Molecular Imaging

Head-to-Head Comparison of Inflammation and Neovascularization in Human Carotid Plaques
Implications for the Imaging of Vulnerable Plaques

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Background—Inflammation and intraplaque neovascularization are acknowledged to be 2 features of plaque vulnerability, although their temporal expression and their respective value in predicting clinical events are poorly understood. To determine their respective temporal associations, we conducted a comprehensive assessment of inflammation and intraplaque neovascularization in the carotid plaque of symptomatic and asymptomatic patients.

Methods and Results—Thirty patients with severe carotid stenosis underwent 18F-fluorodeoxyglucose-positron emission tomography/computed tomographic imaging. Plaque 18F-fluorodeoxyglucose-uptake, indicative of inflammation, was measured by calculating the target:background ratio. The presence of intraplaque neovascularization during contrast-enhanced ultrasound was judged semiquantitatively; low-grade contrast enhancement (CE) suggested its absence, and high-grade CE, the presence of neovascularization. Carotid surgery was performed 1.6±1.8 days after completing both imaging modalities in all patients, and the presence of macrophages and neovessels was quantified by immunohistochemistry. We identified a significant correlation between the target:background ratio and macrophage quantification (R=0.78; P<0.001). The number of vessels was also significantly higher in carotid plaque with high-CE (P<0.001). Surprisingly, immunohistochemistry showed that high-CE and vessel number were neither associated with an elevated target:background ratio (P=0.28 and P=0.60, respectively) nor macrophage infiltration (P=0.59 and P=0.40, respectively). Finally, macrophage infiltration and target:background ratio were higher in the carotid plaque of asymptomatic patients (P=0.021 and P=0.05, respectively), whereas CE grade and the presence of neovessels were not.

Conclusions—Inflammation and intraplaque neovascularization are not systematically associated in carotid plaques, suggesting a temporal separation between the 2 processes. Inflammation seems more pronounced when symptoms are present. These data highlight the challenges that face any imaging strategy designed to assess plaque vulnerability. (Circ Cardiovasc Imaging. 2017;10:e005846. DOI: 10.1161/CIRCIMAGING.116.005846.)

Key Words: contrast ultrasound ▪ plaque inflammation ▪ plaque neovascularization ▪ positron-emission tomography

In the majority of cases, acute vascular events such as myocardial infarction or stroke are caused by the disruption of a vulnerable atherosclerotic plaque. Compared with stable lesions, rupture-prone or vulnerable plaques typically exhibit inflammation and intraplaque neovascularization (IPN).1 The ability to identify which atherosclerotic plaques are at risk of rupture would constitute a major advance in the management of atherosclerotic disease. Accordingly, both invasive and noninvasive imaging techniques are being developed to achieve this and to ensure the timely treatment of patients.2,3

Unfortunately, despite these objectives, robust predictors of plaque rupture remain to be determined.

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See Clinical Perspective

Atherosclerotic plaques develop in response to local inflammatory processes. Cardiovascular risk factors cause endothelial cell dysfunction and a proinflammatory state that promotes the expression of cellular adhesion molecules and consequently the recruitment of inflammatory cells, mainly...
monocytes and lymphocytes. Monocytes differentiate into
macrophages, the principal inflammatory cell constituent of
the atherosclerotic plaque. Macrophages may themselves contribute to the destabilization of the plaque by their secretion of metalloproteinases that degrade the fibrous cap separating the lipid-rich core from the arterial lumen. A strong correlation exists between macrophage plaque infiltration and ischemic symptoms, with this association persisting for several months after becoming symptomatic.

Numerous validation studies have proven 18F-fluorodeoxyglucose (FDG), a glucose analog that accumulates in cells with a high glycolytic rate, to be the most robust positron emission tomographic (PET) tracer with which to image plaque inflammation. FDG-PET/computed tomographic (CT) imaging is therefore a reliable and reproducible measure of vascular inflammation and provides valuable prognostic data. As the plaque size increases, oxygen levels deep within the plaque diminish, local hypoxia ensues, and microvessel formation from adventitia is triggered. Abnormal vascular development, characterized by leaky immature endothelial vessels, can lead to intraplaque hemorrhage, which promotes the transition from a stable to vulnerable plaque. This process may therefore predict future clinical outcomes.

