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ORIGINAL RESEARCH

Alternative Complement Pathway in Carotid Atherosclerosis: Low Plasma Properdin Levels Associate With Long-Term Cardiovascular Mortality

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BACKGROUND: Complement activation may promote atherosclerosis. Yet, data on the to which extent complement, and more specifically the alternative complement pathway, is activated in patients with carotid atherosclerosis and related to adverse outcome in these patients, are scarce.

METHODS AND RESULTS: We measured, by ELISA, plasma levels of factor D, properdin, C3bBbP (C3 convertase), and factor H in patients with advanced carotid atherosclerosis in a *Discovery* (n=324) and in a *Validation* (n=206) cohort in relation to adverse outcome (mean follow-up 7.8 and 6.6 years, respectively). Our major findings were as follows. Compared with healthy controls, patients with carotid atherosclerosis had increased plasma levels of factor D, properdin, and C3bBbP ($P<0.001$), but not factor H, an inhibitor of the alternative complement pathway, compared with controls. Although patients with carotid atherosclerosis had elevated levels of properdin compared with controls, within these patients, low plasma levels of properdin (ie, <median levels of properdin in the patient group) were significantly associated with cardiovascular mortality. This was seen in both the *Discovery* (HR 2.31, $P=0.019$) and the *Validation* cohort (hazard ratio [HR], 2.81, $P=0.014$). In contrast to the low circulating levels, high intraplaque properdin levels (assessed by ELISA) correlated with markers of plaque vulnerability and symptomatology.

CONCLUSIONS: We show a strong and independent association of low plasma properdin levels with cardiovascular mortality in 2 cohorts. Conversely, the plaque properdin levels linked to features of plaque vulnerability, potentially reflecting increased deposition at the site of inflammation or local production of properdin in the atherosclerotic lesion indicating local enhanced alternative complement pathway activation.

Key Words: alternative complement pathway ■ carotid atherosclerosis ■ complement ■ plaque vulnerability ■ properdin ■ stroke

See Editorial by Regal and Fleming

Inflammation characterizes carotid atherosclerosis, a common cause of ischemic stroke.[1] The complement system, a part of the innate immune system that senses exogenous and endogenous danger, can contribute to the inflammatory arm of atherogenesis.[2] Thus, although the complement system is an important part of the host defense against invading pathogens, acute overwhelming or low-grade persistent complement activation

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CLINICAL PERSPECTIVE

What Is New?

- Patients with advanced carotid atherosclerosis patients have increased plasma levels of circulating components of the alternative pathway for complement activation as compared with healthy controls, except for the inhibitory molecule factor H.
- Plasma levels of the alternative pathway component properdin below median levels in the patient group as a whole associated independently with long-term cardiovascular mortality in 2 independent cohorts from 2 different countries.
- In contrast, intraplaque properdin levels were associated with a vulnerable plaque character and symptomatology, potentially reflecting increased deposition of this enhancer of the alternative complement pathway within the inflammatory and atherosclerotic lesion.

What Are the Clinical Implications?

- Our finding should encourage further studies of components of the alternative complement pathway and in particular properdin in other cardiovascular and inflammatory disorders.
- If possible, the studies should preferentially combine systemic and local measurements.
- Targeting the alternative complement pathway could represent a novel therapeutic approach in carotid atherosclerosis and ischemic stroke.

Nonstandard Abbreviations and Acronyms

AP	alternative pathway
C3	complement factor 3
CFP	complement factor properdin
FD	factor D
FH	factor H

could prove harmful rather than protective for the host.[3] However, whereas there are several reports on the complement system in relation to atherosclerosis,[2] if and to what extent the complement system is activated in patients with carotid atherosclerosis and its relation to outcome has received less attention.

Three distinct routes can activate complement, the classical, lectin, or alternative pathway (AP). All 3 pathways merge at C3 (complement factor 3), which is cleaved into C3a and C3b by a convertase. Activation typically continues into the cleavage of C5 into C5a and C5b, and finally the assembly of the TCC (terminal complement complex).[4] Unlike the classical and lectin pathway, the AP does not require a recognition molecule for its activation, instead, the AP is activated by surface-bound C3b, thereby amplifying activation initiated by the classical and lectin pathway (Figure 1). The regulation of the AP is rather complex, initiated by the active circulating FD (factor D) that cleaves FB (factor B) resulting in the C3bBb (C3 convertase) of the AP. The AP also involves a properdin-mediated stabilization of the labile C3bBb complex.[5] In fact, properdin is the only positive regulator in the complement system, whereas several negative regulators can continuously hold the cascade in check, including other AP components such as FH (factor H) and FI (factor I) (Figure 1).

Whether complement promotes or dampens lesion formation in atherosclerosis largely depends on the degree of AP amplification.[2] Activation of the classical and lectin pathway, without AP amplification, may at least partly be beneficial, via the removal of C3b/iC3b-opsonized apoptotic cells and cell debris from the plaque.[2,6] However, activation of the alternative and terminal pathway, which is the common pathway after the merger of the 3 main pathways, seems to have a potent proinflammatory effect via the formation of anaphylatoxins and promotion of plaque instability.[7] Patients with ischemic stroke may have increased plasma levels of C3a, C5a, and TCC.[8] Additionally, gene variations in C3 and C5 indicate an increased risk for ischemic stroke.[6,9,10] Specific studies on carotid atherosclerosis and ischemic stroke in relation to complement are, however, somewhat limited and have mainly investigated TCC, the end-product of the terminal pathway.[11–13] Data on the AP are scarcer, in particular regarding properdin levels in patients with carotid atherosclerosis in relation to long-term outcome, where data so far are nearly nonexistent.

We hypothesized that complement components of the AP are altered in plasma from patients with carotid atherosclerosis and that these members of the complement cascade could link to cardiovascular mortality in these patients. The hypotheses were investigated in 324 well-characterized patients with advanced carotid atherosclerosis from Oslo University Hospital, Oslo, Norway, most of whom had undergone a cerebral ischemic event before blood sampling and with follow-up data including long-term (mean 7.8 years) clinical outcome (the *Discovery cohort*). The outcome data were validated in an additional cohort consisting of 206 patients with advanced carotid atherosclerosis from Skåne University Hospital, Malmö, Sweden (mean follow-up 6.6 years; the *Validation cohort*).

METHODS

Data Availability Statement

Deidentified data from the discovery cohort underlying this article will be shared on reasonable request

