Peripheral Artery Disease Is Associated With a High CD163/TWEAK Plasma Ratio

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Objective—In addition to its role in the clearance of haptoglobin-hemoglobin (Hp-Hb) complexes, CD163 is a macrophage scavenger receptor for tumor necrosis factor–like weak inducer of apoptosis (TWEAK). We recently reported that the CD163/TWEAK plasma ratio could be a potential biomarker of atherothrombosis in asymptomatic subjects. In this study, we assessed soluble TWEAK (sTWEAK) and soluble CD163 (sCD163) plasma levels in white males with peripheral artery disease (PAD) and in atherothrombotic femoral plaques to evaluate their relationship with disease. We also analyzed whether Hp-Hb complexes could compete for CD163-mediated TWEAK uptake.

Methods and Results—Patients with PAD (n=155) showed a trend toward lower sTWEAK (median [interquartile range]: 134 [110–204] versus 147 [119–205] pg/mL; P=0.067) and higher sCD163 (median [interquartile range]: 367 [269–506] versus 288 [234–369] ng/mL; P<0.001) plasma concentrations than age-matched controls (n=251). sCD163 and sTWEAK plasma levels were negatively correlated in both patients and controls. After stratification according to the severity of disease, sCD163/sTWEAK ratio was significantly increased in patients with more severe disease relative to the other groups (P=0.049). Analysis of conditioned medium obtained from cultured human atherothrombotic femoral plaque samples (n=36) and healthy aortas (n=14) revealed that high amounts of sCD163 were released by the atherothrombotic tissue, whereas sTWEAK presented the opposite trend (P<0.05). Finally, we report a potential association between CD163 shedding and oxidative stress.

Conclusion—Our results suggest that the sCD163/sTWEAK plasma ratio may be associated with atherothrombosis burden in PAD. We hypothesize that an imbalance between TWEAK and CD163 could reflect the progression of atherothrombosis. (Arterioscler Thromb Vasc Biol. 2010;30:1253-1262.)

Key Words: atherosclerosis ■ cytokines ■ hemoglobin ■ macrophages ■ peripheral arterial disease

Peripheral artery disease (PAD) is an important manifestation of atherothrombosis, in which several proinflammatory cytokines are increased. Cytokine-mediated monocyte recruitment and differentiation into macrophages play a key role in atherothrombotic lesion progression and vulnerability. However, recent evidence describes atheroprotective properties of a subtype of macrophages (type II) able to scavenge haptoglobin-hemoglobin (Hp-Hb) complexes via the CD163 receptor from lesions with intraplaque hemorrhage.

Intraplaque hemorrhage is a common event in advanced atherothrombotic lesions. Hemolysis of extravasated red blood cells leads to accumulation of pro-oxidant Hb. CD163 is expressed mainly at the membrane of resident tissue macrophages; however, a soluble form of the protein (sCD163) has also been identified in both human plasma and cell culture supernatants, resulting from a proteolytic cleavage of the cell surface protein. sCD163 plasma levels are increased in diseases associated with macrophage activation, including coronary artery disease. CD163 has been recently described as a scavenger receptor for tumor necrosis factor–like weak inducer of apoptosis (TWEAK); this provides new insights into the importance of CD163 beyond its role in the clearance of Hp-Hb complexes.

TWEAK is a member of the tumor necrosis factor superfamily of structurally related cytokines, produced mainly by macrophages. It induces the production of proinflammatory cytokines and the proliferation and migration of cells present in atherothrombotic plaques, and it stimulates expression of metalloproteinases. Surprisingly, sTWEAK plasma levels are decreased in patients with carotid atherothrombosis or...
atherothrombosis-associated diseases, such as type 2 diabetes and end-stage or chronic kidney disease, and are negatively associated with carotid artery intima/media thickness in asymptomatic subjects. Our hypothesis is that decreased circulating sTWEAK could result from its clearance through CD163-expressing macrophages in atherothrombotic plaques. We have recently observed that CD163 and TWEAK were expressed reciprocally in human carotid atherothrombotic plaques and that sCD163 plasma levels were negatively associated with sTWEAK concentrations in asymptomatic subjects. In the present study, we assessed sTWEAK and associated with sCD163 plasma levels were negatively expressed reciprocally in human carotid atherothrombotic lesions. The adjacent area, considered NSP, corresponded to type III or IV lesions. Nonatherothrombotic control endarteries were obtained by removal of the adventitia from whole aortic segments.

SPs were separated into hemorrhagic/noncalcified (n=14), nonhemorrhagic/calcified (n=13), or those sharing both criteria (n=9). The presence of heme was assessed by a chromogenic assay, by the addition of formic acid to the conditioned media (vol/vol 10/90) and was considered as being proportional to hemoglobin release. After the binding of formate to heme, optical density was monitored at 405 nm (see Figure 4B). The macroscopic evaluation at the time of the dissection (grading the presence of hemoglobin from 1 to 5) fitted well with the results of the biochemical determination of hemoglobin content (in 98% of cases).

