Myocardial interaction of apixaban after experimental acute volume overload

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Abstract

Objective: Acute volume overload (AVO) induces early ischemia-like changes in intramyocardial arteries. We investigated whether the Factor Xa (FXa) inhibitor apixaban interacts with the myocardium early after AVO.

Methods: Fifty-five syngeneic Fisher rats underwent surgical abdominal aortocaval fistula to induce AVO. Among them, 17 rats were treated with apixaban (10 mg/kg/day). The myocardial outcome was studied using histological analysis and by measuring atrial natriuretic peptide (ANP) and matrix metalloprotease 9 (MMP9) gene expression.

Results: After 3 days, the total number of intramyocardial arteries was significantly increased in the AVO+apixaban (AVO+A) group compared with that in the AVO group (12.0 ± 1.2 and 10.2 ± 1.5, point score units, respectively). In the AVO+A group, there were significantly more edematous nuclei in myocardial arteries in the right and left ventricle compared with that in the AVO group. ANP and MMP9 expression levels continued to increase significantly in the AVO+A group compared with those in the AVO group.

Conclusion: Apixaban interacts with intramyocardial arteries in the left and right ventricles after AVO and ANP and MMP9 expression levels increased. Thus, the myocardial effect of Factor Xa inhibition needs to be monitored after AVO.
Keywords
Factor Xa inhibition, apixaban, acute volume overload, myocardial artery, rat, atrial natriuretic peptide, matrix metalloprotease 9

Date received: 16 March 2022; accepted: 20 October 2022

Introduction
Acute atrial and ventricular rupture of the cardiac septum induce acute volume overload (AVO) of the heart leading to increased cardiac oxygen consumption. Concomitant low coronary perfusion pressure and acute right-sided heart congestion impacts cardiac remodeling and induces thrombosis through circulating blood stagnation. Anticoagulant treatment aims at protecting the blood circulation from consequences of early ischemia and thrombosis triggered by acute ventricle volume overload. However, the effect of anticoagulant treatment on the myocardium after AVO remains unknown.

Factor Xa (FXa) inhibitors prevent thrombosis, and these anticoagulants also have a direct effect on smooth muscle cells by attenuating the development of ischemia-induced myocardial fibrosis through inhibition of Gq/PKC signaling and cell proliferation. The mechanism may involve inactivation of activated protease receptors on cardiomyocytes. FXa-induced mitogenesis and migration require sphingosine kinase activity and sphingosine 1-phosphate formation in human vascular smooth muscle cells, which signals angiogenesis.

We hypothesized that the FXa inhibitor apixaban impacts cardiac remodeling after AVO in a rat model that simulates the clinical scenario of acute cardiac insufficiency. The aim of this study was to investigate the effect of apixaban on acute myocardial vascular changes after experimental AVO because intramyocardial artery injury may be mediated by FXa. Changes in intramyocardial arteries reflect the onset of ischemia-like injury because the heart is dependent on circulating blood in intramyocardial arteries that are vulnerable to an ischemia-like insult during AVO. Early apixaban administration may thus be observed by investigating early intramyocardial artery histology. The effect of apixaban on myocardial remodeling was investigated via E-selectin, vascular endothelial growth factor α (VEGFα), matrix metalloprotease 9 (MMP9), chitinase-3-like glycoprotein (YKL-40), hypoxia inducible factor 1α (HIF1α), and inducible nitric oxide synthase (iNOS) gene expression. To confirm the ischemic state in the heart after AVO and AVO+A, HIF1α and iNOS gene expression was also investigated. Compensation capacity after congestive heart failure was evaluated by atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) gene expression, which reflect cell vasorelaxation and antiproliferation.

Materials and methods
Rats
Male Fischer 344 rats (F344/NHsd, Harlan Laboratories, The Netherlands) weighing 200 to 350 g underwent surgical abdominal arterial–venous fistula to induce AVO, and normal hearts from non-operated rats served as controls. The rats were kept in the University vivarium and received
humane care including daily chow, water, and care in compliance with the “Principles of Laboratory Animal Care” from the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” from the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996). The study was approved by the Finnish State Provincial Office (ESAVI/7430/04.10.07/2014).

