

1 **Increased complement factor B and Bb levels are associated with mortality**
2 **in patients with severe aortic stenosis**

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4 **Running title:** Complement factor B in aortic stenosis

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1 **Abstract**

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3 Inflammation is involved in initiation and progression of aortic stenosis (AS). However, the
4 role of the complement system, a crucial component of innate immunity in AS is unclear. We
5 hypothesized that circulating levels of complement factor B (FB), an important component of
6 the alternative pathway, are upregulated and could predict outcome in patients with severe
7 symptomatic AS. Therefore, plasma levels of FB, Bb and terminal complement complex
8 (TCC), were analyzed in three cohorts of patients with severe symptomatic AS and mild-
9 moderate or severe asymptomatic AS (*population 1*, n=123; *population 2*, n=436; *population*
10 *3*, n=61), and in healthy controls by enzyme immunoassays. Compared to controls,
11 symptomatic AS patients had significantly elevated levels of FB (2.9- and 2.8-fold increase in
12 population 1 and 2, respectively). FB levels in symptomatic and asymptomatic AS patients
13 were comparable (population 2 and 3), and in asymptomatic patients FB correlated inversely
14 with valve area. FB levels in population 1 and 2 correlated with TCC levels and measures of
15 systemic inflammation (*i.e.* CRP), cardiac function (*i.e.* NT-proBNP) and cardiac necrosis
16 (*i.e.* troponin T). High FB levels were significantly associated with mortality also after
17 adjusting for clinical and biochemical covariates (hazard ratio 1.37; p=0.028, population 2).
18 Plasma levels of the Bb fragment showed a similar pattern in relation to mortality. We
19 concluded that elevated levels of FB and Bb are associated with adverse outcome in patients
20 with symptomatic AS. Increased levels of FB in asymptomatic patients suggest the
21 involvement of FB from the early phase of the disease.

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1 **Key points**

- 2 • Plasma FB levels are elevated in patients with symptomatic and asymptomatic AS
- 3 • High FB and Bb levels are associated with adverse outcome in severe symptomatic
- 4 AS
- 5 • FB may be involved in AS pathogenesis, and could operate in the early disease phase

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1 **Key words**

2 complement factor B; complement system; inflammation; aortic stenosis; biomarker.

1 **Introduction**

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

14 The complement system is a crucial arm of innate immunity and consists of more than
15 50 soluble and membrane-bound proteins (7). This system can be activated through three
16 different pathways: the classical, the lectin and the alternative pathways which all can initiate
17 a rapid self-amplifying loop via the alternative pathway cascade and eventually formation of
18 effector molecules, including the anaphylatoxins C3a and C5a, and the terminal complement
19 complex (TCC) (7). The complement system is activated in HF and associated with adverse
20 clinical outcome (8-11), and we recently showed dysregulation of the alternative pathway in
21 HF patients (12). The complement system is also activated in stenotic aortic valves (13), but
22 little is known about systemic complement activation in patients with AS.

23 Complement factor B (FB) is a crucial component of the alternative pathway. Upon
24 activation of this pathway, FB is cleaved to Ba and Bb, and through a Bb-dependent
25 amplification loop C3- and C5-convertases are generated, eventually leading to formation of

1 TCC (Figure 1)(7). Increased circulating levels of FB have been associated with endothelium
2 damage and risk of coronary heart disease (14). Moreover, cardiac FB expression increases in
3 models of cardiac stress and damage, and FB deficiency reduces cardiac inflammation,
4 ischemic damage and cardiac hypertrophy during myocardial infarction in mice (15-17).
5 However, the role and regulation of FB in AS patients is not known.

6 We hypothesized that AS patients have increased complement activation and that FB
7 could contribute to disease development. Specifically, we evaluated if plasma FB, Bb and
8 TCC were (I) increased in AS patients vs. healthy controls, (II) correlated with
9 echocardiographic or biochemical measures of disease severity and (III) could provide
10 independent prognostic information on adverse outcomes in two independently collected AS
11 cohorts adjusting for predefined established predictors including N-terminal pro-brain
12 natriuretic peptide (NT-proBNP), C-reactive protein (CRP) and Troponin T (TnT).

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1 **Materials and methods**

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3 **Study populations and design**

4 Patients from two previously reported clinical studies at our tertiary center (Department of
5 Cardiology, Oslo University Hospital, Rikshospitalet) were investigated in order to explore
6 levels of FB in patients with AS. *Patient population 1* consisted of 123 patients (Table 1)
7 with confirmed symptomatic AS, evaluated for aortic valve replacement (AVR) surgery, and
8 consecutively enrolled between May 2005 and January 2007(18). For comparison, blood
9 samples were collected from 49 sex- and age-matched healthy control subjects (average age
10 69, 43% female). A combination endpoint for survival analysis consisting of all-cause
11 mortality and heart transplantation was used. *Patient population 2* consisted of 436 patients
12 (Table 1) with confirmed symptomatic and asymptomatic AS, evaluated for AVR surgery,
13 and consecutively enrolled between May 2010 and January 2013. This cohort was registered
14 at Clinicaltrials.gov (NCT01794832). More detailed information of these patients is given by
15 Auensen *et al.* (6). For comparison, blood samples were collected from 39 sex- and age-
16 matched healthy control subjects (average age 64, 49% females). We performed two sets of
17 follow-up analysis in patient population 2 including; one-year major adverse cardiovascular
18 events (MACE) and three-year all-cause mortality. In the analysis focused on MACE, patients
19 were followed from the date of inclusion (operation day for operated, or day of outpatient
20 evaluation for un-operated patients) to the date of MACE. For survival analysis consisting of
21 all-cause mortality, patients were followed from date of inclusion to their date of death or
22 censored after three years. A third population was included to perform comparisons between
23 patients with asymptomatic and symptomatic AS. *Patient population 3* consisted of 61
24 patients (Table 1) with confirmed symptomatic and asymptomatic AS, evaluated for AVR
25 surgery, and consecutively enrolled between January and November 2018 at St Olav's

