a main diagnostic variable, other characteristics of the plaque geometry, which have strong mechanical implications, should be characterized as well to improve the diagnostic power of vascular imaging.

In this study, we immunohistochemically analyzed the carotid specimens to determine whether the activation of proteolytic, apoptotic, and angiogenic processes within the plaque is characterized by longitudinal asymmetry. Furthermore, we followed up the analyses of protein markers of vulnerability with the analysis of structural aspects of plaque morphology and geometry to identify the mechanisms of increased vulnerability of the upstream plaque region. The combination of these 2 approaches provides a better insight into the clinically relevant interplay of biochemical processes and local hemodynamic conditions along atherosclerotic plaque, which operate in concert contributing to plaque rupture at the upstream region.

Methods

Antibodies

Mouse monoclonal antibodies against cathepsin L (1:100) and Bax (1:100) were from GeneTex (Irvine, CA), and monoclonal antibody against mast cell chymase (1:500) was from NeoMarkers (Fremont, CA). To detect neovascularization, endothelial cells were stained with a mouse monoclonal antibody against human von Willebrand factor (1:25, DAKO, Hamburg, Germany). Mouse monoclonal antibody against vascular endothelial growth factor was from Abcam (Cambridge, United Kingdom). A highly specific affinity-purified rabbit antihuman connective tissue growth factor (CTGF) IgG (1.92 μg/mL) was a kind gift from Dr. David Brigstock, (Nationwide Children’s Hospital, Columbus, OH).\textsuperscript{14}

Patients and Arterial Specimens

Carotid specimens were obtained from 104 consecutive patients undergoing carotid endarterectomy. Preoperative duplex scanning, MRI, and/or angiography of the carotid arteries were performed. The indications for endarterectomy were stenosis of the internal carotid artery of >70% for symptomatic patients and stenosis of >80% for asymptomatic patients. Our investigation conformed to the principles outlined in the Declaration of Helsinki. The clinical data of patients were recorded (Supplemental Table I; http://stroke.ahajournals.org). The study was approved by local ethics committee, and informed consent was obtained from all of the patients.

Endarterectomy specimens, containing the entire intima and a part of the media of the vessel wall, were fixed in formalin and decalcified in EDTA buffer. After macroscopic inspection, the part of the internal carotid artery containing a plaque was removed when high-grade stenosis was visible and occlusion was absent. Plaques were embedded in paraffin in the longitudinal direction, which was indicated on the paraffin block mounting support. Only specimens with clearly visible upstream and downstream plaque shoulders were used for the immunohistochemical study (Supplemental Figure I). The inclusion criteria were fulfilled by 80 plaques. On the basis of the morphological characteristics, the carotid plaques used in our study were of advanced stage (type IV to VI) according to the American Heart Association classification.\textsuperscript{15}

Immunohistochemical Staining

Paraffin-embedded serial longitudinal sections of 4-μm thickness were placed on silane-coated slides, with the downstream direction indicated on each slide. The sections were dewaxed in xylene, rehydrated in ethanol, and washed with Tris-buffered saline containing 0.1% Tween 20. The sections were stained as described previously\textsuperscript{9} using the DAKO catalyzed signal amplification system with diamobenzidine as a chromogene, followed by counterstaining with hematoxylin (DAKO). Irrelevant isotype-matched antibodies were used to obtain negative controls. To identify cell types expressing cathepsin L, Bax, and growth factors, serial sections of 2-μm thickness were stained in parallel for anti-CD68 (macrophages), anti-von Willebrand factor (endothelial cells), anti-CD3 (T lymphocytes), antiplasma cell, or for anti-smooth muscle actin (SMCs). All of the cell-specific antibodies were obtained from DAKO.