**Surgical procedure**

Rats, including the controls, were anesthetized using sevoflurane (Baxter, Bloomington, IN, USA) for inhalation and pentobarbiturate (Mebunat vet®; Orion, Espoo, Finland; 50 mg/kg) intraperitoneally. The abdominal cavity was surgically opened, and the inferior vena cava and the aorta were exposed. An aorto-venous fistula was constructed intra-abdominally by vertically incising 5 mm of both the abdominal aorta and the adjacent inferior vena cava and joining these vessels surgically using a 7-0 running vascular suture. After surgery, 100 U Heparin Leo, (Vianex S.A., Pallini, Greece) was administered intravenously. From the abdominal aorta, oxygenated blood was introduced into the abdominal vena cava, which resulted in AVO of the heart. The surgical procedure was confirmed by observing an immediate color change in the circulating blood in the inferior vena cava from dark blue to pale blue proximal to the created aorto-venous fistula. Arterial blood pulsation was both visualized and palpated on the inferior vena cava proximally adjacent to the fistula. This model allows the study of extensive AVO and associated myocardial changes in vivo. Because AVO was introduced without direct surgical intervention to the heart, the model simulates the clinical set-up for acute cardiac insufficiency. After the procedure, buprenorphine (Vetergesic®; Orion; 0.1 mg/100 g) and carprofen (Norocarp®; Norbrook Laboratories Limited, Newry, Northern Ireland; 0.5 mg/100 g) was administered subcutaneously for pain relief. Fifty-five syngeneic Fisher rats underwent surgical abdominal aortocaval fistula to induce AVO. Four animals died during surgery and were excluded from the analysis. AVO+A rats were treated with apixaban at a dose of 10 mg/kg/day by subcutaneous injection (AVO+A).

**Tissue samples**

The rats were sacrificed 1 or 3 days after AVO, and their hearts were obtained at each time point. The distant abdominal wound causes a temporary local inflammation, which subsides before day 1 after surgery, and thus, seven normal hearts from non-operated rats were also procured after sevoflurane and pentobarbiturate anesthesia. The basal part of the hearts was separated and stored in RNA Later® (Ambio, Thermo Fisher Scientific, Waltham, MA, USA) for quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. The apex part of the heart was fixed in formalin and embedded in paraffin.

**Histology**

For histology, 5-μm sections were cut and stained with hematoxylin and eosin. The following variables were evaluated from all samples: presence of subendocardial and myocardial edema, hemorrhage, and ischemia. Nuclear vacuolization in the media layer of intramyocardial arteries reflects edema, and thus, representative cross-sectional intramyocardial arteries were chosen randomly from the left anterior, septum, and right posterior ventricular walls. Normal, edematous, and sharp-edged media cell nuclei were manually counted separately. The presence of periventricular
inflammatory cells was graded using an arbitrary scale from 0 to 1, as follows: 0, no inflammation; and 1, presence of inflammation. The total number of intramyocardial arteries was manually counted from the histological sections together with the normal, edematous, and ischemic media cell nuclei of these arteries. Histological evaluation was performed by two investigators who were blinded to the study protocol.

**Immunohistochemistry**

Immunohistochemistry was performed on three untreated AVO and three AVO+ hearts using Ventana Lifesciences Benchmark XT© Staining module (Tucson, AZ, USA). The paraffin-embedded slides were deparaffinized three times in xylene, rehydrated in a graded ethanol series, and rinsed well under running distilled water. Slides were placed in a preheated retrieval buffer that was at pH 8.0 and included 0.1 mmol ethylenediaminetetraacetic acid for 30 minutes. The slides were then cooled in the buffer for 5 minutes, followed by a 5-minute rinse under running distilled water. After heat-induced epitope retrieval, slides were placed on an autostainer (DAKO Corp., Carpinteria, CA, USA). Sections were incubated with 3% hydrogen peroxide in ethanol for 5 minutes to inactivate the endogenous peroxides and incubated with YKL-40 antibody (dilution 1:100) (Biomedica Gruppe, Vienna, Austria) for 30 minutes, followed by rinsing with Tris-buffered saline solution with Tween 20 (TBST) wash buffer. Secondary incubation was performed using DUAL-labeled polymer horseradish peroxidase (K4061; DAKO Corp.) for 15 minutes. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3-diaminobenzidine (K3467, DAKO Corp.) for 5 minutes, counterstained with modified Schmidt hematoxylin for 5 minutes, and rinsed for 3 minutes under tap water to reveal the blue sections. They were then dehydrated using graded alcohol concentrations and cleared in xylene three times before mounting. Positively stained YKL-40 depositions were evaluated using a representative cross-sectional intramyocardial artery that was chosen randomly from the left anterior ventricular wall, right ventricular wall, and septum. The manually calculated number of positively stained YKL-40 depositions was divided by the total number of the intramyocardial arteries in each histological section. The evaluation was performed by two investigators (AM and NK) who were blinded to the study protocol.

