Carotid Atheroma From Men Has Significantly Higher Levels of Inflammation and Iron Metabolism Enabled by Macrophages

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Background and Purpose—Men differ from women in the manifestation of atherosclerosis and iron metabolism. Intraplaque hemorrhage and hemoglobin (Hb) catabolism by macrophages are associated with atherosclerotic lesion instability. The study aims were to investigate sex differences in (1) lesion severity in relation to blood Hb, (2) iron homeostasis in human carotid plaques, and (3) macrophage polarization within atheroma.

Methods—The carotid artery samples from 39 men and 23 women were immunostained with cell markers for macrophages, smooth muscle cells, ferritin, and TfR1 (transferrin receptor 1), which were further analyzed according to sex in relation to iron, Hb, and lipids in circulation. Additionally, samples of predefined regions from human carotid atherosclerotic lesions, including internal controls, were used for proteomic analysis by mass spectrometry.

Results—Male patients, compared with women, had larger necrotic cores and more plaque rupture, which were associated with higher levels of Hb. Atheroma of male patients had significantly higher levels of Hb in circulation and CD68 macrophages, ferritin, and TfR1 in lesions. CD68 macrophages were significantly correlated with ferritin and TfR1. Plaques from male patients comparatively possessed higher levels of inflammatory macrophage subsets, CD86 (M1) and CD163 (M2), but lower levels of STF (serotransferrin) and HPX (hemopexin).

Conclusions—Male patients with carotid atheroma had more advanced and ruptured lesions associated with significantly higher levels of inflammatory macrophage infiltration and high iron stores in the blood and in their plaques. These findings help to understand sex differences and iron metabolism in atherosclerosis and factors related to atheroma progression. **(*Stroke*. 2018;49:419-425. DOI: 10.1161/STROKEAHA.117.018724.)**

Key Words: atherosclerosis ■ ferritins ■ hemoglobins ■ hemopexin ■ macrophages ■ male

Atherosclerosis-related cardiovascular disease (CVD) is the leading cause of mortality and morbidity in many countries. It is well known that men differ from women in epidemiology, symptoms, and progression of CVD. The recent trends show a reduction of incidence and mortality for CVD at all age groups, except for middle-aged women, where there has been no decline. The clinical outcomes, including myocardial infarction mortality, all-cause mortality, and reinfarction rates, are worse in women with CVD than in men.[1] Moreover, the stroke-protective effect of carotid endarterectomy is greater in men, and the recurrence rate of ipsilateral stroke 5 years after the surgery is lower in men than in women.[2]

As one of alternative explanation for the sex difference in the incidence and mortality of atherosclerosis, Dr Sullivan in 1981 proposes that the greater incidence of heart diseases in men and postmenopausal women compared with the incidence in premenopausal women is because of higher levels of stored iron.[3] However, it cannot be excluded that the cardiovascular effects are a consequence of the older age of menopausal women.[4] Based on several conclusive experimental studies, and conflicting epidemiological studies, Dr Sullivan further suggests that the development of atherosclerosis may associate with accumulated iron in arterial plaque as a modifiable risk factor.[5] This, in turn, may lead to iron-driven oxidative stress and activation of signaling in atherosclerotic vessel walls,[6] where we have further revised the hypothesis.

Recent data suggest that intraplaque hemorrhage is associated with the development of atherosclerotic lesions and plaque instability. Histological observations and magnetic resonance imaging have identified intraplaque hemorrhage as the main determinant of plaque evolution toward rupture.[7] Iron accumulation because of hemoglobin (Hb) breakdown in

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the process can act as a catalyst in the formation of free radicals that modify low-density lipoprotein cholesterol and foam cells. Thus, iron accumulation deposits are not only a histological marker of previous hemorrhage but also an important catalyst of reactive oxygen species, which could activate the redox state inducing inflammatory reaction, leading to coronary plaque instability.[8] However, it is unknown whether Hb in the circulation is related to the progression of human atherosclerotic lesions in men and women.

Several iron metabolism proteins in atherosclerotic lesions have been suggested as potential markers to investigate the involvement of iron in atherogenesis, including ferritin, transferrin, and TfR1 (transferrin receptor 1).[5] However, it remains unknown whether the lesion-related cellular iron uptake by macrophages differs between men and women.

Macrophages are central immune cells in atherosclerosis and are of importance in the progression of the atherosclerotic plaque. The phenotype of macrophages in atherosclerotic lesions can be polarized by the local microenvironment in the lesion as proinflammatory (M1) and anti-inflammatory (M2).[9] Although lower levels of CD68-positive macrophages have been found in carotid atheroma in plaques obtained from women,[10] it is unknown whether macrophage subtypes in human carotid plaques differ between men and women.

In the present study, we aimed to investigate (1) whether levels of blood Hb are related to plaque severity in men and women, (2) whether human carotid plaques from men and women differ in protein expression of iron homeostasis, and (3) whether macrophage polarization differs in human carotid plaques between men and women.

Materials and Methods

The data that support the findings of this study are available from the corresponding authors on reasonable request.

Linköping Carotid Study and Human Carotid Atheroma

The Linköping Carotid Study is a prospective clinical pathology study in which atherosclerotic carotid arteries are collected from patients who undergo carotid endarterectomy at Linköping University Hospital. The local ethics committee of Linköping University Hospital has approved this study, and all procedures were carried in accordance to accepted guidelines. Written informed consent was obtained from all patients included within the study.

