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Increased Complement Factor B and Bb Levels Are Associated with Mortality in Patients with Severe Aortic Stenosis

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Inflammation is involved in initiation and progression of aortic stenosis (AS). However, the role of the complement system, a crucial component of innate immunity in AS, is unclear. We hypothesized that circulating levels of complement factor B (FB), an important component of the alternative pathway, are upregulated and could predict outcome in patients with severe symptomatic AS. Therefore, plasma levels of FB, Bb, and terminal complement complex were analyzed in three cohorts of patients with severe symptomatic AS and mild-to-moderate or severe asymptomatic AS (population 1, n = 123; population 2, n = 436; population 3, n = 61) and in healthy controls by enzyme immunoassays. Compared with controls, symptomatic AS patients had significantly elevated levels of FB (2.9- and 2.8-fold increase in population 1 and 2, respectively). FB levels in symptomatic and asymptomatic AS patients were comparable (population 2 and 3), and in asymptomatic patients FB correlated inversely with valve area. FB levels in population 1 and 2 correlated with terminal complement complex levels and measures of systemic inflammation (i.e., CRP), cardiac function (i.e., NT-proBNP), and cardiac necrosis (i.e., Troponin T). High FB levels were significantly associated with mortality also after adjusting for clinical and biochemical covariates (hazard ratio 1.37; p = 0.028, population 2). Plasma levels of the Bb fragment showed a similar pattern in relation to mortality. We concluded that elevated levels of FB and Bb are associated with adverse outcome in patients with symptomatic AS. Increased levels of FB in asymptomatic patients suggest the involvement of FB from the early phase of the disease. ***The Journal of Immunology*, 2019, 203: 000–000.**

Aortic stenosis (AS), caused by progressive calcification of the aortic valve, is the most common of all valvular diseases, and its prevalence increases with age (1). The progression of AS is actively regulated, and inflammatory pathways are suggested to be involved (2, 3). Narrowing of the aortic orifice induces cardiac pressure overload, and the left ventricle (LV) remodels to maintain normal wall stress. However, without aortic valve repair, excessive LV remodeling turns maladaptive, with development of interstitial fibrosis, a progressive LV systolic and diastolic dysfunction, and ultimately heart failure (HF) (4). Chronic HF is associated with systemic inflammation (5), and this also seems to be the case in patients with AS (6). However, whether inflammation in AS patients is related to AS itself, accompanying HF, or both is not clear. The inflammatory processes during cardiac remodeling in response to pressure overload in AS patients is not fully understood, but the innate immune system could be involved.

The complement system is a crucial arm of innate immunity and consists of more than 50 soluble and membrane-bound proteins (7).

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Abbreviations used in this article: AS, aortic stenosis; AVR, aortic valve replacement; CI, confidence interval; CRP, C-reactive protein; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FB, complement factor B; HF, heart failure; HR, hazard ratio; LV, left ventricle; LVEF, left ventricular ejection fraction; MACE, major adverse cardiovascular event; NT-proBNP, N-terminal probrain natriuretic peptide; NYHA, New York Heart Association; TCC, terminal complement complex; TnT, Troponin T.

This system can be activated through three different pathways: the classical, the lectin, and the alternative pathways, which all can initiate a rapid self-amplifying loop via the alternative pathway cascade and eventually formation of effector molecules, including the anaphylatoxins C3a and C5a and the terminal complement complex (TCC) (7). The complement system is activated in HF and associated with adverse clinical outcome (8–11), and we recently showed dysregulation of the alternative pathway in HF patients (12). The complement system is also activated in stenotic aortic valves (13), but little is known about systemic complement activation in patients with AS.

Complement factor B (FB) is a crucial component of the alternative pathway. Upon activation of this pathway, FB is cleaved to Ba and Bb, and through a Bb-dependent amplification loop C3 and C5 convertases are generated, eventually leading to formation of TCC (Fig. 1) (7). Increased circulating levels of FB have been associated with endothelium damage and risk of coronary heart disease (14). Moreover, cardiac FB expression increases in models of cardiac stress and damage, and FB deficiency reduces cardiac inflammation, ischemic damage, and cardiac hypertrophy during myocardial infarction in mice (15–17). However, the role and regulation of FB in AS patients is not known.

We hypothesized that AS patients have increased complement activation and that FB could contribute to disease development. Specifically, we evaluated if plasma FB, Bb, and TCC were 1) increased in AS patients versus healthy controls, 2) correlated with echocardiographic or biochemical measures of disease severity, and 3) could provide independent prognostic information on adverse outcomes in two independently collected AS cohorts adjusting for predefined established predictors, including N-terminal probrain natriuretic peptide (NT-proBNP), C-reactive protein (CRP), and Troponin T (TnT).