**RNA extraction and quantitative reverse transcription polymerase chain reaction analysis**

Immediately after collection, the tissue from the base of the heart was placed into RNA Later® and stored at −80°C. For RNA extraction of six randomly chosen hearts from each group, the samples were homogenized using a Qiashredder (Qiagen Inc., Hilden, Germany), and RNA extraction was performed using an RNasy Mini Kit with on-column DNase digestion (Qiagen). Total RNA was then reverse-transcribed to cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) in 10 μL reaction volume and diluted 1:5 with RNase-free water. Quantitative PCR was performed using QuantiTect® Primer Assays (Qiagen, Valencia, CA, USA) for HIF1α, iNOS, E-selectin, ANP, BNP, VEGFα, MMP9, YKL-40, transforming growth factor (TGF)β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific); and the ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reaction parameters for
SYBR® Green detection were as follows: incubation at 50°C for 2 minutes, incubation at 95°C for 10 minutes, and thereafter, 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 minute. Ct values were determined, and the relative quantification was calculated using the $2^{-\Delta\Delta C_t}$ method.\textsuperscript{12} HIF1α, iNOS, E-selectin, ANP, BNP, VEGFα, MMP9, YKL-40, and TGFβ expression were normalized against GAPDH.

**Statistical analysis**

The data are presented as the mean ± standard deviation. Statistical analyses were performed using SPSS version 24.0 statistical software (IBM Corp., Armonk, NY, USA). Comparisons between groups were analyzed using the non-parametric Kruskall–Wallis and Mann–Whitney U tests. The number of hearts within each experimental group was greater than five, and the normality of the data could not be confirmed. Additionally, $p$-values <0.05 were considered significant.

**Results**

The AVO model was established in 55 male Fischer 344 rats (F344/NHsd, Harlan Laboratories), and seven non-operated rats were used as controls. Four rats that died during surgery were excluded. Among the 51 rats with AVO, 17 rats were treated with apixaban (10 mg/kg/day)\textsuperscript{11} subcutaneously (AVO+A), and 34 rats with AVO were the untreated controls. On day 1, hearts were obtained from nine AVO+A rats and 25 AVO rats. On day 3, hearts were obtained from eight AVO+A rats and nine AVO rats. Seven non-operated control hearts were also obtained.

**Histology**

The rats were sacrificed 1 (n = 34) or 3 (n = 17) days after AVO, and among them, nine and eight AVO+A hearts were obtained at 1 and 3 days, respectively. The presence of subendocardial and myocardial edema, hemorrhage, myocardial inflammation, and ventricular ischemia did not differ in hearts with AVO+A compared with those in AVO hearts. The total number of intramyocardial arteries after 1 day was 11.0 ± 0.0 and 10.3 ± 2.5 in the AVO+A and AVO groups, respectively. After 1 day, the absolute number of normal nuclei in intramyocardial arteries in the right ventricle was increased in the AVO+A group compared with that in the AVO and control groups (54.3 ± 19.7 vs. 34.4 ± 15.8 and 29.3 ± 6.8 point score units [PSU], respectively, $p = 0.003$, Table 1). There was also an increased absolute number of ischemic nuclei in intramyocardial arteries in the left ventricle (7.9 ± 6.2 vs. 4.3 ± 4.9 and 1.7 ± 0.5 PSU, respectively, $p = 0.024$, Figure 1).

