High Neutrophil Numbers in Human Carotid Atherosclerotic Plaques Are Associated With Characteristics of Rupture-Prone Lesions

Mihaela G. Ionita, Pleunie van den Borne, Louise M. Catanzariti, Frans L. Moll, Jean-Paul P.M. de Vries, Gerard Pasterkamp, Aryan Vink, Dominique P.V. de Kleijn

Objective—To score the number of plaque neutrophils and relate the score to plaque morphology and inflammatory status. Methods and Results—Neutrophils are inflammatory cells with tissue destruction capabilities that have been found at the site of an atherosclerotic plaque rupture or erosion. Poor evidence exists for neutrophil infiltration in human carotid atherosclerotic plaques, and its association with plaque morphology has not yet been described. A set of 355 human carotid plaques was stained for the neutrophil marker CD66b. High neutrophil numbers were found in plaques with a large lipid core, high macrophage numbers, and low collagen amount and smooth muscle cell numbers. High neutrophil numbers were associated with high interleukin 8 (P<0.001) and matrix metalloproteinases 8 (P=0.005) and 9 (P<0.001) plaque levels. High microvessel density within plaques was correlated with high neutrophil numbers (P=0.01). In addition, low numbers of neutrophils were associated with female sex and use of β-blockers.

Conclusion—For the first time to our knowledge, these results show that neutrophil numbers are strongly associated with the histopathologic features of rupture-prone atherosclerotic lesions and suggest a role for neutrophils in plaque destabilization. (Arterioscler Thromb Vasc Biol. 2010;30:1842-1848.)

Key Words: atherosclerosis ■ β-adrenergic receptor blockers ■ carotid arteries ■ stroke ■ surgery ■ vascular biology ■ vascular surgery ■ MMP ■ neutrophil

C hronic inflammation plays a key role in the pathogenesis and progression of atherosclerosis and, later, in the destabilization and rupture of an atherosclerotic plaque, leading to adverse cardiovascular events. The involvement of inflammatory cells, such as macrophages and T cells, in atherogenesis is well documented, whereas neutrophil granulocytes, also present in atherosclerotic lesions, are detected in much lower numbers.

Neutrophils have been observed at the site of plaque erosion or rupture in atherectomy specimens from patients with unstable angina and in autopsy samples from patients with acute myocardial infarction.1,2 Histological analysis of plaques from cerebral arteries has shown that the expression of neutrophil elastase is increased in late-stage plaques.3 Epidemiological studies have shown that neutrophil counts in peripheral blood positively correlate with coronary atherosclerotic risk4 and acute myocardial infarction risk.5

Mouse studies reveal the accumulation of neutrophils in the luminal plaque region and adventitia of aortic plaques of mice that lack apolipoprotein E6 and in lesions of low-density lipoprotein receptor–deficient mice.7 Neutrophils exert most of their functions via preformed granule proteins, mostly found in atherosclerotic lesions (eg, alarmins, human neutrophil peptides, elastase, cathespins G, and proteinase 3).8 Neutrophils, similar to monocytes, are phagocytic cells involved in innate immunity.9 Next to pathogen recognition and destruction, neutrophils contribute to tissue damage by secreting enzymes, such as myeloperoxidase (MPO), elastase, esterase, and matrix metalloproteinase (MMP) 9,10 MPO and MMP-9, expressed in mouse and human atherosclerotic lesions,11 have been shown to have prognostic value in atherothrombosis.12-14

Although the presence of neutrophils in atherosclerotic specimens (carotid, coronary, and femoral) has been previously reported, no study so far has strictly and specifically investigated neutrophil localization within plaques, their association with plaque characteristics, and clinical data. We hypothesized that the number of neutrophils per plaque correlates with the features of high-risk, rupture-prone, atherosclerotic lesions.

In this observational study, we determined the total number of neutrophils per plaque in a large human cohort and
assessed their localization within plaques and the correlation with plaque characteristics. We also studied the association of neutrophils with sex and β-blocker therapy and report an association between high neutrophil plaque numbers and the features of the rupture-prone plaque. Low neutrophil numbers were associated with female sex and history of β-blocker medication. These results might imply a role for neutrophils in rupture-prone plaque development and provide evidence for in vivo β-blocker therapy–induced neutrophil-dependent plaque stabilization.

