Sex Is Associated With the Presence of Atherosclerotic Plaque Hemorrhage and Modifies the Relation Between Plaque Hemorrhage and Cardiovascular Outcome

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Background and Purpose—Plaque hemorrhage (PH) may lead to accelerated progression of atherosclerotic disease. The presence of local PH in the carotid plaque predicts future cardiovascular events in any vascular territory. We investigated the prevalence of local PH and the predictive value of PH for the occurrence of cardiovascular events in men and women separately.

Methods—Atherosclerotic plaques from 1422 patients (969 men, 453 women) who underwent carotid endarterectomy were analyzed histologically for the presence of PH. Patients were monitored for 3 years for cardiovascular events (nonfatal stroke, nonfatal myocardial infarction, vascular death, and vascular intervention).

Results—Plaques from men showed a significantly higher prevalence of PH compared with women (67% versus 54%; \(P<0.001\)). In 1353 patients with available follow-up data, with a median duration of 2.9 years, 270 events had occurred in men (29%) and 94 in women (22%). Stratified by presence of PH, the event rate was 32% in men with PH versus 23% in men without PH, and 23% in women with PH versus 21% in women without PH. A multivariable Cox proportional hazards model found a significant interaction between sex and PH. PH was significantly associated with events in men (adjusted hazard ratio, 1.9; 95% CI, 1.2–2.8) but not in women (adjusted hazard ratio, 1.0; 95% CI, 0.6–1.7).

Conclusions—Atherosclerotic carotid plaques obtained from men reveal a higher prevalence of PH compared with women. Local PH is strongly related to secondary manifestations of cardiovascular disease in men but not in women. (Stroke. 2013;44:3318-3323.)

Key Words: atherosclerosis • carotid artery plaque • endarterectomy, carotid • epidemiology • prognosis • sex
Methods

Patient Selection

All studied patients were included in the Athero-Express biobank, a longitudinal study (observational, prospective) with patients undergoing CEA, as described in detail previously. In summary, all patients undergoing CEA at the University Medical Center, Utrecht, and St Antonius Hospital, Nieuwegein, the Netherlands, were asked to participate consecutively during the study period. There were no exclusion criteria. Indications for CEA were reviewed by a multidisciplinary vascular team and were based on recommended criteria of the Asymptomatic Carotid Atherosclerosis Study, the North American Symptomatic Carotid Endarterectomy Trial (NASCET), and the European Carotid Surgery Trial (ECST). Patients completed a questionnaire at baseline regarding medication use, cardiovascular risk factors, and medical history. The institutional review boards of the 2 participating hospitals approved the study, and all patients provided informed consent. For the present study, we examined available data from all unique patients included in the Athero-Express biobank from March 26, 2002, until December 31, 2010.

Tissue Collection, Tissue Processing, and Histological Examination

Atherosclerotic plaques were collected during conventional CEA and immediately brought to the laboratory for processing. According to a standardized protocol, plaques were divided into 5-mm segments along the longitudinal axis. The segment with the largest plaque burden was defined as the culprit lesion and was fixed in formaldehyde (4%), embedded in paraffin, and histologically examined. The remaining segments were immediately frozen using liquid nitrogen before storage at −80ºC and used for protein isolation.

Plaque characteristics were scored previously by 2 independent observers blinded to clinical outcome, with a good intraobserver and interobserver reproducibility. PH was analyzed by hematoxylin and eosin staining at original magnification ×20 and ×100. Because of fragmentation of the tissue, it was difficult to discriminate luminal thrombus from intraplaque hemorrhage; therefore, intraplaque hemorrhage and thrombus were both included in the definition of PH (Figure 1) as described previously. Erythrocytes at the border of the specimen were regarded artifacts and not included in the definition. Microvessel density (CD34) was measured quantitatively by a computerized protocol, by averaging the number of vessels in 3 fields with high density (×40 magnification), and counted on a grid to improve reproducibility and avoid double-counting.

Follow-Up and Clinical Outcome

Patients were monitored from day of inclusion for 3 years after surgery, with an ultimate follow-up in June 2012, using annual questionnaires. In addition, the patient records in the electronic hospital database were reviewed for cardiovascular end points. In case of no response or if a response suggested any vascular event, the general practitioner or specialist was contacted for further information. Outcome was defined as a composite end point, including any vascular death (including fatal myocardial infarction, fatal stroke, fatal abdominal aortic aneurysm rupture, fatal heart failure, and sudden death that was not otherwise specified), nonfatal myocardial infarction or nonfatal stroke, and any vascular intervention (both interventions for peripheral arterial disease, including lower limb amputations, and coronary interventions) that had not already been planned at the time of surgery. Two independent researchers validated the outcome. Lost to follow-up was defined as missing data on all follow-up moments.