After 3 days, the total number of intramyocardial arteries remained increased in the AVO+A group compared with that in the AVO group (12.0 ± 1.2 and 10.2 ± 1.5, respectively, $p = 0.013$), but there were no longer a difference in the absolute number of normal or ischemic nuclei in intramyocardial arteries in the AVO+A group compared with that in the AVO group. The presence of edematous nuclei in myocardial arteries in the right and left ventricles remained increased in the AVO+A group compared with that in the AVO group (3.0 ± 1.9 vs. 0.5 ± 0.9 PSU, $p = 0.005$; and 2.5 ± 2.7 vs. 0.6 ± 0.7 PSU, $p = 0.046$).

**Quantitative reverse-transcription polymerase chain reaction analysis**

After 1 day, there were no differences in the expression of any genes in the AVO+A group compared with that in the AVO group. After 3 days, ANP and MMP9 levels were increased in the AVO+A group compared with those in the AVO group (5.0 ± 2.2 vs. 2.1 ± 1.9 and
After 3 days, YKL-40 was equally distributed in the control, AVO, and AVO+A groups (0.4 ± 0.1 vs. 0.4 ± 0.2 vs. 0.5 ± 0.1 PSU, respectively, Figures 2 and 3).

**Discussion**

In this study, apixaban preserved the total number of intramyocardial arteries after AVO. The number of normal cell nuclei in the right ventricle and the number of intramyocardial arteries at risk for ischemia were maintained in the left ventricle at day 1 in the AVO+A group compared with that in the AVO group. At 3 days, edematous nuclei in myocardial arteries in the right and left ventricle remained increased in the AVO+A group compared with that in the AVO group.

Net cardiac remodeling of the ventricles and septum are interdependent during AVO.13 In this model, acute volume overload leads to a sudden backflow of blood to the right side of the heart that compresses the septum towards the left side of the heart.8 Compensating mechanisms associated with the onset of acute cardiac failure include intramyocardial artery ischemia along with ventricular cellular swelling. Early vacuolization of nuclei in the cells and swollen mitochondria are associated with edema, which eventually may also signify ongoing ischemia before plausible apoptosis and cell death.14,15 The entire heart eventually undergoes early myocardial remodeling associated with ischemia. HIF1α and iNOS expression mirrors the cardiac global ischemic state during the early time period when the hearts were subjected to AVO.9

The thrombotic risk is attenuated by anticoagulation drugs such as FXa inhibitors (e.g., apixaban).3 The coagulation protease FXa plays a key role in coagulation, and it also has a direct effect on smooth muscle cells that enhance cell proliferation and migration at sites of vascular and tissue injury.16 FXa also participates in the

### Table 1. Histology of medial cell nuclei in intramyocardial arteries of the hearts with AVO after 1 or 3 days compared with that of AVO mice treated with apixaban (AVO+A).

|                  | Day 1          | Day 3          | p-value Day 1 | p-value Day 3 |
|------------------|---------------|---------------|---------------|---------------|
|                  | Control (n = 7) | AVO (n = 25)  | AVO+A (n = 9) | p-value (n = 9) | AVO+A (n = 8) | p-value |
| Right ventricle  |               |               |               |               |               |         |
| Normal nuclei    | 29.3 ± 6.8    | 34.4 ± 15.8   | 54.3 ± 19.7   | 0.003*        | 42.1 ± 18.9   | 62.8 ± 29.4 | 0.114 |
| Ischemic nuclei  | 1.7 ± 2.1     | 3.2 ± 3.0     | 4.3 ± 5.5     | 0.813         | 2.6 ± 1.8     | 5.1 ± 4.7   | 0.263 |
| Edematous nuclei | 3.0 ± 2.9     | 4.5 ± 5.1     | 6.0 ± 5.8     | 0.441         | 0.5 ± 0.9     | 3.0 ± 1.9   | 0.005* |
| Left ventricle   |               |               |               |               |               |         |
| Normal nuclei    | 47.5 ± 19.2   | 47.1 ± 17.6   | 55.2 ± 18.7   | 0.249         | 55.9 ± 14.3   | 54.1 ± 26.2 | 0.700 |
| Ischemic nuclei  | 1.7 ± 0.5     | 4.3 ± 4.9     | 7.9 ± 6.2     | 0.024*        | 6.0 ± 3.1     | 4.3 ± 3.2   | 0.207 |
| Edematous nuclei | 5.2 ± 5.0     | 5.3 ± 10.7    | 4.8 ± 3.5     | 0.237         | 0.6 ± 0.7     | 2.5 ± 2.7   | 0.046* |
| Septum           |               |               |               |               |               |         |
| Normal nuclei    | 31.3 ± 12.1   | 37.4 ± 19.2   | 28.6 ± 9.9    | 0.273         | 42.7 ± 16.0   | 35.0 ± 6.8 | 0.246 |
| Ischemic nuclei  | 1.2 ± 1.2     | 5.6 ± 4.6     | 3.5 ± 3.7     | 0.143         | 5.7 ± 2.4     | 3.3 ± 1.8  | 0.058 |
| Edematous nuclei | 3.2 ± 3.1     | 5.8 ± 6.2     | 4.0 ± 4.5     | 0.409         | 1.6 ± 2.5     | 4.5 ± 3.8  | 0.139 |