Materials and Methods

Study populations and design

Patients from two previously reported clinical studies at our tertiary center (Department of Cardiology, Oslo University Hospital, Rikshospitalet) were investigated to explore levels of FB in patients with AS. Patient population 1 consisted of 123 patients (Table I) with confirmed symptomatic AS evaluated for aortic valve replacement (AVR) surgery and consecutively enrolled between May 2005 and January 2007 (18). For comparison, blood samples were collected from 49 sex- and age-matched healthy control subjects (average age 69, 43% female). A combination endpoint for survival analysis consisting of all-cause mortality and heart transplantation was used. Patient population 2 consisted of 436 patients (Table I) with confirmed symptomatic and asymptomatic AS evaluated for AVR surgery and consecutively enrolled between May 2010 and January 2013. This cohort was registered at clinicaltrials.gov (NCT01794832). More detailed information of these patients is given by Auensen et al. (6). For comparison, blood samples were collected from 39 sex- and age-matched healthy control subjects (average age 64, 49% females). We performed two sets of follow-up analysis in patient population 2, including 1-y major adverse cardiovascular events (MACE) and 3-y all-cause mortality. In the analysis focused on MACE, patients were followed from the date of inclusion (operation day for operated or day of outpatient evaluation for unoperated patients) to the date of MACE. For survival analysis consisting of all-cause mortality, patients were followed from date of inclusion to their date of death or censored after 3 y. A third population was included to perform comparisons between patients with asymptomatic and symptomatic AS. Patient population 3 consisted of 61 patients (Table I) with confirmed symptomatic and asymptomatic AS evaluated for AVR surgery and consecutively enrolled between January and November 2018 at St Olav's Hospital in Trondheim, Norway. This cohort was registered at clinicaltrials.gov (NCT03422770).

In all study populations, all patients underwent clinical and physical examinations such as blood pressure evaluation, standard resting 12-lead electrocardiography, angiographic examination, transthoracic echocardiography, 6-min walk distance, and peripheral blood sampling. All patients were clinically stable and none had severe comorbidities such as malignancies, infections, and autoimmune disorders. Exclusion criteria were severe (grade III) aortic or mitral regulation, serum creatinine >150 μmol/liter, unwillingness to participate, or previous AVR.

All studies were approved by the Regional Committee for Ethics in Medicine of South-Eastern Norway and conducted according to the ethical guidelines outlined in the Declaration of Helsinki for use of human tissue. All participants signed a written informed consent before study participation.

Echocardiography

Doppler echocardiographic calculations of stroke volume and cardiac output were performed on the basis of the cross-sectional area of LV outflow tract and aortic annular flow velocity data. Echocardiography was performed using Vivid 7, E9, or E95 ultrasound scanners (GE Vingmed Ultrasound, Horten, Norway). Continuous wave Doppler from multiple positions was used to obtain the maximum aortic annular blood flow velocities, and aortic valve area was calculated by using the continuity equation (19). Left ventricular ejection fraction (LVEF) was obtained by using the biplane Simpson method (20). To obtain a semiquantitative measure of the morphology of the stenotic aortic valve, ultrasound backscatter data analysis was performed as previously described (21). Observers were blinded to the clinical patient status and the standard echo findings.

Biochemistry and blood sampling

Peripheral venous blood was drawn into pyrogen-free tubes with EDTA as anticoagulant from all patients in all three study populations at baseline, before AVR. The tubes were immediately immersed in melting ice and centrifuged within 30 min at 2000 × *g* for 20 min to obtain platelet-poor plasma. All samples were stored at −80°C in multiple aliquots and had been thawed once prior to assay. NT-proBNP and CRP were assayed on a MODULAR platform (high-sensitivity assay for CRP; Roche Diagnostics, Basel, Switzerland). Estimated glomerular filtration rate (eGFR) was calculated according to the Modification of Diet in Renal Disease formula. TnT was measured by electrochemiluminescence immunoassay (hsTnT, Elecsys Troponin T high sensitive; Roche Diagnostics).

Measurements of plasma FB, Bb, and TCC

ELISA was used to measure levels of FB in plasma diluted 1:400. A mAb (clone P21/15; catalog no. HM2254; Hycult Biotech, Uden, the Netherlands) with specificity for a common epitope on both native FB and the activated Ba fragment of FB was used as a coating Ab. A monoclonal FB/Ba Ab (clone M20/6; catalog no. HM2255; Hycult Biotech) biotinylated according to manufacturer's instructions (Long Arm NHS-Biotin, catalog no. 1210; Vector Laboratories) was used for detection of bound FB/Ba (22, 23). FB concentration was determined by relating the absorbance to a standard curve of pooled human plasma with known FB concentration, determined via radial immunodiffusion. MicroVue Bb Plus Fragment Enzyme Immunoassay was used to measured Bb in plasma, diluted 1:10, according to manufacturer's instructions (Quidel, San Diego, CA). TCC was measured in plasma diluted 1:5 by an in-house ELISA as previously described (24). The results are given in CAU/mL related to a standard that was human serum activated by zymosan-and heat-aggregated IgG and defined to contain 1000 CAU/ml.

Statistical analysis

Differences between controls and AS patients or asymptomatic and symptomatic patients were analyzed with the use of Mann–Whitney *U* tests. Associations between variables were assessed by means of Spearman correlation coefficient. Kaplan–Meier analysis with log-rank test was performed to visualize and evaluate differences in survival. Follow-up time for all-cause mortality in population 1 and 2 was calculated from time of inclusion to death from any cause. Multivariate Cox regression analysis was used to evaluate the association between covariates and the risk of 3-y all-cause mortality or the composite endpoint, MACE within 1 y from inclusion. All biochemical measures displayed a skewed distribution and were log-transformed and then presented as Z-scores. Hazard ratios (HR) from the Cox regression are therefore expressed as log per SD change. Confounding factors for multivariate analysis were as following: gender, age at inclusion, diabetes mellitus (DM), ejection fraction, CRP, TnT, eGFR, NT-proBNP, and New York Heart Association (NYHA) class. The *p* values are two-sided and considered significant when <0.05. All analyses were performed with SPSS for Windows version 24.