Methods

Athero-Express Biobank and Plaque Processing

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by 2 Dutch hospitals: the University Medical Center Utrecht and the St Antonius Hospital in Nieuwegein. Details are included in the supplemental material (available online at http://atvb.ahajournals.org).

All 355 carotid plaques used in this study were carefully dissected from the carotid arteries and immediately transferred to the laboratory for further processing, as previously described. In the laboratory, the atherosclerotic fragments were dissected into 0.5-cm-thick cross-sectional segments along the longitudinal axis of the vessel. The plaque segment showing the largest plaque burden was called the culprit lesion and was used for histological analysis to determine plaque morphology. The adjacent segments were used for protein isolation.

Human Carotid Endarterectomy Specimens and (Immunohistochemistry

Patients included for carotid endarterectomy (CEA) were asymptomatic (ie, no clinical symptoms related to carotid luminal stenosis >75%, n=46) and symptomatic (n=309), with minor clinical presentations (eg, transient ischemic attack, amaurosis fugax, and retinal infarction; n=234) or major presentations (eg, stroke; n=75). Plaque segments were fixed in formalin and embedded in paraffin. Consecutive sections were immunostained with hematoxylin-eosin, elastin von Giesen, picrosiris red, α-actin, CD68, and CD34,15,16 To determine the plaque phenotype, sections were scored as previously described.

To visualize neutrophils, consecutive sections were boiled in citrate buffer and stained with mouse anti–human CD66b (dilution, 1:100; AbD Serotec, Oxford, England) and MPO (dilution, 1:10 000; Dako, Glostrup, Denmark) monoclonal antibodies. Power vision poly–horseradish peroxidase–anti-mouse IgG (Immunologic, Duiven, the Netherlands) was used as the secondary antibody. Mouse IgG of the same isotype and subclass as the primary antibodies was used as a negative control. The signals were visualized using diaminobenzidine. Sections were counterstained with hematoxylin.

In addition to the immunostains, we used a third staining to detect intraplaque hemorrhaging. Intraplaque hemorrhaging was scored using hematoxylin-eosin and fibrin (Mallory phosphotungstic acid–hematoxylin) and anti–smooth muscle actin immunostains.

Protein Isolation

Segments adjacent to those used for histology were used for protein isolation, as previously described. In short, plaque segments were frozen in liquid nitrogen and stored at −80°C until further use; protein extraction was performed according to a standard protocol using Tris. Levels of interleukin (IL) 8 were measured by a multiplex suspension array system according to the manufacturer’s protocol (Bender Med Systems, Vienna, Austria). MMP 8 and 9 activities were measured using the Biotrak activity assays RPN 2635 and RPN 2634 (Amersham Biosciences, Buckinghamshire, England), respectively. Measurements of IL-8, MMP-8, and MMP-9 are standard for ACS patients; a portion of these data (8.5%) has been used in another article.

Table 1. Patient Characteristics Related to Number of Neutrophils in Common CEA Specimens*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low (n=178)</th>
<th>High (n=177)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of neutrophils, median (IQR)</td>
<td>8 (2–17)</td>
<td>110 (58–240)</td>
<td>NA</td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
<td>72 (10)</td>
<td>73 (9)</td>
<td>0.83</td>
</tr>
<tr>
<td>Male sex</td>
<td>102</td>
<td>134</td>
<td>0.05</td>
</tr>
<tr>
<td>Hypertension</td>
<td>115</td>
<td>124</td>
<td>0.94</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>41</td>
<td>37</td>
<td>0.56</td>
</tr>
<tr>
<td>Current smoker</td>
<td>46</td>
<td>37</td>
<td>0.22</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>113</td>
<td>117</td>
<td>0.94</td>
</tr>
<tr>
<td>History of angina pectoris</td>
<td>60</td>
<td>51</td>
<td>0.17</td>
</tr>
<tr>
<td>History of myocardial infarction</td>
<td>27</td>
<td>31</td>
<td>0.68</td>
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<tr>
<td>History of vascular intervention</td>
<td>29</td>
<td>24</td>
<td>0.65</td>
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<tr>
<td>History of carotid intervention</td>
<td>16</td>
<td>9</td>
<td>0.25</td>
</tr>
<tr>
<td>Symptomatic carotid stenosis</td>
<td>149</td>
<td>159</td>
<td>0.08</td>
</tr>
<tr>
<td>Time between TIA or stroke</td>
<td>57 (24–110)</td>
<td>58 (21–106)</td>
<td>0.88</td>
</tr>
<tr>
<td>and CEA, median (IQR)</td>
<td></td>
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<tr>
<td>Medication</td>
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<tr>
<td>Statins</td>
<td>151</td>
<td>147</td>
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<tr>
<td>Aspirin</td>
<td>47</td>
<td>36</td>
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<tr>
<td>ACE inhibitors</td>
<td>55</td>
<td>56</td>
<td>0.90</td>
</tr>
<tr>
<td>β-blockers</td>
<td>100</td>
<td>83</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Intraplaque hemorrhaging was scored using hematoxylin-eosin and fibrin (Mallory phosphotungstic acid–hematoxylin) and anti–smooth muscle actin immunostains.