1 Hospital in Trondheim, Norway. This cohort was registered at clinicaltrials.gov
2 (NCT03422770).

3 In all study populations, all patients underwent clinical and physical examinations
4 such as blood pressure evaluation, standard resting 12-lead electrocardiography (ECG),
5 angiographic examination, transthoracic echocardiography, 6-minute walk distance, and
6 peripheral blood sampling. All patients were clinically stable and none had severe
7 comorbidities such as malignancies, infections and autoimmune disorders. Exclusion criteria
8 were severe (grade III) aortic or mitral regurgitation, serum creatinine $>150 \mu\text{mol/L}$,
9 unwillingness to participate, or previous AVR.

10 All studies were approved by the Regional committee for ethics in medicine of South-
11 Eastern Norway and conducted according to the ethical guidelines outlined in the Declaration
12 of Helsinki for use of human tissue. All participants signed a written informed consent before
13 study participation.

14 **Echocardiography**

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

1 **Biochemistry and blood sampling**

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

12 **Measurements of plasma FB, Bb and TCC**

13 Enzyme-linked immunosorbent assay (ELISA) was used to measure levels of FB in plasma
14 diluted 1:400. A monoclonal antibody (clone P21/15; catalog#HM2254, Hycult Biotech,
15 Uden, the Netherlands) with specificity for a common epitope on both native FB and the
16 activated Ba-fragment of FB was used as a coating antibody. A monoclonal FB/Ba antibody
17 (clone M20/6; catalog#HM2255, Hycult Biotech, Uden, the Netherlands), biotinylated
18 according to manufacturer's instructions (Long arm NHS-biotin, catalog#1210, Vector
19 Laboratories), was used for detection of bound FB/Ba(22, 23). FB concentration was
20 determined by relating the absorbance to a standard curve of pooled human plasma with
21 known FB concentration, determined via radial immunodiffusion. MicroVue Bb Plus
22 Fragment Enzyme Immunoassay was used to measured Bb in plasma, diluted 1:10, according
23 to manufacturer's instructions (Quidel, San Diego, CA). TCC was measured in plasma diluted
24 1:5 by an in-house ELISA as previously described (24). The results are given in complement

1 arbitrary units (CAU) per mL, related to a standard that was human serum activated by
2 zymosan-and heat-aggregated IgG, and defined to contain 1000 CAU/mL.

3 **Statistical Analysis**

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

18

1 **Results**

2

3 **Circulating levels of FB are increased in patients with symptomatic AS – Patient** 4 **population 1 (n=123)**

5 Baseline characteristics of patients with AS are shown in Table 1. Plasma levels of FB, an
6 essential component required for activation of the alternative pathway of the complement
7 system, were markedly elevated (2.9-fold) in patients with symptomatic AS (n=123)
8 compared to healthy sex- and age-matched controls (n=49, average age 69, 43% females)
9 (Figure 2A). There was no significant association between FB levels and aortic valve area or
10 echocardiographic measures of cardiac function and structure (Table 2). However, we found
11 that plasma levels of FB were positively correlated with NT-proBNP, TnT and CRP,
12 reflecting associations with cardiac wall stress, myocardial injury and systemic inflammation,
13 respectively (Table 2). There was also a significant negative correlation between FB levels
14 and eGFR. During follow-up, 29 patients died and in univariate Cox regression analysis,
15 levels of FB were significantly associated with mortality (HR 1.69, 95% CI [1.24-2.31],
16 $p<0.001$) after a mean follow-up of 4.1 years (range 1-5.6 years). The association between FB
17 levels and mortality was statistically significant also after adjustment for AVR (HR 1.52, 95%
18 CI [1.08-2.13], $p=0.016$).

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20 **Elevated levels of FB are associated with severity of the disease in patients with** 21 **symptomatic AS – Patient population 2 (n=436)**

22 Baseline characteristics of patient population 2 are shown in Table 1. To validate and extend
23 the findings on FB in patient population 1, we measured FB in a larger population of patients
24 with AS (n=436). The population included patients scheduled for AVR (n=344) and 92
25 patients that did not undergo surgery, due to either lack of symptoms (n=34), a high risk-

1 benefit ratio (n=38), or patient refusal (n=20). Similar to patient population 1, FB levels were
2 also significantly elevated (2.8-fold) in these AS patients compared to healthy controls (n=39,
3 average age 64, 49% females) (Figure 2B).

4 FB showed no significant correlation with aortic valve area, aortic peak velocity and
5 mean aortic gradient or with echocardiographic measures of cardiac function and structure.
6 Moreover, as in population 1, we found positive correlations between levels of FB and levels
7 of NT-proBNP, CRP and TnT and a negative correlation between levels of FB and eGFR
8 (Table 2). Furthermore, we found a negative correlation between FB and the 6-minute walk
9 test ($r=-0.31$, $p<0.001$). Forward stepwise regression identified TnT, CRP and eGFR as the
10 strongest predictors of plasma levels of FB.