The study included 62 atherosclerotic carotid samples obtained from consecutive patients (39 men and 23 women). A detailed clinical history of each patient was taken, and patient characteristics are presented in Table I in the online-only Data Supplement. All patients underwent preoperative carotid artery duplex ultrasound scanning and had ≥50% stenosis. The average maximum peak in-flow velocity of the internal carotid artery was 3.95 m/s at 60° insonation.

Plaque Processing and Immunohistochemistry

After surgical excision, carotid artery samples were collected and equally cut into 2 parts. One part was snap-frozen and saved at −80°C for biochemical analysis. Another part was dissected into 3 to 5 segments (≈5 mm), fixed in 4% formaldehyde and embedded in paraffin for serial sections.

Immunohistochemistry was performed on serial sections, as described previously.[11] The primary antibodies used were CD68 clone PG-M1 (Dako, Denmark), smooth muscle actin clone 1A4 (Dako), CD86 (R&D Systems, United Kingdom), and CD163 (Santa Cruz Biotechnology, TX), ferritin (Dako), and TfR1 (Alpha Diagnostic International, TX). The immunoreactions were visualized using the EnVision+/HRP (horseradish peroxidase; Dako) method and ChemMate EnVision Detection Kit (Dako). Omission of the primary antibodies or isotype controls served as negative controls.

Classification of the Plaques

To examine whether men and women differ in plaque severity, the carotid sections were classified into 3 groups based on their morphology and collagen staining, as described previously.[11] In brief, type 1 plaques were intact plaques without necrotic cores; type 2 plaques were also intact plaques with necrotic cores; and type 3 plaques were ruptured plaques, often containing a large necrotic core, cholesterol crystals, intraplaque hemorrhage, or thrombosis.

Image Analysis

All immunostained samples (3–5 sections per sample) were digitalized with the same setting for all the samples (8 images per section) and analyzed with Adobe Photoshop as described previously.[12] Immunostained sections with CD86 and CD163 were initially classified by microscopy with grids under a 10× microscopic field, as negative expression (<5%), low expression (5%–25%), moderate expression (≥25% and <75%), and high expression (≥75%). For statistical analysis, we combined the negative- with low-expression sections as a group named low expression and moderate- and high-expression sections as a group named high expression. Histological assessments were divided between 2 observers who were blinded to plaque classification and patient characteristics; both observers were trained to perform the same methodology in terms of region selection and analysis.

Mass Spectrometry Analysis

Carotid atherosclerotic lesions from 10 men and 10 women were used for mass spectrometry analysis. Lesion sampling was performed by intraplaque biopsies of predefined pathologically distinct regions, including internal control, fatty streak, and plaque core. Proteins were extracted from each biopsy, as described previously.[13]

Protein extracts from 60 biopsies (3 biopsies per carotid lesion from each patient) were prepared for mass spectrometry analysis as described previously.[13] Separation and analysis of protein samples were performed using liquid chromatography (Easy nLC; Thermo Scientific, MA) and tandem mass spectrometry (Orbitrap Velos Pro; Thermo Scientific). Spectra were processed using MaxQuant v1.5.3 (Max Planck Institute of Biochemistry, Germany) to search against the UniProt human protein database, using previously described parameters.[13]

Statistical Analysis

Statistical analysis were performed using SPSS 24.0 (IBM, United Kingdom). Continuous data are expressed as mean±SEM. Differences were compared by nonparametric Mann–Whitney *U* test. Nominal data were examined by a χ^2 test. Correlations between immunohistochemical-positive areas for CD68, CD86, CD163, TfR1, and ferritin were examined by the Spearman correlation test and presented as the Spearman correlation coefficient (ρ). A P value <0.05 was considered statistically significant, and all tests were 2 tailed.

The influence of potential confounders on the levels of Hb in circulation and CD68, ferritin, TfR1, and smooth muscle actin in lesions, was accessed by multivariable logistic regression. These outcome variables were grouped into 2 groups, low and high levels, using a median as a cutoff. A binary logistic model with a probability for stepwise entry (0.05) and removal (0.10) was performed. After a Bonferroni correction for multiple comparisons, a P value <0.008 was considered significant.

Mass spectrometry data were analyzed by Mann–Whitney *U* test, with P<0.05 considered significant. Sex differences were analyzed using a cumulative protein abundance per patient, summing the 3

biopsies. Plaque region differences were analyzed using individual biopsy values.

Results

Male Patients With Advanced Carotid Atherosclerotic Lesions Had Significantly Higher Levels of Hb in the Blood and Lesions

Human carotid plaques from 62 patients were divided into 2 groups according to sex. Serum levels of iron, Hb, and iron-related proteins were measured (Table II in the online-only Data Supplement). Levels of serum Hb, and mean cell Hb concentration were significantly higher in male patients although still within the normal range. There was no significant statistical difference in serum iron levels between men and women. The levels of iron transport protein transferrin were significantly lower, whereas simultaneously levels of transferrin saturation were higher in the male patients (Table II in the online-only Data Supplement).