Statistical Analysis

Variables were compared between men and women using the t test and Mann–Whitney U test, where applicable, for continuous variables and the χ² test for dichotomous variables. Event rates were presented as descriptive rates during the total follow-up period. Life table survival analysis was used to obtain annualized event rates. If a patient experienced multiple events during follow-up, only the first event was included in survival analyses. A probability value <0.05 (2-sided) was defined as statistically significant in all analyses.

A multivariable Cox proportional hazards model was used to obtain hazard ratios (HR) for PH in relation to outcome in men and women, adjusted for potential confounders. This model was built by starting with backward stepwise elimination of nonsignificant variables in an analysis with previously determined potential confounders for the relation between PH and outcome (age, body mass index, current smoking, hypertension, diabetes mellitus, glomerulator filtration rate, use of statins or dipyridamole or acetylsalicylic acid, contralateral carotid stenosis >50%, history of coronary artery disease, symptomatic carotid disease, plaque microvessel density). In this first model, a multiplicative interaction term between sex and PH was included. The remaining significant variables (including sex and PH separately) were subsequently entered in a Cox regression model simultaneously. To test the Cox proportional hazards assumption, a time-dependent variable of time×PH was added in this model. Thereafter, this analysis was repeated in the same way as before, but now stratified on sex (including all significant variables from the previous analysis and excluding sex, the interaction term sex×PH, and the time-dependent variable). This eventually led to 2 final models, 1 for men and 1 for women. In all intermediate (backward and enter) models, P<0.25 was used as cutoff, based on the likelihood ratio, to exclude variables from the model. In the final models, a 95% confidence interval (CI) not including 1 was regarded statistically significant. All statistical analyses were performed using SPSS version 19.0 (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY). All analyses were performed by the first author, who had full access to the primary data and takes responsibility for the integrity of the data and accuracy of data analysis.

Results

The study included 1422 patients, comprising 969 men and 453 women. Compared with men, women used acetylsalicylic acid more frequently (89% versus 83%; P<0.01); reported hypertension more often (81% versus 71%; P<0.001); had higher concentrations of total cholesterol (4.9 versus 4.6 mmol/L; P<0.001), low-density lipoprotein (2.9 versus 2.7 mmol/L; P=0.006), and high-density lipoprotein (1.3 versus 1.1 mmol/L; P<0.001); and a lower glomerular filtration rate...
(68 versus 77 mL/min; \(P<0.001\)). Previous coronary artery disease and bilateral carotid stenosis were observed less frequently in women (23% and 40%), versus 35% and 48% in men. The percentage of symptomatic patients at time of CEA was similar between men and women: 84% and 88%. All clinical characteristics are summarized in Table 1. The prevalence of PH was significantly higher in men than in women (67% versus 54%; \(P<0.001\)).

The total follow-up period in all patients had a median of 2.9 years (interquartile range, 1.3–3.1). Follow-up data were available for ≥1 follow-up moment in 1353 patients. Forty-two men were lost to follow-up (14 went to intramural care or were terminally ill, 5 moved abroad, 3 withdrew from follow-up, and no reason known for 20 patients). Among women patients, 27 were lost to follow-up (17 went to intramural care or were terminally ill, 2 withdrew from follow-up, and no reason known for 8 patients). In the remaining 927 men and 426 women, 270 events of the composite outcome occurred in men (29%) and 94 in women (22%) during total follow-up (Table 2). Annual event rates (number of events divided by number at risk entering each yearly interval) were 15%, 10%, and 7% in men, and 12%, 5%, and 5% in women. Most events were peripheral interventions (13% in men, 10% in women), followed by nonfatal stroke (5% in both sexes; Table 2).

Stratified by presence of PH, the composite event rate was 32% in men with PH and 23% in men without PH. In women, the event rate was 23% versus 21%. Total person-years of follow-up were 2091 in men and 993 in women.