11

12 **Comparable levels of FB in symptomatic and asymptomatic AS patients – Patient** 13 **population 2 (n=436) and population 3 (n=61)**

14 Baseline characteristics for patient population 3 are shown in Table 1. To investigate if there
15 was a difference in FB levels between symptomatic and asymptomatic AS patients we
16 measured FB in a subset of population 2 consisting of patients with severe asymptomatic AS
17 (n=34) as well as in population 3 consisting of patients with mild to moderate or severe
18 asymptomatic AS (n=26 and n=13, respectively) and compared them with their respective
19 symptomatic patients (n=402 and n=22, respectively). Plasma levels of FB were elevated in
20 asymptomatic patients, but notably, with no differences between asymptomatic and
21 symptomatic AS patients in neither patient population 2 with severe patients nor in patient
22 population 3 with mild to moderate and severe patients (Figure 3, $p=0.84$, population 2;
23 $p=0.17$ and $p=0.79$, population 3). Moreover, the negative correlation between FB and valve
24 area was stronger in the asymptomatic patients compared with the symptomatic patients in
25 population 2 ($r=-0.39$, $p=0.026$; $r=-0.05$, $p=0.31$, respectively; Table 2, Supplemental Table

1 1). A similar pattern was seen in population 3, although the correlation did not reach
2 statistical significance, potentially reflecting a low number of patients in this cohort ($r=-0.23$,
3 $p=0.14$; $r=0.34$, $p=0.11$, asymptomatic [$n=39$] and symptomatic [$n=22$] patients, respectively)
4 (Supplemental Table 1).

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6 **FB levels are associated with 1-year MACE and 3-year mortality in patients with**
7 **symptomatic AS - Patient population 2**

8 We next analyzed the association between FB and clinical outcome in more detail in the
9 larger patient population 2. The composite endpoint MACE was met by 42 patients referred
10 for AVR and 16 patients referred for continued medical treatment during one-year follow-up
11 from inclusion. Kaplan-Meier analysis for MACE based on quartile levels of FB indicated an
12 association between MACE and FB ($p=0.019$; Figure 4A). In univariate Cox regression
13 analysis, high levels of FB were significantly associated with MACE (HR 1.50, 95% CI
14 [1.20-1.86], $p<0.001$), and this association was only marginally weakened with adjustment for
15 AVR (HR 1.44, 95% CI [1.17-1.76], $p<0.001$), but not statistically significant following
16 further adjustment for clinical and biochemical variables ($p=0.10$; Figure 4C).

17 Three-year mortality was 10% ($n=34$) among patients who underwent AVR, and 49%
18 ($n=34$) among patients referred for continued medical treatment. Kaplan-Meier analysis
19 revealed a clear association between high levels of FB and all-cause mortality (Figure 4B),
20 which was also evident in univariate Cox regression analysis (HR 1.79, 95% CI [1.42-2.25],
21 $p<0.001$) and when adjusted for AVR (HR 0.17, 95% CI [0.11-0.27], $p<0.001$). The
22 association remained significant, also after adjustment for clinical and biochemical variables
23 including TnT, NT-proBNP and CRP (HR 1.37, 95% CI [1.02-1.83], $p=0.036$) (Figure 4D).

24 To further evaluate whether AVR affected the association between FB and outcome,
25 we analyzed the two groups, *i.e.* non-operated ($n=92$) and AVR ($n=344$) separately.

1 Importantly, Kaplan-Meier analysis showed a significant association between high
2 levels of FB and all-cause mortality in AVR (p=0.002) but not non-operated (p=0.539) groups
3 (Figure 5A and 5B, respectively). This association was further establish in univariate Cox
4 regression in the AVR group (HR 1.63 [1.18-2.26], p=0.003). Moreover, the univariate Cox
5 regression revealed similar associations between higher levels of FB and MACE in both the
6 AVR (HR 1.38 [1.08-1.77], p=0.011) and non-operated (HR 1.40 [0.95-2.06], p=0.087)
7 groups.

8

9 **Plasma levels of TCC are increased in patients with symptomatic AS, but not associated**
10 **with clinical outcome - Patient population 2**

11 Herein we found a significant correlation between FB and TCC (p<0.001; Figure 6A),
12 suggesting that high FB levels trigger the activation of the final common pathway of the
13 complement cascade. However, whereas plasma levels of TCC were significantly elevated
14 (2.1-fold) as compared to the control group (p<0.0001; Figure 6B), Kaplan-Meier curve for
15 MACE according to quartile levels of TCC, showed no significant association between TCC
16 and adverse outcome of the disease (p=0.133; Figure 7A). The same non-significant
17 association was revealed when using Kaplan-Meier analysis for all-cause mortality on quartile
18 levels of TCC (p=0.269; Figure 7B) with the same pattern in AVR (univariate Cox regression:
19 HR 1.12 [0.81-1.55], p=0.47) and non-operated patients (HR 1.12, [0.76-1.64], p=0.55).

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21 **Plasma levels of the FB activation product Bb reflected FB in outcome, but contributed**
22 **marginally to native FB levels – Patient population 2**

23 Since our antibody against FB cannot distinguish between native FB and the activated Ba or
24 Bb-fragment, we measured Bb levels in patient population 2. Kaplan-Meier survival analysis
25 of three years all-cause mortality revealed that patients with the highest and lowest levels of

1 the Bb-fragment have the highest and lowest all-cause mortality, respectively (p=0.007,
2 Figure 8). Bb levels largely showed the same pattern in relation to outcome analyses as
3 compared with FB levels, and in this population of symptomatic AS patients these factors
4 were significantly correlated (r=0.22, p<0.000). However, the level of Bb in our FB-assay
5 was negligible (approx. 1%).

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1 **Discussion**

2 Complement activation has been found in several forms of acute and chronic cardiovascular
3 disease (9, 25). Herein, for the first time, we have demonstrated that patients with
4 symptomatic AS have increased complement activation, detected by TCC levels, representing
5 the activation of the complement cascade to its final stage. Moreover, FB levels as a marker
6 of activation of the alternative pathway were correlated with measures of systemic
7 inflammation, cardiac wall stress, and cardiac injury. Most importantly, elevated levels of FB
8 were significantly associated with increased risk of MACE and all-cause mortality. Our
9 findings suggest the involvement of complement activation in the progression of AS, and FB
10 could potentially represent a novel marker for risk stratification in these patients.