Table I. Clinical characteristics of patients with symptomatic AS

	Patient Population 1	Patient Population 2		Patient Population 3	
	Symp (n = 123)	Asymp (n = 34)	Symp (n = 402)	Asymp (n = 39)	Symp (n = 22)
Demography/medical history					
Age (y)	77 (69, 81)	83 (75, 87)	76 (67, 82)	69 (63, 789)	71 (61, 76)
Male sex (%)	57	59	56	66	58
Hypertension (%)	23	59	44	46	27
Coronary artery disease (%)	45	24	14	5	47
DM (%)	11	21	20	2	10
NYHA classification (%)					
Class III–IV	63	18	45	0	63
Echocardiographic measures					
LVEF (%)	63 (56, 72)	55 (46, 61)	56 (50, 61)	52 (48, 56)	54 (49, 63)
Aortic valve area (cm^2)	0.6 (0.5, 0.8)	0.7 (0.5, 0.9)	0.7 (0.5, 0.8)	1.2 (0.8, 1.6)	0.7 (0.6, 1.0)
Mean aortic gradient (mmHg)	55 (39.1, 67.1)	46 (40, 57)	52 (43, 64)	26 (17, 46)	56 (47, 77)
Biochemistry					
NT-proBNP (ng $liter^{-1}$)	812 (330, 2114)	955 (346, 2332)	761 (270, 1944)	97 (58, 239)	378 (198, 733)
TnT (ng $liter^{-1}$)	13 (8, 25)	16 (10, 18)	13 (10, 25)	11 (10, 20)	11 (10, 20)
CRP (mg $liter^{-1}$)	1.7 (0.9, 4.61)	2.3 (0.7, 3.4)	2.0 (0.8, 5.7)	1.1 (0.7, 2.2)	1.4 (0.6, 2.5)
eGFR (ml min^{-1} 1.73 m^{-2})	73 ± 33	66 ± 34	74 ± 32	87 (74, 90)	82 (77, 90)
ALT (U $liter^{-1}$)	na	21 ± 11	25 ± 14	23 ± 9	24 ± 11
Medication (%)					
β-blocker	50	56	53	2	48
ACE inhibitor/ARB	33	65	38	30	34
Statins	48	50	53	23	50
Ca^{2+} antagonist	8	29	19	18	11
ASA	48	47	53	11	39
Warfarin	20	27	18	0	20
Loop diuretics	33	27	22	0	31

Values are presented as (%), mean ± SD, or median (interquartile range).

ACE, angiotensin-converting enzyme; ALT, alanine transaminase; ARB, angiotensin receptor blocker; ASA, acetylsalicylic acid; Asymp, asymptomatic; na, not available; Symp, symptomatic.

Results

*Circulating levels of FB are increased in patients with symptomatic AS: patient population 1 (*n *= 123)*

Baseline characteristics of patients with AS are shown in Table I. Plasma levels of FB, an essential component required for activation of the alternative pathway of the complement system (Fig. 1), were markedly elevated (2.9-fold) in patients with symptomatic AS (n = 123) compared with healthy sex- and age-matched controls (n = 49, average age 69, 43% females) (Fig. 2A). There was no significant association between FB levels and aortic valve area or echocardiographic measures of cardiac function and structure (Table II). However, we found that plasma levels of FB were positively correlated with NT-proBNP, TnT, and CRP, reflecting associations with cardiac wall stress, myocardial injury, and systemic inflammation, respectively (Table II). There was also a significant negative correlation between FB levels and eGFR. During follow-up, 29 patients died, and in univariate Cox regression analysis, levels of FB were significantly associated with mortality (HR 1.69, 95% confidence interval [CI] [1.24–2.31], $p < 0.001$) after a mean follow-up of 4.1 y (range 1–5.6 y). The association between FB levels and mortality was statistically significant also after adjustment for AVR (HR 1.52, 95% CI [1.08–2.13], $p = 0.016$).

*Elevated levels of FB are associated with severity of the disease in patients with symptomatic AS: patient population 2 (*n *= 436)*

Baseline characteristics of patient population 2 are shown in Table I. To validate and extend the findings on FB in patient population 1, we measured FB in a larger population of patients with AS (n = 436). The population included patients scheduled for AVR (n = 344) and 92 patients that did not undergo surgery because of either lack of symptoms (n = 34), a high risk–benefit ratio (n = 38), or patient refusal (n = 20). Similar to patient population 1, FB levels were also significantly elevated (2.8-fold) in these AS patients compared with healthy controls (n = 39, average age 64, 49% females) (Fig. 2B).

FB showed no significant correlation with aortic valve area, aortic peak velocity, and mean aortic gradient or with echocardiographic measures of cardiac function and structure. Moreover, as in population 1, we found positive correlations between levels of FB and levels of NT-proBNP, CRP, and TnT and a negative correlation between levels of FB and eGFR (Table II). Furthermore, we found a negative correlation between FB and the 6-min walk test ($r = -0.31$, $p < 0.001$). Forward stepwise regression identified TnT, CRP, and eGFR as the strongest predictors of plasma levels of FB.

