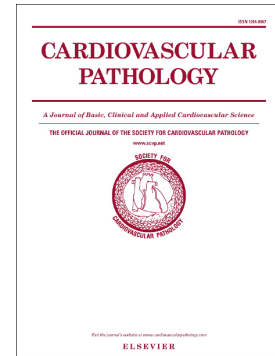


## Accepted Manuscript

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PII: S1054-8807(18)30266-7  
DOI: <https://doi.org/10.1016/j.carpath.2018.11.006>  
Reference: CVP 7100  
To appear in: *Cardiovascular Pathology*  
Received date: 5 September 2018  
Revised date: 23 October 2018  
Accepted date: 30 November 2018

Please cite this article as: Juha Näpänkangas, Pasi Ohtonen, Pauli Ohukainen, Jonna Weisell, Timo Väisänen, Tuomas Peltonen, Panu Taskinen, Jaana Rysä, Increased mesenchymal podoplanin expression is associated with calcification in aortic valves. *Cvp* (2018), <https://doi.org/10.1016/j.carpath.2018.11.006>

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**Increased mesenchymal podoplanin expression is associated with calcification in aortic valves**

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Short title: Podoplanin in aortic valves

**Keywords:** CAVD; aortic valve stenosis; calcification; bicuspid valve; podoplanin

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**Abstract**

Background and aim of the study: Calcific aortic valve disease (CAVD) is a progressive disease starting from mild valvular sclerosis and progressing to severe aortic stenosis (AS) with calcified valves. The origin of the calcification is proposed to be mesenchymal cells which have differentiated towards an osteoblastic phenotype. Podoplanin is a glycoprotein expressed in the endothelium of lymphatic vessels and in osteoblasts and osteocytes, mesenchymal cells, as well as in many carcinomas and aortic atherosclerotic lesions. In CAVD, its expression has been evaluated only as a marker of the lymphatic vasculature.

Materials and methods: We determined podoplanin expression in human aortic valves in four patient groups: control (C, n=7), aortic regurgitation (AR, n=8), aortic regurgitation and fibrosis (AR+f, n=15) and AS (n=49) by immunohistochemistry and quantitative real-time PCR (RT-PCR).

Results: Immunohistochemically, podoplanin expression was significantly increased in AR+f and AS groups when compared with the control and AR groups and the level of expression positively correlated with the extent of calcification and vascularity. Podoplanin mRNA levels were 1.7-fold higher in the AS group as compared with the control group ( $p=0.05$ ). Podoplanin-positivity was present not only in lymphatic vessel endothelium but also in osteoblasts, osteocytes, chondrocytes, macrophages and extracellular matrix. The majority of the podoplanin-positivity was in spindle cells with a myofibroblastic phenotype, often associated with calcifications.

Tricuspid valves had more calcification-associated podoplanin than bi/unicuspid valves (median 1.52 vs 1.16,  $p<0.001$ ).

Conclusions: CAVD is characterized by an increased expression of podoplanin; this is associated with the differentiation of valvular interstitial cells into calcium-producing, myofibroblast-like cells. In addition, tricuspid valves express relatively more podoplanin than bi/unicuspid valves.

## 1. Introduction

Calcific aortic valve disease (CAVD) represents a spectrum of disease which range from mild aortic valve sclerosis to severe aortic stenosis (AS) [1]. CAVD is associated with a high risk of myocardial infarction and cardiovascular death [2]. CAVD is a common disease in the US and Europe with the reported prevalence of AS in the elderly ranging between 2.6 to 22.8 % and severe AS between 1.2 – 6.1 % [3]. As the population of Western countries ages, the prevalence of CAVD is likely to rise. The only known treatment for symptomatic CAVD is aortic valve replacement [4]. Pharmacological treatments have proven unsuccessful in slowing the progression of the disease (for review, see [5]).

The aortic valve is an avascular tricuspid outflow valve in the left ventricle. Normally it is under 1 mm thick and has three major layers: fibrosa, spongiosa and ventricularis, consisting mainly of valvular interstitial cells (VICs) and extracellular matrix [6], covered by valvular endothelial cells (VECs). It is known that anatomical, genetic and clinical factors contribute to the pathogenesis of CAVD (for review, see [7]). The most significant risk factor for CAVD is a bicuspid aortic valve [8]. Individuals with a bicuspid aortic valve are more likely to develop CAVD and suffer cardiovascular events requiring surgical interventions at a younger age than those with a tricuspid valve [9].