Novel noninvasive imaging modalities capable of detecting neovascularization include carotid contrast-enhanced ultrasound (CEUS). This promising imaging tool detects IPN with a good histological correlation. One advantage of this technique is that the ultrasound contrast media is purely intravascular, and its tissue uptake therefore reflects the total number of microvessels, rather than permeability of the activated endothelium. Furthermore, IPN as assessed by CEUS has been associated with past ischemic neurological events.

Figure 1. Quantification of the area percentage of CD68 staining on digitized immunohistochemically stained slides by using a computer-assisted planimetry. For each slice (A), the software identified the immunohistochemical staining to be quantified by minimizing background-staining artifacts using image filters (B). Macrophage staining was reported as a percentage (in this example: 7.16%) of the total plaque area (C). The box indicates the region corresponding to the high-powered CD68 stain (D). FC indicates fibrous cap; L, lumen; and NC, necrotic core.
Although a pathophysiologic link exists between the presence of inflamed cells and the triggering of neovascularization, the temporal association between inflammation and neovascularization within the plaque has yet to be firmly established. Therefore, the aim of this study was to systematically assess the presence of inflammation and neovascularization using FDG-PET/CT and CEUS, respectively, in a population of consecutive asymptomatic and symptomatic patients scheduled for carotid surgery. Imaging findings were then systematically compared with histology.

### Methods

#### Patient Recruitment and Study Protocol

Patients referred for carotid endarterectomy were prospectively recruited for this study. After obtaining written informed consent and completing a baseline questionnaire, patients were invited to attend a same-day combined assessment of carotid plaque inflammation by 18F-FDG PET/CT, and carotid IPN by CEUS. Imaging was performed by a physician blinded to the patient’s history and with a maximal delay of 72 hours from the carotid surgery. The inclusion criteria were as follows: age >18 years, >70% carotid stenosis as determined by duplex ultrasound, and computed tomographic angiography. The indication for surgery was based on the referring physician’s decision. The exclusion criteria were pregnancy, inability to provide informed consent, and a known allergy to sulfur-containing drugs. Both asymptomatic and symptomatic patients were recruited. Symptoms were defined as a carotid-territory–related transient ischemic attack or stroke within 6 months of imaging. The study protocol was approved by the ethical committee of our institution and was conducted in the Cliniques Universitaires Saint-Luc, which obtained full Association for the Accreditation of Human Research Protection Programs accreditation for clinical research in 2015.

#### 18F-FDG-PET/CT Imaging Protocol and Analyses

After a fasting period of >6 hours, patients received an intravenous injection of 300±20 MBq of 18F-FDG. Imaging was performed after a circulation time of 150±22 minutes. The patient’s head was secured in a head-holder, with arms placed to either side. Images were acquired using a Gemini PET/CT system (Philips Healthcare, The Netherlands). The patients breathed normally while CT imaging was performed with a 16-slice multidetector scanner (adaptive dose in Z-DOM mode; voltage, 120 kV; rotation, 500 ms; pitch, 0.813). One 15-minute bed position PET image (voxel size 2×2×2 mm³) of the neck was obtained with the 3-dimensional (3D) mode and iterative reconstruction (3D line of response time-of-flight algorithm; Philips Healthcare) using CT-based attenuation correction, random, and scatter corrections. Patients were kept in a quiet environment before and after injection.

On the basis of anatomic boundaries defined by CT, carotid FDG uptake was measured at 2-mm intervals along the long axis of the carotid artery within a region of 6 mm above and below the bifurcation point. At each axial cross section, a single region of interest was drawn around the wall of the common or the internal carotid artery, and the maximum standardized uptake value measured. Standardized uptake value is the decay-corrected tissue concentration of FDG (kBq/mL) divided by the injected dose per body weight (kBq/g).