**Conditioned Media**

Small pieces of tissue (~5 mm³) were weighed and incubated for 24 hours in RPMI medium 1640 containing 1% L-glutamine, 1% penicillin, streptomycin, and amphotericin at 37°C and without serum. For standardization, the volume of medium was adjusted to sample wet weight (6 mL per gram). Conditioned media from SPs and NSPs were collected and then centrifuged (3000g for 10 minutes at 20°C) and stored at −80°C until further analysis.

**Enzyme-Linked Immunosorbent Assay**

Plasma and conditioned media concentrations of sTWEAK and sCD163 were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (BMS2006INST, Bender MedSystems; DC1630, R&D). Intra- and interassay coefficients of variation were 5.1% and 7.9% (sTWEAK) and 3.7% and 6.8% (sCD163), respectively.

**Immunofluorescence**

Specimens were fixed in paraformaldehyde for 24 hours and stored in ethanol until paraffin-embedded. Immunofluorescence was performed on 4-μm-thick sections as previously described. Primary antibodies were goat anti-TWEAK polyclonal antibody (AF1090; R&D Systems) and mouse anti-CD163 monoclonal antibody (EDhu-1; Serotec) followed by a secondary antibody respectively conjugated with Alexa 488 and Alexa 455 (Invitrogen). Nuclei were stained with 4′,6-diamidino-2-phenylindole (0.1 μg/mL). Negative controls using the corresponding nonimmune IgG were included in each set of experiments to check for nonspecific staining.

**In Vitro Studies**

**Reagents**

RPMI medium 1640, penicillin, and streptomycin were obtained from BioWhittaker. Fetal bovine serum was from Gibco. Recombinant soluble human TWEAK (rHuTWEAK) was from Millipore (GF102). H₂O₂, lipopolysaccharide, myeloperoxidase (MPO), hemoglobin (lyophilized stabilized purified Ao ferrous hemoglobin, H0267), and haptoglobin phenotype 1 to 1 (lyophilized purified, H0138) were from Sigma-Aldrich. Hb and Hp were reconstituted in PBS at 1 mg/mL. Hb was labeled with Alexa Fluor 633 (protein labeling kit A20170) following the instructions of the manufacturer. Hp-Hb complexes were generated by dissolving equimolar amounts of labeled Hb and Hp in culture medium. Recombinant CD163 and TWEAK were from R&D Systems. Oxidized low-density lipoproteins were obtained as described.
Cell Culture
Human THP-1 monocytes (American Type Culture Collection; TIB-202) were cultured in RPMI medium 1640 supplemented with 10% decomplemented fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO2. Cells were differentiated to macrophages using phorbol 12-myristate 13-acetate (10−7 M) for 48 hours and then treated for 24 hours with dexamethasone to induce maximal CD163 expression (2.5×10−7 M). For uptake studies, 50 μg/mL of labeled Hp-Hb complexes (approximately 3×10−7 mol/L), r-HuTWEAK (1 ng/mL), or both were added to cells for 30 minutes at 37°C. Macrophages were then washed twice with PBS and harvested. Cells were centrifuged at 1,200 rpm for 60 seconds in a microcentrifuge and resuspended in 400 μL of PBS for flow cytometry analysis. The cells were read in an LSRII cytometer (Becton Dickinson), and r-HuTWEAK was then assessed by ELISA in the supernatants. Red blood cells were obtained from healthy volunteers with informed consent, as previously described.31

Western Blot
Western blots were performed on conditioned medium from tissues after different experimental conditions, as previously described.34 The blots were incubated with goat anti-TWEAK polyclonal antibody (AF1090; R&D Systems) or mouse anti-CD163 monoclonal antibody (EDhu-1; Serotec).

RNA Extraction and Real-Time Polymerase Chain Reaction
Total RNA was obtained from macrophage-derived cells by the Trizol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. Real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated Taq DNA polymerase (Amplitaq Gold). After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. GAPDH served as a housekeeping gene and was amplified in parallel with the genes of interest. All primers, probes, and reagents were obtained from Applied Biosystems. All measurements were performed in duplicate.

Statistical Analysis
Data are presented as the mean and standard deviation or medians and interquartile ranges for continuous variables according to their distribution and the percentage (count) for categorical variables. Because of the skewed distribution of sTWEAK and sCD163 plasma levels, comparison of sTWEAK, sCD163, and sCD163/sTWEAK ratio between PAD cases and controls was performed using nonparametric analysis of covariance adjusted to a matching variable (ie, age). The linearity of the association of PAD with sTWEAK, sCD163, and sCD163/sTWEAK ratio was tested using logistic regression adjusted for age. After categorization of sTWEAK, sCD163, and sCD163/sTWEAK ratio into tertiles, the odds ratios (ORs) and 95% confidence interval (CI) of PAD for the upper 2 tertiles relative to the lowest tertile were calculated. Additional adjustments were made for cardiovascular risk factors (hypertension, hypercholesterolemia, diabetes, and smoking) and lipid-lowering treatment. The homogeneity of the relationship between sTWEAK, sCD163, and sCD163/sTWEAK ratio across the conventional cardiovascular risk factors was tested by introducing the corresponding interaction term into the model. After pooling PAD cases and controls, we studied the association of sTWEAK, sCD163, and sCD163/sTWEAK ratio with several patients’ characteristics using nonparametric analysis of covariance adjusted for age and case-control status. Finally, among the PAD cases, we investigated the association of sTWEAK, sCD163, and sCD163/sTWEAK ratio with the characteristics and severity of PAD using nonparametric analysis of covariance corrected for age. Age-adjusted partial Spearman correlation coefficients were calculated for continuous characteristics. In vitro experiments were performed at least 3 times. Differences between SPs and NSPs from the same endarterectomy sample were evaluated by the Wilcoxon paired nonparametric test. Differences between endarterectomy samples and controls or between femoral SPs and NSPs, with or without intraplaque hemorrhage, were assessed by the Mann–Whitney nonparametric test. Statistical testing was done with a 2-tailed α level of 0.05. Data were analyzed using the SAS package.