*Comparable levels of FB in symptomatic and asymptomatic AS patients: patient population 2 (*n *= 436) and population 3 (*n *= 61)*

Baseline characteristics for patient population 3 are shown in Table I. To investigate if there was a difference in FB levels between symptomatic and asymptomatic AS patients, we measured FB in a subset of population 2, consisting of patients with severe asymptomatic AS (n = 34) as well as in population 3, consisting of patients with mild-to-moderate or severe asymptomatic AS (n = 26 and n = 13, respectively) and compared them with their respective symptomatic patients (n = 402 and n = 22, respectively). Plasma levels of FB were elevated in asymptomatic patients but notably with no differences between asymptomatic and symptomatic AS patients in neither patient population 2 with severe patients nor in patient population 3 with mild-to-moderate and severe patients (Fig. 3, $p = 0.84$, population 2; $p = 0.17$ and $p = 0.79$, population 3). Moreover, the negative correlation between FB and valve area was stronger in the asymptomatic patients compared with the symptomatic patients in population 2

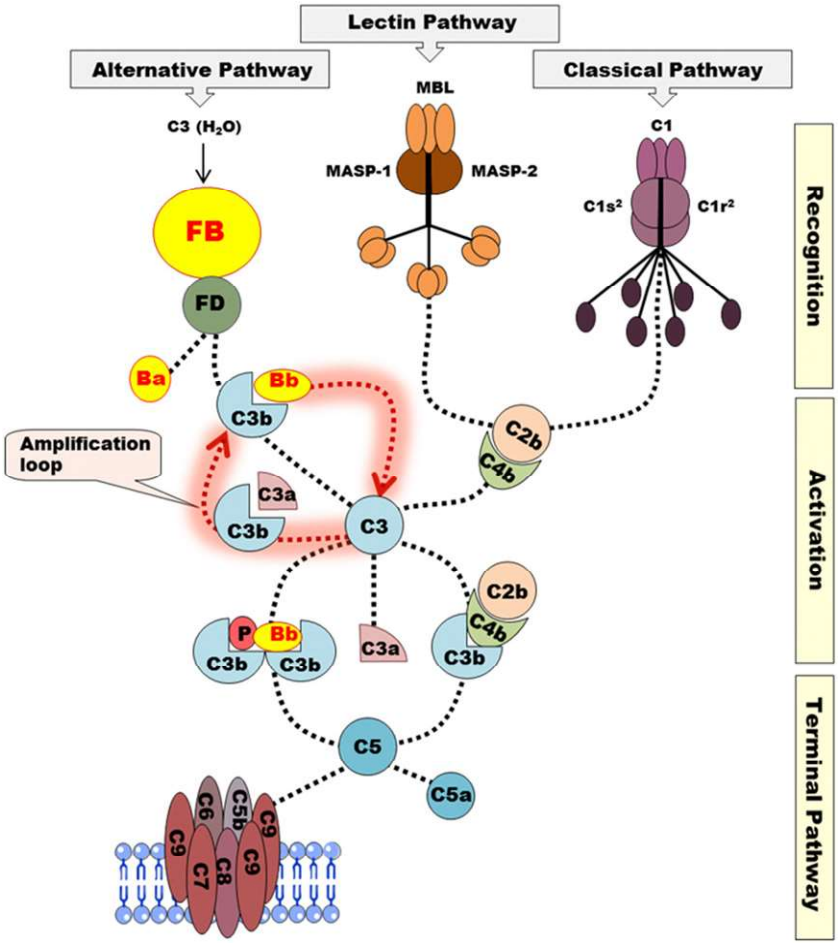

FIGURE 1. Schematic overview of the complement system. Complement can be activated through the lectin, the classical, and the alternative pathway. Alternative pathway is a dominant contributor to overall complement activation due to the amplification loop, and its activation requires activated FB (i.e., Bb) to bind to C3b. The C3bBb complex will be stabilized by properdin, which contributes to the formation of the terminal complement pathway. Mannose-binding lectin-associated serine protease (MASP), mannose-binding lectin (MBL), complement component 3 (C3), FB, complement component 5 (C5), properdin (p), complement factor D (FD), complement component 6 (C6), complement component 7 (C7), complement component 8 (C8), complement component 9 (C9).

($r = -0.39$, $p = 0.026$; $r = -0.05$, $p = 0.31$, respectively; Supplemental Table I, Table II). A similar pattern was seen in population 3, although the correlation did not reach statistical significance, potentially reflecting a low number of patients in this cohort ($r = -0.23$, $p = 0.14$; $r = 0.34$, $p = 0.11$, asymptomatic [$n = 39$] and symptomatic [$n = 22$] patients, respectively) (Supplemental Table I).

FB levels are associated with 1-y MACE and 3-y mortality in patients with symptomatic AS: patient population 2

We next analyzed the association between FB and clinical outcome in more detail in the larger patient population 2. The composite endpoint MACE was met by 42 patients referred for AVR and 16 patients referred for continued medical treatment during 1-y follow-up from inclusion. Kaplan–Meier analysis for MACE based on quartile levels of FB indicated an association between MACE and FB ($p = 0.019$; Fig. 4A). In univariate Cox regression analysis, high levels of FB were significantly associated with MACE (HR 1.50, 95% CI [1.20–1.86], $p < 0.001$), and this association was only marginally weakened with adjustment for AVR (HR 1.44, 95% CI [1.17–1.76], $p < 0.001$) but not statistically significant following further adjustment for clinical and biochemical variables ($p = 0.10$; Fig. 4C).

Three-year mortality was 10% ($n = 34$) among patients who underwent AVR, and 49% ($n = 34$) among patients referred for continued medical treatment. Kaplan–Meier analysis revealed a clear association between high levels of FB and all-cause mortality (Fig. 4B), which was also evident in univariate Cox regression analysis (HR 1.79, 95% CI [1.42–2.25], $p < 0.001$) and when adjusted for AVR (HR 0.17, 95% CI [0.11–0.27], $p < 0.001$).