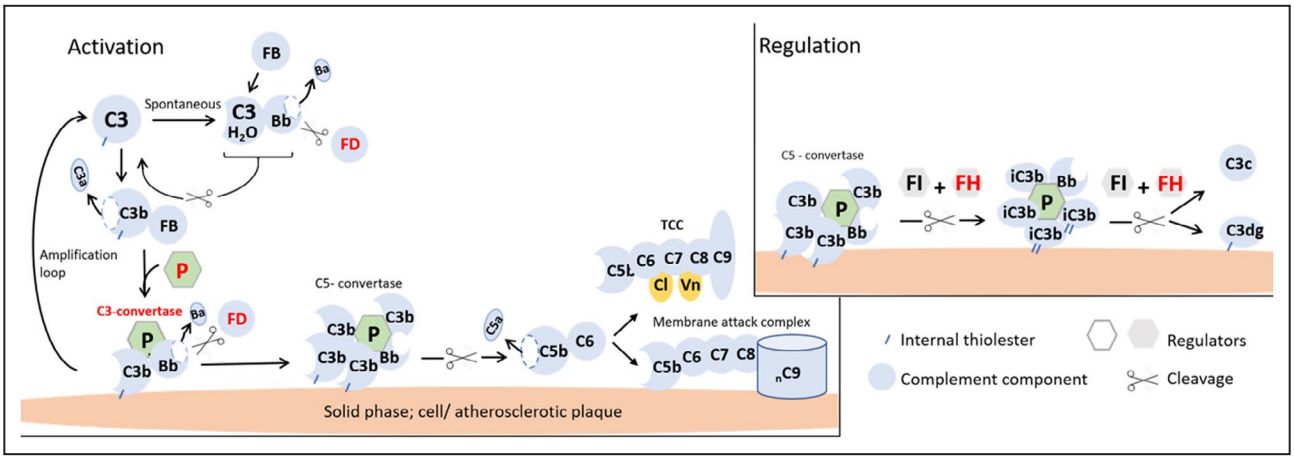


Figure 1. Simplified overview of the activation and regulation of the alternative complement pathway.
The measured AP markers in the present study are marked in red: FD, FH, P, and C3bBbP. The alternative pathway can be activated by a continuous low-grade spontaneous hydrolysis of C3. This conformational change of the C3 molecule results in its activated form, C3(H_2O). FB then connects with C3(H_2O), and is cleaved by FD, resulting in the fluid phase AP C3-convertase, C3(H_2O)Bb, which cleaves C3 into C3a and C3b. C3b can, together with another Bb molecule, with or without the stabilizer properdin, form a surface-bound AP C3-convertase, C3bBb/C3bBbP, which induces an amplification loop where more C3b is formed that will surround the C3-convertase. This $C3b_nBbP$ complex (ie, C5-convertase) will cleave C5 into C5a and C5b. If C5b, together with C6 attached to it, is close to a surface, it can attach to form with C7, C8 and C9 the membrane attack complex leading to osmotic cell lysis of sensitive cells. The other option is the formation of a soluble TCC in the fluid phase, which associates with clusterin and vitronectin coating the lipophilic sites of the complex. Negative regulators, like FH, play an important role in regulating complement activity since they promote FI-dependent degradation of C3b into iC3b, as well as iC3b into C3c and C3dg. C3bBbP indicates C3 convertase; Cl, clusterin; FB, complement factor B; FD, complement factor D; FH, complement factor H; FI, factor I; P, properdin; TCC, terminal complement complex; and Vn, vitronectin.

to the corresponding author. For the validation cohort, no individual human data can be shared due to ethical and General Data Protection Regulation regulations, but aggregated summary may be shared on reasonable request to the corresponding author after adequate ethical and legal requirements are fulfilled according to the Lund University, Region Skåne and Swedish laws.

Study Population

Discovery Cohort

In total, 324 patients with carotid stenosis were consecutively recruited between 2004 and 2020 at the Department of Neurology, Oslo University Hospital Rikshospitalet (Oslo, Norway) (Table 1). Patients were selected according to predefined inclusion criteria that included moderate (50%–69%) to severe (>70%) internal carotid stenosis who were treated with carotid endarterectomy, carotid artery stenting, or medically according to established guidelines.[14] The carotid stenosis were classified by color triplex ultrasound and computed tomography angiography as previously described.[15] Patients with severe concomitant disease like infections, connective tissue disease, malignancies, heart failure, and liver or kidney disease were excluded. Patients were categorized according to the time of onset of disease (ie, transient ischemic attack, amaurosis fugax, or ischemic stroke): 2 months or less (n=199), between 2 and 6 months (n=35), or over 6 months or no clinical events (n=89). For 1 patient, these data were missing. Patients without any events were recruited by performing examination of the carotid arteries during investigation of coronary or peripheral artery disease or hypercholesterolemia. Hypertension and dyslipidemia were defined as taking antihypertensive medication or medications against hyperlipidemia (mostly statins), respectively. For comparison, blood samples were collected from 69 age- and sex-matched healthy controls, recruited from the same area (southeastern part) of Norway as the patients, during the same period and with the same blood sampling guidelines. All controls were healthy based on disease history, none took any regular medication including statins, and none was diagnosed with any cardiovascular disease including hypertension. Levels of CRP (C-reactive protein) in the healthy controls were 1.3 (0.6–72.5) mg/L (mean and interquartile range).

Informed written consent was obtained from all participants and the study was approved by the Regional Committee for Medical and Research ds (2015/2175 and 2014/2078) and complied with the Declaration of Helsinki. Data from the Norwegian Cause of Death Registry were used to track cardiovascular mortality among the patients over time.

Table 1. Total Characteristics of the Discovery Cohort, Baseline Variables in Patients and Healthy Controls

	Healthy controls (n=69)	Discovery cohort (n=324)	*P* value
Clinical characteristics			
Male sex, n (%)	38 (53.5)	199 (61.4)	0.22
Age, y	68 (5)	68 (10)	0.33
Hypertension, n (%)	…	197 (61.4)	…
Diabetes, n (%)	…	46 (14.2)	…
Current smoking, n (%)	6 (8.5)	109 (43.8)	<0.001
Biochemistry Median (interquartile range)			
White blood cell count, 10^9/L	5.6 (1.2)	8.4 (2.7)	<0.001
Total cholesterol, mmol/L	5.90 (0.98)	4.27 (1.13)	<0.001
Low-density lipoprotein cholesterol, mmol/L	3.74 (0.89)	2.50 (0.98)	<0.001
High-density lipoprotein cholesterol, mmol/L	1.74 (0.46)	1.30 (0.47)	<0.001
Triglycerides, mmol/L	1.22 (0.68)	1.41 (0.83)	0.048
Platelets, 10^9/L	262 (62)	261 (82)	0.57
C-reactive protein, mg/L	1.3 (0.6, 2.5)	3.0 (1.1, 7.0)	<0.001
Hemoglobin A1c, %	37.3 (5.3)	42.2 (13.9)	0.050
Creatinine, mmmol/L	74 (13)	80 (27)	0.14
Medication, n (%)			
Anticoagulant/antiplatelet therapy	…	236 (73.1)	…
Statins	…	224 (69.6)	…

Validation Cohort

Carotid plaques (n=186) and plasma (n=206) from patients included in the CPIP (Carotid Plaque Imaging Project) biobank at the Vascular Department of Skåne University Hospital (Malmö, Sweden) were included as a validation cohort (Table 2 and Table S1). The inclusion criteria consisted of patients with symptomatic carotid plaques, indicated by conditions such as stroke, transient ischemic attack, or amaurosis fugax, within 1 month before surgery and with a stenosis degree >70%. For asymptomatic plaques, the required stenosis was >80%. The degree of stenosis was determined by duplex ultrasound, and all patients were preoperatively assessed by a neurologist.

Plaques were immediately snap-frozen in liquid nitrogen upon surgical removal. Histological analyses to calculate a plaque vulnerability index were performed in 1-mm-thick plaque fragments from the most stenotic region of the plaques.[16] Plaque tissue homogenates for properdin measurements were prepared, as described previously.[17] Clinical characteristics were recorded at the time of inclusion, while plasma samples to assess circulating components were collected the day before surgery.

The study was approved by the local ethical review board (472/2005; September 8, 2005, 2014/904; January 13, 2015) and were carried out following the principles of the Declaration of Helsinki. Data from the Swedish National Inpatient Health Register was used to track cardiovascular mortality among the patients over time.

Table 2. Total Characteristics of Validation Cohort, Baseline Variables in Patients (Total n=206)

	Validation cohort (n=206)
Clinical characteristics	
Male sex, n (%)	138 (67.0)
Age, y	71 (11)
Hypertension, n (%)	156 (76.1)
Diabetes, n (%)	67 (32.5)
Current smoking, n (%)	66 (32.0)
Biochemistry Median (interquartile range)	
White blood cell count, 10^9/L	7.80 (2.80)
Total cholesterol, mmol/L	4.20 (1.5)
Low-density lipoprotein cholesterol, mmol/L	2.40 (1.22)
High-density lipoprotein cholesterol, mmol/L	1.09 (0.44)
Triglycerides, mmol/L	1.20 (0.80)
Platelets, 10^9/L	247.5 (92.5)
C-reactive protein, mg/L	3.00 (4.60)
Hemoglobin A1c, mmol/mol	43.8440 (15.09)
Creatinine, mmol/L	86.0 (20.0)
Medication, n (%)	
Anticoagulant/antiplatelet therapy	198 (96.1)
Statins	183 (88.8)

Blood Sampling Protocol

Forearm venipuncture was performed with minimal stasis. Blood was drawn into pyrogen-free EDTA tubes, immediately immersed in melting ice, centrifuged within 30 minutes at 2500*g* for 20 minutes to obtain platelet-poor plasma that was stored in multiple aliquots at −80 °C and thawed <2 times before analysis. The same blood sampling protocol was used for patients and healthy controls.

Measurements of Complement Factors in Plasma and Plaque Homogenates

ELISA was used to measure circulating levels of FD (Catalog #DY1824, R&D Systems, Minneapolis, MN), properdin (HYB039-40 and HYB039-04B, Statens seruminstitutt, Denmark, as originally described by Hertle et al.[18]), C3bBbP as originally described by Mollnes et al.[19,20] and FH (Catalog #DY4779, R&D Systems). The results for C3bBbP are given in complement activating units (CAU)/mL related to a standard of complement activated human serum defined to contain 1000 CAU/mL as detailed in Bergseth et al.[19] The properdin levels are expressed as arbitrary units (AU). Thus, we used serially diluted human plasma from healthy controls (Catalog #A7472, Sigma Diagnostic, Burlington, MA) to get a relative standard curve and based on this curve we calculated the actual relative concentration of properdin. Plaque tissue homogenate levels of properdin are given in relation to g of wet weight plaque (ie, AU/g wet weight plaque).