Protein Isolation

Segments adjacent to those used for histology were used for protein isolation, as previously described. In short, plaque segments were frozen in liquid nitrogen and stored at −80°C until further use; protein extraction was performed according to a standard protocol using Tris. Levels of interleukin (IL) 8 were measured by a multiplex suspension array system according to the manufacturer’s protocol (Bender Med Systems, Vienna, Austria). MMP 8 and 9 activities were measured using the Biotrak activity assays RPN 2635 and RPN 2634 (Amersham Biosciences, Buckinghamshire, England), respectively. Measurements of IL-8, MMP-8, and MMP-9 are standard for all Athero-Express patients; a portion of these data (8.5%) has been used in another article.

Statistical Analysis

Statistics were performed using commercially available software (SPSS 15.0). Correlations between plaque characteristics, cytokines, MMPs, and patient clinical data were assessed using the Spearman correlation test; and the difference between 2 groups was determined using the Mann-Whitney test. For these analyses, the number of neutrophils per plaque was used as a continuous variable.

For the associations mentioned in Table 1, the number of neutrophils per plaque (as a continuous variable) was divided into 2 equal groups using the median (31.0) as a cutoff. Multivariate analysis was used to adjust for confounders (eg, β-blockers and sex): the neutrophil data were binned into 2 groups (low and high) using the median as a cutoff; a binary logistic model with a probability for stepwise
entry (0.01) and removal (0.05) was performed. \( P<0.05 \) was considered significant.

**Results**

**Neutrophil Identification and Localization Within Plaques**

The clinical characteristic of the study population in relation to neutrophil numbers are shown in Table 1 and Table 2.

Three staining methods were used to identify the neutrophils in sections of atherosclerotic plaques (CD66b, Leder, and MPO). The analysis showed that CD66b positivity was detected in neutrophils and not in other cell types within plaques. Leder staining was mainly found in neutrophils and resembled the CD66b distribution, and MPO expression was found in neutrophils and in a subset of plaque macrophages (supplemental Figure I).

Further analysis of the CD66b positivity of plaque neutrophils showed a heterogeneous distribution for neutrophils within plaque tissue. Neutrophils were found in different regions of the plaque: in the fibrous cap or in the shoulder, in the interface to media (also called the base of a plaque), or in areas with intraplaque bleeding (hemorrhaging) (Figure 1A). In addition, neutrophils were observed lying underneath the luminal endothelium (Figure 1B) or in the vicinity of microvessels in the plaque (Figure 1C).

**Neutrophils and Plaque Histology**

The number of neutrophils per plaque was assessed and compared with different plaque histological characteristics: neutrophil numbers were positively correlated with the size of the lipid core (\( P<0.001 \)) and the amount of macrophages (\( P<0.001 \)) and microvessels (\( P=0.01 \)); and negatively correlated with the amount of collagen (\( P<0.001 \)) and smooth muscle cells (\( P<0.001 \)) (Figure 2).