In the multivariable Cox proportional hazards model with backward stepwise introduction of the selected potential

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**Table 1. Clinical and Plaque Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>All Patients (n=1422)</th>
<th>Men (n=969)</th>
<th>Women (n=453)</th>
<th>(P) Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>68.6 (9.2, 35–92)</td>
<td>68.4 (8.9, 40–88)</td>
<td>69.1 (9.8, 35–92)</td>
<td>0.17</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.4 (3.8, 15–52)</td>
<td>26.4 (3.3, 16–40)</td>
<td>26.4 (4.8, 15–52)</td>
<td>0.83</td>
</tr>
<tr>
<td>Current smoking</td>
<td>474/1357 (35)</td>
<td>310/929 (33)</td>
<td>164/428 (38)</td>
<td>0.08</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>314/1404 (22)</td>
<td>216/956 (23)</td>
<td>98/448 (22)</td>
<td>0.76</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1003/1357 (74)</td>
<td>652/921 (71)</td>
<td>351/436 (81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Use of ≥1 antihypertensive drugs</td>
<td>1068/1390 (77)</td>
<td>732/947 (77)</td>
<td>336/443 (76)</td>
<td>0.55</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>863/1298 (67)</td>
<td>581/886 (66)</td>
<td>282/412 (68)</td>
<td>0.31</td>
</tr>
<tr>
<td>History of peripheral intervention</td>
<td>251/1398 (18)</td>
<td>163/947 (17)</td>
<td>88/442 (20)</td>
<td>0.224</td>
</tr>
<tr>
<td>History of coronary artery disease or intervention</td>
<td>435/1392 (31)</td>
<td>331/948 (35)</td>
<td>104/444 (23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>Total</td>
<td>4.7 (1.2, 1.9–9.8)</td>
<td>4.6 (1.2, 1.9–9.3)</td>
<td>4.9 (1.3, 1.9–9.8)</td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>2.8 (1.0, 0.2–7.7)</td>
<td>2.7 (1.0, 0.6–7.7)</td>
<td>2.9 (1.1, 0.2–6.8)</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>1.2 (1.1, 0.2–6.2)</td>
<td>1.1 (0.4, 0.3–6.2)</td>
<td>1.3 (0.4, 0.2–2.8)</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>1.7 (1.0, 0.3–8.9)</td>
<td>1.7 (1.0, 0.3–8.9)</td>
<td>1.6 (1.0, 0.3–6.4)</td>
</tr>
<tr>
<td>Glomerular filtration rate, CG, mL/min</td>
<td>74.0 (26.5, 7.0–185.9)</td>
<td>76.6 (26.9, 7.0–185.1)</td>
<td>68.3 (24.8, 7.0–185.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein, median (IQR), nmol/L</td>
<td>29 (12–58)</td>
<td>28 (11–56)</td>
<td>31 (13–63)</td>
<td>0.08</td>
</tr>
<tr>
<td>Acetylsalicylic acid use</td>
<td>1182/1390 (85)</td>
<td>790/947 (83)</td>
<td>392/443 (89)</td>
<td>0.01</td>
</tr>
<tr>
<td>Dipiridamole use</td>
<td>736/1389 (53)</td>
<td>499/946 (53)</td>
<td>237/443 (54)</td>
<td>0.79</td>
</tr>
<tr>
<td>Statin use</td>
<td>1043/1389 (75)</td>
<td>704/946 (74)</td>
<td>339/443 (77)</td>
<td>0.40</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td></td>
<td></td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>212/1414 (15)</td>
<td>156/962 (16)</td>
<td>56/452 (12)</td>
<td></td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>208/1414 (15)</td>
<td>134/962 (14)</td>
<td>74/452 (16)</td>
<td></td>
</tr>
<tr>
<td>Transient ischemic attack</td>
<td>635/1414 (45)</td>
<td>431/962 (45)</td>
<td>204/452 (45)</td>
<td></td>
</tr>
<tr>
<td>Stroke</td>
<td>359/1414 (25)</td>
<td>241/962 (25)</td>
<td>118/452 (26)</td>
<td></td>
</tr>
<tr>
<td>Degree of ipsilateral stenosis</td>
<td></td>
<td></td>
<td></td>
<td>0.91</td>
</tr>
<tr>
<td>50%–70%</td>
<td>86/1377 (6)</td>
<td>60/936 (6)</td>
<td>26/441 (6)</td>
<td></td>
</tr>
<tr>
<td>70%–99%</td>
<td>1291/1377 (94)</td>
<td>876/936 (94)</td>
<td>415/441 (94)</td>
<td></td>
</tr>
<tr>
<td>Days from clinical event to CEA, if symptomatic, median (IQR)</td>
<td>45 (18–95)</td>
<td>45 (19–102)</td>
<td>44 (17–87)</td>
<td>0.08</td>
</tr>
<tr>
<td>Bilateral carotid stenosis (&gt;50%)</td>
<td>594/1302 (46)</td>
<td>429/893 (48)</td>
<td>165/409 (40)</td>
<td>0.01</td>
</tr>
<tr>
<td>Presence of plaque hemorrhage</td>
<td>884/1404 (63)</td>
<td>642/959 (67)</td>
<td>242/445 (54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Increased microvessel density†</td>
<td>628/1297 (48)</td>
<td>429/883 (49)</td>
<td>199/414 (48)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Continuous values are means, with SD and limits in parentheses, unless specified otherwise. Dichotomous variables are numbers of total (percentage). CEA indicates carotid endarterectomy; CG, Cockroft–Gault; HDL, high-density lipoprotein; IQR, interquartile range; and LDL, low-density lipoprotein.