*p-value < 0.05.

AVO, acute volume overload.

12.0 ± 7.3 vs. 5.4 ± 3.3 fold change, p = 0.012 and p = 0.028, respectively, Table 2).
pathogenesis of tissue remodeling and fibrosis through its effect on fibroproliferation.\textsuperscript{17,18} An increase in ANP levels during AVO\textsuperscript{+}A in this study suggests compensation for congestive heart failure upon vasodilation and antiproliferation.\textsuperscript{10,19} FXa inhibition may interact with the myocardium including early onset of fibrogenesis and scar formation.\textsuperscript{19} The mechanisms may be mediated by the sphingosine pathway, and an important messenger of cellular functions includes sphingosine-1-phosphate, which regulates endothelial permeability, cell proliferation, migration, angiogenesis, and inflammation.\textsuperscript{15,20,21} Sphingosine-1-phosphate has been associated with controlling smooth muscle cell proliferation and migration\textsuperscript{20} and down-regulating cell apoptosis.\textsuperscript{21,22} In our study, ischemic nuclei were decreased and the total number of cells was preserved during apixaban treatment. Cell death leads to fewer cell nuclei, and the increased number of vacuolized cells with the increased total number of intramyocardial arteries suggested ongoing cellular preservation.

There was no net impact on inflammatory gene expression, such as E-selectin,
Table 2. Gene expression in hearts with AVO treated with apixaban (AVO+A) at 1 or 3 days compared with AVO without treatment.

|          | Control (n = 7) | AVO (n = 13) | AVO+A (n = 8) | p-value | AVO (n = 8) | AVO+A (n = 8) | p-value |
|----------|----------------|--------------|---------------|---------|------------|--------------|---------|
| HIF1α    | 1.0 ± 0.2      | 1.9 ± 1.5    | 1.2 ± 0.2     | 0.585   | 1.5 ± 1.0  | 1.3 ± 0.3    | 0.834   |
| iNOS     | 1.0 ± 0.7      | 2.4 ± 2.4    | 1.3 ± 0.7     | 0.869   | 0.9 ± 0.4  | 1.5 ± 0.8    | 0.083   |
| E-selectin | 1.0 ± 0.6   | 5.1 ± 5.4    | 4.5 ± 3.6     | 0.929   | 2.0 ± 0.9  | 2.1 ± 0.5    | 0.661   |
| ANP      | 1.0 ± 0.6      | 2.6 ± 2.0    | 2.0 ± 0.7     | 0.562   | 2.1 ± 1.9  | 5.0 ± 2.2    | 0.012*  |
| BNP      | 1.0 ± 0.2      | 2.0 ± 2.2    | 1.8 ± 1.6     | 0.828   | 0.8 ± 0.4  | 1.1 ± 0.5    | 0.203   |
| VEGFα    | 1.0 ± 0.3      | 0.6 ± 0.3    | 0.4 ± 0.1     | 0.099   | 1.2 ± 0.5  | 0.9 ± 0.3    | 0.093   |
| MMP9     | 1.0 ± 0.9      | 9.6 ± 7.4    | 8.3 ± 3.5     | 0.929   | 5.4 ± 3.3  | 12.0 ± 7.3   | 0.028*  |
| YKL-40   | 1.0 ± 0.7      | 10.8 ± 5.4   | 9.4 ± 2.7     | 0.534   | 3.9 ± 1.6  | 6.3 ± 3.9    | 0.247   |
| TGFβ     | 1.0 ± 0.3      | 4.1 ± 2.9    | 2.1 ± 0.5     | 0.051   | 3.0 ± 0.8  | 3.1 ± 0.9    | 0.796   |

*p-value < 0.05.