Male patients showed significantly high levels of Hb in the serum, which was independent to potential confounders (Table); thus we classified the plaques according to serum Hb levels. We found that the majority of male patients had Hb levels >140 g/L in contrast female patients (Figure 1A). Mass spectrometry analysis of plaque samples revealed that male patients had higher levels of Hb (α and β subunits) in their carotid atherosclerotic lesions (Figure 1B). Plaques from male patients had predominately type 3 lesions, ruptured plaques (Figure 1C), and among these patients, serum Hb levels were predominately >140 g/L (Figure 1D).

Plaques of Male Patients Have Significantly Higher Levels of Ferritin and TfR1 and Significantly Lower Levels of STF and HPX

We next investigated iron storage protein ferritin and iron transport protein TfR1 in carotid plaques from men and women. Plaques from male patients showed significantly higher levels of ferritin and TfR1 as compared with women as assayed by immunohistochemistry (Figure 2A). Subsequent regression analyses did not show a significant independent relationship between the male sex and TfR1 or ferritin; however, a trend is evident with TfR1 (Table). The ferritin subtype was further quantified by mass spectrometry, and lesions of men had lesion-dependent increases in expression of ferritin heavy and light chains (Figure 2B). A higher level of ferritin light chain was found in the fatty streak in lesions from male patients. The similar tendency of increased ferritin heavy chain was determined in the fatty streak and plaque centers from male lesions (Figure 2B).

Moreover, assay of plaque samples by mass spectrometry showed significantly lower levels of STF (serotransferrin) and HPX (hemopexin) in atherosclerotic tissues from male patients (Figure 3A). The differences in STF and HPX were already

Table. Associations Between Sex and Serum Hb, CD68, Ferritin, TfR1, and Smooth Muscle Actin, Adjusted for Potential Confounders by Multivariable Logistic Regression

	CD68	Ferritin	TfR1	SMC	Hb
	OR (CI)	OR (CI)	OR (CI)	OR (CI)	OR (CI)
Sex					
Female	Reference	Reference	Reference	Reference	Reference
Male	5.32 (0.98–28.95)	2.02 (0.43–9.57)	5.02 (0.87–29.04)	0.43 (0.09–2.05)	8.17 (1.28–52.18)*
Age, y					
50–69	Reference	Reference	Reference	Reference	Reference
70+	0.67 (0.12–3.66)	0.65 (0.12–3.42)	0.41 (0.08–2.17)	0.50 (0.10–2.42)	0.64 (0.12–3.46)
Hypertension					
No	Reference	Reference	Reference	Reference	Reference
Yes	0.74 (0.12–4.66)	0.42 (0.06–2.70)	0.72 (0.12–4.18)	0.72 (0.13–3.93)	1.14 (0.18–7.09)
Statin					
No	Reference	Reference	Reference	Reference	Reference
Yes	0.51 (0.10–2.50)	0.36 (0.07–1.57)	1.10 (0.22–5.45)	0.83 (0.19–3.71)	2.88 (0.51–16.28)
Diabetes mellitus					
No	Reference	Reference	Reference	Reference	Reference
Yes	0.95 (0.13–7.22)	1.19 (0.18–8.01)	3.47 (0.42–28.44)	0.44 (0.07–2.98)	2.25 (0.19–26.86)
Smoking					
No	Reference	Reference	Reference	Reference	Reference
Yes	0.64 (0.12–3.37)	0.94 (0.19–4.63)	2.58 (0.48–13.83)	0.39 (0.08–1.94)	1.31 (0.23–7.43)

CIs adjusted for multiple comparisons by Bonferroni correction. CI indicates confidence intervals; Hb, hemoglobin; OR, odds ratio; SMC, smooth muscle cell; and TfR1, transferrin receptor 1.

*P=0.004.