**Neutrophils and IL-8, MMP-8, and MMP-9**

Next, we investigated the association of plaque neutrophils with IL-8, a neutrophil chemoattractant protein; and MMPs 8 and 9, 2 proteases expressed by neutrophils. A positive correlation was observed between the number of neutrophils and the levels of IL-8 (Spearman correlation coefficient \( r=0.41, P<0.001 \)), active MMP-8 (Spearman correlation coefficient \( r=0.24, P=0.005 \)), and active MMP-9 (Spearman correlation coefficient \( r=0.30, P<0.001 \)).

**Neutrophils and Clinical Patient Characteristics**

Having established that neutrophils are associated with characteristics of rupture-prone plaques, we investigated whether clinical parameters differed between patients with high and low levels of neutrophils (equal groups, using the median 31.0 as a cutoff) within plaques (Table 1).

Table 2 shows the number of neutrophils in plaques for each patient characteristic. The number of plaque neutrophils was significantly different between men and women. Lower neutrophil numbers were observed in plaques from women compared with plaques from men (\( P<0.001 \)) (Figure 3A). In addition, an association between the number of neutrophils per plaque and the use of \( \beta \)-blockers was found (Table 2). The number of neutrophils was lower in plaques from patients treated with \( \beta \)-blockers before their CEA compared with plaques from untreated patients (\( P=0.04 \)) (Figure 3B).

In addition, patients receiving \( \beta \)-blocker treatment for longer than 1 year had lower plaque neutrophil numbers compared with patients receiving treatment for less than 1 year (\( P=0.049 \)) (supplemental Figure II). A trend toward
lower neutrophil numbers in plaques from patients treated with selective β1-blockers (eg, metoprolol, bisoprolol, and atenolol) than in plaques from patients treated with nonselective drugs (eg, sotalol) was observed (supplemental Figure II). Baseline patient characteristics in relation to β-blocker therapy are presented in supplemental Table I.

Multivariate analysis showed that the association between neutrophil numbers and the characteristics of rupture-prone plaque is independent of β-blocker therapy and sex (Table 3).

No difference in neutrophil plaque numbers between patients presenting with clinical manifestations of the carotid stenosis (symptomatic) and those without symptoms (asymptomatic) was observed (P = 0.08) (Table 2 and supplemental Figure III).

Discussion
Atherosclerosis is a chronic inflammatory disease. Different inflammatory cell types infiltrate through a vessel’s damaged endothelium into the intima, where they initiate a chronic inflammatory process. The role of macrophages and T lymphocytes in the process of atherogenesis is already well established. A wide range of functions relevant to atherosclerosis are attributed to mast cells and dendritic cells.11 Neutrophils, although present in much lower numbers within an atherosclerotic lesion, are regaining interest in respect to atherosclerosis development and progression.

We used different markers to identify the neutrophils in human CEA specimens: CD66b, MPO, and esterase. The analysis of consecutive sections stained for CD66b, anti-MPO, and anti-esterase showed that the CD66b-positive cells expressed MPO and esterase, demonstrating that the CD66b-positive cells in plaques are neutrophils (as previously reported1).

The localization of neutrophils within the plaque proved to be heterogeneous; neutrophils were found infiltrated in the cap, in the shoulder, and in areas toward the media (also known as the base of the plaque) (Figure 1D). In these areas, neutrophils were mainly found around microvessels (Figure 1C and E); in the cap, neutrophils were also observed underneath the luminal endothelium (Figure 1B and E). These may represent 2 distinct routes by which neutrophils infiltrate into the plaque. In apolipoprotein E–deficient mice (by use of flow cytometry on the whole aorta and confocal microscopy on whole mounted plaques), Rotzius et al19 recently showed how neutrophils infiltrate and abundantly accumulate in the shoulder regions of the plaque, where they outnumber the amount of infiltrating monocytes. In addition, they observed that neutrophils were the main leukocyte subset that interacted with the lesion endothelium. Naruko et al,1 in coronary arteries, and Leclercq et al,20 in carotid arteries, reported the localization of neutrophils in the vicinity of intraplaque vessels. In our study, an association between high numbers of neutrophils and high vessel density per plaque was observed (Figure 2E), providing further support for neutrophil infiltration through small vessels. The presence of (neo)vessels, expressing adhesion molecules (intercellular adhesion and vascular cell adhesion molecules) within an atherosclerotic lesion, may facilitate the influx of inflammatory cells, thus possibly contributing to plaque destabilization and rupture.21