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>All Patients (n=30)</th>
<th>Asymptomatic Patients (n=19)</th>
<th>Symptomatic Patients (n=11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>72±9</td>
<td>71.5±9</td>
<td>73±10</td>
<td>0.6</td>
</tr>
<tr>
<td>Male sex</td>
<td>22/8 (73)</td>
<td>14/5 (74)</td>
<td>8/3 (73)</td>
<td>0.97</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.7±4.5</td>
<td>27.5±4.1</td>
<td>28.3±5.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7 (23)</td>
<td>5 (26)</td>
<td>2 (18)</td>
<td>0.73</td>
</tr>
<tr>
<td>Smoking</td>
<td>5 (17)</td>
<td>4 (21)</td>
<td>1 (9)</td>
<td>0.6</td>
</tr>
<tr>
<td>Hypertension</td>
<td>28 (93)</td>
<td>17 (90)</td>
<td>11 (100)</td>
<td>0.64</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>24 (80)</td>
<td>15 (79)</td>
<td>9 (82)</td>
<td>0.9</td>
</tr>
<tr>
<td>Statin use</td>
<td>24 (80)</td>
<td>15 (79)</td>
<td>9 (82)</td>
<td>0.9</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>16 (53)</td>
<td>10 (53)</td>
<td>6 (54.5)</td>
<td>0.93</td>
</tr>
<tr>
<td>Carotid stenosis, %</td>
<td>84±9</td>
<td>85±9</td>
<td>82±10</td>
<td>0.46</td>
</tr>
<tr>
<td>Glucose pre FDG-PET/CT, mg/dL</td>
<td>106±25</td>
<td>100±16</td>
<td>114±34</td>
<td>0.32</td>
</tr>
<tr>
<td>Delay between FDG-PET/CT and CEUS, h</td>
<td>3 (2–17)</td>
<td>3 (2–4)</td>
<td>5 (3–20)</td>
<td>0.97</td>
</tr>
<tr>
<td>Delay between imaging and surgery, d</td>
<td>1 (1–1.2)</td>
<td>1 (1–1)</td>
<td>1(0–2)</td>
<td>0.52</td>
</tr>
<tr>
<td>Delay between symptoms and surgery, d</td>
<td>NA</td>
<td>NA</td>
<td>25 (10–36)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD or n (%) or as median with interquartile range.
FDG-uptake was measured within the internal jugular vein on 4 consecutive cross sections. Target/background ratio (TBR) was calculated by dividing the carotid plaque maximum standardized uptake value by the venous blood maximum standardized uptake value.

Plaque calcium score was evaluated by the Agatston method on an independent workstation (Osirix v5.7; Geneva, Switzerland). A calcification plaque was defined as a radiation-attenuating structure above an attenuation threshold of 130 HU. The same region of interests that we used for PET analysis were used for the calcium score.

**Immunohistochemical Analyses**

At the time of carotid endarterectomy, the atherosclerotic plaques were immediately fixed in 10% buffered formalin and decalcified in the standard fashion. Specimens were transversally sectioned at 2-mm intervals. Each interval section was embedded in paraffin block, from which 5-µm sections were collected. Sections were then mounted on gelatin-coated slides and stained with the macrophage and endothelial cell markers, anti-CD68 (monoclonal antibody diluted 1/100; Dako), and anti-CD34 (monoclonal antibody diluted 1/500; Biocare Medical), respectively. A total of 8×2 slices covering the entire length of the plaque burden were analyzed per patient and per staining. All slices were scanned using Digital Image Hub 4.0 (Leica) for signal quantification. To evaluate CD68-staining intensity, computer-assisted planimetry (Tissue Image Analysis 2.0; Leica) was used. For each slice, the software identified the immunohistochemical staining to be quantified by minimizing background-staining artifacts using image filters (Figure 1). The same filter was used for all slices. Vasa-vasora were identified by CD34/CD31-staining in 10 randomly selected patients participating in our study (Data Supplement).
Intraobserver and interobserver agreement were assessed for the quantification of microvessels. Finally, intraplaque hemorrhage was semiquantitatively assessed on histological samples.

Statistical Analyses

Continuous variables are presented as mean±SD or as median with interquartile range. Continuous variables were compared between 2 groups using the Mann–Whitney U test. The Spearman method was used to assess correlations between TBR and the histopathologic assessment of inflammation (CD68-staining) and the number of neovessels and between CD68-staining and the number of neovessels. To determine intra- and interobserver variability, CD34-stained images were reanalyzed separately by 2 observers. The interval between the initial analyses and those made for variability assessment were >3 months. Intra- and interobserver variability in quantitative parameters were assessed by computing intraclass correlation coefficients and by calculating the limits of agreement between measurements using Bland–Altman analysis. Kendall τ coefficients were used to determine the levels of agreement between the visual grading scores of the CEUS readers. Kendall τ coefficient was used to measure the association between TBR and the CE-grade and between CE-grade and symptoms. A 2-sided value of P<0.05 was considered significant. All statistical analyses were performed using SPSS version 21.0 statistical software (Chicago, IL).