Results
PAD Case-Control Study
General characteristics of study subjects are presented in Supplemental Table I, available online at http://atvb.ahajournals.org. All patients were male, with a mean age of 58 years. Smoking, hypertension, and diabetes were significantly more frequent in patients with PAD than in the controls. Cases were 5 times more likely to be on lipid-lowering drugs; as a result, they had lower total cholesterol and low-density lipoprotein–cholesterol levels than the controls. Triglyceride levels were higher and HDL-cholesterol levels were lower in the cases, and 77% of cases had intermittent claudication, with a median walking distance of 200 m.

sTWEAK Plasma Levels and PAD
PAD cases showed a trend toward lower plasma sTWEAK concentrations than controls (median [interquartile range]: 134 [110–204] versus 147 [119–205] pg/mL; age-adjusted P=0.067) (Figure 1A). This trend became significant after analysis of covariance on log-transformed values (geometric means [95% CI]: 144 [132–158] versus 165 [153–180] pg/mL; age-adjusted P=0.035). After categorization in tertiles of plasma levels of sTWEAK, we found a modest association of TWEAK with PAD that was attenuated with adjustment (Table). Individuals in the upper 2 tertiles of sTWEAK presented a lower incidence of PAD relative to the lowest one (age-adjusted OR [95% CI]: 0.55 [0.33 to 0.90] and 0.63 [0.38 to 1.02], P=0.04), suggesting a protective effect of higher sTWEAK levels on PAD. Similar results were found after adjustment for common PAD risk factors (hypertension, hypercholesterolemia, diabetes, and smoking status) and lipid-lowering treatment. Using the 33rd percentile as the cutoff point, the age-adjusted OR (95% CI) of PAD for sTWEAK ≥124 pg/mL was 0.59 (0.38 to 0.89; P=0.013), supporting that elevated sTWEAK levels may be protective in PAD. This association was of borderline significance in further multivariate analysis (OR [95% CI]: 0.63 [0.37 to 1.07], P=0.085). No significant heterogeneity in this association was found across traditional PAD risk factors. We next assessed whether common PAD risk factors affected sTWEAK concentration. sTWEAK concentration did not vary significantly with hypertension, hypercholesterolemia, diabetes, smoking status, or lipid-lowering treatment. Taken together, our results show a weak relation between sTWEAK concentration and the presence of PAD, where subjects with higher sTWEAK levels have a decreased PAD risk.

sCD163 Plasma Levels and PAD
sCD163 plasma levels were significantly higher in PAD cases compared with control individuals (median [interquartile...
found after further adjustment for PAD risk factors and lipid-lowering treatment (OR [95% CI], 1.49 [1.23 to 1.79]; \(P<0.001\)). Because sCD163 and sTWEAK show opposite trends, we calculated the sCD163/sTWEAK ratio and tested its association with clinical PAD. Similarly to sCD163 results, a significant difference in sCD163/sTWEAK ratio was found between PAD cases and controls (Figure 1C and Table). The association of the sCD163/sTWEAK ratio with PAD was driven primarily by sCD163. After adjustment on PAD risk factors and lipid-lowering treatment, the OR (95% CI) of PAD per 1-point increase in sCD163/sTWEAK ratio was 1.21 (1.06 to 1.37).

Diabetic individuals presented higher sCD163 plasma concentrations than nondiabetics (405 [281–558] versus 302 [243–397]; \(P=0.008\)). No differences in sCD163 levels were found to be associated with smoking status, the presence of hyperlipidemia or hypertension, or the prescription of hypolipemiant or antiagregants (Supplemental Table II). On the other hand, we found a significant interaction between sCD163 levels and hypertension on PAD risk (\(P<0.013\)), whereas no significant interaction was observed for hypercholesterolemia, diabetes, or smoking status. The association between sCD163 and PAD risk was stronger in presence of hypertension than in normotensive individuals (age-adjusted OR [95% CI] per 100 ng/mL, 2.17 [1.50 to 3.14] versus 1.21 [0.96 to 1.53], respectively).

sTWEAK and sCD163 Plasma Levels, sCD163/sTWEAK Plasma Ratio, and Severity of PAD
To analyze whether sCD163 and sTWEAK could be potential markers reflecting the progression of disease, we classified cases according to clinical criteria of PAD severity (Fontaine-Leriche stages, WHO classification): grade 1=intermittent claudication with maximal walking distance <100 meters; grade 2=intermittent claudication with maximal walking distance >100 meters; grade 3=critical leg ischemia. We found no significant difference in sTWEAK plasma levels among the 3 grades of severity (Supplemental Table III). However, we observed a trend toward a positive correlation between sCD163 levels and the severity of PAD (age-adjusted \(P\) for trend, 0.06). Interestingly, we observed a significant association between sCD163 and PAD risk stages, where the sCD163/sTWEAK ratio significantly increased with the severity of PAD (age-adjusted \(P=0.049\)).