Historically, CAVD was considered to be a degenerative process and even though mechanical stress and injury are important factors, today it is recognized that complex and actively regulated processes are the key drivers of the pathogenesis of CAVD [10]. Mechanical stress initiates the disease progression by injuring the endothelium on the aortic side of the valve, leading to a disruption of the underlying basement membrane that can be seen histologically as a subendothelial thickening with lipid accumulation [11]. Subsequently, the lipids become oxidized

and inflammatory cells infiltrate the valve. Different inflammatory pathways are activated and this process is followed by activation of VICs leading to calcification, starting in the fibrosa layer [12]. In addition, angiogenesis is involved in the pathogenesis of CAVD: a neovasculature starts to form during the fibrosclerotic phase but is more pronounced in AS [13].

Podoplanin is a glycoprotein expressed in the endothelium of lymphatic vessels [14]. More recently, it has been shown to be expressed in many types of cancer cells, including vascular tumors, malignant mesothelioma, tumors of the central nervous system, germ cell tumors and squamous cell carcinomas [15] and in cancer-associated fibroblasts in several carcinoma types [16]. Podoplanin can also be detected in fibroblast-like synoviocytes from patients with rheumatoid arthritis [17]. Furthermore, it is expressed in macrophages and the  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) positive cells present in atherosclerotic lesions of human aorta, especially in advanced conditions but to a lesser extent, also in early lesions [18]. Kholova and co-workers [19] also demonstrated the presence of podoplanin in the endothelium of lymphatic vessels in heart valves in different cardiovascular conditions. Podoplanin is also expressed by periarterial stromal cells in mouse bone marrow and it activates platelets through the C-type lectin-like receptor 2 (CLEC-2) [20].

Since podoplanin has been studied in calcified valves only as a marker of lymphatic vessels [19] and increased expression of podoplanin has been described in a gene expression profiling screen of human AS [21], we have now investigated in detail the expression of podoplanin in aortic valve calcification.

## 2. Methods

### 2.1. Patients

Aortic valves were obtained from 79 patients (59 male and 20 female) during aortic valve or aortic root surgery (Table 1) using normal surgical procedures. The study protocol was approved by the Research Ethics Committee of Oulu University Hospital and it conformed to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from the patients included in the study. After dissection, parts of aortic valve cusps were immersed immediately in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Some parts of the samples were fixed in 10% buffered formalin.

Patients were divided into four groups based on the clinical evaluation. Patients in control (C) group ( $n=7$ ) were operated because of root pathology of the ascending aorta with normal aortic annulus diameter, i.e. the aortic valve leaflets were normal and non-regurgitant. In the aortic regurgitation (AR) group ( $n=8$ ), patients had regurgitant, but pliable, smooth and non-calcified valves. The aortic regurgitation + fibrosis group (AR+f) consisted of 15 patients whose aortic valves were macroscopically thickened ( $n=15$ ). The aortic stenosis (AS) group consisted of 49 patients with severe aortic valve sclerosis and at least one of the following features: mean transvalvular gradient  $> 40$  mmHg, peak transvalvular gradient  $> 50$  mmHg, aortic valve area  $< 1\text{ cm}^2$ , maximal aortic velocity  $> 4$  m/s. None of the patients had rheumatic fever. The demographics of the patients are shown in Table 1.

### 2.2. Isolation and analysis of RNA

Total RNA was extracted from aortic valve cusps (C and AS groups) with RNeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer's protocols. Quantitative real-time PCR (qRT-PCR) analyses were performed as previously described [22] using Taqman Real Time PCR assays (Thermo Fisher Scientific): podoplanin (Hs00366766\_m1), C-Type Lectin Domain Family 1, Member

B (Hs00212925\_m1) and endogenous control 18s rRNA (4352930E). From each tissue sample, duplicate technical replicates were analyzed, and the gene expression levels were normalized against 18s measured from the same sample.