Image Quantification

Digital images of upstream and downstream plaque regions were obtained using a CCD camera (Nikon DXM-1200, Duesseldorf, Germany) at a magnification of ×150. For analyses of protein expression, the color threshold for immunostained cells was manually adjusted in the images until the computerized detection matched the visual interpretation. The numbers of immunoreactive cells were digitally counted (Lucia Software version 4.2.1, Laboratory Imaging Ltd, Nuremberg, Germany) in the representative areas of 0.25 mm$^2$ in the upstream and the downstream plaque shoulders. The expression of Bax and cathepsin L was additionally analyzed at the upstream and the downstream borders of the lipid core (Figure IB). Analyses were carried out by 3 independent observers (I.C., A.W., K.U.). The intraobserver and interobserver variabilities were <10%. The minimum FC thicknesses at the upstream slope, at the top of the plaque, and at the downstream slope of each plaque were measured for each plaque (Figure IB) using the images obtained at ×150 magnification.

Plaque Geometry Analysis

Digital images of atherosclerotic plaques taken at a magnification ×20 were used for the analyses of plaque geometry. Measurements were performed in 15 randomly selected plaques that were ruptured upstream and 15 nonruptured ones. Lumen diameter, plaque length, and the thickness of total intima, thickness of lipid core, and thickness of FC were measured at 5 locations along the plaques, using computer-aided planimetry (Lucia Software). The first location (counting from the upstream end of the sample) corresponds with the upstream shoulder slope. The following 4 locations were taken equidistantly, so as to cover the length of the plaque. The data were used to produce averaged geometric profiles for upstream-ruptured and nonruptured plaques.

Computational Fluid Dynamics Analysis

To obtain information about the loads locally affecting the FC regions and globally affecting each single plaque, numeric simulations were performed. In our study, the geometric information about overall plaque shapes and structure was limited by the strict 2-dimensionality of the images and the strong natural deviation of those shapes from a hypothetical ellipsoid. Because of these limitations imposed by the 2D samples, the present study used only the steady-state computational fluid dynamics (CFD). The standard commercially available CFD software, ANSYS Fluent (Darmstadt, Germany), was used. Three-dimensional geometric models were constructed for the samples from the upstream-ruptured group and from the nonruptured group. For each selected histological section, 5
locations along the longitudinal axis were specified, as described above. At each location, the height of the plaque relative to a baseline corresponding with the upstream vessel wall was measured. The data were interpolated by an order-2 spline to obtain a model of the plaque section boundary. This section was then imbedded along the axis of a straight pipe segment and then extruded in the direction perpendicular to the slice. Unstructured tetrahedral meshes were generated in the vicinity of the plaque and structured meshes in the far upstream and downstream regions. The 3D plaque models had sharp edges at both the upstream and the downstream shoulder ends. The resulting nonsmoothness of the computed flow was detectable only in a very small region (comparable to 1 finite volume of the mesh) at the upstream and downstream shoulder ends. Eliminating it produced a smooth and physically consistent set of hydrodynamic fields for each of the analyzed geometries. Inflow rate and standard outflow boundary conditions were applied, and the computed pressure drop along the plaque was recorded. Stresses along the complete surface of the 3D plaque model were computed using the corresponding functionality in ANSYS Fluent software.

Statistical Analysis

Data were expressed as median±SEM unless stated otherwise. \( P<0.05 \) was considered statistically significant. The nonparametric Wilcoxon test was used to compare the numbers of immunoreactive cells in upstream and downstream shoulders of the plaques. The correlations between the immunohistochemistry results and patient clinical data were analyzed by Spearman rank-order correlation test. Proportions were calculated using Fisher exact test for number of events <=5 or \( \chi^2 \) test. Geometric differences between upstream-ruptured and nonruptured plaques were analyzed using Mann-Whitney \( U \) rank-sum test.

Clinical Data and Morphological Plaque Characteristics

In the present study, 80 patients with a median age of 72 years were included, 55 (68%) of whom were men. Acute ischemic cerebral symptoms within 6 weeks preceding surgery were recorded in 63% of all of the patients. Clinical data of the patients are shown in Supplemental Table I.