sCD163 and sTWEAK Are Differently Released by Human Atherothrombotic Femoral Plaques
We have evaluated whether sCD163 and sTWEAK plasma levels could reflect such a differential release from diseased versus nonpathologic arterial wall. We incubated atherothrombotic endarterectomy femoral plaques separated into stenosing and adjacent noncomplicated area (\(n=36\)) (Figure 2A) or control aortic media (\(n=14\)) in serum-free culture medium to harvest the released proteins. Western blot analysis of the supernatants obtained revealed that sCD163 was released in higher amounts by SP relative to NSP or healthy aortas, whereas sTWEAK showed the opposite pattern (Figure 2B). These data were confirmed by quantitative ELISA:
sCD163 (ng/mg of tissue), 792.4±90.3 (SP) versus 288.1±48.5 (aorta) and 534.6±132.5 (NSP), P<0.05; sTWEAK (pg/mg of tissue), 4200±378 (SP) versus 7308±1294 (NSP) and 8040±726 (aorta), P<0.05 (Figure 3C). Furthermore, we have observed the presence of sCD163/sTWEAK complexes in the conditioned medium from femoral plaques where exposed to Hp-Hb complexes (3 mol/L); however, TWEAK mRNA expression remained unchanged for up to 24 hours (data not shown), indicating that the increase in sTWEAK after incubation with exogenous Hp-Hb complexes was not due to an increase in TWEAK synthesis. To confirm the competition between Hp-Hb complexes and sTWEAK for CD163-mediated uptake, macrophages were exposed to labeled Hp-Hb complexes or r-HuTWEAK or coincubated with both ligands for 30 minutes. As shown in Figure 5C, a decrease in the uptake of labeled Hp-Hb complexes, as determined by flow cytometry, was observed.

## Table. Odds Ratios of Peripheral Arterial Disease According to the Tertiles of sTWEAK and sCD163 Plasma Levels

<table>
<thead>
<tr>
<th>sTWEAK</th>
<th>Cases (n=155)</th>
<th>Controls (n=251)</th>
<th>P*</th>
<th>OR (95% CI)*</th>
<th>OR (95% CI)†</th>
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<tr>
<td>&lt;124</td>
<td>40.6 (63)</td>
<td>28.7 (72)</td>
<td>0.040</td>
<td>Ref. Ref.</td>
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<tr>
<td>124–179</td>
<td>28.4 (44)</td>
<td>36.6 (92)</td>
<td></td>
<td>0.55 (0.33–0.90)</td>
<td>0.66 (0.36–1.22)</td>
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<tr>
<td>&gt;179</td>
<td>31.0 (48)</td>
<td>34.7 (87)</td>
<td></td>
<td>0.63 (0.38–1.02)</td>
<td>0.59 (0.31–1.11)</td>
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<tr>
<th>sCD163</th>
<th>&lt;267</th>
<th>22.6 (35)</th>
<th>39.8 (100)</th>
<th>&lt;0.001</th>
<th>Ref.</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>267–373</td>
<td>29.0 (45)</td>
<td>36.3 (91)</td>
<td>1.44 (0.85–2.44)</td>
<td>2.17 (1.11–4.23)</td>
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<tr>
<td>&gt;373</td>
<td>48.4 (75)</td>
<td>23.9 (60)</td>
<td>3.78 (2.24–6.38)</td>
<td>4.67 (2.33–9.34)</td>
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<table>
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<tr>
<th>sCD163/sTWEAK</th>
<th>&lt;1.6</th>
<th>25.8 (40)</th>
<th>37.9 (95)</th>
<th>&lt;0.001</th>
<th>Ref.</th>
<th>Ref.</th>
</tr>
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<tr>
<td>1.6–2.9</td>
<td>28.4 (44)</td>
<td>36.7 (92)</td>
<td>1.16 (0.69–1.95)</td>
<td>1.98 (1.03–3.81)</td>
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<tr>
<td>&gt;2.9</td>
<td>45.8 (71)</td>
<td>25.5 (64)</td>
<td>2.72 (1.64–4.51)</td>
<td>2.74 (1.42–5.22)</td>
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Ref indicates reference value of 1.
*Computed using logistic regression analysis adjusted for age.
†Additional adjustment for PAD risk factors (hypertension, hypercholesterolemia, diabetes, and smoking status) and lipid-lowering treatment.

**CD163 and TWEAK Colocalize in Human Atherothrombotic Femoral Plaques**

To evaluate the relevance of testing the competition between hemoglobin and sTWEAK for CD163 in vitro, we first analyzed their expression in a hemorrhagic femoral plaque. As shown in Figure 4, double immunostaining revealed that TWEAK and CD163 colocalized in some but not all cells, suggesting that the interaction between TWEAK and CD163 is possible in vivo.