AVO, acute volume overload; HIF1α, hypoxia inducible factor 1α; iNOS, inducible nitric oxide synthase; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; VEGFα, vascular endothelial growth factor α; MMP9, matrix metalloproteinase 9; YKL-40, chitinase 3-like protein; TGFβ, tumor growth factor β.

VEGFα, YKL40, and TGFβ, and an increase in myocardial MMP9 levels was observed during AVO+A. MMP9 may also participate in fibrogenesis through interfering with the activation of tissue inhibitors of metalloprotease.22,23 Subsequently, all hearts with AVO showed positive immunohistochemical staining for YKL-40 after 3 days, suggesting ongoing cardiac remodeling. YKL40 is an inflammatory marker that interacts with cell proliferation, differentiation, inflammation, and extracellular matrix remodeling, and it inhibits apoptosis.23,24 However, the exact mechanisms associated with FXa inhibition during AVO remain to be explored.

Cardiac function analysis would add important information to the experimental study, especially after a longer follow-up time if the animals survive the initial insult caused by AVO. Myocardial protein expression analysis is also relevant for investigating chronic histological changes such as possible fibrosis onset associated with AVO. The results of our study suggest that apixaban-associated FXa inhibition may preserve the number and cells of intramyocardial arteries early after AVO, which may delay fibrinogenesis, irreversible changes associated with fibrosis, and the risk for cellular death at later time points. MMP9 reflects inflammation, but it may also have a temporary and dual effect on enhancing and decreasing developing fibrosis.23,24

An interaction between macrophages and MMP9 may also explain the presence and induction of YKL-40.24,25 YKL-40 is
expressed in arteries, which makes the molecule attractive to study during myocardial ischemia-like injury. YKL-40 expression has been observed during induction of inflammation, and its deletion may enhance stroke development. The role of YKL-40 during induction of an ischemia-like insult and related inflammation, especially mediated by macrophages and metalloproteases, remains to be explored.

**Limitations**

There are limitations to the experimental approach used in this study. We did not measure the left ventricular ejection fraction to describe cardiac function. AVO includes histological findings, and careful statistical interpretation of the results is required. The results should be cautiously interpreted because the findings and conclusions should not be simply translated to humans in a clinical setting. In the future, sham-operated controls may also be included, although the effect of a distant abdominal wound causing temporary local inflammation resolves within 1 day after surgery. However, a safe and effective apixaban dose was selected for this study on the basis of a previous investigation. The methods and data presented in this study provide preliminary results and, therefore,
the effect of apixaban on AVO cannot be definitively confirmed. The results suggest that apixaban interacts with intramyocardial arteries during AVO, and in future research, we intend to investigate whether apixaban has a sustainable impact on chronic cardiac changes after AVO.

Conclusion
Apixaban interacts with intramyocardial arteries in the left and right ventricles after AVO. Although anticoagulants prevent thrombosis, further studies are required to delineate the direct myocardial effect of FXa inhibition after AVO.

Author contributions
Conception: AM and CH; Design: AM, EM, and TP; Supervision: AM, EM, and TP; Funding: AM, EM, and TP; Materials: AM, EM, TP, and MH; Data collection and processing: AM, CH, NK, and MH; Analysis and interpretation: AM, CH, NK, EM, and MH; Literature review: AM, CH, NK, EM, and MH; Writer: AM, CH, NK, EM, and MH; Critical review: AM, CH, NK, EM, MH, and TP.

Data availability statement
The data are available upon reasonable request.

Declaration of conflicting interests
The authors of this manuscript have no conflicts of interest.

Funding
The authors disclosed receipt (pending publication) of the following financial support for the research, authorship, and/or publication of this article: This study was financially supported by the Competitive State Research Financing of the Expert Responsibility Area of Tampere
University Hospital, the unit of Heart Center Co. Grant Z60078, Tampere Tuberculosis Foundation, and The Finnish Heart Association.

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