In the majority of analyzed plaques, significant morphological differences were observed between the upstream and the downstream plaque regions (Figure 1A). Strong thinning of the FC was observed at the upstream side of the lesions as compared with the other plaque regions. Minimum FC thickness upstream was 100.5±15.8 \( \mu \)m, as compared with 265.5±17 \( \mu \)m at the top of the plaques (\( P<0.001 \)), and 264.5±20 \( \mu \)m downstream (\( P<0.001 \); Figure 1B).

Neovascularization was observed in 58 plaques, in 43 of which the newly formed vessels accumulated particularly at the upstream side of the lesions. As shown in Figure 1B, neovascularization density at the upstream vs downstream side of atherosclerotic plaque, defined as the number of vasa vasorum per millimeter squared. Graphs show the median, 10th, 25th, 75th, and 90th percentiles. The statistical differences between the regions were calculated using Wilcoxon test; \( n=80 \).
versus 43% in negative group; \( P=0.032 \). Moreover, 92% patients in the neovascularization-positive group were hypertensive as compared with 74% patients without plaque neovascularization \( (P=0.05) \).

Intraplaque hemorrhage was detected in 51 plaques, 31 (61%) of which were hemorrhagic only at the upstream side of the plaque, whereas 20 had hemorrhages at both sides (Figure 1C). The presence of hemorrhage was associated with male sex \( (77\% \) males in hemorrhage group versus 53.6\% among patients with nonhemorrhagic plaques; \( P=0.044 \)) and the occurrence of neovascularization \( (P=0.005) \). In the hemorrhage group, significantly lower FC thickness upstream was observed \( (78\pm22.1 \text{ versus } 194.5\pm18.9 \mu m \text{ in nonhemorrhagic plaques}; \ P=0.03) \).

Endothelial erosion, observed in the majority of analyzed plaques, was more frequent downstream. In 62 plaques, endothelial erosion was present at the downstream side, as compared with 37 cases of erosion upstream \( (P<0.001) \). In some cases, erosion could be detected at both sides of the lesions.

Of 80 analyzed plaques, ruptures were detected in 28 specimens. In 24 \( (86\%) \) of them, rupture took place at the upstream side (Figure 1A and 1C). At the downstream side, rupture was present in only 3 plaques, whereas 1 plaque was ruptured on top, near to the point of maximum stenosis. In addition, 37 plaques had superficial ulcerations/fissures (defined as superficial tearing of the FC, without the exposure of lipid core material to the bloodstream), which were localized upstream in 24 plaques, whereas only 7 plaques were superficially fissured at the downstream side. Seventy-eight percent of ruptured plaques were clinically symptomatic, as compared with 57% symptomatic cases among nonruptured plaques \( (P=0.14) \). Moreover, cap thinning on the upstream side was significantly more pronounced in ruptured plaques \( (\text{minimum FC thickness: } 36\pm10 \text{ versus } 180\pm19 \mu m \text{ in nonruptured plaques}; \ P<0.001) \). Rupture was also significantly associated with the presence of intraplaque hemorrhage \( (P=0.041) \).

Expression of Proteolytic Enzymes at the Upstream and the Downstream Plaque Shoulders

Matrix breakdown by proteolytic enzymes produced by macrophages and mast cells reduces tissue strength.\(^{16}\) Mast cell chymase and cathepsin L have been strongly implicated in plaque vulnerability.\(^{17-19}\) Therefore, we investigated the local expression of mast cell chymase and cathepsin L with respect to blood flow direction. The mean numbers of cells positive for chymase were significantly higher upstream \( (12\pm4.2 \text{ as compared with the downstream shoulder } (7\pm2.6; P<0.001) \) of the atherosclerotic lesions (Figure 2A). As shown in Figure 2B, the expression of cathepsin L was also significantly increased in the upstream shoulder region of the plaques \( (50.5\pm4.6 \text{ as compared with the downstream plaque shoulder } (23\pm3.7; P<0.001) \). Interestingly, a significant increase in the numbers of cathepsin L-expressing cells, in particular, macrophages and endothelial cells, was observed at the upstream shoulder of ruptured plaques \( (58\pm11.3) \), as compared with nonruptured plaques \( (42\pm4.2; P=0.044) \). Moreover, cathepsin L expression was inversely correlated with minimum FC thickness upstream \( (r=-0.3; P=0.013; \text{ Figure } 2C) \).