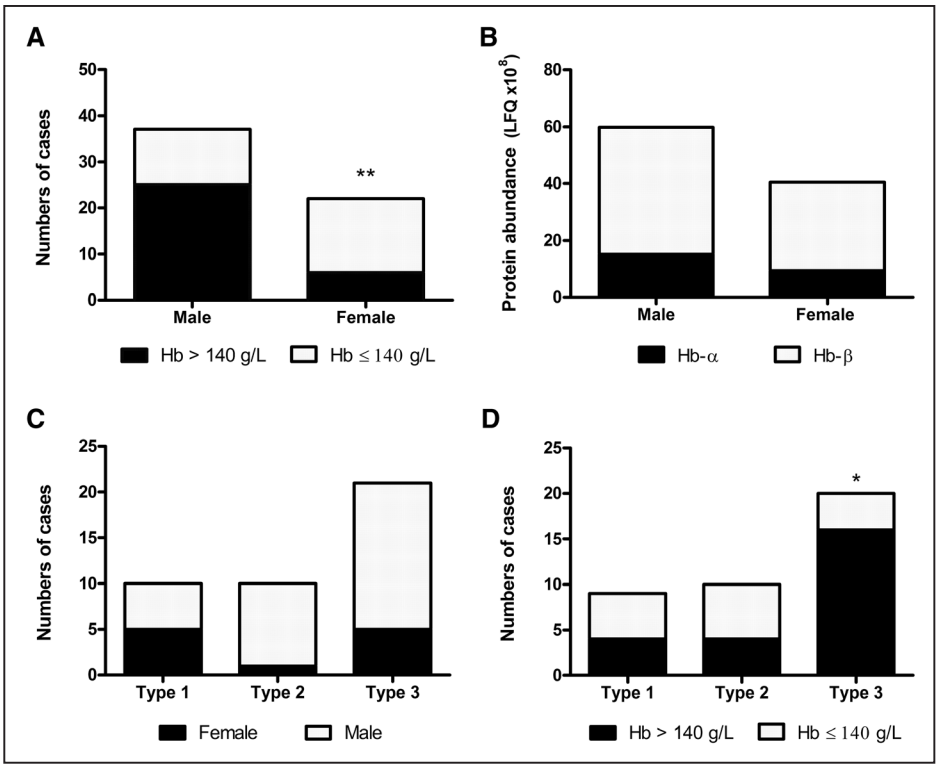

Figure 1. Male patients with advanced carotid atherosclerotic lesions had significantly higher levels of Hb (hemoglobin) in the blood and plaques. **A**, Male patients had significantly higher levels of serum Hb (high/low Hb: n=25/12 for men, n=6/16 for women). ***P*<0.01. **B**, Male patients had a lesion-dependent increase of Hb (α+β) in their plaques, assayed by mass spectrometry (n=10 for both men and women). **C**, Plaques from male patients had predominately type 3 plaques (type 1/2/3: n=5/9/16 for men, n=5/1/5 for women). **D**, Among patients with type 3 plaques, serum Hb levels were predominately >140 g/L (type 1/2/3: n=4/4/16 with high Hb, n=5/6/4 with low Hb). **P*=0.05 vs type 1 and type 2 plaques. LFQ indicates label-free quantification.

seen in the internal control samples, as well as fatty streak and plaque center of lesions (Figure 3B). Prominently, there was a clear lesion-dependent increase in LTF (lactotransferrin) detected in plaque centers, and the level was higher in lesions from male patients (Figure 3B). Moreover, there was a lesion-dependent decrease in HPX in male patients (Figure 3B).

We previously demonstrated that ferritin and TfR1 were positively correlated with macrophage infiltration and severity of human carotid plaques.[11] Here, we further questioned whether the correlation between CD68 and ferritin or TfR1 differ between men and women. We found that the levels of CD68-positive macrophages were significantly correlated with levels of ferritin and TfR1 in the lesions from both male and female patients (Figure I in the online-only Data Supplement).

Plaques From Male Patients Possess Significantly Higher Levels of CD68 Macrophages

Macrophages and smooth muscle cells are 2 major types of cells in human carotid plaques. Plaques from male patients frequently had pronounced accumulation of CD68-positive macrophages (Figure IIA in the online-only Data Supplement) but lower levels of smooth muscle actin-positive cells in the serial sections (Figure IIA in the online-only Data Supplement). The exemplified photographs of carotid plaques were taken from asymptomatic patients in both men and women. In contrast to men, plaques from female patients showed few diffuse CD68-positive macrophages in the intima and clearly more smooth muscle actin-positive cells in the cap and even in the intima areas (Figure IIA in the online-only Data Supplement). The image analysis results show that the atheroma of men had significantly higher levels of CD68-positive macrophages, which were >2× higher than women (Figure IIB in the online-only Data Supplement). Multivariable logistic regression showed a trend toward a relationship between high CD68 levels and the male sex, albeit not significant on adjustments for multiple comparisons with Bonferroni correction (Table). The levels of positive areas of smooth muscle cells were slightly lower in the plaques from male patients as compared with women, although the difference was statistically insignificant (Figure IIC in the online-only Data Supplement).

Plaques From Male Patients Possess Higher Levels of Inflammatory Macrophage Subsets, CD86 (M1) and CD163 (M2)

Macrophages identified in human atheroma are heterogeneous and are classified as a classical proinflammatory profile (M1) or an alternative anti-inflammatory and reparatory phenotype (M2).[14] We further investigated whether plaques from men and women differ in levels of M1 and M2 macrophages as determined by markers CD86 and CD163, respectively, and whether they are related to levels of CD68-positive cells. Generally, positive immunohistochemical staining of CD68, CD86, and CD163 showed a close correlation in the same areas among most of plaques. The percentage of positive CD86 in CD68-positive

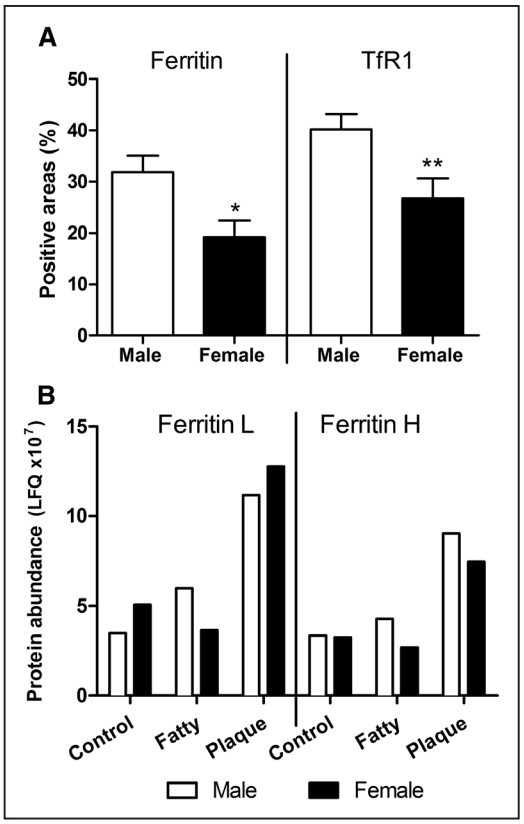

Figure 2. Expression levels of ferritin and TfR1 (transferrin receptor 1) were significantly higher in male plaques than female ones. **A**, Quantification of the expression levels of ferritin and TfR1 in human carotid atherosclerotic lesions by image analysis of immunohistochemistry (n=39 for men and 23 for women). **B**, Expression of ferritin-H and ferritin-L was lesion dependently increased in human carotid atherosclerotic lesions, assayed by mass spectrometry (n=10 for both men and women). LFQ indicates label-free quantification.