*Comparison of men and women by univariable analysis.

†Above the median of vessel density.
that our results regarding PH and sex were not modified by symptom status before CEA. Furthermore, timing from event until surgery could potentially affect prevalence and difference of PH between the sexes, and delay between the clinical event and CEA has significantly decreased during the inclusion period. Therefore, we analyzed the influence of timing until surgery and observed that this did not affect our outcomes (data not shown).

Discussion
In cardiovascular disease, a validated difference exists in disease presentation, progress, and outcome between the sexes.\(^\text{17–19}\) In the presence of carotid artery disease, women have less benefit from CEA compared with men.\(^\text{1–4}\) The underlying pathophysiologic mechanisms that explain these sex-specific differences are poorly understood.\(^\text{5}\) The stability of the atherosclerotic plaque may be one of the factors that explain these findings. In line with previous observations showing a more unstable plaque phenotype in men,\(^\text{6}\) we observed that PH in the atherosclerotic plaque is more prevalent in men with carotid artery stenosis.

We previously hypothesized and confirmed that a local plaque can carry information about the systemic vascular status of a patient\(^\text{10,14,20}\) and specifically that PH in carotid plaques is predictive for events in all vascular territories.\(^\text{10}\) In the current study, we demonstrate that, independent of cardiovascular risk factors and symptom status before CEA, PH does not predict outcome in women, whereas in men, PH predicts a substantially and significantly increased risk of an event in any vascular territory after CEA. This further supports the view that the pathophysiology of disease progression in patients with atherosclerosis probably also differs between the sexes.

The mechanism that explains these sex-related observed differences is yet to be unraveled. The dissimilarity between the sexes in the predictive value of PH might point toward the existence of different pathways contributing to vulnerability and progression of disease in women. In women with suspected myocardial ischemia included in the Women’s Ischemia Syndrome Evaluation (WISE) study, levels of certain confounders (age, body mass index, current smoking, hypertension, diabetes mellitus, glomerulator filtration rate, use of statins or dipyridamole or acetylsalicylic acid, contralateral carotid stenosis >50%, history of coronary artery disease, symptomatic carotid disease, plaque microvessel density), there was a significant interaction between sex and PH on a multiplicative scale (\(P<0.001\)). In the subsequent model with simultaneous introduction of all remaining potential confounders from the backward stepwise model, PH was significantly associated with the composite end point (HR, 1.5; 95% CI, 1.1–2.1). The time-dependent variable of PH was not significant in this model (\(P=0.297\)), indicating that the Cox proportional hazards assumption was not violated. In the final multivariable models stratified for sex, the adjusted HRs for PH and outcome were 1.9 (95% CI, 1.2–2.8) in men and 1.0 (95% CI, 0.6–1.7) in women (Figure 2).

Symptomatic carotid stenosis was not associated with outcome because it had already been eliminated in the backward stepwise model with \(P=0.872\). In addition, when adding a multiplicative interaction term for symptoms and PH, this was also eliminated with a probability value of 0.753, indicating

Table 2. Rates of Individual End Points Contributing to the Composite End Point

<table>
<thead>
<tr>
<th></th>
<th>Men (n=927)</th>
<th>Women (n=426)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral interventions (PAD)</td>
<td>13%</td>
<td>10%</td>
</tr>
<tr>
<td>Coronary interventions</td>
<td>3.2%</td>
<td>2.8%</td>
</tr>
<tr>
<td>Nonfatal myocardial infarction</td>
<td>3.0%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Nonfatal stroke</td>
<td>5.4%</td>
<td>4.9%</td>
</tr>
<tr>
<td>Cardiovascular death</td>
<td>4.9%</td>
<td>3.1%</td>
</tr>
<tr>
<td>Composite end point</td>
<td>29%</td>
<td>22%</td>
</tr>
</tbody>
</table>

All event rates include only the first event per patient as recorded in the composite end point. Peripheral interventions include all interventions for PAD, both percutaneous and surgical, that were not planned at time of inclusion in this study. Coronary interventions include all interventions for coronary artery disease, including percutaneous coronary interventions and coronary artery bypass grafting, which were not planned at time of inclusion in this study. PAD indicates peripheral arterial disease.