**Hp-Hb Complexes Compete for CD163-Mediated TWEAK Uptake Ex Vivo and In Vitro**

To test whether Hp-Hb complexes could compete for CD163-mediated uptake, both hemorrhagic and nonhemorrhagic SPs were incubated with or without exogenous Hp-Hb complexes (Figure 5). Addition of exogenous Hp-Hb complexes increased sTWEAK release by nonhemorrhagic samples (NSP and SP), as shown by Western blot and ELISA performed on conditioned medium. These observations indicate that Hp-Hb complexes may compete with CD163-mediated sTWEAK uptake or increase TWEAK expression. To analyze whether Hp-Hb complexes upregulate TWEAK mRNA expression, THP-1 cells were differentiated to macrophages using phorbol 12-myrisate 13-acetate for 48 hours and then treated with dexamethasone to induce maximal CD163 expression. At this time (0 hours), macrophages where exposed to labeled Hp-Hb complexes (3×10⁻⁷ mol/L); however, TWEAK mRNA expression remained unchanged for up to 24 hours (data not shown), indicating that the increase in sTWEAK after incubation with exogenous Hp-Hb complexes was not due to an increase in TWEAK synthesis.
when the cells were coincubated with r-HuTWEAK. In a second experiment, macrophages were incubated with r-HuTWEAK with or without Hp-Hb complexes (Figure 5D). Quantification of sTWEAK in the supernatants showed a lower clearance of r-HuTWEAK when incubated with Hp-Hb complexes, indicating a direct competition between r-HuTWEAK and Hp-Hb complexes for CD163.

Oxidative Stress Evoked by Hemoglobin May Participate in CD163 Shedding

The mechanisms leading to CD163 shedding are not yet fully understood. For this reason, we incubated THP-1 cells expressing CD163 in the presence of different proinflammatory and pro-oxidant stimuli and subsequently quantified sCD163 in the supernatant by ELISA (Supplemental Figure II). As previously reported, lipopolysaccharide and hydrogen peroxide (1 mmol/L) were able to increase CD163 shedding. Among all other stimuli tested, only hemoglobin and aged red blood cells were able to promote CD163 shedding (MPO, oxidized or native low-density lipoprotein, and soluble TWEAK did not produce any effect on CD163 solubilization). This suggests that oxidative stress evoked by hemoglobin may participate in CD163 shedding. To corroborate this hypothesis, we assessed the amount of advanced oxidation protein products in both conditioned media obtained from femoral atherosclerotic plaques and plasma of patients with PAD. Our results show a positive correlation between advanced oxidation protein products and sCD163 levels in the plasma of patients ($r=0.24$, $P<0.001$) and in femoral condi-

Figure 2. sTWEAK and sCD163 concentrations in conditioned media from healthy aortas and atherothrombotic femoral plaques. A, Human endarterectomy femoral plaques were dissected, separating the SP from the adjacent NSP. B, Representative Western blot of conditioned media from 4 different individuals. C, sCD163 and sTWEAK concentrations in conditioned medium by ELISA. sTWEAK and sCD163 concentrations were normalized by the wet weight of the tissue sample used to obtain conditioned medium. Results are expressed as mean±SD. *$P<0.05$ as compared with healthy aorta; #$P<0.05$ as compared with NSP.

Figure 3. sTWEAK and sCD163 concentrations in conditioned media according to the presence of intraplaque hemorrhages. SPs were classified as hemorrhagic (H; n=14), intermediate hemorrhage (I; n=9), or nonhemorrhagic (NH; n=13). A, Representative images of healthy aorta, nonhemorrhagic, intermediate hemorrhage, and hemorrhagic plaques. B, Quantitative determination of heme content in conditioned media. C, Conditioned media were then analyzed for sCD163 and sTWEAK concentration by Western blot and ELISA. Median sTWEAK and sCD163 concentrations were normalized by quantity of tissue. Results are expressed as mean±SD. *$P<0.05$ vs healthy aorta, #$P<0.05$ vs non-hemorrhagic SP, †$P<0.05$ vs intermediate hemorrhage.
tioned medium ($r=0.379$, $P=0.024$). Along this line, immunostaining for CD163, MPO, and the p22phox-NADPH subunit revealed that CD163 localized in areas with elevated expression of these pro-oxidant enzymes in serial sections of hemorrhagic human femoral atherosclerotic plaques (Supplemental Figure III).

Discussion
This is the first study to demonstrate increased plasma levels of sCD163 and decreased sTWEAK concentrations in white males with clinical PAD. An increased sCD163 and decreased sTWEAK release was also observed in cultured human atherothrombotic femoral plaques relative to control arteries. By immunohistology, we report that CD163 and TWEAK are both expressed in human hemorrhagic femoral atherothrombotic plaques and can colocalize. Ex vivo and in vitro, we show a competition between TWEAK and Hp-Hb complexes for the CD163 scavenger receptor. Our results suggest that the plasma sCD163/sTWEAK ratio may reflect peripheral atherothrombosis progression because this ratio increases with the severity of PAD. Finally, we show that solubilization of CD163 is stimulated by hemoglobin and aged red blood cells and is associated with markers of oxidative stress ex vivo and in plasma of patients.