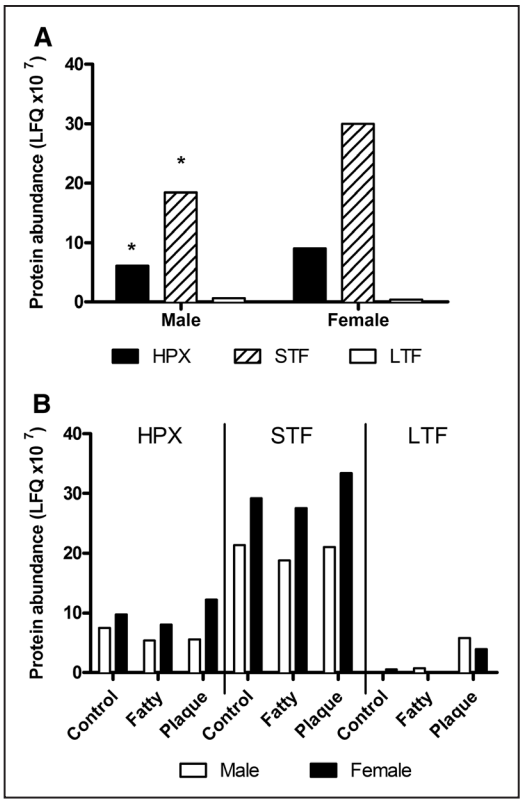

Figure 3. Expression levels of HPX (hemopexin) and STF (serotransferrin) were significantly lower in carotid atherosclerotic lesions of male patients, and the difference was lesion independent. Samples from 10 male and 10 female patients were assayed by mass spectrometry. **A**, Quantification of the expression levels of STF, HPX, and LTF (lactotransferrin). *P<0.05 vs women. **B**, The differences in STF and HPX abundance between men and women was seen across all lesion regions. Prominently, there was a clear increase in LTF within the plaque center of lesions. LFQ indicates label-free quantification.

areas was 46.5%, and the percentage of positive CD163 in CD68-positive areas was 53.8%. The expression levels of CD68-positive macrophages were significantly correlated with the positive areas of CD86 (Figure 4A) and CD163 (Figure 4B). The plaques from male patients contained more pronounced infiltration of CD68 (Figure IIB in the online-only Data Supplement), CD86, and CD163 (Figure 4C and 4D), as compared with plaques of women. Scoring analysis of plaque immunohistochemistry showed that compared with women, more plaques from male patients had remarkably higher levels of CD86 M1 and CD163 M2 macrophages (Figure 4C). Furthermore, results of image analysis from matched plaque areas revealed that plaques of male patients had significantly higher levels of CD86 and CD163 than female patients (Figure 4D).

Discussion

Although men and women do differ in mortality and morbidity in atherosclerosis-related diseases, little is known on sex differences in plaque iron metabolism in atherosclerosis. In the present study, we demonstrate for the first time that high levels of Hb in the circulation associate with advanced and ruptured atherosclerotic lesions and that lesions from male patients have significantly higher levels of CD68-positive macrophages together with high levels of TfR1 and ferritin. Moreover, lesions from male patients have low levels of STF, which is consistent with the lower levels of transferrin in blood sample in male patients.

Hb—an iron-containing oxygen-transport metalloprotein in red blood cells—has been considered as a risk factor of atherosclerosis-related CVD.[15,16] Although Hb levels in the present study are within the normal ranges in both sexes, men indeed have a significantly higher level of Hb. In the process of atherosclerosis, extracellular Hb[17] contributes to the proinflammatory nature of high-density lipoprotein.[18] Serum-free Hb may serve as a novel potential biomarker for the diagnosis of acute ischemic stroke[16] and myocardial infarction.[19]

HPX—a known heme-binding protein—has been considered as antiatherogenic because of its capacity to prevent oxidation of low-density lipoprotein and atheroma lipids mediated by Hb.[20] It plays a protective role in alleviating heme-induced oxidative stress, improving inflammatory properties of high-density lipoprotein, and inhibiting the development of atherosclerosis in apoE$^{-/-}$ mice.[21] HPX by scavenging heme liberated from Hb in atherosclerotic lesions defends against oxidative stress and related inflammatory events in atherosclerosis. Therapeutic administration of HPX is beneficial to counteract heme-driven macrophage-mediated inflammation.[22] Our