Atherosclerotic Plaque Hemorrhage and Sex

**Figure 2.** Adjusted Cox regression survival curves of plaque hemorrhage (PH) and sex. Continuous lines represent patients with presence of PH; dashed lines, patients with absence of PH in men (A) and women (B). Numbers at risk at each moment in follow-up are depicted below the x axis. \(P\) value men, 0.002; women, 0.894. Survival was plotted from the Cox regression analysis, adjusted for previously determined potential confounders for the relation between PH and outcome (age, body mass index, current smoking, hypertension, diabetes mellitus, glomerulator filtration rate, use of statins or dipyridamole or acetylsalicylic acid, contralateral carotid stenosis >50%, history of coronary artery disease, symptomatic carotid disease, plaque microvessel density).
proinflammatory markers (high sensitivity C-reactive protein, serum amyloid A, and interleukin 6) were strongly associated with adverse cardiovascular events <5 years of follow-up. However, only a minor association was found with the extent of angiographic coronary artery disease, suggesting that inflammatory markers may contribute to disease progression without directly affecting atherogenesis.21 Naturally, this cannot be generalized to a male population with coronary artery disease. Besides, plaques from women demonstrate more superficial erosions22,23 that could increase coagulability via apoptosis of endothelial cells and activation of circulating tissue factor.24,25 Furthermore, genetic variations in tissue factor genes seem to be associated with sex. In a coronary heart disease population, the prevalence of tissue factor 5466A/G single nucleotide polymorphism was higher in women than in men.26 In another study, this single-nucleotide polymorphism was associated with an increase of tissue factor activity in monocytes from healthy donors after stimulation with lipopolysaccharide through higher expression of mRNA levels.27 This may also be a mechanism resulting in a hypercoagulable state in women, which could be independent of plaque-related mechanisms. Relating to this hypercoagulability in a clinical setting, women show a higher embolic rate after CEA,28,29 which is associated with cardiovascular complications after this surgical procedure.29 In addition, microvascular disease may play a bigger role in women in predicting adverse cardiovascular events. For instance, in women with suspected myocardial ischemia, an impaired endothelium-independent microvascular response toward adenosine was shown to be a strong predictor for adverse events.30 Moreover, by using indicators of small vessel disease in the brain (silent brain infarcts and white matter lesions) in a population-based elderly cohort, stroke prediction was significantly improved, especially in women.31

Possible Future Clinical Implications
This prospective atherosclerotic biobank study has shown that PH is a marker for progression of the disease in men only. This underlines the need for research that acknowledges the heterogeneity imposed by sex. A promising diagnostic and prognostic tool to implement our observation in sex-specific risk stratification is noninvasive plaque imaging. Imaging studies have previously shown that MRI-detected PH was associated with recurrent ipsilateral cerebrovascular events in patients with high-grade symptomatic carotid stenosis11 as well as in asymptomatic men with moderate stenosis.12 Our findings put these studies into perspective, because detection of high-risk patients for clinical prediction based on this marker may not be applicable to women.

Limitations
This study has some limitations. First, the definition of the presence of PH is a challenge, because intraplaque hemorrhage and luminal thrombus cannot always be distinguished reliably because of surgical artifacts (removal of the plaque by length arteriotomy whereby damage and fragmentation of the plaque can occur). We avoided this uncertainty in histological characterization by using a composite of intraplaque hemorrhage and thrombus. For better understanding of the mechanisms behind the effects of PH and sex differences, the distinction could be important. However, this is not a limitation for the sex-associated differences we found, because the same definition was used in both sexes. Second, this study could not assess racial differences because information about ethnicity is not recorded in our biobank. However, nearly all patients were assumed to be white. Finally, a possible change of cardiovascular risk factors, used as confounding variables in our multivariable analysis, was not recorded during follow-up and could thus not be taken into account.

Conclusions
From this study, we conclude that atherosclerotic carotid plaques obtained from men reveal a higher prevalence of PH than those from women. Local PH is significantly related to adverse cardiovascular outcome in men but not in women. This contributes to our understanding of sex differences in cardiovascular disease that can currently play a role in noninvasive imaging and help to target sex-specific therapies in the future, such as selecting patients for CEA based on plaque characteristics.

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Disclosures
Drs Pasterkamp, de Kleijn, and Moll are cofounders of Cavadis BV, a biomarker company. The other authors have no conflicts to report.

References


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