Results

Patient Variables

A total of 30 patients were included in this study; their characteristics are displayed in Table 1. The mean age was 72±9 years, 22 patients were men, with 11 patients symptomatic. Symptoms consisted of transient ischemic attack for 4 patients and stroke for 7 patients. The mean delay between symptoms and surgery was 25 days (10–36 days).

Imaging Data Validated by Histology

As expected, we found a good correlation between the plaque 18F-FDG-uptake and the number of macrophages (Figure 3A, \( r=0.7 \); \( P<0.001 \)). 18F-FDG-uptake and macrophage number were inversely associated with calcium score (\( r=-0.43, P=0.017 \) and \( r=-0.37, P=0.041 \), respectively). Plaques with more intense CE showed a significantly higher amount of neovessels as assessed by histology (mean vessel density: 4.84±2.41/mm² in plaques with grade 0 contrast enhancement versus 9.92±3.53/mm² in plaques with grade 1 contrast enhancement [Figure 3B;
We could find no correlation between vessel number and the calcium score ($r = -0.24$, $P = 0.21$).

**Inflammation and Neovascularization Are Not Systematically Associated Within the Plaque**

As illustrated in Figures 4 and 5, respectively, grade-1 CEUS did not correlate with a high 18F-FDG uptake, and inflammation could be observed in carotid plaques with a grade 0 CEUS. When patients were categorized according to their inflammatory status based on the median TBR value (1.81), no association was found between inflammation and IPN (Table 2, Kendall coefficient $\tau$: 0.0; $P > 0.99$). TBR observed for grades 0 and 1 CEUS plaques were comparable (1.69±0.39 versus 1.87±0.31, respectively; $P = 0.28$; Figure 6A). Similarly, macrophage infiltration as determined by immunohistochemistry was comparable for grade 0 versus grade 1 CEUS plaques (CD68% 9.77±6.47 versus 12.38±9.5, respectively; $P = 0.59$; Figure 6B). Finally, neither 18F-FDG uptake nor CD68% were found to correlate with the degree of IPN (Figure 7A and 7B) or the presence of hemorrhage ($P = 0.4$ and $P = 0.23$, respectively).

**Features Associated With Symptoms**

TBR was higher in symptomatic patients than in asymptomatic patients (1.97±0.28 versus 1.68±0.35; Figure 8A; $P = 0.05$). Similarly, macrophage infiltration was significantly higher in the carotid plaque of symptomatic patients than in the asymptomatic patients (14.16±6.2 versus 8.1±6.6 %CD68, respectively;
Inflammation and Neo vascularization in Carotid Plaques

![Figure 8B; $P=0.021$]. In contrast, the degree of neovascularization was comparable for asymptomatic and symptomatic patients ($8.24\pm4$ versus $6.82\pm4$ vessels/mm$^2$, respectively; Figure 8C; $P=0.35$). When patients were categorized according to their symptomatic status, no agreement was found between symptoms and IPN based on CEUS-grade (Kendall coefficient $\tau$: 0.085; $P=0.65$; Table 3). Finally, we could identify no difference in the grading of carotid calcification (calcium score) according to the symptomatic status ($P=0.98$).

Intra- and Interobserver Agreement
We found an intraobserver reproducibility (intraclass correlation coefficients) of 0.77 with a bias of $80\pm74$, and an interobserver reproducibility (intraclass correlation coefficients) of 0.83 with a bias of $93\pm81$ for microvessel measurements. The concordance for the qualitative assessment of CEUS was determined by grading IPN by 2 different readers and by 1 reader at an interval of $>3$ months using CEUS cine-loops. The intraobserver agreement was 91% ($\kappa=0.82$), with an interobserver agreement for the same parameter of 77% ($\kappa=0.51$).

Discussion
Plaque inflammation and neovascularization are 2 recognized features of plaque vulnerability. Here, we demonstrate that inflammation is not systematically associated with the degree of neovascularization within the plaque, suggesting a temporal separation of these processes. Furthermore, intraplaque inflammation was found to be associated with clinical symptoms whereas IPN was not.