### 2.3. Histological and immunohistological analysis

After formalin fixation, aortic valve samples were embedded in paraffin. When necessary, EDTA was used for decalcification. In the quantification of the calcified area, 5- $\mu$ m sections were cut and stained with hematoxylin and eosin. In the area calculations (total valve area and calcified area, including mature cartilage and bone occasionally seen in the calcified areas), slides were photographed with a Leica DFC420 camera (Wetzlar). Area measurements were performed with the image analysis program ImageJ (<http://rsbweb.nih.gov/ij/>). Blood vessel density was determined by counting Factor VIII-positive vessels (polyclonal rabbit anti-human Factor VIII, #A0082, DakoCytomation, dilution 1:50) and dividing the count by tissue area. In the other immunohistochemical stainings, monoclonal mouse anti-human antibodies against podoplanin (clone D2-40, #M3619, DakoCytomation, 1:50),  $\alpha$ -SMA (#M0851, DakoCytomation, 1:500), CD31 (#M0823, DakoCytomation, 1:300), CD34 (NCL-L-END, Leica Biosystems, 1:500) and CD68 (clone KP1, #M0814, DakoCytomation, 1:10000) were used. Histochemical visualization of the calcifications in aortic valves was done using Masson's trichrome staining, Lillie's modification (Dako Artisan Masson's Trichrome staining kit, AR173) [23].

In the quantification of podoplanin expression, the stained slides were digitized with a Leica Aperio AT2 slide scanner with a 40x objective. The resulting virtual slides were analyzed using the Qupath program [24]. An experienced pathologist manually annotated areas with positive staining in different groups: lymphatic vessels (Lymph v), areas not associated with calcifications (Non-

calc), and areas associated with calcifications (Calc). Furthermore, the latter group was divided into two groups: edges of calcifications i.e. the positive reaction tightly surrounding the calcifications (Edge), and areas around the calcifications, but not in a direct contact with them (Non-edge). The region identification module of Qupath for cytokeratin annotations was used (Downsample factor 4.0, Gaussian sigma 0.01, Tissue threshold 0, DAB threshold 0.35, Separation distance 0). The areas of the resulting annotations with positive staining reactions were calculated by Qupath and divided by the total valvular tissue area to obtain the relative podoplanin expression. In addition, podoplanin-positive lymph vessels were counted.

#### 2.4. Statistical analysis

Summary measurements are presented as mean and standard deviation (SD) or as median with 25<sup>th</sup>–75<sup>th</sup> percentiles. Age, sex, comorbidities (no/yes), number of cusps (1-2 or 3), left ventricular ejection fraction (LVEF) and usage of statin medication were set as covariates in the analysis of covariance (ANCOVA) to control their influence on measurements only if they had a significant (>10%) impact on the models coefficient of determination ( $R^2$ ). If the analysis of covariance showed a significant group effect, the adjusted means between groups were compared pair-wise. In the case of right-skewed distribution (natural base) logarithmic transformation was used. Pearson's  $\chi^2$ -test or Fisher's exact test were used for categorical data. Spearman's correlation coefficient ( $\rho$ ) was calculated. The analyses were performed with SPSS statistics (IBM Corp). Two-tailed p-values <0.05 were considered statistically significant.

In the qRT-PCR-analysis, statistical analysis was performed by Student's t-test. A value of  $p < 0.05$  was considered statistically significant.



### 3. Results

#### 3.1. Valve calcification, vascular density, lymphatic vessel count and topography in CAVD

Aortic valve calcification and vascular density were higher in the AS and AR+f groups than in the control or AR groups, as we have shown before [13], [25], [26]. In the control group, no lymphatic vessels were found, as expected (Fig.1A). In the AR group, only one valve and in the AR+f group, three of the 15 valves had lymphatic vessels. In the AS group, most valves (40 out of 49) had lymphatic vessels, but the count varied considerably. The lymphatic vessels were most often, almost exclusively, seen close to the calcifications, in the fibrotic areas between them. The lymphatic vessels weren't directly associated with inflammation: of the 44 valves that showed lymphatic vessels, only seven had them in the inflamed areas. Furthermore, in four of those cases, the great majority of the lymph vessels were in non-inflamed areas.