Numbers of cathepsin L-expressing macrophages were also compared at the upstream and the downstream borders of the lipid core. Activated macrophages expressing high levels of cathepsin L were observed more frequently at the downstream border of the lipid core \( (50\pm4.2 \text{ versus } 38\pm3.0 \text{ upstream}; \ P<0.001; \text{ Figure } 2C) \). Activation of proteolytic processes in this area may serve to create space for the growing core and indicates enlargement of the plaque in downstream direction.

Increased Numbers of Apoptotic Cells at the Upstream Region of the Plaque

Apoptotic cell death has recently been proposed to contribute to plaque vulnerability by inducing thinning of the FC.\(^{20,21}\) In our study, the proapoptotic protein Bax was detected more frequently at the upstream plaque shoulder and localized mainly to SMCs and macrophages (Figure 3A). On the contrary, apoptotic cells were barely detectable at the downstream plaque shoulder \( (7\pm1.6 \text{ versus } 17.5\pm3.3 \text{ upstream}; P<0.001) \). The increased expression of Bax upstream showed positive correlations with cathepsin L \( (P=0.036) \) and with mast cell chymase \( (P<0.001) \), which was shown previously to induce SMC apoptosis in vitro.\(^{22}\) In subsequent analyses, we also compared Bax expression between upstream and downstream borders of the lipid core. Significantly higher numbers of apoptotic macrophages were detected on the upstream side of lipid core as compared with downstream, where very low numbers of Bax-expressing cells were present (Figure 3B).

Angiogenic Growth Factor Expression at the Upstream and the Downstream Plaque Shoulders

The protein levels of angiogenic growth factors showed a very high degree of variability between the individuals, being nearly undetectable in some plaques, whereas other lesions were characterized by a very high expression of vascular endothelial growth factor and CTGF, in some cases at both plaque shoulders. In the overall statistical analyses, vascular endothelial growth factor immunoreactivity, mainly localized to macrophages and endothelial cells, was significantly higher upstream \( (23\pm5.3) \) as compared with the downstream region of the atherosclerotic lesions \( (15\pm4; P<0.005; \text{ Figure } 3C) \). Vascular endothelial growth factor expression at the upstream shoulder correlated positively with the expression of mast cell chymase \( (P=0.021) \) and with intimal neovascularization \( (P=0.03) \).

CTGF immunoreactivity in the plaques localized, as reported before,\(^{23}\) to macrophages, a subpopulation of SMCs, and endothelial cells. In the majority of analyzed plaques, median numbers of CTGF-positive cells were increased at the upstream shoulder \( (17\pm3.8 \text{ versus } 10\pm3.2 \text{ downstream}; P<0.001; \text{ Figure } 3C) \). At the upstream plaque shoulder, the strongest positive correlation was observed between CTGF and mast cell chymase expression \( (P<0.0001) \). The expression of Bax in this region also showed positive correlation with CTGF expression \( (P<0.001) \), which may reflect the assumed contribution of CTGF to SMC apoptosis at the upstream shoulder.\(^{24}\)