Atherosclerosis is a chronic inflammatory disease characterized by lipid-containing lesions of large- and medium-sized arteries. Vasa vasora participate in the pathogenesis of the disease and proliferate to meet the nutritional needs of the artery’s outer medial layer when metabolic demands exceed oxygen delivery from the luminal blood. Hypoxia is the most potent stimulus for angiogenesis, predominantly by its activation of the hypoxia-inducible factor/vascular endothelial growth factor pathway. A molecular link exists between the expansion of adventitial vasa vasora, neointima formation, and atherosclerotic plaque development. Notably, their regression is accompanied by reduced plaque growth. Neovessels may serve as a pathway for the recruitment of leucocytes to high-risk areas of plaque. Microvessel density has been shown to increase in inflamed lesions and correlates with plaque rupture. Newly formed microvessels may promote intraplaque bleeding, which increases the levels of free cholesterol, and leads to rapid necrotic core expansion. This mechanism contributes to the vulnerability of the plaque. Fortunately, not all plaques will progress to destabilization and a substantial number will heal spontaneously. Some of the relevant mechanisms have been elucidated and promising strategies to stabilize atherosclerotic plaques are under development. Atherosclerotic lesions evolve in successive phases during which inflammation and neovascularization are intertwined. However, it must be acknowledged that our ability to predict which plaques will progress to destabilization...
Inflammation and Neovascularization in Carotid Plaques and cause an unstable syndrome such as transient ischemic attack, stroke, or acute coronary syndrome is currently very limited. Identifying vulnerable plaques before their rupture is the Holy Grail for clinicians. In this study, we identify patients with plaque inflammation without evidence of neovascularization, as well as the opposite. Our imaging findings have been validated by a systematic immunohistochemical evaluation covering the entire length of the surgical specimen.

Conflicting data for the temporal association of intraplaque inflammation and neovascularization currently exist in the literature. Some authors have found a weak correlation between the 2 processes, which is contradicted by others. We could find no correlation between either of these major determinants of plaque vulnerability, highlighting the practical challenges in defining imaging strategies with which to assess plaque vulnerability. Furthermore, plaque characteristics also seem to be dependent on general factors such as sex and age. Several outstanding questions have yet to be satisfactorily addressed. For example, which plaque feature is the best predictor of a clinical event? When should we image, and should imaging be repeated? Can we base our imaging strategy on one plaque feature alone, and will we be in a position to predict the natural history of an atherosclerotic plaque? Ultimately, it is likely that imaging based on CEUS-grade (Kendall coefficient: \( r: 0.085; P=0.65 \)). CEUS indicates contrast-enhanced ultrasound.

<table>
<thead>
<tr>
<th></th>
<th>CEUS Grade 0</th>
<th>CEUS Grade 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>7</td>
<td>12</td>
</tr>
</tbody>
</table>

Patients were categorized according to their symptomatic status. No association was found between symptoms and intraplaque neovascularization based on CEUS-grade (Kendall coefficient: \( r: 0.085; P=0.65 \)). CEUS indicates contrast-enhanced ultrasound.
on multiple structural and molecular targets will be required to identify atherosclerotic plaques at risk of rupture. Shear stress imaging, active calcification imaging (18F-NaF), or other imaging tracers could also be helpful in achieving this goal.

In previous studies to image vulnerable plaques, multimodality imaging has been investigated. In particular, the combined assessment of inflammation by 18F-FDG PET-CT and neovascularization by dynamic contrast-enhanced magnetic resonance imaging has been evaluated in patients with carotid plaques,20,25,26 with divergent results reported (inverse, weak, or a moderate correlation between inflammation and neovascularization). However, systematic histological validation was either lacking in those studies or limited to a comparatively small number of patients.26 Furthermore, the same parameter for assessing neovascularization, \( \kappa^{\text{imm}} \), was used, which reflects microvascular flow and permeability rather than the total number of neovessels. The contrast agent used for CEUS evaluation in our study is purely intravascular and does not bridge the endothelial barrier. Consequently, this approach reveals the total number of neovessels in the plaque and not endothelial permeability, which is linked to inflammation. This approach therefore constitutes the major difference with dynamic contrast-enhanced magnetic resonance imaging, with either technique evaluating different tissue criteria (ie, the number of neovessels versus endothelial permeability). This may explain the discrepancies reported between results obtained using the 2 techniques and the absence of any correlation between inflammation and the total intraplaque number of vessels that we found in our study. The systematic immunohistological evaluation of the entire length of the surgical specimens of our patients confirmed our imaging findings and constitutes, to date, the largest series in which the 2 imaging techniques have been correlated with histology.