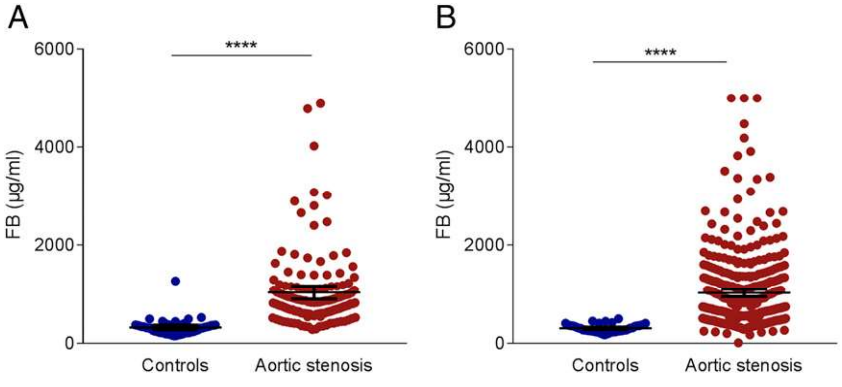

FIGURE 2. Plasma levels of FB are increased in patients with symptomatic AS. Circulating levels of FB in (**A**) 123 AS patients compared with 49 healthy controls and in (**B**), 402 AS patients compared with 39 healthy controls. Lines and error bars are mean with 95% CI. ****$p < 0.0001$.

Table II. Correlation of FB with cardiac function and biochemical parameters in patients with symptomatic AS

	Patient Population 1 (n = 123)		Patient Population 2 (n = 402)	
	r	p value	r	p value
Aortic valve area	−0.18	0.05	−0.05	0.31
CO	−0.12	0.22	−0.10	0.045
LVEF	−0.08	0.34	0.01	0.86
LVEDV	−0.08	0.38	−0.09	0.089
LVESV	−0.01	0.85	−0.06	0.27
6MWT	na	—	**−0.27**	**<0.001**
NT-proBNP	**0.40**	**<0.001**	**0.28**	**<0.001**
TnT	**0.48**	**<0.001**	**0.33**	**<0.001**
eGFR	**−0.49**	**<0.001**	**−0.43**	**<0.001**
ALT	na	-	**−0.11**	**0.037**
CRP	**0.29**	**<0.001**	**0.27**	**<0.001**
WBC	0.18	0.052	0.09	0.075

Bold values indicate statistical significance at the $p < 0.05$ level.

ALT, alanine transaminase; CO, cardiac output; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; 6MWT, 6-min walk test; na, not available.

The association remained significant also after adjustment for clinical and biochemical variables, including TnT, NT-proBNP, and CRP (HR 1.37, 95% CI [1.02–1.83], $p = 0.036$) (Fig. 4D).

To further evaluate whether AVR affected the association between FB and outcome, we analyzed the two groups (i.e., nonoperated [$n = 92$] and AVR [$n = 344$]) separately.

Importantly, Kaplan–Meier analysis showed a significant association between high levels of FB and all-cause mortality in AVR ($p = 0.002$) but not nonoperated ($p = 0.539$) groups (Fig. 5, respectively). This association was further established in univariate Cox regression in the AVR group (HR 1.63 [1.18–2.26], $p = 0.003$). Moreover, the univariate Cox regression revealed similar associations between higher levels of FB and MACE in both the AVR (HR 1.38 [1.08–1.77], $p = 0.011$) and nonoperated (HR 1.40 [0.95–2.06], $p = 0.087$) groups.

Plasma levels of TCC are increased in patients with symptomatic AS but not associated with clinical outcome: patient population 2

In this study, we found a significant correlation between FB and TCC ($p < 0.001$; Fig. 6A), suggesting that high FB levels trigger the activation of the final common pathway of the complement cascade. However, whereas plasma levels of TCC were significantly elevated (2.1-fold) as compared with the control group ($p < 0.0001$; Fig. 6B), Kaplan–Meier curve for MACE, according to quartile levels of TCC, showed no significant association between TCC and adverse outcome of the disease ($p = 0.133$; Fig. 7A). The same nonsignificant association was revealed when using Kaplan–Meier analysis for all-cause mortality on quartile levels of TCC ($p = 0.269$; Fig. 7B) with the same pattern in AVR (univariate Cox regression: HR 1.12 [0.81–1.55], $p = 0.47$) and nonoperated patients (HR 1.12, [0.76–1.64], $p = 0.55$).

Plasma levels of the FB activation product Bb reflected FB in outcome but contributed marginally to native FB levels: patient population 2

Because our Ab against FB cannot distinguish between native FB and the activated Ba or Bb fragment, we measured Bb levels in patient population 2. Kaplan–Meier survival analysis of 3-y all-cause mortality revealed that patients with the highest and lowest levels of the Bb fragment have the highest and lowest all-cause mortality, respectively ($p = 0.007$, Fig. 8). Bb levels largely showed the same pattern in relation to outcome analyses as compared with FB levels, and in this population of symptomatic AS patients, these factors were significantly correlated ($r = 0.22$, $p < 0.000$). However, the level of Bb in our FB assay was negligible (~1%).