Immunostaining of Intracranial Thrombus Material

In the Discovery cohort (Oslo), intracranial thrombus material was obtained by thrombectomy from patients with carotid plaques in a single-center cohort study.[21] Study inclusion criteria were acute stroke with clinical ischemic symptoms corresponding to an angiographically proven large intracranial vessel occlusion, the absence of intracranial hemorrhage on computed tomography scan, and a clearly defined time of symptom onset within 6 hours of inclusion. Mechanical thrombectomy involved arterial catheterization from the femoral artery to the level of the intracranial vessel occlusion and thrombus retraction with the use of a retrievable stent delivered through a microcatheter. After retraction, the thrombi used for immunofluorescence were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen for subsequent cryo-sectioning. Frozen sections (10 μm) were air dried and fixed in ice-cold acetone before staining. Sections were blocked in 0.5% BSA before incubation with the primary antibodies (rabbit antihuman properdin [LifeSpan Bioscience, Shirley, MA] and mouse antihuman Neutrophil Elastase [DAKO, Nowy Sącz, Poland]), for 1 hour at room temperature. After washing, the slides were incubated for 30 minutes with corresponding secondary antibodies (goat antirabbit, Alexa-488 and goat antimouse-IgG1, Alexa-568 [both Invitrogen, Waltham, MA]). Nuclei were stained with Hoechst (1:10 000, Life Technologies, Carlsbad, CA). Isotype control (IgG1, Curida Diatec, Oslo, Norway) served as negative control.

Assessment of the Vulnerability Index in Carotid Plaque

In the Validation cohort (Skåne) serial sections from the most stenotic plaque region were immunostained for smooth muscle cells (alpha-actin), lipids (Oil Red O), macrophages (CD68), and hemorrhagic areas (glycophorin A), following the methodology previously outlined.[16] Russell-Movat pentachrome staining was used to identify the collagen within the plaques. The plaque areas stained for each component (%) were quantitatively assessed using the Biopix iQ 2.1.8 software. The vulnerability index was determined by calculating the ratio of the cumulative percentages of Oil Red O, CD68, and glycophorin A to the combined percentages of alpha-actin and collagen, as previously reported.[16] The researchers determining the vulnerability index were blinded to all variables related to the tissue samples.

High-Density Oligonucleotide Microarrays

Total RNA was isolated from frozen carotid tissue with the use of MagNA Pure Kit III (Roche Applied Science, Indianapolis, IN), quantified spectrophotometrically, and stored at −80 °C. The Human Genome U133A 2.0 Array encoding 14 500 genes was purchased from Affymetrix (Santa Clara, CA), and hybridization was performed according to the manufacturer's 2-cycle target labeling protocol as previously described.[22]

Publicly Available Data Sets

To assess gene expression of properdin (*complement factor properdin* [*CFP*]) across cell types in carotid plaques, we accessed publicly available single-cell RNA-seq data from patients with carotid atherosclerosis undergoing endarterectomy (n=3[23] and n=38[24]) using the PlaqView explorer platform.[25] To assess *CFP* expression in carotid plaques in relation to symptomatology, we used publicly available transcriptome data from carotid plaque related ischemic cerebrovascular event within the previous 5 days (symptomatic, n=6) or an absence of a cerebrovascular event (asymptomatic, n=5).[26]

Statistical Analysis

Differences in demographics and clinical characteristics were compared with *t* test or Mann–Whitney *U*

test depending on the sample distribution. Differences in categorical data were analyzed with the chi-square test. As complement levels were skewed and there were no differences in age and sex distribution between patients and controls, the Mann–Whitney *U* test was used for comparison. Spearman rho correlations were used to determine univariate associations between plaque properdin levels and histological components as well as the vulnerability index. *P* value was adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate.

Kaplan–Meier curves were constructed to visualize and evaluate (log-rank test) differences in survival according to dichotomized (median) levels of complement factors. For properdin the median level was used as a cutoff in the outcome analyses as it was similar in both the Discovery (110.2 AU) and the Validation (107.6 AU) cohort. If significant, the association with outcome (all-cause mortality and cardiovascular death) was assessed by multivariable Cox proportional hazards models, controlling for cardiovascular risk factors[27] (age, sex, total and high-density cholesterol, diabetes, hypertension, and smoking status) and CRP. Two-sided probability values were considered significant at $P<0.05$. All calculations were performed with SPSS statistical software (Version 29.02.0; SPSS Inc, Chicago, IL). R version 4.4.0 was used to generate the correlation graph.

RESULTS

Increased Plasma Levels of Alternative Pathway Components in Carotid Stenosis Patients

In the entire patient population from the Discovery cohort, levels of FD, the essential protease of the AP, were elevated 1.4-fold in patients compared with healthy controls ($P<0.0001$, Figure 2A). Properdin, the stabilizer of the C3-convertase, and the AP C3 convertase C3bBbP, were both elevated in patients compared with healthy controls (1.2-fold, $P<0.0001$ and 1.3-fold, $P<0.001$, respectively; Figure 2B and 2C). In contrast, levels of FH, a negative regulator of the C3-convertase,

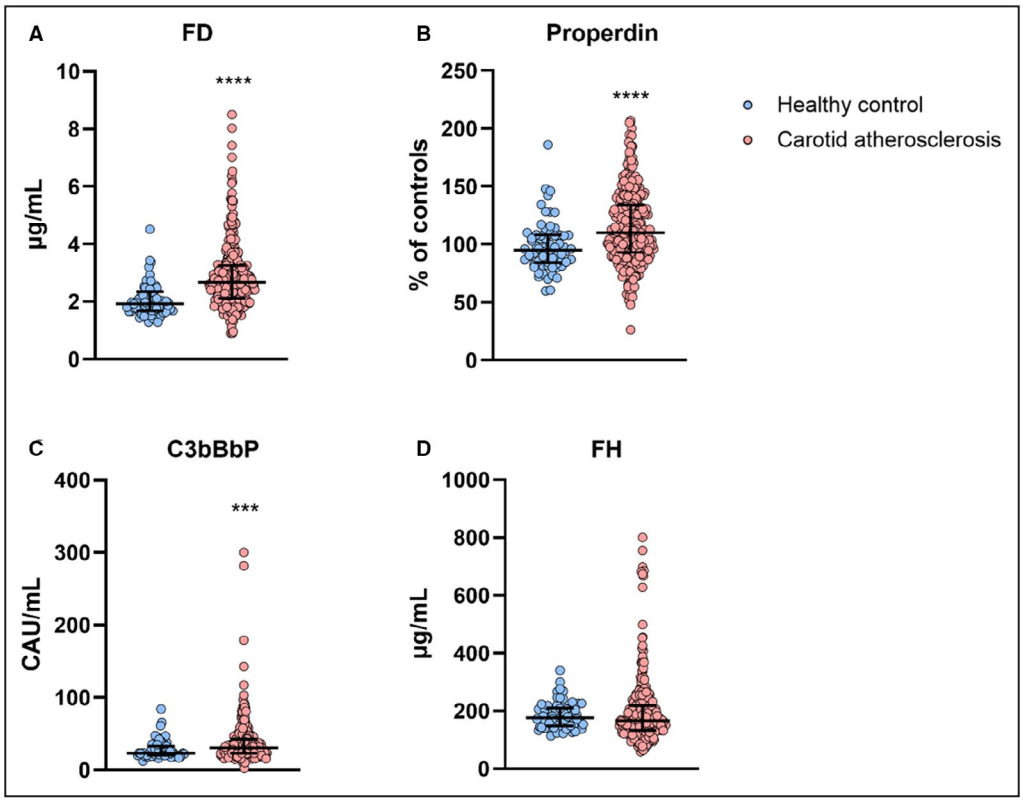


Figure 2. Altered plasma levels of alternative complement pathway components in patients with carotid atherosclerosis: data from the Discovery cohort.
ELISA was used to measure plasma levels of FD (**A**), properdin (**B**), C3bBbP (**C**), and FH (**D**), in 324 patients with advanced carotid atherosclerosis and 69 healthy controls. For properdin, the results are given in arbitrary units in relation to levels in a plasma pool from healthy controls (see Methods). The results for C3bBbP are given in CAU/mL related to a standard of complement activated human serum defined to contain 1000 CAU/mL (see Methods). Values are expressed as single values and median and 25 through 75th percentiles. ***$P<0.001$ and ****$P<0.0001$ vs healthy controls. C3bBbP indicates C3 convertase; CAU, complement activating units; FD, complement factor D; FH, complement factor H.

did not differ between patients and healthy controls (Figure 2D). In a specific sex-subgroup analysis, FD levels were slightly lower in women (P<0.05) compared with men but did not reveal any differences for the other AP markers between men and women (Figure S1).