#### 3.2. Podoplanin expression in calcified valves

Because increased podoplanin mRNA levels had been described before in an expression profiling study of calcified valves [21], we initially measured podoplanin expression in the valves of AS and control groups. The mRNA levels were significantly higher in the AS group (n=10) than in the controls (n=8, 1.7-fold,  $p<0.05$ , Fig.1B). Immunohistochemically analyzed total podoplanin expression was significantly higher in the AS and AR+f groups when compared to the control and AR groups (Fig.1C). Furthermore, podoplanin expression was slightly higher in the AS than in the AR+f group (median 1.55 vs. 0.31,  $p=0.077$ ). The level of podoplanin expression positively correlated with the extent of valve calcification ( $\rho=0.62$ ,  $p<0.001$ ) and vascular density ( $\rho=0.64$ ,  $p<0.001$ ).

### 3.3. Podoplanin expression in valvular cell types

The positivity in podoplanin immunohistochemistry varied extensively in different areas of the valves. In immunohistochemical stainings, some podoplanin-positivity was seen in the endothelium of lymphatic vessels as previously described [19,27] (Fig.2A). However, most of the podoplanin-positivity was located in spindle cells. In non-calcified valves, only a few podoplanin-positive spindle cells were seen, mostly in the middle section, i.e. spongiosa layer (Fig.2B). Some valves showed a nodular proliferation of  $\alpha$ -SMA-positive myofibroblasts, but there was no significant podoplanin-positivity in these areas. In some cases, podoplanin surrounded cholesterol and lipid accumulations (Fig.2C) or degenerative nodules, i.e. areas with tissue degeneration, but no calcification (Fig.2D). Podoplanin was often associated with calcifications – mostly in spindle cells and, to a lesser extent, in the extracellular matrix (Fig.2E). Chondrocytes, osteoblasts, osteocytes and macrophages stained positively for podoplanin (Figs. 2F, 2G), but osteoclasts were negative. The detected chondroid tissue was surrounded by a large calcification and the chondrocytic cells were of the hypertrophic phenotype. The osteoblasts were recognized by their morphology and location at the edges of bone trabeculae. In many of podoplanin-positive and calcification-surrounding spindle cells, podoplanin seemed to co-localize with  $\alpha$ -SMA (Fig.3). It is worthwhile noting that podoplanin-positive cells often tightly surrounded very small calcifications, measuring between 10 to 50  $\mu$ m. CD31 and CD34 were positive in vascular endothelium, but negative in spindle cells.

The total and area-wise relative podoplanin-positivity in different groups are shown in Fig.4A.

Since there was no calcification or any lymphatic vessels in the control group and only very minor calcification and a few lymph vessels in the AR group, the podoplanin-positivity in these groups was almost exclusively restricted to scattered interstitial spindle cells. In the AR+f and AS groups, most of the positivity was associated with calcifications. The expression of calcification-associated

podoplanin (relative area = 0 vs >0) was significantly higher in the AS group when compared to the AR+f group ( $p < 0.001$ , Fig.4.B) as well as when the podoplanin-positivity at the edges of calcifications in the AS group was compared to that in the AR+f group ( $p = 0.002$ , Fig.4.C).

### **3.4. Calcification associated podoplanin expression in tricuspid and bi/unicuspid valves**

There were 37 patients with tricuspid valves, 21 patients with bicuspid valves and 2 patients with unicuspid valves who had calcification-associated podoplanin. Tricuspid valves had more calcification-associated podoplanin than bi/unicuspid valves (median 1.520 vs. 1.157,  $p < 0.001$ ) (Fig.5). The same pattern was seen when only the edges of the calcifications were taken into account (median 0.671 vs 0.351,  $p = 0.007$ , 33 tricuspid, 17 bicuspid and 2 unicuspid valves).