11 Several studies of complement activation point to the activation of the classic and
12 lectin pathway, in chronic HF (8, 11). Moreover, we have recently shown the significance of
13 the alternative pathway by demonstrating enhanced levels of activators factor D and properdin
14 and decreased levels of inhibitory factor H (12). In contrast, data on complement activation in
15 AS patients are scarce. In a small study of patients undergoing AVR surgery (n=24), Helske
16 *et al.* reported up regulation of TCC and the anaphylatoxin receptors C3aR and C5aR (13).
17 Herein we report enhanced systemic complement activation in two populations of AS patients
18 as shown by increased plasma levels of TCC indicating that the complement cascade is
19 activated to the very end. Moreover, high levels of FB were significantly associated with all-
20 cause mortality after three years also after adjustment for both clinical and biochemical
21 variables including TnT, CRP and NT-proBNP that all are established as strong predictors of
22 outcome in various cardiovascular diseases. Although FB was weakly but significantly
23 correlated with TCC, terminal complement activation was not associated with adverse
24 outcome of the disease. In fact, although soluble plasma TCC is important as a marker of
25 overall complement activation, it has not any known function in its soluble form. Tissue

1 deposition of TCC, not evaluated in this study, could also be a better prognostic factor than
2 the fluid phase levels here. Our data indicate an increased level of FB is a triggering factor for
3 increased alternative pathway activation, rather than being a marker of complement activation
4 *per se*. Thus, complement activation, and in particular activation of the alternative pathway
5 with release of Bb, is a good candidate for reflecting the inflammatory phenotype in patients
6 with symptomatic AS, potentially being both a marker and mediator of disease progression.

7 It could be argued that the enhanced systemic levels of FB reflect myocardial
8 remodeling rather than the inflammatory process within the aortic valve itself. However, we
9 found elevated FB levels in patients both with asymptomatic and symptomatic AS, including
10 those with mild to moderate AS. Moreover, while no significant correlation was found
11 between valve area in symptomatic AS patients, FB was inversely correlated with valve area
12 in the asymptomatic patients. These findings suggest that FB and its activation may be
13 involved in the pathogenesis of AS, potentially operating in the early phase of the disorder,
14 and not only elevated as a cause of disease severity and adverse myocardial remodeling.
15 Moreover, FB levels were associated with all-cause mortality and incidence of MACE in
16 patients undergoing AVR but not in the non-operated patients. Although we cannot fully
17 explain this pattern, it may reflect that pathogenic pathways related to adverse outcome of the
18 disease are still active after AVR and that the trigger of FB activation is not “removed” by
19 removing the diseased aortic valve. However, these issues will have to be clarified in larger
20 studies which also have to include patients with asymptomatic AS.

21 Our antibody against FB could not differentiate between native FB and the activated
22 Ba-fragment, which is important when distinguishing whether the increased FB was due to
23 increased native FB or in addition to its activation fragments Ba and Bb. In our study, we
24 concluded that the level of the Bb-fragment contributes less than 1% to the total FB level.
25 Still this might be of pathophysiological importance, since the half-life of the activation

1 products are very short as compared to the native components. Furthermore, the activation
2 products are normally biologically highly active, as compared to their native zymogen.
3 Interestingly, the Bb levels showed the same pattern in relation to outcome analyses as FB,
4 and were significantly correlated with FB levels. Thus, they seem rather equivalent as
5 biomarkers in the clinical setting.

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

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

1 asymptomatic patients was rather small, in particular the number of patients with mild to
2 moderate disease.

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

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1 **Financial support**

2 This work was supported by grants from Helse Sør-Øst Regional Health Authority, Norway
3 [grant number 2012037 to A.Y.]; Norwegian Research Council [grant number 240099/F20 to
4 P.A.]; and the Norwegian Health Association [grant number 1444 to P.A.]. Funding sources
5 had no involvement in study design, analysis, or in writing of the manuscript.

6

7

1 **Figure legends**

2 **Figure 1. Schematic overview of the complement system.**

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

12

13 **Figure 2. Plasma levels of complement factor B (FB) are increased in patients with**
14 **symptomatic aortic stenosis (AS).**

15 Circulating levels of FB in (A) 123 AS patients compared to 49 healthy controls and in (B)
16 402 AS patients compared to 39 healthy controls. Lines and error bars are mean with 95%
17 confidence interval. **** $p < 0.0001$.

18

19 **Figure 3. Plasma levels of complement factor B (FB) are increased in patients with**
20 **asymptomatic aortic stenosis (AS).**

21 Circulating levels of FB in (A) 34 severe asymptomatic AS patients compared to 402
22 symptomatic AS patients and in (B) 26 mild to moderate and 13 severe asymptomatic AS
23 patients compared to 22 symptomatic AS patients. Lines and error bars are mean with 95%
24 confidence interval.

25

1 **Figure 4. Complement factor B is significantly associated with all-cause mortality in**
2 **patients with symptomatic aortic stenosis (AS).**

3 Kaplan-Meier survival analysis of (A) one-year major adverse cardiovascular event (MACE)
4 and (B) three-year all-cause mortality in relation to quartile levels of FB. Adjusted hazard
5 ratio based on FB levels, estimated by cox proportional analysis, for (C) MACE and (D) all-
6 cause mortality. Risk estimates are adjusted for operation, New York Heart Association
7 (NYHA) class, diabetes mellitus (DM), gender, age at inclusion, estimated glomerular
8 filtration rate (eGFR), ejection fraction (EF), C-reactive protein (CRP), troponin T (TnT), NT-
9 proBNP, and FB.