Low Properdin Levels Associate With Cardiovascular Mortality

To elucidate further the relation between alternative complement pathway markers and carotid atherosclerosis, we examined the association of dichotomized plasma levels with mortality in the total study population of the Discovery cohort. During a mean follow-up of 7.8 (±4.4 SD) years, 141 patients died, 52 due to cardiovascular causes. Complement markers associated with mortality in univariate analysis in the discovery cohort, were further assessed in the Validation cohort where 63 patients died during a mean follow-up of 6.6 (±2.7 SD) years, 35 due to cardiovascular causes.

Kaplan–Meier survival analysis demonstrated that below median levels of properdin were associated with increased all-cause mortality in the Discovery cohort (P<0.001), but not in the Validation cohort (P=0.16) (Figure 3A). Further, below median properdin levels were associated with cardiovascular death in both the Discovery (P<0.001) and the Validation cohort (P=0.007) (Figure 3B). In contrast to the association with cardiovascular mortality, there were only minor differences in patients features between characteristics according to properdin levels in these cohorts, with no differences between high and low properdin levels in the, whereas in the Discovery cohort, patients with low properdin levels were somewhat older and had slightly higher levels of white blood cell counts and lower total cholesterol and triglycerides (Table 3).

We next assessed the independence of the association with cardiovascular mortality from established predictors using covariates from the well-known cardiovascular risk scores[27] (ie, age, sex, total and high-density lipoprotein cholesterol, hypertension, diabetes, smoking status) and CRP. As there were substantial missing data on smoking status for the Discovery cohort this was omitted but was used in the Validation cohort. Having below median levels of properdin was associated with a 1.85 (P=0.005) times higher risk of dying (ie, total mortality) in adjusted analysis in the Discovery cohort but did not associate with mortality in univariate or multivariable analysis in the Validation cohort (Figure 3C). For cardiovascular death, low (below median) properdin levels were associated with outcome in adjusted analysis in both the Discovery (hazard ratio [HR], 2.31; P=0.019) and Validation cohort (HR, 2.83; P=0.014).

Finally, when properdin levels at inclusion in the Discovery cohort were presented in relation to the time since the index ischemic stroke, an interesting pattern was seen. Whereas those who survived during long-time follow-up showed a gradual increase in properdin levels along with increasing time since the ischemic stroke, this was not seen in those who died (Figure S2), further supporting indirectly an association of low properdin levels with adverse outcome in these patients.

In contrast, we found no association between dichotomized levels of FD and all-cause (HR, 1.26 [95% CI, 0.90–1.77]; P=0.17) or cardiovascular (HR, 0.95 [95% CI, 0.59–1.76]; P=0.95) mortality, or C3bBbP and all-cause (HR, 0.98 [95% CI, 0.70–1.38]; P=0.91) or cardiovascular (HR, 0.57 [95% CI, 0.32–1.01]; P=0.053) mortality or FH and all-cause (HR, 0.95 [95% CI, 0.69–1.33]; P=0.78) or cardiovascular (95% CI, HR, 1.19 [0.69–2.04]; P=0.54) mortality in univariate analysis in the Discovery cohort and these complement factors were therefore not included in the mortality analyses of the Validation cohort.

Outcome Analyses When Using Properdin as a Continuous Variable

The median was used as a cutoff in the outcome analyses as it was similar in both the Discovery and the Validation cohort. To examine if this association persisted when continuous levels of properdin were tested, we performed outcome (cardiovascular mortality) analyses based on properdin as a continuous variable. In the Discovery cohort the association remained significant in the unadjusted (P=0.019; HR, 0,747 [95% CI, 0.585–0.952]) but not in the adjusted (P=0.169; HR, 0.793 [95% CI, 0.57–1.104]) analyses. In the Validation cohort, the associations were not significant neither in the unadjusted (P=0.289; HR, 0.807 [95% CI, 0.543–1.199]) or adjusted (P=0.134; HR, 0.726 [95% CI, 0.478–1.104]) analyses. Thus, when using properdin as a continuous variable, its prognostic impact was weakened.

Association of Low Plasma Properdin to the Degree of Carotid Stenosis

Data on the degree of stenosis in carotid arteries as assessed by color triplex ultrasound and computed tomography angiography examination were available in the Discovery cohort. To relate plasma properdin to carotid plaque pathology, we analyzed plasma levels of properdin in relation to the degree of carotid plaque stenosis in these patients. As shown in Figure S3, along with the increase in the degree of stenosis, there was a decline in properdin levels, in particular in those who died during follow-up. These finding link low properdin levels in plasma to plaque pathology, in particular in those with adverse clinical outcome.

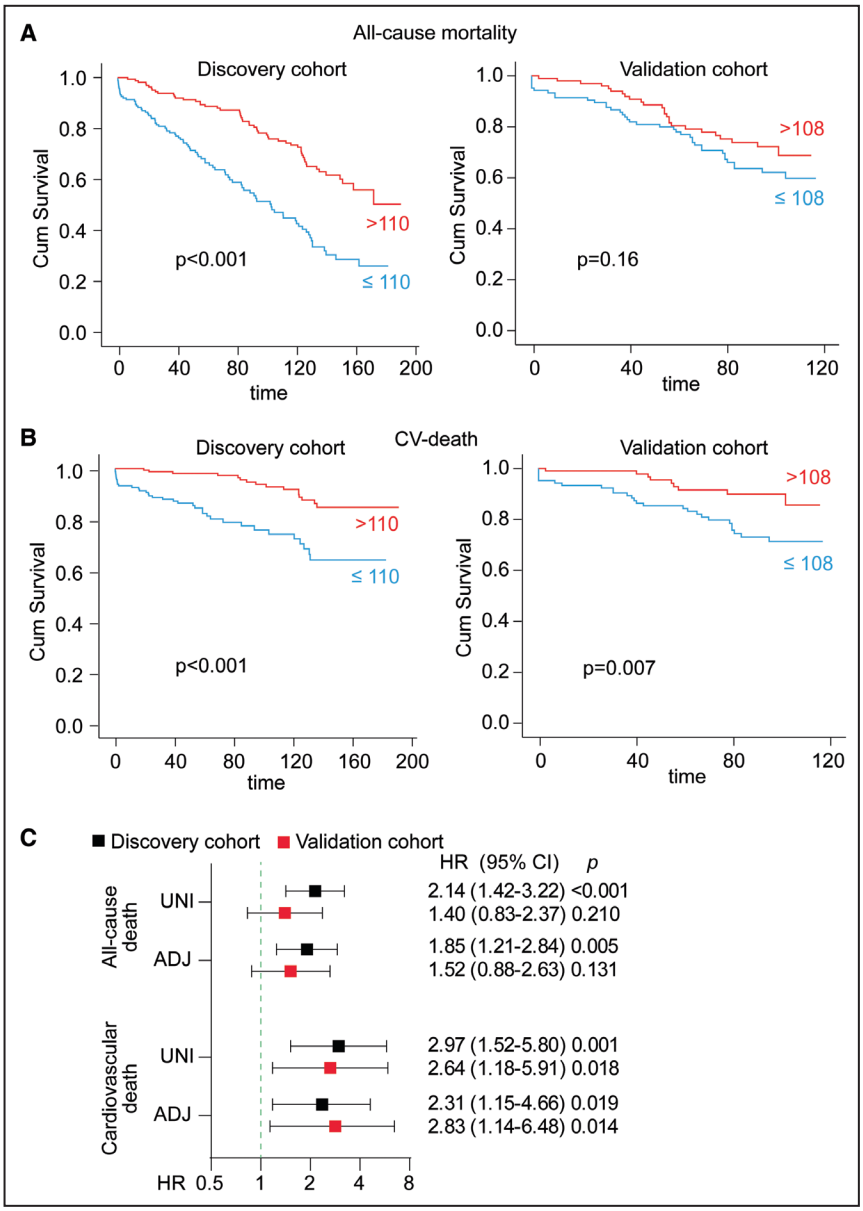


Figure 3. Low properdin levels (below median) are associated with poor outcome. Properdin levels are associated with all-cause and cardiovascular mortality in patients with carotid atherosclerosis. Kaplan–Meier survival curves in relation to dichotomized properdin (blue line, below median; red line, above median) for (**A**) all-cause mortality and (**B**) cardiovascular death in the Discovery (n=324, left panels) and Validation (n=206, right panels) cohort. (**C**) Univariate and multivariable adjusted cox-regression analysis for dichotomized properdin (above median set as reference) in relation to all-cause and cardiovascular mortality in the Discovery (black) and the Validation (red) cohort. Properdin median levels was used as a cutoff dividing the cohorts in high and low properdin as it was similar in both the Discovery (110.2 AU) and the Validation (107.6 AU) cohort. ADJ indicates adjusted; AU, Arbitrary Unit; HR, hazard ratio; and UNI, univariate.