Discussion

Complement activation has been found in several forms of acute and chronic cardiovascular disease (9, 25). In this study, for the first time, to our knowledge, we have demonstrated that patients with symptomatic AS have increased complement activation, detected by TCC levels, representing the activation of the complement cascade to its final stage. Moreover, FB levels as a marker of activation of the alternative pathway were correlated with measures of systemic inflammation, cardiac wall stress, and cardiac injury. Most importantly, elevated levels of FB were significantly associated with increased risk of MACE and all-cause mortality. Our findings suggest the involvement of complement activation in

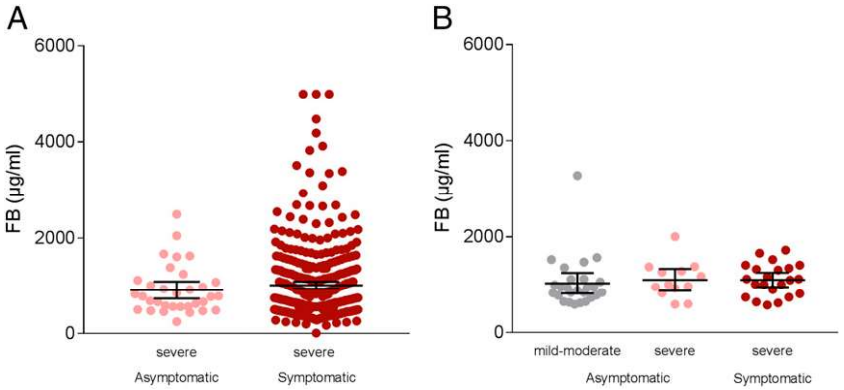

FIGURE 3. Plasma levels of FB are increased in patients with asymptomatic AS. Circulating levels of FB in (**A**) 34 severe asymptomatic AS patients compared with 402 symptomatic AS patients and in (**B**) 26 mild-to-moderate and 13 severe asymptomatic AS patients compared with 22 symptomatic AS patients. Lines and error bars are mean with 95% CI.

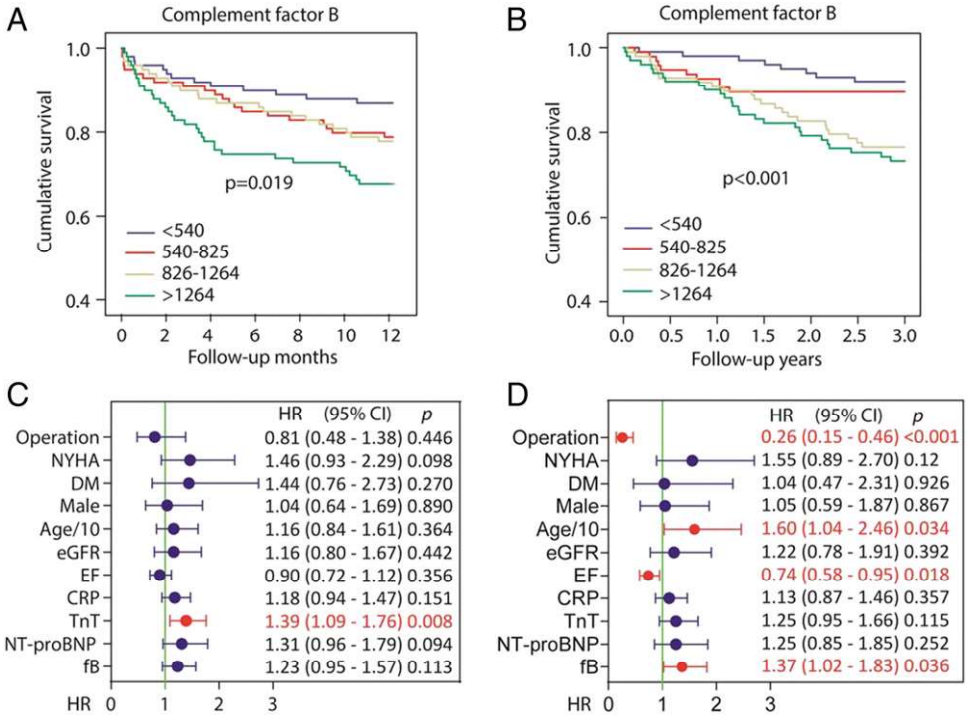

FIGURE 4. FB is significantly associated with all-cause mortality in patients with symptomatic AS. Kaplan–Meier survival analysis of (**A**) 1-y MACE and (**B**) 3-y all-cause mortality in relation to quartile levels of FB. Adjusted HR based on FB levels, estimated by Cox proportional analysis, for (**C**) MACE and (**D**) all-cause mortality. Risk estimates are adjusted for operation, NYHA class, DM, gender, age at inclusion, eGFR, ejection fraction (EF), CRP, TnT, NT-proBNP, and FB.

the progression of AS, and FB could potentially represent a novel marker for risk stratification in these patients.

Several studies of complement activation point to the activation of the classic and lectin pathway in chronic HF (8, 11). Moreover, we have recently shown the significance of the alternative pathway by demonstrating enhanced levels of activators factor D and properdin and decreased levels of inhibitory factor H (12). In contrast, data on complement activation in AS patients are scarce. In a small study of patients undergoing AVR surgery (n = 24), Helske et al. (13) reported upregulation of TCC and the anaphylatoxin receptors C3aR and C5aR. In this study, we report enhanced systemic complement activation in two populations of AS patients as shown by increased plasma levels of TCC, indicating that the complement cascade is activated to the very end. Moreover, high levels of FB were significantly associated with all-cause mortality after 3 y also after adjustment for both clinical and biochemical variables, including TnT, CRP, and NT-proBNP that all are established as strong predictors of outcome in various cardiovascular diseases. Although FB was weakly but significantly correlated with TCC, terminal complement activation was not associated with adverse outcome of the disease. In fact, although soluble plasma TCC is important as a marker of overall complement activation, it does not have any known function in its soluble form. Tissue deposition of TCC, not evaluated in this study, could also be a better prognostic factor than the fluid phase levels in this study. Our data indicate an increased level of FB is a triggering factor for increased alternative pathway activation, rather than being a marker of complement activation per se. Thus, complement activation, and in particular activation of the alternative pathway with release of Bb, is a good candidate for reflecting the inflammatory phenotype in patients with symptomatic AS, potentially being both a marker and mediator of disease progression.