Plaque Geometry

In the subsequent analyses, we determined whether overall plaque dimensions and geometric characteristics of rupture-prone and stable plaques are comparable. For this purpose,
plaque geometric features were measured in 15 randomly selected upstream-ruptured and 15 nonruptured plaques (Figure 4A) and used to create averaged geometric profiles outlining the outer shapes of plaques and shapes of the lipid cores. As shown in Figure 4 and the Table, stable and upstream-ruptured plaques did not differ with respect to length and total intima thickness. However, at the upstream shoulder, all of the stable plaques had an FC thickness \( \geq 200 \mu m \), which was significantly higher than in the upstream-ruptured plaques (Table). In further analyses, the ratio of lipid core thickness:total intima thickness was calculated. For ruptured plaques, the lipid core upstream amounted to a median 83±3% of plaque thickness, which was significantly higher than the values obtained for stable plaques (49±3.5%; Table). Importantly, these differences were observed only at the upstream side of the plaques, whereas stable and ruptured plaques were nearly indistinguishable by the geometric characteristics in their downstream regions. As shown in Figure 4, stable plaques had nearly symmetrical shapes with respect to the point of maximal stenosis, whereas upstream-ruptured plaques exhibited a strongly pronounced longitudinal asymmetry. Significant differences between upstream and downstream shoulders of upstream-ruptured plaques were detected in the FC thickness and the lipid core thickness. These plaques tended to have a “droplet shape,” with the slope of the upstream region in front of the stenosis increasing at high angle and a strongly asymmetrical shape of the lipid core (Figure 4).

**CFD Analysis**

To obtain more detailed and quantitative information about the mechanical implications of the differences between upstream-ruptured and stable groups of plaques, CFD analysis of vessel geometry models obtained with the averaged plaque profile for each group (Figure 5A) was used. As shown in Figure 5B, the upstream-ruptured plaque geometry (top) induced stronger disturbances, including multiple recirculation vortices immediately adjacent to the downstream shoulder. These caused unfavorable, spatially oscillating shear stress, including reversal of direction. Moreover, the levels of shear stress differed by an order of magnitude between the upstream-ruptured and the stable groups, implying a very different rate of remodeling and degenerative processes between the 2 groups.

Local pressure, as a difference in comparison with the average pressure at the outlet, was highest at the beginning of
the upstream shoulder, reflecting the most rupture-prone region. Moreover, in the flow along the upstream-ruptured plaque model, 20% higher pressure drop was observed (Figure 5C). This effect resulted from the shape difference and was related to the higher level of downstream flow disturbances in the upstream-ruptured plaques. These hemodynamic differences produce a net effect of higher (by at least one third, on the average) overall pull force acting on upstream-ruptured plaques as compared with stable plaques. Assuming identical tissue characteristics, this single effect might be sufficient to explain why the upstream-ruptured plaques had ruptured and stable ones had not.

Discussion
Plaque rupture is preconditioned by increasing vulnerability in the presence of unfavorable mechanical loads. Our present findings indicate that positive feedback hemodynamic and biochemical mechanisms interact, leading to increased occurrence of plaque rupture upstream, whereas other plaque regions remain less vulnerable.