10

11 **Figure 5. Circulating levels of FB are associated with all-cause mortality in both AVR**
12 **and non-operated patients with symptomatic aortic stenosis (AS).** Kaplan-Meier survival
13 curves in relation to quartiles levels of FB in (A) 344 patients with aortic valve replacement
14 (AVR) and (B) 92 non-operated patients with AS.

15

16 **Figure 6. Plasma levels of terminal complement complex (TCC) are increased in patients**
17 **with symptomatic aortic stenosis (AS).**

18 Correlation between plasma levels of TCC and FB (A). Circulating levels of TCC in 402
19 patients compared to 39 healthy controls (B). Lines and error bars are mean with 95%
20 confidence interval. ****p<0.0001.

21

22 **Figure 7. Terminal complement complex (TCC) levels are not associated with outcome**
23 **in patients with aortic stenosis (AS).** Kaplan-Meier survival curves in relation to quartiles
24 levels of TCC using (A) major adverse cardiovascular event (MACE) and (B) three year all-
25 cause mortality as endpoint.

1

2 **Figure 8. High and low levels of the Bb fragment have the worst and best outcome in all-**
3 **cause mortality in patients with symptomatic aortic stenosis (AS).** Kaplan-Meier survival
4 analysis of three-year all-cause mortality in relation to quartile levels of Bb-fragment in 402
5 AS patients.

1 **Table 1.** Clinical characteristics of patients with symptomatic aortic stenosis (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 (years)	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	
Diabetes mellitus (%)	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 L ⁻¹)	812 [330, 2114]	955 [346, 2332]	761 [270, 1944]	97 [58, 239]	378 [198, 733]	
TnT (ng L ⁻¹)	13 [8, 25]	16 [10, 18]	13 [10, 25]	11 [10, 20]	11 [10, 20]	
CRP (mg L ⁻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 L ⁻¹)	n.a.	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	

2 NYHA, New York Heart Association functional class; LVEF, left ventricular ejection
3 fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide; TnT, troponin; CRP, C-
4 reactive protein; eGFR, estimated glomerular filtration rate; ALT, alanine transaminase; ACE,
5 angiotensin-converting enzyme; ARB, angiotensin receptor blocker; ASA, acetylsalicylic
6 acid; n.a, not available. Values are presented as (%), mean ± SD, or median [interquartile
7 range].

1 **Table 2.** Correlation of FB with cardiac function and biochemical parameters in patients with
 2 symptomatic aortic stenosis.

	Patient population 1		Patient population 2	
	(n=123)		(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	n.a.	-	-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	n.a.	-	-0.11	0.037
CRP	0.29	<0.001	0.27	<0.001
WBC	0.18	0.052	0.09	0.075

3 CO, cardiac output; LVEF, left ventricular ejection fraction; LVEDV, left ventricular end-
 4 diastolic volume; LVESV, left ventricular end-systolic volume; 6MWT, 6 minute walk test;
 5 NT-proBNP, N-terminal pro-B-type natriuretic peptide; TnT, troponin; eGFR, estimated
 6 glomerular filtration rate; ALT, alanine transaminase; CRP, C-reactive protein; WBC, white
 7 blood cells count; n.a, not available.

Figure 1.

