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Authors: Magdalena Kopytek, Janusz Konstanty-Kalandyk, Michał Ząbczyk, Anetta Undas, Joanna Natorska

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Valvular expression of factor XI correlates with valve calcification and aortic stenosis severity

**Short title:** Valvular FXI presence in aortic stenosis

Magdalena Kopytek¹,², Janusz Konstanty-Kalandyk³,⁴, Michał Ząbczyk¹,², Anetta Undas¹,², Joanna Natorska¹,²

¹Thromboembolic Disorders Department, Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland
²Krakow Centre for Medical Research and Technologies, St. John Paul II Hospital, Kraków, Poland
³Department of Cardiovascular Surgery and Transplantology, Institute of Cardiology, St. John Paul II Hospital, Kraków, Poland
⁴Institute of Cardiology, Jagiellonian University Medical College, Kraków, Poland

**Correspondence to:**
Joanna Natorska, MD, PhD,
Institute of Cardiology,
Jagiellonian University Medical College,
80 Pradnicka, 31–202 Kraków, Poland,
phone: + 48 12 614 21 08,
fax: +48 12 614 21 20,
e-mail: j.natorska@szpitaljp2.krakow.pl

**INTRODUCTION**
Aortic stenosis (AS) is a progressive disease with a pathogenesis similar to atherosclerosis [1]. AS progression is associated with the aortic valve orifice and leaflet mobility reduction, and there is no available pharmacological treatment to prevent or at least retard disease progression [1]. Currently, the only therapeutic options for AS are surgical aortic valve replacement or transcatheter aortic valve implantation [2, 3]. Associated activation of both coagulation and inflammation leading to valvular calcification has been shown in AS [4, 5]. Growing evidence indicates a contribution of factor XI (FXI) to thrombosis [6] and atherogenesis [7]. However, the role of the intrinsic pathway of coagulation, especially FXI expression, in AS progression
has not been studied. FXI plays an important role in blood coagulation and its activation to FXIa is mediated by activated FXII (FXIIa), through the feedback activation by tissue factor (TF)/thrombin, or via autoactivation [8]. FXIa converts FIX to its active form but its activation is also catalyzed by FVIIa-TF complex [8]. Kossmann et al. [9] demonstrated that FXI inhibition in mice, beyond antithrombotic effects, protects also against vascular inflammation, namely reactive oxygen species formation, leukocyte infiltration, and fibrotic remodeling. Importantly, FXIa inhibitors and antibodies to FXI reduced atherogenesis and inflammation in mice [10]. Here we investigated whether FXI is present within stenotic leaflets in severe AS patients and if its expression correlates with the disease severity.

MATERIAL AND METHODS

We enrolled 20 patients between April 2022 and June 2023 with symptomatic severe AS. All patients underwent first-time elective surgical aortic valve replacement at the Department of Cardiovascular Surgery and Transplantology, John Paul II Hospital, Krakow, Poland. Data on demographics, medical history, and current treatment were collected using a standardized questionnaire. Severe AS was defined as the mean transvalvular pressure gradient (PGmean) ≥40 mm Hg, peak transvalvular velocity (Vmax) ≥4.0 m/s, and aortic valve area (AVA) ≤1 cm² on transthoracic echocardiography [11]. Arterial hypertension, hypercholesterolemia, and atherosclerosis were diagnosed as previously described [12]. The exclusion criteria for AS patients included atherosclerotic vascular disease requiring revascularization, acute infection including infective endocarditis, rheumatic AS, diabetes mellitus, advanced chronic kidney disease, need for concomitant valvular surgery (e.g., mitral valve repair), percutaneous coronary intervention, recent (<3 months) acute coronary syndrome or cerebrovascular episode, diagnosed malignancy, and pregnancy. Angiographically documented coronary artery stenosis greater than 20% of the diameter was the exclusion criterion to avoid any influence of nonobstructive atherosclerosis [12, 13].

The ethics committee approved the study (8/KBL/OIL/2019 and 53/KBL/OIL/2022) and all participants provided their written informed consent in accordance with the Declaration of Helsinki.