In our series and others,22,27 a history of neurological symptoms is associated with increased plaque inflammation, whereas neovascularization is not. It is noteworthy that inflammation also seems to be associated with a higher risk of subsequent stroke, unlike neovascularization.8,28,29 Collectively, an assessment of these data leads us to hypothesize that inflammation is probably the more useful parameter (than the presence of neovascularization) in discriminating patients at a high versus low risk for presenting acute syndromes. This approach therefore constitutes the major difference with dynamic contrast-enhanced magnetic resonance imaging, with either technique evaluating different tissue criteria (ie, the number of neovessels versus endothelial permeability). This may explain the discrepancies reported between results obtained using the 2 techniques and the absence of any correlation between inflammation and the total intraplaque number of vessels that we found in our study. The systematic immunohistological evaluation of the entire length of the surgical specimens of our patients confirmed our imaging findings and constitutes, to date, the largest series in which the 2 imaging techniques have been correlated with histology.

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The possible limitations of this study are as follows. First, although the results of the present study are limited by its modest sample size, its strength lies in the simultaneous assessment of PET activity, IPN by CEUS, and histological validation. Nonetheless, subgroup analyses of asymptomatic versus symptomatic patients must be interpreted with caution. Second, we did not coregister imaging with histological data sets. Given the poor spatial resolution of FDG-PET, and the impossibility of coregistering CEUS with histology, we deliberately chose not to perform coregistration. We attempted to overcome this limitation by analyzing the total length of the surgical specimens to collect the entirety of the information contained within the plaque. Furthermore, inherent to the surgical technique, we could not quantify inflammation and neovascularization present in the adventitia by histology. Third, because histological analyses were mandatory for this study, we did not include patients with less advanced disease. Fourth, atherosclerotic plaques were not imaged by a high-resolution imaging modality like magnetic resonance imaging. Thus, components, including fibrous cap thickness, lipid-rich necrotic core, or intraplaque hemorrhage, that could be the physiopathological link between neangiogenesis and inflammation were not evaluated. Finally, no scan–rescan reproducibility was performed.

Conclusions

Inflammation and neovascularization are not systematically associated in the carotid plaques of patients suggesting a temporal separation of these processes. Inflammation seems also more pronounced when symptoms are present, whereas neovascularization seems not. This study highlights the practical challenges in defining imaging strategies with which to assess plaque vulnerability.

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Disclosures

None.

References


20. T primary clinical context. }

21. T primary clinical context. }

22. T primary clinical context. }

23. T primary clinical context. }

24. T primary clinical context. }

25. T primary clinical context. }

26. T primary clinical context. }

27. T primary clinical context. }

28. T primary clinical context. }

29. T primary clinical context. }

30. T primary clinical context. }
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**SUPPLEMENTAL MATERIAL**

**Supplemental Methods.**

Assessment of microvessel density by using a cocktail of endothelial cell markers CD34/CD31. Comparison with the single CD34-staining.

Double CD34/CD31-staining was performed in 10 randomly selected patients participating in our study. Sections were mounted on gelatin-coated slides and stained with the endothelial cell markers anti-CD34 (monoclonal antibody diluted 1/500, Biocare Medical). The signal was quantified as described in the methods section: vasa-vasora were identified by CD34-positive immunostaining and were counted at a magnification of 20X. Afterwards, the same sections were stained with the endothelial cell markers anti-CD31 (monoclonal antibody diluted 1/50, DAKO). The vasa-vasora were identified by CD34/CD31-positive immunostaining and were recounted. The signal was compared to the single CD34 staining. As shown in this figure, we did not observe any visual difference between the single CD34-staining and the double CD34/CD31-signal. The correlation between the 2 different staining was excellent (r= 0.9).

**Supplemental Figure.**
Supplemental Figure legend. As shown in this typical example, no visual difference was observed between the single CD34 staining (A) and the double CD34/CD31 staining (B). The correlation between the 2 different staining was excellent (C).