It has been shown that sCD163 plasma levels reflect the total pool of membrane-bound CD163, which may be elevated in cases of either an increase in CD163-expressing macrophages or upregulation of CD163 gene expression by proinflammatory mediators, such as interleukin-6 or interferon-γ. Our results show that subjects with higher sCD163 plasma levels present an increased PAD risk, independently of conventional risk factors (ie, age, hyperlipidemia, hypertension, and current smoking). In agreement with our data, sCD163 plasma concentrations were also significantly increased in other inflammatory processes, such as diabetes, rheumatoid arthritis, Gaucher disease, hemophagocytosis, sepsis, and myelomonocytic leukemia. Furthermore, the study of Aristoteli et al showed that sCD163 may act as a plasma marker of coronary atherothrombotic burden. In the same study, the authors observed a trend toward higher sCD163 concentrations in hypertensive patients that was not statistically significant. We have observed that the association between sCD163 and PAD risk was stronger in the presence of hypertension. The biological significance of this potential association requires further investigation.

The principal characteristic of CD163 is to act as a scavenger receptor for Hp-Hb complexes in intralesional hemorrhage. The atheroprotective effect of CD163 depends not only on the removal of the pro-oxidant Hb from the vessel but also on the subsequent induction of antiinflammatory pathways through interleukin-10 and heme oxygenase-1. CD163 has also been recently identified as a new receptor for TWEAK. Heretofore, the only reported TWEAK receptor was Fn14 (fibroblast growth factor-inducible molecule 14). The interaction between TWEAK and Fn14 has several proatherogenic effects, because it induces the production of proinflammatory cytokines and metalloproteinases and activates proliferation, migration, and angiogenesis. Paradoxically, we have observed that PAD individuals had lower levels of sTWEAK than control individuals. This is in agreement with previous studies showing that sTWEAK plasma concentrations were decreased in subjects with ca-

![Figure 4. TWEAK and CD163 expression in human femoral plaques. Immunohistofluorescence showing the expression of CD163 (green), TWEAK (red), and nuclei (4',6-diamidino-2-phenylindole [DAPI], blue) in human femoral atherothrombotic plaque (with presence of hemorrhage). Magnification, ×20 and ×40 (insets). Arrows show colocalization of TWEAK and CD163. Triangles show areas where CD163 (green) and TWEAK (red) are not colocalized.](image-url)
rotid atherothrombosis or atherothrombosis-associated diseases, indicating that low levels of this protein could reflect the presence of atherothrombosis. The mechanisms leading to this decrease in sTWEAK plasma concentration in subjects with atherothrombosis are not known; our hypothesis is that it could be related to the scavenging capacity of CD163. Previous data from our group showed that CD163-expressing macrophages can sequester and internalize sTWEAK in vitro. Furthermore, internalization of sTWEAK by CD163 was followed by its degradation in cells that do not express Fn14. In the present work, we observed that sCD163 plasma levels were inversely correlated with sTWEAK concentrations in both subjects with PAD and controls, supporting the hypothesis that decreased circulating sTWEAK could result from its clearance through CD163-expressing macrophages. We found a positive relation between sCD163/sTWEAK ratio and PAD severity, indicating that sCD163/sTWEAK ratio in plasma could be a potential novel biomarker of PAD progression. However, additional studies are necessary to confirm our observations.

Recently, it has been reported that TWEAK protein structure has similarities with the Hp-Hb complex, indicating that Hp-Hb could compete with TWEAK for CD163 binding. Hp-Hb complexes could thus inhibit TWEAK catabolism/degradation and therefore make it available for other targets within the plaque. TWEAK could thus trigger its proatherogenic effects via the Fn14 receptor. Until now, the existence of this interaction in the arterial wall has not been documented. In this study, we demonstrated both ex vivo and in vitro competition between Hp-Hb complexes and TWEAK for CD163 that would promote proatherothrombotic effects of TWEAK in the arterial wall. It is important to note that massive intraplaque hemorrhage is not a hallmark of PAD; indeed, other types of plaque, including calcified plaques, are predominant. We can thus speculate that decreased circulating TWEAK may result from the overall increased presence of macrophages along the vascular tree and subsequent scavenging by CD163-expressing cells.

CD163 expression has been reported in coronary and aortic lesions. In agreement with these reports, we have observed that CD163 is also expressed in human femoral atherothrombotic plaques. In the present study, we showed that TWEAK was present in human femoral atherothrombotic plaques, colocalized with CD163, suggesting a potential in vivo interaction. As was recently published, CD163-expressing macrophages may represent a new atheroprotective intraplaque population acting via hemoglobin clearance but also by scavenging TWEAK, thus thwarting its proinflammatory effects.

The mechanisms leading to CD163 shedding are not yet fully understood, but they seem to involve oxidative stress, proteases, or inflammatory conditions, all of which are associated with the pathogenesis of PAD. Our data show that free hemoglobin or that contained in aged erythrocytes is able to promote CD163 shedding. Hemoglobin is a potent oxidant, and erythrophagocytosis is associated with intracellular oxidative stress. Interestingly, supporting our results obtained in vitro, sCD163 concentration was positively correlated with that of hemoglobin in conditioned medium of femoral endarteries. In addition, a positive correlation between advanced oxidation protein products and sCD163 levels was observed in the plasma of PAD patients and in conditioned medium from femoral atherosclerotic plaques. NADPH oxidase and MPO play key roles in the development and progression of atherosclerotic lesions via production of oxidant.
superoxide and hypochlorous acid in the vessel wall, respectively.\textsuperscript{51,52} Immunostaining for CD163, MPO, and the p22\textsuperscript{phox}-NADPH subunit revealed that CD163 localized in areas with elevated expression of these pro-oxidant enzymes in hemmaggic human femoral atherosclerotic plaques, reinforcing the potential role of oxidation and hemoglobin in the release of sCD163 and therefore in the progression of the atherothrombotic plaque.