Fasting venous blood was drawn before aortic valve replacement. Citrated blood (9:1 of 0.106 M sodium citrate) was centrifuged at 2500 g for 20 minutes at 20°C, while blood drawn into serum tubes was centrifuged at 1600 g for 10 minutes at 4°C. Routine laboratory assays were used to determine glucose, creatinine, lipid profile, C-reactive protein, and fibrinogen.
Aortic valves were collected during open heart surgery, embedded in Cryomatrix (Thermo Scientific, Kalamazoo, MN, US) and sectioned into 5 μm slices with a Leica CM1520 cryostat. Five control valves were obtained at autopsy from apparently healthy individuals of similar age. Activation of FXI was assessed indirectly by double staining of FXI with active FIX (FIXa) or TF. Immunostaining was conducted according to the previously described protocol [12] using primary antibodies against FXI (Santa Cruz Biotechnology, Dallas, TX, US), FIXa (Antibodies-online, Aachen, Germany), TF (Abcam, Cambridge, UK) and bone morphogenetic protein 2 (BMP2; Abcam). The corresponding secondary antibodies conjugated with AlexaFluor 488 or 594 (Abcam) were applied. A negative IgG isotype control was performed routinely. Olympus BX43 microscope (Tokyo, Japan) was used to analyze the images. The percentage of immunopositive areas was calculated as previously [12, 13], and 15 serial step sections were analyzed per each valve by two independent observers blinded to the sample origin.

Statistical analysis
All statistics were performed using the STATISTICA software (Version 13.3, TIBCO Software, Palo Alto, CA, US). Categorical variables were presented as numbers and percentages, while continuous variables were expressed as mean and standard deviation (SD) or median and quartiles Q1–Q3. Normality was analyzed by the Shapiro–Wilk test. Associations between variables were calculated using Pearson’s or Spearman’s correlation coefficients, as appropriate. A P-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION
Baseline characteristics of AS patients are shown in Table S1 (Supplementary material, Table S1). In loco analysis revealed valvular expression of FXI within all studied stenotic valves, but not within control ones (Figure 1A and B). The mean (SD) FXI-immunopositive valve area constituted 21.5 (1.4)% of the total leaflet area, while means (SD) for FIXa and TF were 17.2 (2.5)% and 26.5 (5.1)%, respectively. The mean (SD) BMP2-positive area was 23.9 (4.1)%. The expression of studied proteins was observed at the aortic side of the stenotic leaflets and presented a condensed pattern of fluorescence. Interestingly, the expression of FXI co-expressed with FIXa in 66% suggesting local activation of the intrinsic pathway as well as in 71% with TF, the major component of the extrinsic coagulation pathway (Figure 1C and D). Moreover, FXI co-expressed with BMP2 in 83% (Figure 1E), which supports our previous findings that coagulation activation is involved in leaflet calcification [4, 5, 12, 14]. Importantly, valvular amounts of FXI correlated with the disease severity reflected by $V_{\text{max}}$ (r
both transvalvular pressure gradients (PG_{mean} \ r = 0.49; \ P = 0.03; \ PG_{max} \ r = 0.53; \ P = 0.02) and AVA (r = –0.53; \ P = 0.02) (Supplementary material, Figure S1). Valvular expression of FIXa correlated with AVA (r = –0.49; \ P = 0.03) and V_{max} (r = 0.44; \ P = 0.049) but not transvalvular pressure gradients (both \ P > 0.05).

To the best of our knowledge this report provides the first evidence that the intrinsic coagulation pathway is activated within stenotic aortic valves. A strong co-expression of FXI with FIXa, TF, and BMP2 highlights the involvement of both coagulation pathways in valve calcification. Coagulation activation within stenotic valves is implicated in both valvular inflammation and calcification via the nuclear transcription factor kappa B (NF-κB) pathway [12]. Since the current study showed that abundant FXI valvular expression was associated with the disease severity, it is tempting to speculate that FXIa inhibitors, such as asundexian or milvexian [15] might not only attenuate coagulation activation but also inflammatory response and thus retard AS progression. Importantly, phase II clinical trials showed that FXIa inhibitors prevent stroke and systemic embolism in patients with atrial fibrillation without increasing bleeding risk compared to non-vitamin K antagonist oral anticoagulants [15]. Therefore, FXIa inhibition might offer a novel strategy preventing AS development and/or progression, at least in patients with an indication for anticoagulant therapy. Moreover, taking into account FXI contribution to inflammation in atherosclerosis, targeting FXI might influence the cross-talk between coagulation and inflammation, resulting in retardation of aortic valve leaflets calcification. Clinical relevance of our findings requires further studies.

**Supplementary material**
Supplementary material is available at https://journals.viamedica.pl/kardiologia_polska.

**Article information**

**Conflict of interest:** None declared.

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**Figure 1.** Valvular factor (F)XI expression together with active FIX(a), tissue factor (TF) and bone morphogenetic protein 2 (BMP2). Representative microphotographs of: A. Valvular expression of (FXI) in control leaflets and B. Stenotic leaflets. C–E. Colocalization (orange) of FXI (green) and active FIX(a) (FIXa), TF or bone morphogenetic protein 2 (BMP2) (red) within stenotic leaflets. Red arrowheads indicate aortic side of the leaflet; yellow arrowheads indicate the immunopositive areas. Scale bar 200 µm, original magnification 4×