Detection of Properdin in Thrombi Materials Retrieved From the Site of Vascular Occlusion in Ischemic Stroke

Although weakened when properdin was analyzed in relation to outcome as a continuous variable, our findings so far suggest that low plasma levels of properdin are associated with total mortality (Discovery cohort) and cardiovascular mortality (Discovery and Validation cohort). One possible explanation for this finding is that low properdin levels in the fluid phase could reflect higher properdin levels *in situ* at the

Table 3. Characteristics of the Discovery Cohort and the Validation Cohort According to Plasma Median Levels of Properdin*

Discovery cohort, n=324			
	Low properdin (n=162)	High properdin (n=162)	*P* value
Clinical characteristics			
Male sex, n (%)	104 (64.2)	95 (58.6)	0.304
Age, y	70 (10)	67 (10)	0.003
Hypertension, n (%)	98 (61.3)	99 (61.5)	0.965
Diabetes, n (%)	26 (16.0)	20 (12.3)	0.340
Current smoking, n (%)	51 (45.1)	58 (42.6)	0.694
Biochemistry Median (interquartile range)			
WBC, 10^9/L	8.60 (2.40)	8.00 (2.30)	0.022
Total cholesterol, mmol/L	4.13 (1.1)	4.42 (1.13)	0.033
LDL cholesterol, mmol/L	2.42 (0.99)	2.60 (0.97)	0.138
HDL cholesterol, mmol/L	1.29 (0.46)	1.32 (0.4)	0.507
Triglycerides, mmol/L	1.20 (0.80)	1.40 (0.48)	0.041
Platelets, 10^9/L	255 (85)	267 (79)	0.232
CRP, mg/L	3.3 (6.9)	2.8 (4.4)	0.325
HbA1c, %	42.0 (14.2)	42.9 (13.7)	0.595
Creatinine, mmol/L	82 (32)	78 (20)	0.196
Medication, n (%)			
Anticoagulant/antiplatelet			
Therapy	119 (73.5)	128 (79.0)	0.240
Statins	107 (66.5)	117 (72.7)	0.226
Validation cohort (n=206)			
	Low properdin (n=105)	**High properdin (n=101)**	***P* value**
Clinical characteristics			
Male sex, n (%)	72 (68.6)	66 (65.3)	0.658
Age, y	71 (13)	70 (11)	0.313
Hypertension, n (%)	81 (77.9)	75 (74.3)	0.624
Diabetes, n (%)	35 (33.3)	32 (31.7)	0.882
Current smoking, n (%)	28 (26.7)	38 (37.6)	0.102
Biochemistry Median (interquartile range)			
WBC, 10^9/L	7.60 (2.85)	8.00 (2.80)	0.463
Total cholesterol, mmol/L	4.30 (1.6)	4.15 (1.5)	0.918
LDL cholesterol, mmol/L	2.50 (1.20)	2.38 (1.30)	0.551
HDL Cholesterol, mmol/L	1.10 (0.50)	1.07 (0.39)	0.991
Triglycerides, mmol/L	1.20 (0.80)	1.40 (0.85)	0.376
Platelets, 10^9/L	240 (115.0)	252 (85.0)	0.828
CRP, mg/L	3.00 (5.50)	2.10 (4.40)	0.379
HbA1c, mmol/mol	44.9 (16.7)	41.8 (9.9)	0.063
Creatinine, mmol/L	84.0 (28.0)	88.0 (30.5)	0.203
Medication, n (%)			
Anticoagulant/anti-latelet therapy	100 (95.2)	98 (97.0)	0.683
Statins	94 (89.5)	89 (88.1)	0.827

* Low and high properdin levels reflect below and above median levels in the respective cohort. Properdin median level was used as a cutoff dividing the cohorts in high and low poperdin as it was similar in both the Discovery (110.2 AU) and the Validation (107.6 AU) cohort. AU indicates Arbitrary Units; CRP, C-reactive protein; HbA1c, hemoglobin A1c; HDL, high-density cholesterol; LDL, low-density cholesterol; and WBC, white blood cells. Characteristics of participants are compared using the Wilcoxon rank sum test/Mann–Whitney *U* test for continuous variables and the Pearson chi-square tests for categorical variables.

"scene of the crime" such as a carotid plaque or thrombi from patients undergoing an ischemic stroke. Therefore, in the Discovery cohort, we performed an immunostaining on thrombi obtained during thrombectomy from patients with an acute ischemic stroke associated with advanced carotid atherosclerosis. Strong properdin staining was visible in the area with cellular enrichment, localized to neutrophils but not to platelets (Figure 4). Interestingly, and with relevance to this finding, whereas in the Discovery cohort we found no correlation between CRP, as a reliable marker of up-stream inflammation including interleukin-6 activity,[28] and properdin levels in plasma (r=−0.047, *P*=0.41), properdin levels were negatively correlated with leukocyte counts (r=−0.153, *P*=0.006). This finding could potentially reflect an association between low properdin levels in plasma and leukocyte activation.

Association of Plaque Properdin With Vulnerable Plaque Characteristics: Protein Analyses

In the Validation cohort, plaque ELISA measurements of properdin and a histological vulnerability index were available in 155 patients. This vulnerability index was previously validated to predict cardiovascular events in a carotid endarterectomy cohort.[16] In brief, the vulnerability index represents a ratio between elements that contribute to plaque instability (such as macrophages, hemorrhage, and lipids) and those that promote stability (including smooth muscle cells and collagen). Notably, whereas low properdin plasma levels were associated with cardiovascular mortality and the degree of carotid stenosis, plaque properdin levels, as assessed by ELISA, were positively correlated with the plaque vulnerability index (rho=0.205, *P*=0.021) and