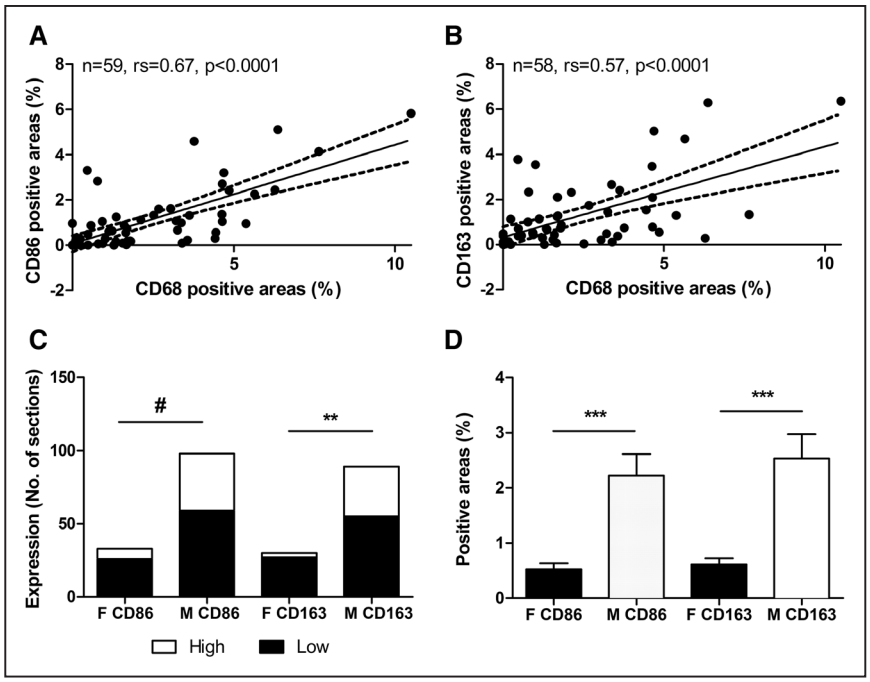

Figure 4. CD68-positive macrophages are significantly related to polarized macrophage subsets, CD86 (M1) and CD163 (M2), which are both significantly increased in carotid lesions from male patients in comparison with women. Human carotid plaques were immunostained with antibodies against CD68, CD86, or CD163, and images of the immunohistochemistry were analyzed as described in Methods. **A** and **B**, The expression of CD86 (**A**) and CD163 (**B**) was significantly correlated with CD68-positive macrophages in human carotid atherosclerotic lesions. **C**, Semiquantification of expression levels of CD86 and CD163 showed significantly higher levels of CD86 and CD163 in atherosclerotic lesions of men (CD86/CD163 sections: n=98/89 for men, n=33/30 for women). #*P*=0.06, ***P*<0.01. **D**, Image analysis of expression levels of CD68, CD86, and CD163 on matched photographs of serial sections (n=19 for men and 40 for women). ****P*<0.001.

results on the lower level of HPX in male patients suggest that the antiatherogenic role of HPX may contribute to sex difference in atherosclerotic process in vessel walls and related vascular diseases.

Iron deposition in the plaque, especially in macrophages, may contribute to plaque development and severity, as demonstrated in several animal models.[23,24] It is rarely investigated whether circulated iron-binding proteins are related to plaque iron metabolism in atherosclerosis. Here, we show that plaques from men have significantly higher levels of cellular iron uptake, in form of parallel increases in ferritin and TfR1. The increased ferritin and TfR1 in macrophages may not only function for cellular iron metabolism but also for inflammatory reactions, as demonstrated in patients with peripheral arterial disease.[25]

Higher levels of macrophage infiltration in form of both M1 and M2 shown in male lesions suggest a higher level of inflammatory activity in carotid plaques that may affect plaque vulnerability. Our results are consistent with previous findings[10] and further propose that plaques obtained from women have less inflammatory phenotypes of macrophages. In addition, CD86-positive M1 and CD163-positive M2 macrophages are localized in same regions of CD68-positive macrophages with higher levels of cellular iron deposit, which suggests that ferritin and TfR1 may be potential markers of macrophage activation in the atherosclerotic process.

Atherosclerotic plaques from male patients tend to develop more vulnerable or risky plaques identified by necrotic core formation and plaque rupture that is supported by previous studies showing that men have a greater plaque area than women at all ages, which has been suggested as a predictor for stroke and myocardial infarction.[26] In our study, this is associated with higher levels of circulating Hb, increased iron uptake, and higher levels of inflammatory activities driven by M1 and M2 macrophages in male plaques. The underlying mechanisms for the increased plaque vulnerability in male subjects call for further investigation.

The present study focusses primarily on the pathology of carotid atherosclerotic lesions from men and women. These carotid lesions have been removed as either the result or as a preventative measure of stroke. Thus, the conclusions presented in this study have their limitations in that they are more indicative as possible risks for plaque stability and rupture, rather than of plaque development. Factors such as age, statin treatment, diabetes mellitus, hypertension, and smoking were accessed, and there were no significant differences between men and women (Table I in the online-only Data Supplement). However, several other factors may also affect atherosclerotic progression, including hormones, epi/genetics, stress, and environmental exposures. Additionally, although the high levels of Hb in men were found to be independent of potential confounders (Table), it must be noted that these regressions are based on a limited number of patients, hence results come with large confidence intervals. The use of the Bonferroni correction, although limiting the risk of false-positive results, also carries the risk of increasing false-negative results. Thus, the trending association of high levels of CD68 and TfR1 with the male sex (Table) should not be fully excluded as possible risk factors. A larger prospective study, with a broader patient characteristic profile, assessing iron import and storage in the vessel wall would further aid in evaluating the results presented in this article.