In the shoulder regions of carotid plaques, Leclercq et al20

Figure 1. A through E, Neutrophil localization in human carotid plaques. A, CD66b-positive neutrophils in the shoulder. B, CD66b-positive neutrophils underneath the luminal endothelium (black arrows). C, CD66b-positive neutrophils around a plaque microvessel. D, Schematic representation of different plaque areas. E, Distribution of CD66b-positive neutrophils within plaque areas. IPH indicates intraplaque hemorrhaging; and the asterisk, mostly observed around microvessels.
observed areas positive for P-selectin (a neutrophil adhesion molecule required for their diapedesis), surrounded by neutrophils. Thus, the infiltration of neutrophils from intraplaque microvessels into atherosclerotic lesions could be an active phenomenon. However, because our study is purely observational, we cannot exclude the possibility that the observed neutrophils around plaque microvessels and underneath the luminal endothelium might be an effect of CEA; it is known that the surgical procedure can induce diapedesis of neutrophils. No correlation between the interval in which the carotid artery was opened and the plaque was removed (on average, $34 \pm 11$ minutes [mean $\pm$ SEM]) and the number of neutrophils per plaque was found (Spearman correlation coefficient, $r = -0.06$, $P = 0.35$).

In 36% of the plaques, neutrophils were observed in areas with intraplaque hemorrhaging (Figure 1E), this being a third possible route by which neutrophils come into the plaque. A histological analysis of human CEA specimens suggested that intraplaque hemorrhaging could convey neutrophils into the lesion. Moreover, intraplaque hemorrhaging and angiogenesis contribute to plaque destabilization and possible rupture.

High neutrophil numbers were found in plaques with features of rupture-prone lesions (bigger lipid core, heavy macrophage influx, and minor collagen and smooth muscle cells). The previously mentioned histological characteristics of rupture-prone plaques originate from cross-sectional observations in coronary lesions; however, these characteristics also apply for carotid plaques.

Different studies reported the presence of neutrophils at sites of rupture of human atherosclerotic lesions (coronary, carotid, and cerebral); and mouse studies suggest a role for neutrophils in plaque formation. Previous human neutrophil studies were based on either autopsy specimens (coronary or cerebral) or atherectomy specimens. In the postmortem coronary specimens, neutrophils were detected in atherosclerotic lesions of patients who died of acute myocardial infarction and not in lesions of patients who died of noncardiovascular disease. A study in CEA specimens compared the culprit zone with the adjacent plaque segments and reported the presence of neutrophils in the culprit lesion and an association between neutrophil infiltration and intraplaque hemorrhaging.

A positive association between the number of neutrophils and plaque levels of IL-8, MMP-8, and MMP-9 was observed. IL-8 is a well-known neutrophil chemoattractant molecule, and high IL-8 levels are found in rupture-prone plaques. It is secreted by active macrophages, endothelial cells, and T cells and may contribute to neutrophil migration into the plaque. IL-8 is also known as a neutrophil stimulator,
Neutrophils in relation to sex and β-blocker therapy. A, The number of neutrophils per plaque and sex (male and female). B, The number of neutrophils per plaque and β-blocker therapy. Bars represent mean±SEM. The number of patients per group is indicated in parentheses. Statistical analyses were performed by the Mann-Whitney test.

inducing the release of MMP-9 from neutrophil’s tertiary granules. In addition, MMP-8 (also known as neutrophil collagenase) is highly expressed by neutrophils. This might explain the strong correlation between the high number of neutrophils and the high levels of IL-8, MMP-8, and MMP-9 in plaques and might point to neutrophils as a source of MMP-8 and MMP-9 in human plaques. IL-8 levels were significantly associated with MMP-8 and MMP-9 levels (data not shown), suggesting that IL-8 might stimulate MMP-8 and MMP-9 expression in those plaques; this finding is in accordance with the finding that MMP-8 and MMP-9 levels are elevated in rupture-prone plaques. However, this study can only measure at 1 point in time, which limits firm conclusions on regulatory mechanisms. Also, the source of these proteases in human plaques is heterogeneous; plaque’s vascular endothelial cells, smooth muscle cells, and macrophages can produce and secrete MMP-8 and MMP-9. Therefore, neutrophils could only be counted as 1 of the cellular sources of MMPs in human plaques, next to macrophages and smooth muscle cells.