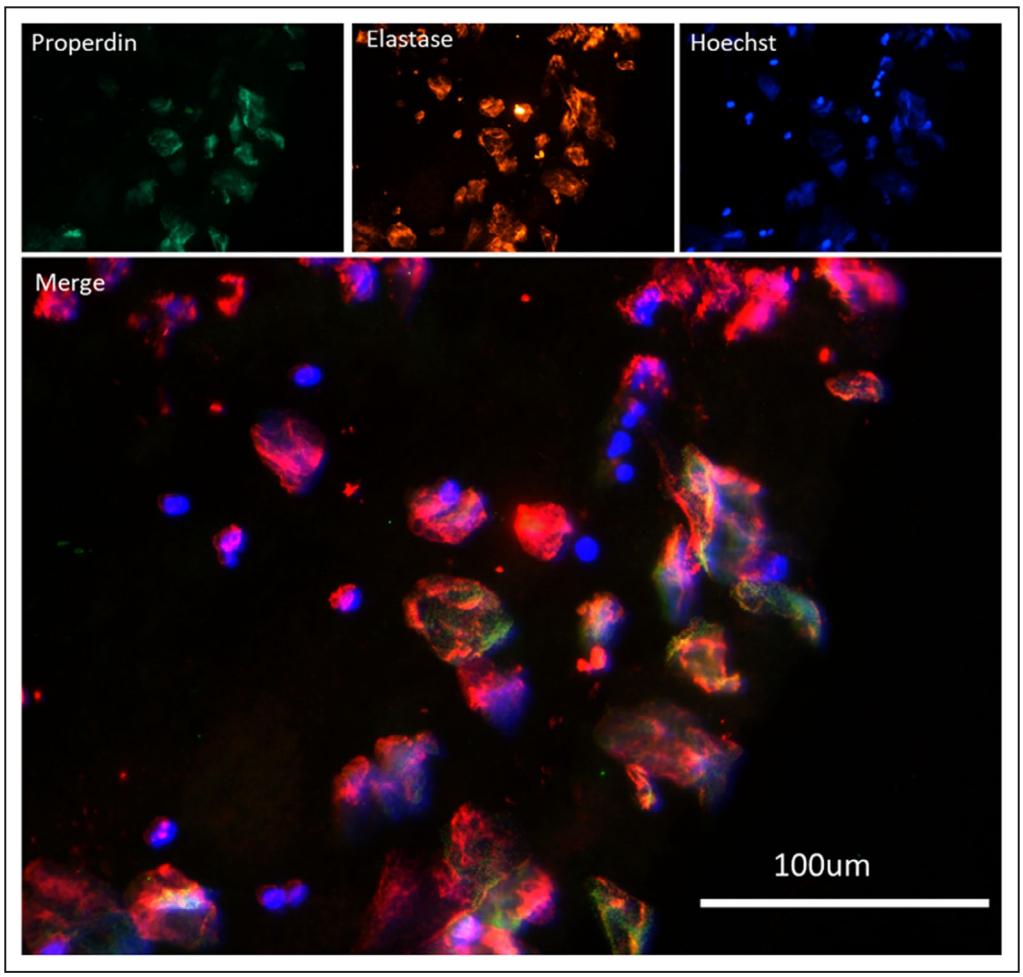


Figure 4. Properdin colocalizes with neutrophils in intracranial thrombi.
Fluorescent double-staining of properdin (green) and elastase (neutrophil marker; red). Nuclei are stained with Hoechst (blue). The individual stainings are merged, and a representative staining is shown (n=4 from the Discovery cohort).

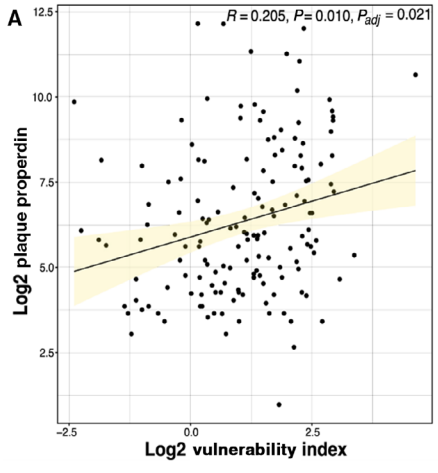
in particular the degree of lipid loading as assessed by Oil-Red-O staining, and the degree of intraplaque hemorrhage, as assessed by Glycophorin A staining, also after adjustment (Figure 5). Moreover, patients with symptomatic carotid lesions (ie, stroke, transient ischemic attack, or amaurosis fugax within 1 month before surgery, n=107) had significantly higher levels of plaque properdin than those without any plaque related symptoms (n=79) (556.7 AU/g wet plaque versus 306.7 AU/g wet plaque; *P*=0.037, Mann–Whitney test).

Plaque Macrophages Express Properdin Transcripts

The levels of properdin within the carotid plaques could reflect deposition of properdin from the fluid (eg, plasma/serum) to the tissue phase when exposed to an inflammatory plaque microenvironment but could also reflect increased production of properdin within the atherosclerotic lesion. We therefore, examined the expression levels of the properdin gene (*CFP*) in relation to cell types in carotid plaques by accessing publicly available single-cell transcriptome data by Pan et al.[23] and Slenders et al.[24] from 3 and 38 patients with carotid atherosclerosis, respectively. Analysis of these available data files showed plaque immune cells, predominantly in macrophages, contained properdin transcripts (Figure S4).

No Difference in Properdin Gene Expression Between Symptomatic and Asymptomatic Carotid Plaques

We have previously used oligonucleotide microarrays encoding 14 500 human genes to analyze the gene expression profiles in specimens from atherosclerotic carotid plaques characterized as symptomatic, that is, carotid plaque related ischemic event within the previous 48 hours (n=4) or asymptomatic (n=4), thatis, no ischemic events; selected randomly from patients at the Department of Neurology, Oslo University Hospital and were not part of the discovery cohort.[22] We localized the expression of *CFP* within the atherosclerotic lesions in these patients (Figure S5A). However, in contrast to the positive association of plaque properdin at the protein level to vulnerable plaque (Figure 5) and recent carotid plaque symptomatology, *CFP* expression did not differ between asymptomatic and symptomatic lesions (*P*=0.69), which suggests a lack of association of properdin to plaque character at the mRNA level. A similar pattern was seen by reanalysing *CFP* expression in publicly available transcriptome data from carotid-related ischemic cerebrovascular event within the previous 5 days (symptomatic, n=6) or an absence of a cerebrovascular event (asymptomatic, n=5)[26] (*P*=0.25; Figure S5B).



B

Variable	Plaque properdin AU/g wet plaque		
	rho	*P*	P_{adj}
Histology (area%)			
Oil red O	0.250	0.002	0.010
Glycophorin A	0.209	0.009	0.021
CD68	0.131	0.103	0.154
Smooth muscle α-actin	-0.049	0.540	0.540
Collagen	-0.052	0.520	0.540
Vulnerability index	0.205	0.010	0.021

Figure 5. Plaque properdin levels were positively correlated with the plaque vulnerability index.
A, Scatterplot showing correlation of plaque properdin and vulnerability index. Spearman's correlation test was used, and the *P* value was adjusted for multiple comparisons using the Benjamini-Hochberg FDR. Variables were log2-transformed for the visualization graph. The vulnerability index was calculated as the ratio of the summed positive staining percentages of Oil Red O, CD68, and glycophorin A to the combined positive staining percentages of alpha-Actin and collagen. **B**, Correlation analyses between plaque properdin levels and histological components of the vulnerability index as well as the vulnerability index. Data from 155 patients from the Validation cohort. *P* value adjusted for multiple comparisons using the Benjamini-Hochberg FDR. AU indicates Arbitrary Unit; and FDR, false discovery rate.

DISCUSSION

Several cardiovascular diseases display an association between an activated complement system and patient outcome.[2,6,29,30] Yet, data on the extent to which complement, and more specifically the AP, is activated in patients with carotid atherosclerosis, and in particular, if complement components of the AP are related to adverse outcome in these patients, are scarce or lacking. The present study, consisting of a discovery and a validation cohort of patients with advanced carotid atherosclerosis, showed that whereas patients with carotid atherosclerosis had elevated plasma levels of most measured regulators and activation products of the AP (ie, FD, properdin, and C3bBbP), the inhibitor of the AP pathway, FH, was not elevated as compared with healthy controls. More interestingly, although patients with carotid atherosclerosis had elevated levels of properdin comparing healthy controls, within these patients, low plasma levels of properdin (ie, below median properdin levels in the patient group) were significantly associated with cardiovascular mortality, also after adjusting for several covariates. Importantly, this pattern was seen in both the discovery and the validation cohort. When using properdin as a continuous variable, its prognostic impact was weakened and additional cutoff levels need to be defined. Nonetheless, our data underscore the pathogenic role for the alternative pathway of complement activation, and particularly properdin, in ischemic stroke.

Unlike most other complement proteins, which are produced mainly in the liver, properdin is primarily synthesized by immune cells,[31] and as shown here, this also includes plaque-related immune cells and, in particular, macrophages. Properdin is a pivotal regulator of the AP, the only "positive" regulator of complement, prolonging the stability of the AP C3-convertase and enhancing the complement activation. Thus, the association of low plasma levels with adverse outcome might seem contradictory. Indeed, Hertle et al. have shown that circulating levels of properdin are associated with adverse cardiovascular outcomes.[18] The reasons for these apparent discrepancies are, at present, not clear. However, whereas the study by Hertle et al. examined the prognostic value of properdin in a stable phase in patients with 1 or more cardiometabolic risk factor,[18] we examined properdin levels in patients with advanced carotid atherosclerosis where most had recently had an ischemic event. Furthermore, Hernández-Díaz et al. have recently shown a positive association of plasma properdin levels and the presence of subclinical carotid atherosclerosis in patients with rheumatoid arthritis.[32] However, this study presented no clinical outcome data such as cardiovascular mortality, which was the most important and novel finding in the present study. Moreover, a beneficial role for properdin in reducing the progression of atherosclerosis emerged from study of atherosclerosis in mice.[33] Also, although properdin deficiency may protect the host against enhanced inflammation in various mice models,[34] an opposite finding pertains to experimental colitis.[35] Thus, the different models, somewhat different diseases and clinical settings as well as different outcome measurements might explain the contrasting findings.