Our study has some limitations. The PAD patients were recruited in a university hospital, often after a second or third referral, suggesting that the case group may not have been fully representative of the general population of PAD patients. In addition, our population consisted of white males. It is important to note that one of the limitations to association studies is the difficulty in corroborating findings observed in populations with different ethnic characteristics. We must be cautious therefore when extrapolating the results to a more general population.

In conclusion, our results indicate that the sCD163/sTWEAK ratio in plasma could be a potential biomarker reflecting the progression of atherothrombosis and in particular, that of PAD. We suggest that decreased levels of circulating TWEAK observed in atherothrombosis may be the result of its trapping by plaque macrophages via the CD163 receptor.

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Disclosures

None.

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Peripheral Artery Disease Is Associated With a High CD163/TWEAK Plasma Ratio
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### SUPPLEMENTAL DATA

| Table I. General characteristics of peripheral arterial disease cases and controls |
|-----------------------------------------------|----------------------|----------------------|----------|
| Age, y, mean ± SD                            | 57.5 ± 7.3           | 57.7 ± 7.9           | 0.6      |
| BMI, kg/m², mean ± SD                        | 24.3 ± 4.2           | 26.3 ± 3.2           | <0.0001  |
| Hypertension, %(n)                           | 55.5 (86)            | 32.3 (81)            | <0.0001  |
| Diabetes, %(n)                               | 23.9 (37)            | 9.2 (23)             | <0.0001  |
| Hypercholesterolemia, %(n)                   | 57.4 (89)            | 54.6 (137)           | 0.8      |
| Current smokers, %(n)                        | 56.8 (88)            | 20.3 (51)            | <0.0001  |
| Lipid-lowering drugs, %(n)                   | 43.1 (65)            | 9.2 (23)             | <0.0001  |
| Total cholesterol, mmol/l, mean ± SD         | 5.0 ± 1.2            | 6.1 ± 0.9            | <0.0001  |
| LDL-C, mmol/l, mean ± SD                     | 3.0 ± 1.0            | 3.9 ± 0.9            | <0.0001  |
| HDL-C, mmol/l, mean ± SD                     | 1.1 ± 0.3            | 1.5 ± 0.4            | <0.0001  |
| Triglycerides, mmol/l, median (IQR)          | 1.6 (1.2-2.3)        | 1.2 (0.8-1.7)        | <0.0001  |
| Fasting glucose, mmol/l, median (IQR)        | 5.7 (5.2-6.6)        | 5.8 (5.5-6.1)        | 0.458    |
| Antiagregants, %(n)                          | 64.9 (101)           | 11.6 (29)            | <0.0001  |
| Asymptomatic, %(n)                           | 9.0 (14)             |                      |          |
| Intermittent claudication, %(n)              | 76.8 (119)           |                      |          |
| Critical ischemia, %(n)                      | 14.2 (22)            |                      |          |
| Disease duration, y, median (IQR)            | 3.7 (1.5-8.3)        |                      |          |
| Prior revascularization, %(n)                | 56.8 (88)            |                      |          |
| Walking distance, m, median (IQR) *          | 200 (100-500)        |                      |          |
| Ankle-brachial systolic pressure index, mean ± SD | 0.65 ± 0.29        |                      |          |
| Coronary heart disease, %(n)                 | 23.9 (37)            |                      |          |
| Cerebrovascular disease, %(n)                | 11.0 (17)            |                      |          |
| Familial PAD, %(n)                           | 20.0 (31)            |                      |          |
| Familial coronary heart disease, %(n)        | 26.5 (41)            |                      |          |
| Familial cerebrovascular disease, %(n)       | 15.5 (24)            |                      |          |

LDL, Low-density lipoprotein. HDL, high-density lipoprotein; BMI, body mass index. Values are given as means ± SD, the percentage (number) or median (interquartile range (IQR)) for each group. Means were compared using the Student t test, medians using the Mann-Whitney U test and percentage using the chi-square test. * data reported among patients with intermittent claudication.
Table II. Comparison of sTWEAK, sCD163 and sCD163/sTWEAK ratio levels across several patients characteristics.