Researchers previously demonstrated that carotid plaques stabilize over time after a stroke; the levels of cytokines and the number of infiltrated macrophages decrease immediately after a stroke or transient ischemic attack until CEA. Therefore, we assessed the relation between neutrophils and time between stroke or transient ischemic attack and CEA; no significant association between the 2 was found (Spearman correlation $r = -0.03$, $P = 0.64$; data not shown).

It would be interesting to know whether the numbers of plaque-infiltrating neutrophils correlate with the numbers of blood-circulating neutrophils. In this study, in a subgroup of 25 patients, no correlation between the number of neutrophils in plaque and the number of circulating granulocytes was observed (Spearman correlation $r = 0.25$, $P = 0.23$; supplemental Figure IV). This might be because of the few patients for whom both plaque and blood were available.

An association between sex and neutrophil numbers in plaques was observed (Figure 3A and Table 2). The plaques from women showed lower neutrophil counts compared with the plaques from men. This is in accordance with previous results in a large cohort of patients undergoing CEA, these results show that women have a more stable and less inflammatory plaque phenotype compared with men. Sex differences in cardiovascular disease are being investigated. Premenopausal women are protected against atherosclerosis and its complications; however, postmenopausally, their risk of cardiovascular complications increases by a factor of 2 or 3. Women have smaller arteries and, therefore, might have smaller plaques. In the current study, the degree of carotid stenosis (partially representing the plaque size) did not differ between women and men (supplemental Table II).

An association between the number of neutrophils per plaque and the use of β-blockers was observed. Plaques from patients with records of β-blocker use had significantly lower neutrophil numbers compared with plaques from patients without a history of β-blocker use (Figure 3B and Table 2). The use of β-blockers showed no association with the different plaque characteristics (ie, size of the lipid core, amount of collagen, macrophages, and smooth muscle cells) or with the levels of IL-8, MMP-8, and MMP-9 (data not shown). The use of β-blockers for longer than 1 year might induce a significant reduction in the number of infiltrating neutrophils because an inverse association between the time of β-blocker treatment and the number of plaque neutrophils was observed (supplemental Figure II). β-Blockers have accessory effects on neutrophils; several in vitro studies demonstrated β-blocker–induced inhibition of neutrophil chemotaxis and release of cytoplasmic products. Our observation is in line with these previous findings, suggesting that β-blockers could reduce neutrophil influx and might, therefore, reduce progression to rupture-prone plaque lesions. However, new prospective clinical studies are necessary to confirm this hypothesis.

In summary, we show that, within human carotid atherosclerotic plaques, many neutrophils are associated with the morphological characteristics and inflammatory status of rupture-prone lesions. This points to neutrophils, known for their tissue destruction capabilities, as potential contributors...
to the destabilization and subsequent rupture of an atherosclerotic plaque.

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We thank Petra van der Kraak-Homoet, BS, for her excellent technical assistance.

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Disclosures
None.

References
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Methods:

Athero-Express biobank

The study has been approved by the institutional boards of both hospitals and written informed consent was obtained from all participants. The study is designed to investigate the expression of atherosclerotic tissue derived biological markers in relation to plaque phenotype of patients undergoing CEA and adverse cardiovascular events during follow up. Patients who undergo CEA fill in an extensive questionnaire and diagnostic examinations are performed. The indication for CEA for asymptomatic patients was based on the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST) and for symptomatic patients was based on recommendations based on the North American Symptomatic Carotid Endarterectomy Trial (NASCET)\(^1\)\(^2\). All patients were reviewed by the vascular surgeon or neurologist before CEA to assess the nature and timing of clinical symptoms.