It could be argued that the enhanced systemic levels of FB reflect myocardial remodeling rather than the inflammatory process within the aortic valve itself. However, we found elevated FB levels in patients both with asymptomatic and symptomatic AS, including those with mild-to-moderate AS. Moreover, although no significant correlation was found between valve area in symptomatic AS

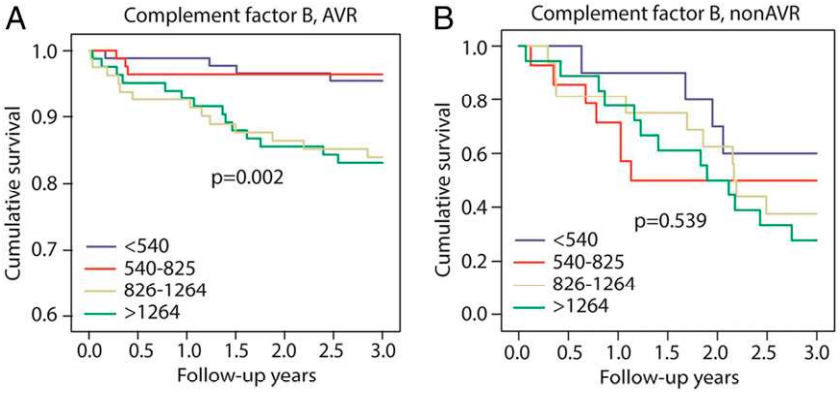

FIGURE 5. Circulating levels of FB are associated with all-cause mortality in both AVR and nonoperated patients with symptomatic AS. Kaplan–Meier survival curves in relation to quartile levels of FB in (**A**) 344 patients with AVR and (**B**) 92 nonoperated patients with AS.

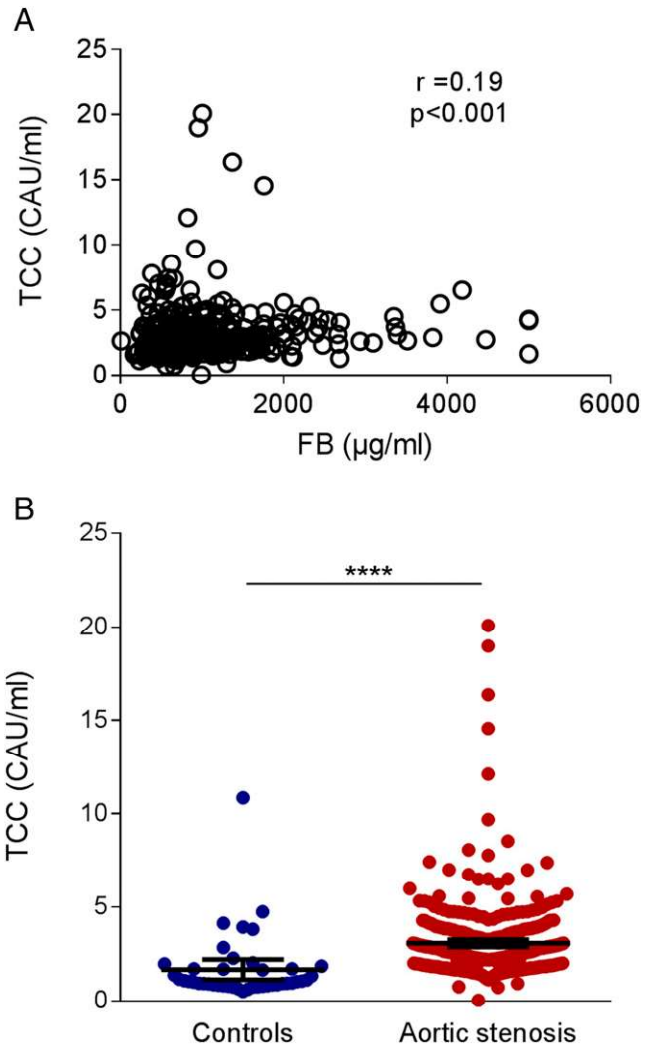

FIGURE 6. Plasma levels of TCC are increased in patients with symptomatic AS. Correlation between plasma levels of TCC and FB (**A**). Circulating levels of TCC in 402 patients compared with 39 healthy controls (**B**). Lines and error bars are mean with 95% CI. ****$p < 0.0001$.

patients, FB was inversely correlated with valve area in the asymptomatic patients. These findings suggest that FB and its activation may be involved in the pathogenesis of AS, potentially operating in the early phase of the disorder and not only elevated as a cause of disease severity and adverse myocardial remodeling. Moreover, FB levels were associated with all-cause mortality and incidence of MACE in patients undergoing AVR but not in the nonoperated patients. Although we cannot fully explain this pattern, it may reflect that pathogenic pathways related to adverse outcome of the disease are still active after AVR and that the trigger of FB activation is not "removed" by removing the diseased aortic valve. However, these issues will have to be clarified in larger studies that also have to include patients with asymptomatic AS.