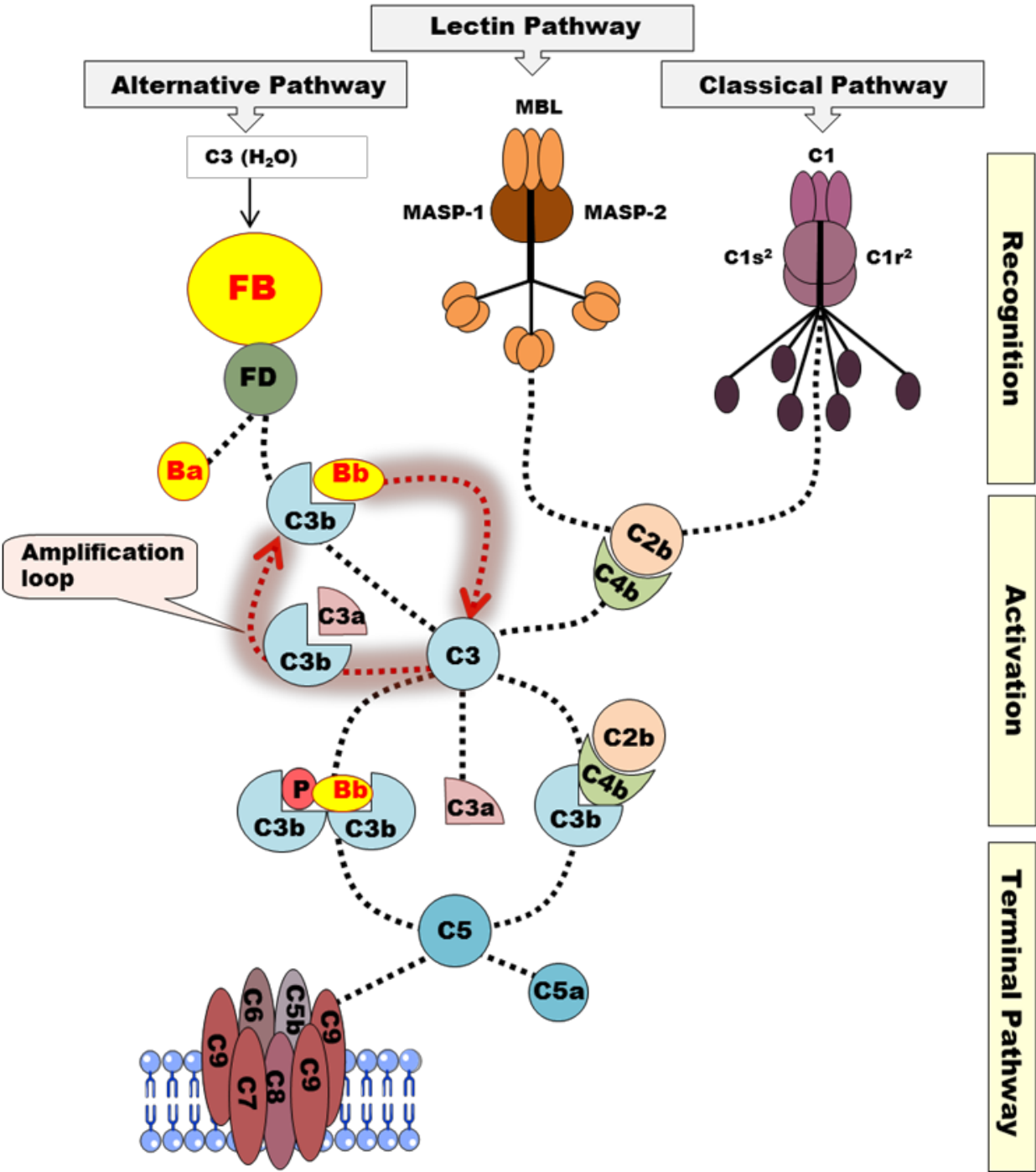


Figure 2.

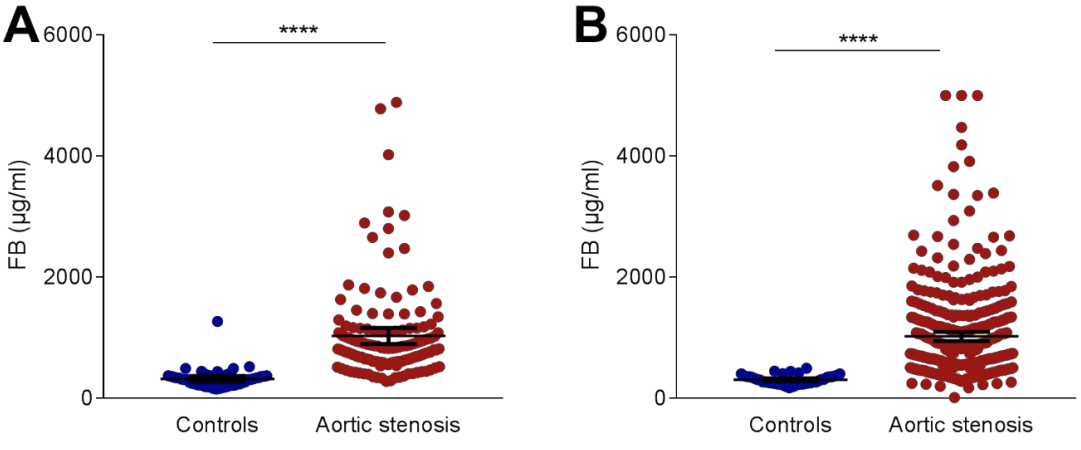


Figure 3.

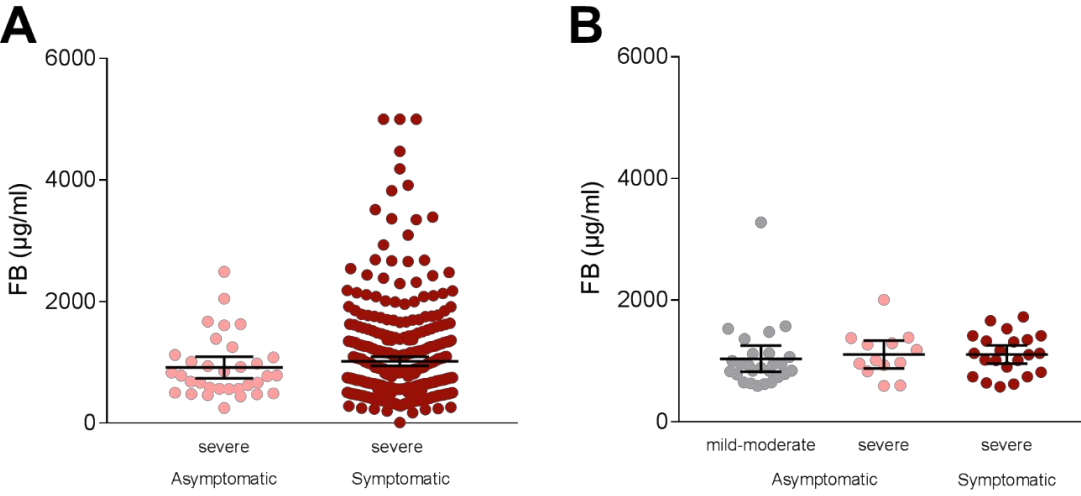


Figure 4.