Blood and granulocyte blood count

From 25 patients included in this study, EDTA blood (10 mL) was withdrawn prior to the surgical incision for carotid endarterectomy. Immediately after collection, a total blood count was performed (by a hematology analyzer Cell-Dyn 800, Abbott) and the total granulocyte number was documented. The average granulocyte count was 69\% (4.9x10\(^6\)/mL) from total white blood count, with values ranging from 60.2\% (2.8x10\(^6\)/mL) to 82.7\% (8.8x10\(^6\)/mL).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>β-blocker naïve (n = 183)</th>
<th>β-blocker treatment (n = 158)</th>
<th>P</th>
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<td>70.3 ± 0.8</td>
<td>72.5 ± 0.7</td>
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<tr>
<td>Gender, n (%)</td>
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<tr>
<td>Male</td>
<td>121 (69.9)</td>
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<tr>
<td>Female</td>
<td>52 (30.1)</td>
<td>53 (34.5)</td>
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<tr>
<td>Smoker, n (%)</td>
<td>39 (22.5)</td>
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<td>103 (66.8)</td>
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<td>41 (23.6)</td>
<td>65 (42.2)</td>
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<td>Myocardial infarction, n (%)</td>
<td>14 (8.09)</td>
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<td>16 (9.2)</td>
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<td>Vascular intervention, n (%)</td>
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<td>Carotid intervention, n (%)</td>
<td>43 (24.8)</td>
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<td>4.2 ± 1.2</td>
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<td>1.1 ± 0.4</td>
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<td>LDL, mmol/L</td>
<td>2.5 ± 0.9</td>
<td>2.4 ± 0.9</td>
<td>0.93</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.3 ± 0.6</td>
<td>1.4 ± 0.7</td>
<td>0.70</td>
</tr>
</tbody>
</table>
1 **Medication**

2 Statins, n (%)  142 (82)  130 (84.4)  0.35

3 Aspirin, n (%)  33 (19)  43 (28)  0.12

4 ACE inhibitors, n (%)  41 (24)  60 (39)  0.38

5 **Plaque histology**

6 Small lipid core

7 (0 - 40% of plaque area), n (%)  117 (67.3)  115 (74.6)  0.89

8 Minor macrophage staining  61 (35.2)  67 (43.5)  0.59

9 Minor collagen staining  31 (17.9)  26 (16.8)  0.50

10 Minor smooth muscle cell staining  45 (31.2)  44 (28.5)  0.91

11 **Table II.** Degree of carotid stenosis (as percentage) among females and males

<table>
<thead>
<tr>
<th>Degree carotid stenosis</th>
<th><strong>65 – 89 %</strong></th>
<th><strong>90 – 99 %</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Females (n = 107), n (%)</td>
<td>72 (67%)</td>
<td>35 (33%)</td>
</tr>
<tr>
<td>Males (n = 236), n (%)</td>
<td>154 (65%)</td>
<td>82 (35%)</td>
</tr>
</tbody>
</table>

12 **Table III.** Localization of neutrophils in different plaque area in relation to clinical manifestation of carotid stenosis prior to carotid endarterectomy

<table>
<thead>
<tr>
<th>Plaque area</th>
<th>Percentage plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-plaque hemorrhage</td>
<td>Asymptomatic (n=46)</td>
</tr>
<tr>
<td></td>
<td>26%</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Interface to media</td>
<td>39%</td>
</tr>
<tr>
<td>Shoulder and/or cap</td>
<td>48%</td>
</tr>
<tr>
<td>Underneath luminal endothelium</td>
<td>17%</td>
</tr>
</tbody>
</table>

**Supplemental Figure Legends:**

**Figure I.** Sections of rupture-prone plaque (A-C) showing CD66b (A), esterase (B) and MPO (C) expression in neutrophils.
Figure II. Neutrophils and β-blocker therapy. (A) Number of Neutrophils per plaque in relation to different beta-blockers and (B) to the time of treatment (less or more than 1 year). Bars represent means ± SEM; below each bar, the number of patients per group is indicated; statistics Mann-Whitney test, significance at p < 0.05.
Figure III. Number of neutrophils in plaque in relation to clinical presentation: asymptomatic (46) versus symptomatic (309), reported as minor or major events. TIA = transient ischemic attack; AF = amaurosis fugax; RI = retinal infarct. Kruskal-Wallis test, significance at p < 0.05.
Figure IV. Number of neutrophils in plaque in relation to the number of blood circulating granulocytes.

Reference List