In conclusion, men differ from women in macrophage-driven inflammation and iron metabolism in both the circulation and the lesions, which is significantly associated with the progression of carotid atherosclerosis. These findings improve our understanding of sex difference in atherosclerosis and related clinical relevance.

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Disclosures

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SUPPLEMENTAL MATERIAL

Carotid atheroma from men have significantly higher levels of inflammation and iron metabolism enabled by macrophages

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Supplemental Table I. Basic clinical information of male and female patients with carotid atherosclerosis

Supplemental Table II. Serum haemoglobin (Hb), mean cell volume (MCV), mean cell Hb concentration (MCHC), serum iron, and lipid profiles in male and female patients with carotid atherosclerosis.

Supplement Figure I. The expression of ferritin and TfR1 in carotid atherosclerotic lesions was significantly correlated to levels of CD68 positive macrophages in both sexes. Quantitative analysis of the immuno-stained sections showed that ferritin (A and C) or TfR1 (B and D) are significantly correlated with levels of CD68 positive macrophages in carotid atherosclerotic lesions in males (A and B) and females (C and D), analysed by means of image analysis of immunohistochemistry of CD68, ferritin or TfR1 on serial sections, as described in Methods.

Supplement Figure II. Carotid atherosclerotic lesions from male patients had significant higher levels of CD68 positive macrophages than the ones from female patients. The serial sections of human carotid plaques were immuno-stained with antibodies against CD68 or SMA and analysed by means of image analysis of immunohistochemistry. (**A)** Representative photographs of CD68 and SMA from male (indicated by M) or female (indicated by F) patients. Bars = 100 μm. (**B** and **C)** Image analysis of expression levels of CD68 (**B**) or SMA (**C**) in atherosclerotic lesions from males and females (n = 39 for males, n = 23 for females). Note: CD68 positive areas were significantly higher in the lesions of male patients (**$p < 0.01$), while SMA levels were slightly decreased.

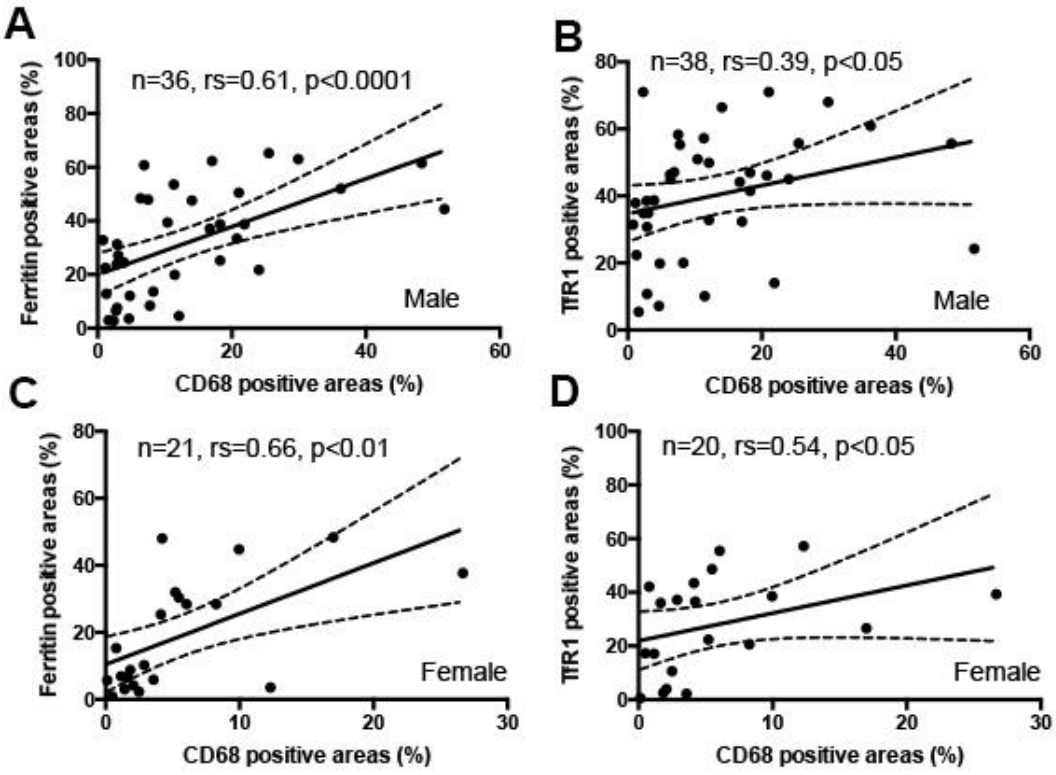
Supplemental Table I. Basic clinical information of male and female patients with carotid atherosclerosis.

	Male	**Female**	***P* value**
n	39	23	
Age, years ± SEM	72.0 ± 1.2	71.5 ± 1.8	0.95
Statin treatment, % (n)	41 (16)	52.2 (12)	0.55
Diabetes mellitus, % (n)	15.4 (6)	21.7 (5)	0.53
Hypertension, % (n)	71.8 (28)	78.3 (18)	0.68
Smoking, % (n)	30.8 (12)	52.2 (12)	0.24
Duplex ilat, m/s ± SEM	3.8 ± 0.2	4.19 ± 0.3	0.25
Time from symptom to surgery, days ± SEM	48.5 ± 5.6	64.6 ± 13.7	0.55

Supplemental Table II. Serum haemoglobin, mean cell volume (MCV), mean cell Hb concentration (MCHC), serum iron, and lipid profiles in male and female patients with carotid atherosclerosis.