The high velocity flow along vessel section narrowed by a grown plaque\(^4\)–\(^6\) is hydrodynamically more stable than the flow at the entry (upstream) and the exit (downstream) parts of the stenosis. The exposure to a chronic, stable mechanical load was shown to maintain FC thickness along the vessel-narrowing plaque relatively constant.\(^25\) In the specimens analyzed here, the FC thickness increased with the distance from the upstream plaque shoulder, reaching a stable thickness along the point of maximal stenosis, characterized by higher than normal wall shear stress levels. In the vicinity of the downstream end, the widening of vessel diameter leads to flow instabilities manifested through vortex formation and low shear stress\(^4\)–\(^6\) (Figure 5), creating a hemodynamic environment that enhances SMC proliferation, either directly or via mural thrombotic deposits. In our study, endothelial erosion was significantly more frequent at the downstream side of the analyzed plaques. This finding suggests that extensive exposure of the subendothelial matrix because of erosion can trigger platelet adhesion in this region and lead to release of growth factors that induce a profibrotic response, such as SMC proliferation and matrix synthesis. This is also in agreement with the observation that no SMC apoptosis is observed in the downstream plaque region. Moreover, the presence of large numbers of cathepsin L-producing macrophages at the downstream lipid core border indicates that further enlargement of the plaque occurs in that direction.
Cap. Moreover, the upstream region was characterized by a steeply increasing wall shear stress. In this study, we observed a significant increase in the expression of proteolytic enzymes and the proapoptotic protein Bax at the upstream plaque shoulder. In particular, cathepsin L expression was found to inversely correlate with minimum FC thickness minus (FC thickness - intima thickness minus FC thickness; closed triangles, total intima thickness) and overall dimensions of rupture-prone and stable plaques are comparable. Structurally, the comparison of averaged shapes of 15 randomly selected nonruptured and 15 upstream-ruptured plaques (Figure 4) indicates that the asymmetry of the FC thickness and the differences in lipid core thickness can be predictive for rupture. In stable plaques, the FC thickness at the upstream plaque shoulder was 200 μm, which is in a good agreement with intravascular ultrasound results reported by Imoto et al. The FC along the top of the stable plaque had a thickness of 450 to 500 μm, and, not less importantly, the distance between the upstream plaque shoulder and the point at which this “safe” FC thickness was achieved was small, typically ≤1 mm. In contrast, rupture-prone plaques were characterized by both significantly thinner FC upstream and simultaneously an increased distance over the upstream slope at which FC was extremely thinned (≈2 to 3 times longer than in stable plaques). This extreme upstream FC thinning in the ruptured plaques was in agreement with the model of flow-plaque interaction and a study based on realistic arterial plaque geometry of 13 carotid bifurcation cases, where FC thickness was shown to critically affect the plaque stability. In this context, our CFD analyses demonstrated that the upstream-ruptured plaque geometry induced a 20% higher pressure drop along the stenosis, producing an increased overall pull force acting on the upstream-ruptured plaques as compared with stable ones. This growing pressure difference between the upstream and the downstream shoulders, that can reach 10 to 100 times the magnitude of wall shear stress, acting on a gradually thinning cap that protects the upstream slope of the lesion, makes the plaque most vulnerable precisely where the me-

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stable (n=15)</th>
<th>Ruptured (n=15)</th>
<th>P</th>
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<tbody>
<tr>
<td>Plaque length, mm</td>
<td>12.3±1</td>
<td>11.9±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Maximal intima thickness, mm</td>
<td>3.7±0.25</td>
<td>4.6±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>FC thickness upstream, μm</td>
<td>456±74</td>
<td>65±35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FC thickness downstream, μm</td>
<td>443±77</td>
<td>546±106</td>
<td>NS</td>
</tr>
<tr>
<td>Lipid core:intima upstream, %</td>
<td>49</td>
<td>83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipid core:intima downstream, %</td>
<td>47</td>
<td>58</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are given as median ± SEM or as absolute numbers of patients (n) and percentage (%).

The differences between the 2 groups were analyzed using Mann-Whitney U rank-sum test.

FC indicates fibrous cap; NS, not significant.

Increased occurrence of neovascularization and hemorrhage, both of which contribute to weakening the structural integrity of the plaque tissue. In agreement with this, FC was thinnest at the upstream side as compared with other plaque regions in the majority of the analyzed plaques, and this FC thinning was even more pronounced in hemorrhagic plaques. These findings provide consistent evidence that matrix degradation, apoptosis, leaky vessel formation, and hemorrhage affect the structural integrity of the FC at the upstream region to a higher extent than in the rest of the plaque.

Not all advanced plaques rupture, even if stenosis grade and overall dimensions of rupture-prone and stable plaques are comparable. Structurally, the comparison of averaged shapes of 15 randomly selected nonruptured and 15 upstream-ruptured plaques (Figure 4) indicates that the asymmetry of the FC thickness and the differences in lipid core thickness can be predictive for rupture. In stable plaques, the FC thickness at the upstream plaque shoulder was >200 μm, which is in a good agreement with intravascular ultrasound results reported by Imoto et al. The FC along the top of the stable plaque had a thickness of 400 to 500 μm, and, not less importantly, the distance between the upstream plaque shoulder and the point at which this “safe” FC thickness was achieved was small, typically ≤1 mm. In contrast, rupture-prone plaques were characterized by both significantly thinner FC upstream and simultaneously an increased distance over the upstream slope at which FC was extremely thinned (≈2 to 3 times longer than in stable plaques). This extreme upstream FC thinning in the ruptured plaques was in agreement with the model of flow-plaque interaction and a study based on realistic arterial plaque geometry of 13 carotid bifurcation cases, where FC thickness was shown to critically affect the plaque stability. In this context, our CFD analyses demonstrated that the upstream-ruptured plaque geometry induced a 20% higher pressure drop along the stenosis, producing an increased overall pull force acting on the upstream-ruptured plaques as compared with stable ones. This growing pressure difference between the upstream and the downstream shoulders, that can reach 10 to 100 times the magnitude of wall shear stress, acting on a gradually thinning cap that protects the upstream slope of the lesion, makes the plaque most vulnerable precisely where the me-