Our Ab against FB could not differentiate between native FB and the activated Ba fragment, which is important when distinguishing whether the increased FB was due to increased native FB or in addition to its activation fragments Ba and Bb. In our study, we concluded that the level of the Bb fragment contributes <1% to the total FB level. Still this might be of pathophysiological importance, because the $t_{1/2}$ of the activation products are very short as compared with the native components. Furthermore, the activation products are normally biologically highly active as compared with their native zymogen. Interestingly, the Bb levels showed the same pattern in relation to outcome analyses, as FB and were significantly correlated with FB levels. Thus, they seem rather equivalent as biomarkers in the clinical setting.

Whereas the enhanced levels of FB could be a potential promising marker of adverse outcome in patients with symptomatic AS, FB could potentially also be a mediator of disease progression in these patients. FB is primarily synthesized in the liver (26); however, recent studies have also suggested that FB can be produced locally in the heart by cardiomyocytes, cardiac fibroblasts, and macrophages (17). Several lines of evidence have indicated that local increased mRNA expression levels of FB in cardiac cells during cardiac stress are involved in AS progression (15–17). Experimental studies have revealed that activation of TLR signaling, particularly TLR4, induces FB production in cardiac cells and increases alternative pathway activation (17, 27). As deletion of FB is associated with a 50% reduction of total complement activation, it is tempting to hypothesize that some of the TLR-mediated effects within the heart, including their effects on aortic valve calcification and inflammation, may be mediated through induction of FB (28, 29).

Some limitations should be considered when interpreting the current study. First, it could be of a great value to measure the complement activation at different time points by consecutive inclusion of the patients in different stages of disease severity. This would allow us to draw a conclusion and evaluate the possible relationship between systemic complement activation and FB in particular and disease progression of patients with AS. Second, the group of nonoperated patients was small and included both high-risk and low-risk patients; therefore, they were not adequate for multivariate analysis. Finally, the number of asymptomatic patients was rather small, in particular the number of patients with mild-to-moderate disease.

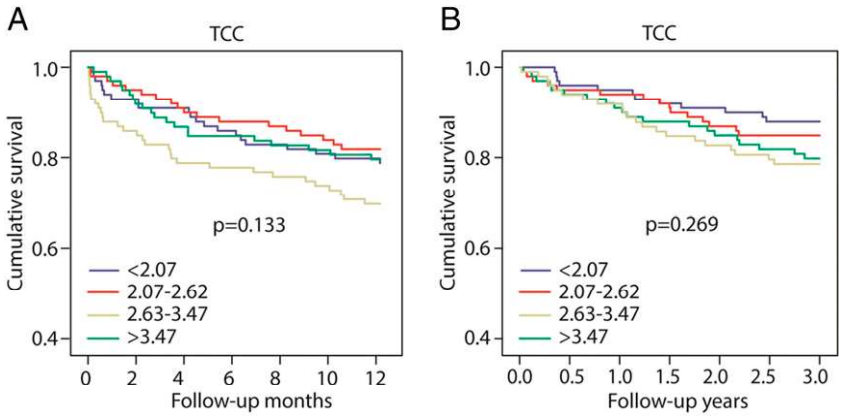

FIGURE 7. TCC levels are not associated with outcome in patients with AS. Kaplan–Meier survival curves in relation to quartiles levels of TCC using (**A**) MACE and (**B**) 3-y all-cause mortality as endpoint.

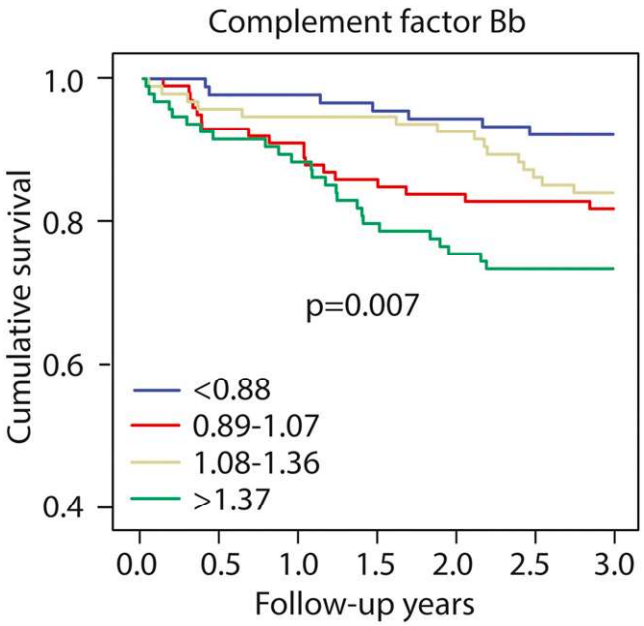

FIGURE 8. High and low levels of the Bb fragment have the worst and best outcome in all-cause mortality in patients with symptomatic AS. Kaplan–Meier survival analysis of 3-y all-cause mortality in relation to quartile levels of Bb fragment in 402 AS patients.

In conclusion, our results show that circulating levels of FB, Bb, and TCC are increased in patients with symptomatic AS, and for FB we found a significant association with all-cause mortality also after adjustment for other prognostic factors of AS, including TnT, CRP, and NT-proBNP. Moreover, our data also suggest that FB may be involved in the pathogenesis of AS, potentially operating in the early phase of the disorder and is not only elevated as a consequence of disease severity and adverse myocardial remodeling. Our findings show that complement activation and increased levels of FB are part of the inflammatory pathways that are active in both asymptomatic and symptomatic AS patients, potentially also contributing to disease progression in these patients.

Disclosures

The authors have no financial conflicts of interest.

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