	Male (mean ± SEM)	**Female (mean ± SEM)**	***P* value**
Haemoglobin, g/L	144.7 ± 1.7	135.2 ± 2.5	**0.003***
MCV, fL	93.2 ± 0.7	88.4 ± 3.0	0.072
MCHC, g/L	333.5 ± 1.9	312.9 ± 14	**0.015***
Iron, µmol/L	13.5 ± 1.1	13.5 ± 1.8	0.880
Transferrin, µmol/L	2.0 ± 0.1	2.4 ± 0.1	**0.031***
Transferrin saturation, %	29.2 ± 2.4	22.9 ± 3.2	0.091
Total cholesterol, mmol/L	4.99 ± 0.3	5.24 ± 0.2	0.385
LDL cholesterol, mmol/L	2.58 ± 0.2	3.12 ± 0.3	0.095
HDL cholesterol, mmol/L	1.22 ± 0.1	1.26 ± 0.1	0.617
Triglycerides, mmol/L	2.74 ± 0.6	1.99 ± 0.2	0.520

Significance ***$p < 0.05$



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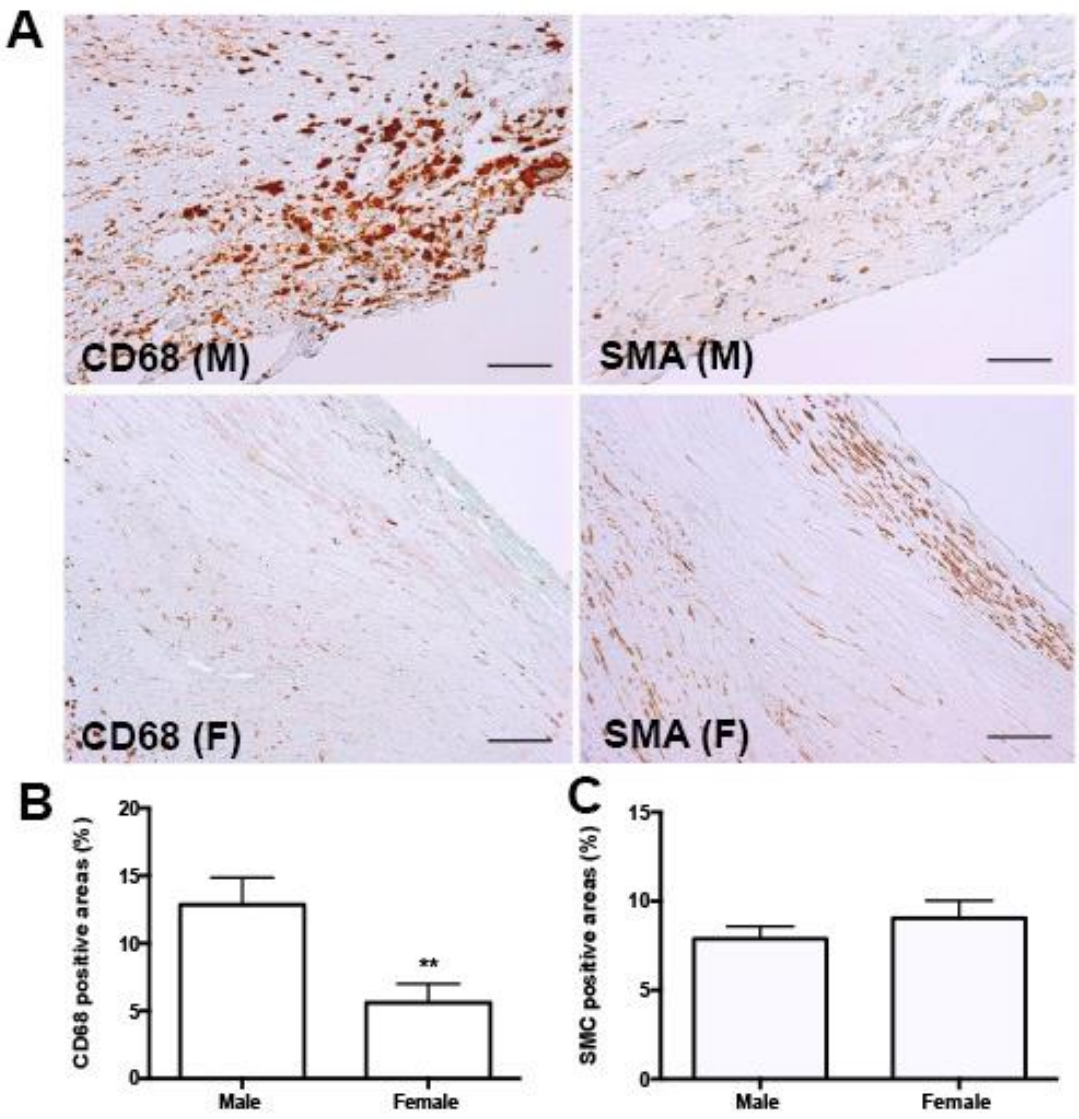

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