<table>
<thead>
<tr>
<th></th>
<th>sTWEAK, pg/mL</th>
<th>P *</th>
<th>sCD163, ng/mL</th>
<th>P *</th>
<th>ratio</th>
<th>P *</th>
</tr>
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<tr>
<td><strong>Diabetes</strong></td>
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<td></td>
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<tr>
<td>No</td>
<td>142 (113-200)</td>
<td>0.221</td>
<td>302 (243-397)</td>
<td>0.008</td>
<td>2.15 (1.32-3.15)</td>
<td>0.370</td>
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<tr>
<td>Yes</td>
<td>141 (114-268)</td>
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<td>405 (281-558)</td>
<td></td>
<td>2.86 (1.28-4.57)</td>
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<tr>
<td><strong>Current smoking</strong></td>
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<tr>
<td>No</td>
<td>145 (119-206)</td>
<td>0.124</td>
<td>313 (247-419)</td>
<td>0.108</td>
<td>2.15 (1.35-3.18)</td>
<td>0.999</td>
</tr>
<tr>
<td>Yes</td>
<td>135 (105-201)</td>
<td></td>
<td>305 (242-420)</td>
<td></td>
<td>2.25 (1.23-3.97)</td>
<td></td>
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<tr>
<td><strong>Hypertension</strong></td>
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<tr>
<td>No</td>
<td>142 (116-198)</td>
<td>0.359</td>
<td>299 (234-373)</td>
<td>0.068</td>
<td>2.01 (1.26-2.92)</td>
<td>0.251</td>
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<tr>
<td>Yes</td>
<td>144 (112-210)</td>
<td></td>
<td>349 (266-483)</td>
<td></td>
<td>2.54 (1.39-3.89)</td>
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<tr>
<td><strong>Hyperlipidemia</strong></td>
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<tr>
<td>No</td>
<td>141 (114-204)</td>
<td>0.678</td>
<td>306 (242-420)</td>
<td>0.759</td>
<td>2.18 (1.29-3.37)</td>
<td>0.695</td>
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<tr>
<td>Yes</td>
<td>142 (113-206)</td>
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<td>314 (250-414)</td>
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<td>2.19 (1.34-3.22)</td>
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<td><strong>Lipid-lowering treatment</strong></td>
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<td>No</td>
<td>141 (115-205)</td>
<td>0.425</td>
<td>305 (243-400)</td>
<td>0.463</td>
<td>2.14 (1.30-3.16)</td>
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<tr>
<td>Yes</td>
<td>144 (107-212)</td>
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<td>326 (256-503)</td>
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<td>2.49 (1.29-3.85)</td>
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<tr>
<td><strong>Antiagregants</strong></td>
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<td></td>
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</tr>
<tr>
<td>No</td>
<td>144 (117-206)</td>
<td>0.600</td>
<td>298 (240-398)</td>
<td>0.653</td>
<td>2.06 (1.26-3.13)</td>
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<tr>
<td>Yes</td>
<td>135 (275-483)</td>
<td></td>
<td>349 (275-483)</td>
<td></td>
<td>2.68 (1.46-3.99)</td>
<td></td>
</tr>
</tbody>
</table>

Median (interquartile range) are shown after pooled PAD cases and controls together.
* p adjusted on age and case-control status (nonparametric analysis of covariance).
Table III. sTWEAK and sCD163 plasma levels in cases according to severity of peripheral arterial disease.

<table>
<thead>
<tr>
<th>Grade of Severity</th>
<th>1, n=85</th>
<th>2, n=40</th>
<th>3, n=30</th>
<th>P * / †</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>sTWEAK, pg/mL</strong></td>
<td>145 (112-206)</td>
<td>120 (83-248)</td>
<td>131 (117-156)</td>
<td>0.266 / 0.291</td>
</tr>
<tr>
<td><strong>sCD163, ng/mL</strong></td>
<td>335 (267-464)</td>
<td>384 (273-557)</td>
<td>425 (305-516)</td>
<td>0.162 / 0.060</td>
</tr>
<tr>
<td><strong>sCD163/sTWEAK</strong></td>
<td>2.44 (1.36-3.58)</td>
<td>2.91 (1.76-4.99)</td>
<td>3.15 (2.10-4.55)</td>
<td>0.089 / 0.049</td>
</tr>
</tbody>
</table>

* p for comparison between three groups; † p for trend test.

Results are expressed as medians (interquartile range)
Supplemental Figure I: sCD163/sTWEAK complexes in conditioned medium from femoral plaques. Western-blot of conditioned medium from three different human atherosclerotic femoral plaques in both, de-naturing and native polyacrylamide gel electrophoresis (SDS-PAGE and PAGE, respectively). The blots were hybridated with anti-CD163 (A) or anti-TWEAK (B). In SDS-PAGE, we observed specific bands corresponding to sCD163 (around 130 KDa) and sTWEAK (around 25 KDa). However, in PAGE an additional band (around 160 KDa) was observed for TWEAK or CD163, which could correspond to the presence of sCD163/sTWEAK complexes.
Supplemental Figure II: Soluble CD163 concentrations in supernatants of THP1 cells differentiated with PMA and dexamethasone. Results are expressed as percentage of release by untreated cells. LPS: 1µg/mL, hemoglobin (Hb): 10µg/mL, RBCs: red blood cells (10 per 1 THP1 cell), H₂O₂: 1mM, MPO: myeloperoxidase 52nmol/L, LDL: 400µg/mL, oxidized LDL: 400µg/mL, TWEAK 500 pg/mL.
Supplemental Figure III: Expression of CD163 and pro-oxidant enzymes in serial sections of hemorrhagic human femoral atherosclerotic plaques. Myeloperoxidase (MPO), p22 is a subunit of NADPH oxidase complex.