Chronologically, the upstream region represents the older part of any plaque. Because of specific distribution of hemodynamic load in the stenosed arteries, the upstream shoulder of the plaque experiences the highest local pressure and a steeply increasing wall shear stress. In this study, we observed a significant increase in the expression of proteolytic enzymes and the proapoptotic protein Bax at the upstream plaque shoulder. In particular, cathepsin L expression was found to inversely correlate with minimum FC thickness, implicating its involvement in the thinning of the cap. Moreover, the upstream region was characterized by
mechanical load is maximal. Monitoring the longitudinal asymmetry of the FC thickness, in particular in asymptomatic patients, could thus contribute to the detection of progressive plaque vulnerability and improve patient management.

Taken together, the results of our study provide evidence supporting the role of local hemodynamic factors in plaque destabilization and in the structural differentiation between stable and rupture-prone plaques. An effective control of plaque growth through the modification of systemic factors is achievable with current therapy but it critically depends on an early diagnosis. Our findings concerning the structural asymmetry of the vulnerable plaques thus provide the means of improved identification of rupture-prone plaques using the available imaging techniques, such as intravascular ultrasound, intravascular ultrasound elastography, high-resolution MRI, and optical coherence tomography. These techniques, capable of discriminating between soft lipid-rich and harder (fibrous, collagen-containing) plaque components, can be expected to reliably and accurately estimate the differences in plaque composition between its upstream and downstream regions. Clinical studies using one of the above-mentioned imaging techniques for geometric analyses on a large sample
will be necessary to confirm the diagnostic value of structural asymmetry as a plaque vulnerability indicator and to demonstrate its ability to discriminate between plaques at immediate risk of rupture and stable plaques.

In conclusion, our findings indicate that the processes leading to the rupture of atherosclerotic plaque should be perceived as a complex network of feedback mechanisms between local hemodynamic and biochemical, as well as systemic, factors. In addition to systemic markers, the key structural characteristics of the rupture-prone plaques can be translated into a powerful diagnostic tool, allowing targeted pharmacological or endovascular intervention to prevent carotid plaque growth and destabilization.

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Disclosures

None.

References

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Supplemental Table I: Clinical data

<table>
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<tr>
<th></th>
<th>Median ± SEM or n</th>
<th>Range or %</th>
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<td>Age (y)</td>
<td>72±1</td>
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<td>Body mass index (BMI)</td>
<td>26.4±0.6 (29 overweight, 11 obese)</td>
<td>19.7-46.1</td>
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<td>Sex (male / female)</td>
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<td>68 /32%</td>
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<td>Smoking (current / ex / never)</td>
<td>25 / 22 / 33</td>
<td>31 / 27 / 41%</td>
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<tr>
<td>Acute ischemic cerebral symptoms</td>
<td>51</td>
<td>63%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>31</td>
<td>38%</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>51</td>
<td>63%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>70</td>
<td>88%</td>
</tr>
</tbody>
</table>

Values are given as median ± SEM of n=80 patients (age, BMI), or as absolute numbers of patients (n) and percent (%).
Supplemental Figure I

Schematic representation of the longitudinal section of atherosclerotic plaque. A. Shear stress distribution along the atherosclerotic plaque; B. Schematic representation of the analyzed regions at the upstream and the downstream side of the plaque.