In addition, tissue samples may show increased properdin content, accompanied by low circulating levels,[34] potentially further explaining the different results of circulating properdin levels in relation to inflammatory diseases. Bousier et al. showed high RNA expression of properdin in whole blood accompanied by low protein levels of properdin in serum in patients with severe COVID-19 disease, suggesting its deposition at the sites of complement activation in inflamed tissue at least partly by infiltrating immune cells.[36] In septicemia, low properdin levels in serum portend decreased survival in patients at the intensive care unit.[37] We have previously observed a similar pattern in patients heart failure, with decreased properdin levels significantly associated with clinical, neurohormonal and echocardiographic measures of disease severity, as well as poor prognosis. In that study, our supplementary in ex vivo experiments showed that during complement activation properdin is depleted from the fluid phase (plasma) with binding of properdin to an activating surface.[38] We hypothesize that similar mechanisms could contribute to the observation in the present study. Indeed, we show that whereas low circulating properdin levels were associated with adverse outcome, properdin levels within the carotid plaques were positively correlated with symptomatology and plaque vulnerability as assessed by a predefined histological vulnerability index. Moreover, despite lacking quantitative assessment, thrombus material obtained at the site of intracerebral occlusion in patients with acute ischemic stroke secondary to carotid atherosclerosis showed strong properdin staining colocalized to intrathrombus neutrophils. Indeed, properdin seems to play a major role in thromboinflammation, contributing to formation of platelet/neutrophil aggregates.[5]

Taken together, we suggest that the lower properdin levels in patients with poor prognosis result from increased properdin deposition at the site of inflammation (ie, the atherosclerotic lesion), representing a shift from the fluid to the tissue phase. Moreover, our data from available single cell transcriptome analyses of carotid plaques suggest that infiltrating or resident macrophages within the lesion could also contribute to plaque properdin levels via local production. In addition, as properdin is produced in immune cells, including leukocytes, it is possible that activated leukocytes during inflammation, such as an ischemic stroke, are attracted to the inflammatory culprit, resulting in increased local rather than systemic levels of properdin. In particular,

whereas mature neutrophils do not synthesize properdin, these cells contain large stores of properdin in secondary granules, which will be released upon contact with an inflammatory lesion,[5] and notably, this increase in local protein levels such as within an inflammatory plaque, will not be accompanied by a similar increase in RNA levels. Nonetheless, although the possible mechanisms resulting in the association of low and not high plasma levels of properdin with adverse outcome is still not clear, properdin is a pivotal regulator of the alternative pathway of complement activation, the only "positive" regulator of complement, prolonging the stability of the alternative pathways C3-convertase and enhancing the complement activation. Notably, these properties operate locally and not systemically, underscoring the pathogenic relevance of our findings herein.

In contrast to properdin, FD, the essential protease of AP, the AP C3 convertase C3bBbP and FH, did not associate with adverse outcome in patents with carotid atherosclerosis. However, contrary to FH, a negative regulator of the AP, circulating levels of FD and C3bBbP were significantly higher in patients with carotid atherosclerosis than in healthy controls. These findings suggest an imbalance between inhibitory and activating factors of the AP in patients with carotid atherosclerosis.

Some limitations merit consideration when interpreting the current data. We lack a few clinical characteristics from some of the patients. Furthermore, not all measurements were available in both cohorts. Moreover, the precise mechanisms of properdin's potential pathogenic role in ischemic stroke remain incompletely investigated. Serial samples from the patients are lacking and the number of patients in the carotid plaque properdin gene analyses were restricted by availability. Furthermore, in some of the subgroup analyses, such as those related to time since the index stroke and the degree of carotid stenosis, the number of patients was low, in particular in those with cardiovascular mortality. The strengths of the study include a reasonably high number of patients in the plasma and plaque protein analyses and the use of a validation cohort, the use of both all-cause and cardiovascular mortality and the combination of data from circulation and data from carotid plaques and intracranial thrombi.

Conclusions

In conclusion, our findings of a strong and independent association of low circulating properdin levels (ie, below median levels within the carotid plaque cohort) with cardiovascular mortality in both the discovery and the validation cohort may reflect increased deposition and, at least in some degree, production by plaque immune cells such as macrophages production of properdin locally within the atherosclerotic lesion indicating enhanced alternative pathway activation. Our finding encourages further studies of this marker in other cardiovascular diseases and studies on the pathogenic role of properdin in these processes.

ARTICLE INFORMATION

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Disclosures

Dr Libby is an unpaid consultant to, or involved in clinical trials for Amgen, Baim Institute, Beren Therapeutics, Esperion Therapeutics, Genentech, Kancera, Kowa Pharmaceuticals, Novo Nordisk, Novartis, and Sanofi-Regeneron. Dr Libby is a member of the scientific advisory board for Amgen, Caristo Diagnostics, CSL Behring, Elucid Bioimaging, Kancera, Kowa Pharmaceuticals, Olatec Therapeutics, Novartis, PlaqueTec, Polygon Therapeutics, TenSixteen Bio, Soley Thereapeutics, and XBiotech, Inc. Dr Libby's laboratory has received research funding in the past 2 years from Novartis, Novo Nordisk and Genentech. Dr Libby is on the Board of Directors of XBiotech, Inc. Dr Libby has a financial interest in Xbiotech, in TenSixteen Bio, and in Soley Therapeutics. Dr Libby's interests were reviewed and are managed by Brigham and Women's Hospital and Mass General Brigham in accordance with their conflict-of-interest policies. The remaining authors have no disclosures to report.

Supplemental Material

Table S1
Figures S1–S5

REFERENCES

1. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105:1135–1143. doi: 10.1161/hc0902.104353