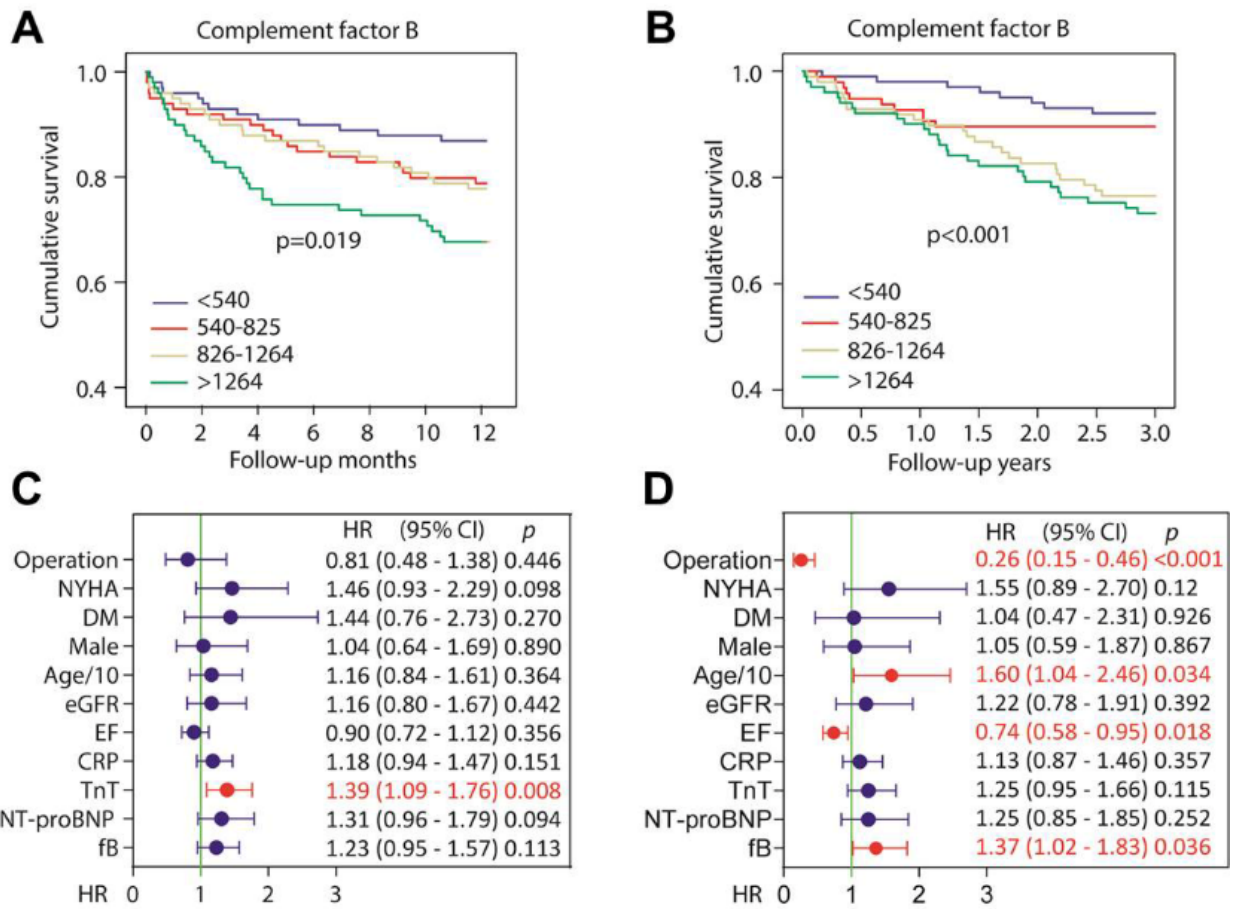


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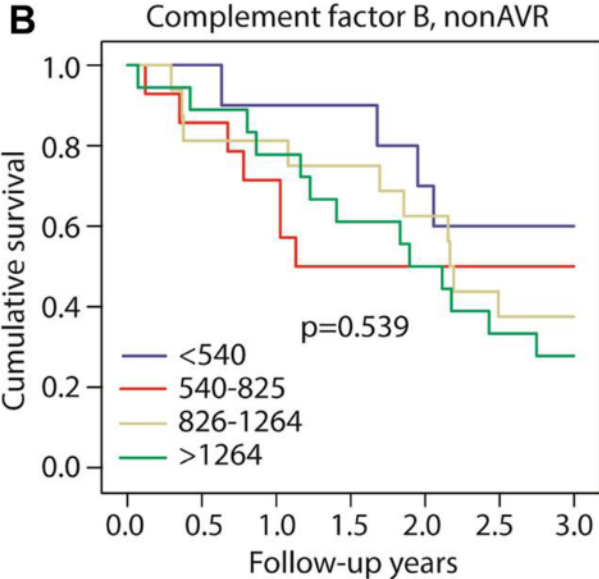
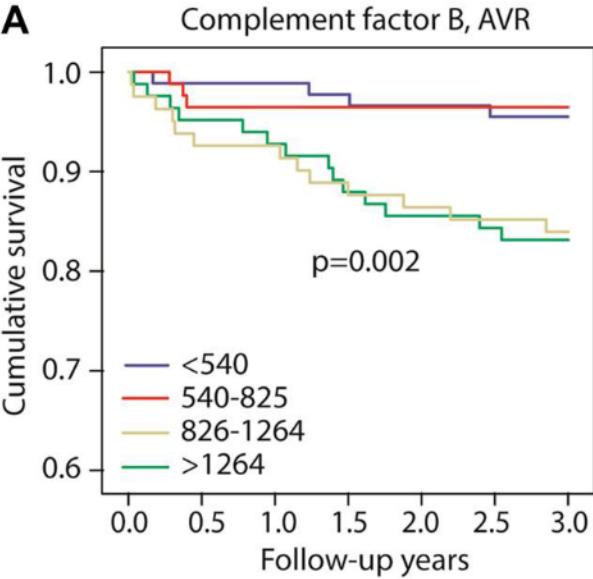


Figure 6.