2. Kiss MG, Binder CJ. The multifaceted impact of complement on atherosclerosis. *Atherosclerosis*. 2022;351:29–40. doi: 10.1016/j.atherosclerosis.2022.03.014
3. Ricklin D, Lambris JD. Complement in immune and inflammatory disorders: pathophysiological mechanisms. *J Immunol*. 2013;190:3831–3838. doi: 10.4049/jimmunol.1203487
4. Harboe M, Mollnes TE. The alternative complement pathway revisited. *J Cell Mol Med*. 2008;12:1074–1084. doi: 10.1111/j.1582-4934.2008.00350.x
5. Blatt AZ, Pathan S, Ferreira VP. Properdin: a tightly regulated critical inflammatory modulator. *Immunol Rev*. 2016;274:172–190. doi: 10.1111/imr.12466
6. Maffia P, Mauro C, Case A, Kemper C. Canonical and non-canonical roles of complement in atherosclerosis. *Nat Rev Cardiol*. 2024;21:743–761. doi: 10.1038/s41569-024-01016-y
7. Wezel A, de Vries MR, Lagraauw HM, Foks AC, Kuiper J, Quax PH, Bot I. Complement factor C5a induces atherosclerotic plaque disruptions. *J Cell Mol Med*. 2014;18:2020–2030. doi: 10.1111/jcmm.12357
8. Ma Y, Liu Y, Zhang Z, Yang GY. Significance of complement system in ischemic stroke: a comprehensive review. *Aging Dis*. 2019;10:429–462. doi: 10.14336/AD.2019.0119
9. Casas JP, Hingorani AD, Bautista LE, Sharma P. Meta-analysis of genetic studies in ischemic stroke: thirty-two genes involving approximately 18,000 cases and 58,000 controls. *Arch Neurol*. 2004;61:1652–1661. doi: 10.1001/archneur.61.11.1652
10. Olsson S, Stokowska A, Holmegaard L, Jood K, Blomstrand C, Pekna M, Jern C. Genetic variation in complement component C3 shows association with ischaemic stroke. *Eur J Neurol*. 2011;18:1272–1274. doi: 10.1111/j.1468-1331.2011.03377.x
11. Niyonzima N, Bakke SS, Gregersen I, Holm S, Sandanger Ø, Orrem HL, Sporsheim B, Ryan L, Kong XY, Dahl TB, et al. Cholesterol crystals use complement to increase NLRP3 signaling pathways in coronary and carotid atherosclerosis. *EBioMedicine*. 2020;60:102985. doi: 10.1016/j.ebiom.2020.102985
12. Si W, He P, Wang Y, Fu Y, Li X, Lin X, Chen F, Cao G, Zhang H. Complement complex C5b-9 levels are associated with the clinical outcomes of acute ischemic stroke and carotid plaque stability. *Transl Stroke Res*. 2019;10:279–286. doi: 10.1007/s12975-018-0658-3
13. Sandvig HV, Aam S, Alme KN, Askim T, Beyer MK, Ellekjaer H, Ihle-Hansen H, Lydersen S, Mollnes TE, Munthe-Kaas R, et al. Plasma inflammatory biomarkers are associated with Poststroke cognitive impairment: the nor-COAST study. *Stroke*. 2023;54:1303–1311. doi: 10.1161/STROKEAHA.122.041965
14. Bonati LH, Kakkos S, Berkefeld J, de Borst GJ, Bulbulia R, Halliday A, van Herzeele I, Koncar I, McCabe DJ, Lal A, et al. European stroke organisation guideline on endarterectomy and stenting for carotid artery stenosis. *Eur Stroke J*. 2021;6:I–XLVII. doi: 10.1177/23969873211012121
15. Skarpengland T, Skjelland M, Kong XY, Skagen K, Holm S, Otterdal K, Dahl CP, Krohg-Sorensen K, Sagen EL, Bjerkeli V, et al. Increased levels of lectin-like oxidized low-density lipoprotein Receptor-1 in ischemic stroke and transient ischemic attack. *J Am Heart Assoc*. 2018;7:e006479. doi: 10.1161/JAHA.117.006479
16. Goncalves I, Sun J, Tengryd C, Nitulescu M, Persson AF, Nilsson J, Edsfeldt A. Plaque vulnerability index predicts cardiovascular events: a histological study of an endarterectomy cohort. *J Am Heart Assoc*. 2021;10:e021038. doi: 10.1161/JAHA.120.021038
17. Goncalves I, Moses J, Dias N, Pedro LM, Fernandes e Fernandes J, Nilsson J, Ares MP. Changes related to age and cerebrovascular symptoms in the extracellular matrix of human carotid plaques. *Stroke*. 2003;34:616–622. doi: 10.1161/01.STR.0000058157.69113.F6
18. Hertle E, Arts IC, van der Kallen CJ, Feskens EJ, Schalkwijk CG, Stehouwer CD, van Greevenbroek MM. The alternative complement pathway is longitudinally associated with adverse cardiovascular outcomes. The CODAM Study. *Thromb Haemost*. 2016;115:446–457. doi: 10.1160/th15-05-0439
19. Bergseth G, Ludviksen JK, Kirschfink M, Giclas PC, Nilsson B, Mollnes TE. An international serum standard for application in assays to detect human complement activation products. *Mol Immunol*. 2013;56:232–239. doi: 10.1016/j.molimm.2013.05.221
20. Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, Videm V, Lappegård KT, Köhl J, Lambris JD. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. *Blood*. 2002;100:1869–1877.
21. Skagen K, Skjelland M, Russell D, Jacobsen EA. Large-vessel occlusion stroke: effect of recanalization on outcome depends on the National Institutes of Health stroke scale score. *J Stroke Cerebrovasc Dis*. 2015;24:1532–1539. doi: 10.1016/j.jstrokecerebrovasdis.2015.03.020
22. Dahl TB, Yndestad A, Skjelland M, Oie E, Dahl A, Michelsen A, Damas JK, Tunheim SH, Ueland T, Smith C, et al. Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization. *Circulation*. 2007;115:972–980. doi: 10.1161/CIRCULATIONAHA.106.665893
23. Pan H, Xue C, Auerbach BJ, Fan J, Bashore AC, Cui J, Yang DY, Trignano SB, Liu W, Shi J, et al. Single-cell genomics reveals a novel cell state during smooth muscle cell phenotypic switching and potential therapeutic targets for atherosclerosis in mouse and human. *Circulation*. 2020;142:2060–2075. doi: 10.1161/CIRCULATIONAHA.120.048378
24. Slenders L, Landsmeer LPL, Cui K, Depuydt MAC, Verwer M, Mekke J, Timmerman N, van den Dungen NAM, Kuiper J, de Winther MPJ, et al. Intersecting single-cell transcriptomics and genome-wide association studies identifies crucial cell populations and candidate genes for atherosclerosis. *Eur Heart J Open*. 2022;2:oeab043. doi: 10.1093/ehjopen/oeab043
25. Ma WF, Hodonsky CJ, Turner AW, Wong D, Song Y, Mosquera JV, Ligay AV, Slenders L, Gancayco C, Pan H, et al. Enhanced single-cell RNA-seq workflow reveals coronary artery disease cellular cross-talk and candidate drug targets. *Atherosclerosis*. 2022;340:12–22. doi: 10.1016/j.atherosclerosis.2021.11.025
26. Bazan HA, Brooks AJ, Vongbunyong K, Tee C, Douglas HF, Klingenberg NC, Woods TC. A pro-inflammatory and fibrous cap thinning transcriptome profile accompanies carotid plaque rupture leading to stroke. *Sci Rep*. 2022;12:13499. doi: 10.1038/s41598-022-17546-9
27. D'Agostino RB Sr, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, Kannel WB. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation*. 2008;117:743–753. doi: 10.1161/CIRCULATIONAHA.107.699579
28. Luan YY, Yao YM. The clinical significance and potential role of C-reactive protein in chronic inflammatory and neurodegenerative diseases. *Front Immunol*. 2018;9:1302. doi: 10.3389/fimmu.2018.01302
29. Shernan SK, Fitch JC, Nussmeier NA, Chen JC, Rollins SA, Mojcik CF, Malloy KJ, Todaro TG, Filloon T, Boyce SW, et al. Impact of pexelizumab, an anti-C5 complement antibody, on total mortality and adverse cardiovascular outcomes in cardiac surgical patients undergoing cardiopulmonary bypass. *Ann Thorac Surg*. 2004;77:942–949; discussion 9–50. doi: 10.1016/j.athoracsur.2003.08.054
30. Speidl WS, Kastl SP, Huber K, Wojta J. Complement in atherosclerosis: friend or foe? *J Thromb Haemost*. 2011;9:428–440. doi: 10.1111/j.1538-7836.2010.04172.x
31. Cortes C, Ohtola JA, Saggu G, Ferreira VP. Local release of properdin in the cellular microenvironment: role in pattern recognition and amplification of the alternative pathway of complement. *Front Immunol*. 2012;3:412.
32. Hernandez-Diaz M, Rodriguez-Gonzalez D, Heras-Recuero E, Gomez-Bernal F, Quevedo-Abeledo JC, Gonzalez-Rivero AF, Gonzalez-Lopez E, Ocejo-Vinyals JG, Jimenez-Sosa A, Gonzalez-Gay MA, et al. The relationship between the complement system and subclinical carotid atherosclerosis in patients with rheumatoid arthritis. *Arthritis Res Ther*. 2024;26:127. doi: 10.1186/s13075-024-03360-3
33. Steiner T, Francescut L, Byrne S, Hughes T, Jayanthi A, Guschina I, Harwood J, Cianflone K, Stover C, Francis S. Protective role for properdin in progression of experimental murine atherosclerosis. *PLoS One*. 2014;9:e92404. doi: 10.1371/journal.pone.0092404
34. Chen JY, Cortes C, Ferreira VP. Properdin: a multifaceted molecule involved in inflammation and diseases. *Mol Immunol*. 2018;102:58–72. doi: 10.1016/j.molimm.2018.05.018
35. Jain U, Midgen CA, Schwaeble WJ, Stover CM, Stadnyk AW. Properdin regulation of complement activation affects colitis in interleukin 10 gene-deficient mice. *Inflamm Bowel Dis*. 2015;21:1519–1528. doi: 10.1097/MIB.0000000000000398
36. Boussier J, Yatim N, Marchal A, Hadjadj J, Charbit B, El Sissy C, Carlier N, Pène F, Mouthon L, Tharaux PL, et al. Severe COVID-19 is associated with hyperactivation of the alternative complement pathway. *J Allergy Clin Immunol*. 2022;149:550–556. doi: 10.1016/j.jaci.2021.11.004
37. Stover CM, McDonald J, Byrne S, Lambert DG, Thompson JP. Properdin levels in human sepsis. *Front Immunol*. 2015;6:24. doi: 10.3389/fimmu.2015.00024
38. Shahini N, Michelsen AE, Nilsson PH, Ekholt K, Gullestad L, Broch K, Dahl CP, Aukrust P, Ueland T, Mollnes TE, et al. The alternative complement pathway is dysregulated in patients with chronic heart failure. *Sci Rep*. 2017;7:42532. doi: 10.1038/srep42532