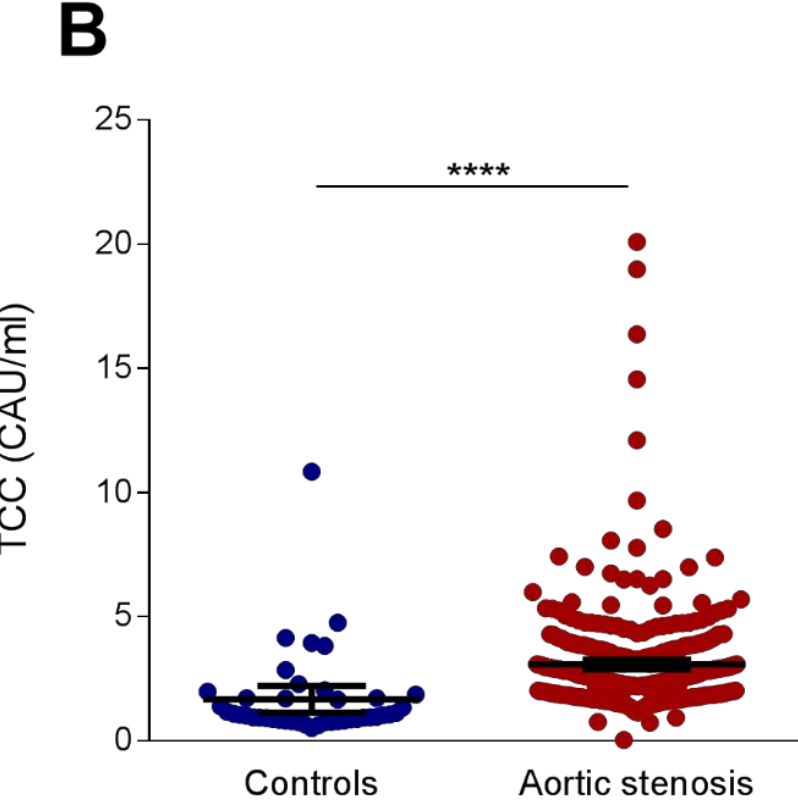
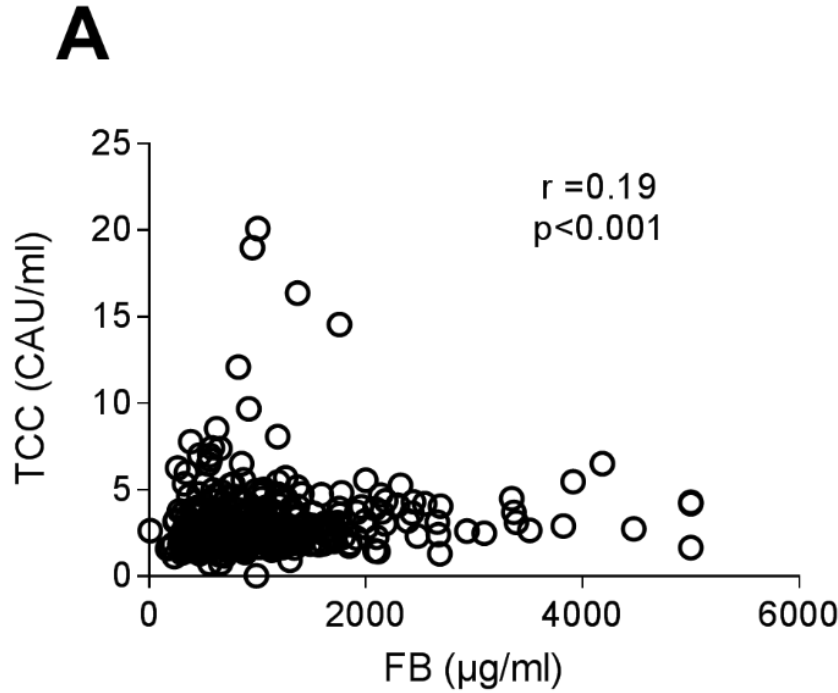


Figure 7.

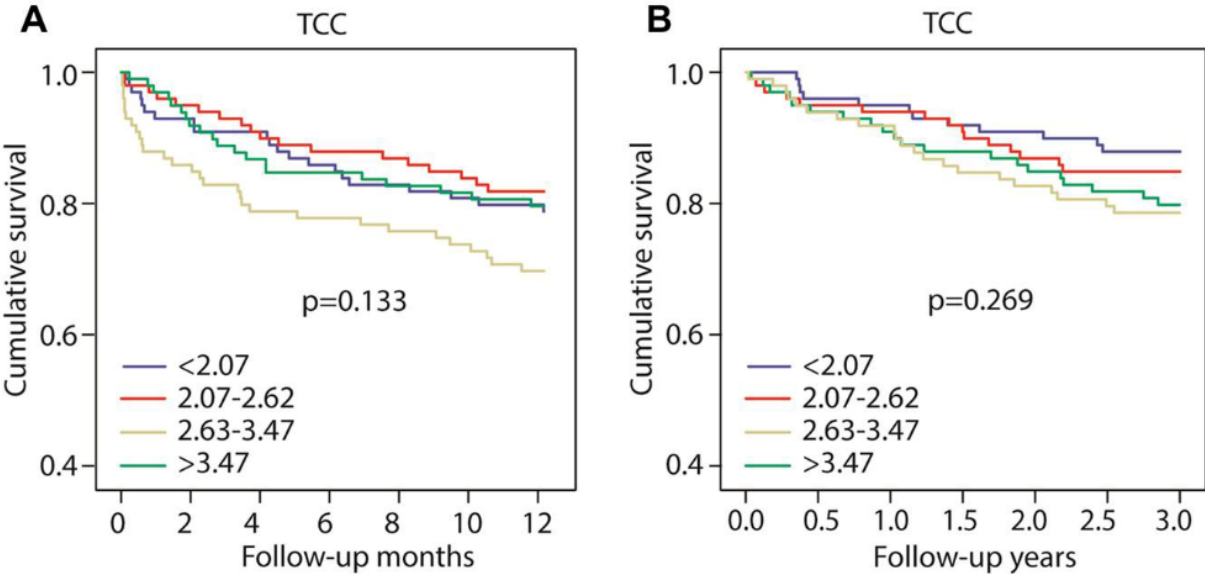


Figure 8.