## **4. Discussion**

In aortic stenosis, there are elevated numbers of lymphatic vessels, visualized with LYVE-1 antibody against lymphatic endothelium, and these correlated positively with that of the neovasculature [28]. Podoplanin has been used for visualization of the lymphatic vessels in the calcified valves in two previous studies; these reported a strongly increased density of lymphatic vessels in the aortic valves of endocarditis patients [27] and, to a lesser extent, in calcified aortic stenosis [19]. We demonstrate here that the expression of podoplanin in aortic valves was not restricted to lymphatic vessels; while podoplanin stained only a modest amount of identifiable lymphatic vessels with an open lumina, it was much more often positive in spindle cells. In our experiments, podoplanin expression was significantly increased in the fibrotic and calcified valves and its level positively correlated with the extent of calcification and vascularity.

In our study, the podoplanin-positive spindle cells often seemed to be also positive with  $\alpha$ -SMA, indicating that these cells are of the myofibroblastic type. Indeed, in an osteogenic environment in a 3D hydrogel culture, VICs first differentiate into myofibroblasts, then into osteoblasts [29]. We stained a set of the valves also with CD31, which was negative in these spindle cells. Most of the lymphatic vessels are known to be CD31-positive [30], so these podoplanin-positive spindle cells most probably are not lymphatic vessels. However, podoplanin-positive myofibroblasts in cancer stroma have been speculated to have an angiogenic potential [31], so it cannot be ruled out that some of these could be maturing lymphatic vessels.

Podoplanin was also intensely expressed in chondrocytes, osteoblasts and osteocytes in calcification-associated cartilage and bone in stenotic valves, whereas before it has been detected only in normal cartilage and bone [32]. Much of the positivity was seen specifically at the edges of small calcifications, 10 to 50  $\mu$ m in diameter, tightly surrounding them and possibly representing active foci of calcification. Nomura and co-workers [33] have isolated mesenchymal stem-like cells (MSLCs) from both calcified and non-calcified human aortic valves. The CD34-negative MSLCs were more numerous in calcified valves and more sensitive to high inorganic phosphate-induced calcification than their CD34-positive counterparts, leading to the suggestion that these MSLCs are responsible for the calcification of the aortic valve. We identified podoplanin-positive cells specifically around smaller calcifications and while CD34 was negative in these cells, it can be speculated that they represent the CD34-negative MSLCs. It should be noted that as podoplanin was expressed also in osteoblasts, our findings support the suggestion that there is osteoblastic differentiation of myofibroblasts in CAVD [29].

We observed a significant difference in the expression of calcification-associated and calcification edge-associated podoplanin between tricuspid and bi/unicuspid valves. Bicuspid valves lead to complications at a younger age [9], but this alone cannot explain the difference. Clinically, non-calcified bicuspid valves have an abnormal collagen fiber arrangement [34] and calcified bicuspid valves are thicker than calcified tricuspid valves [35]. These anatomical differences might affect the movement of myofibroblasts and thus the arrangement of calcifications. The difference might also be due to a different phase in the disease – i.e. CAVD might have advanced further in the bicuspid valves and the active process would be slowing down, meaning that podoplanin-positivity could reflect disease activity. In addition, the genetic alterations in bicuspid valves, e.g. a NOTCH1 receptor mutation, probably play a role in the calcification process occurring in CAVD (for review, see [36]). Often one leaflet of a bicuspid valve is clearly larger with the other being smaller and resembling a normal leaflet of a tricuspid valve. We speculate that it seems that it is the larger leaflet, rather than the smaller leaflet, that is more severely affected with considerably larger calcifications. This might imply that the anatomic variation and thus that mechanical stress is a major player in the calcification process. However, the reason for differential calcification-associated podoplanin expression between tricuspid and bi/unicuspid valves remains to be clarified.

Although podoplanin is a widely expressed glycoprotein its role is poorly understood. Knockout of the mouse podoplanin gene results in increased embryonic lethality in homozygotes [37,38]. Embryos show many cardiac abnormalities, including hypoplastic atrioventricular cushions, from which also aortic valve is developed. This is in accordance with the finding that podoplanin promotes epithelial-mesenchymal transformation (EMT) [39] that is needed in the aortic valve development [40]. Increased endothelial-mesenchymal transition (EndMT), a specialized form of

EMT [41], has been linked to CAVD [42]. In vascular calcification, endothelial cells undergoing EndMT have been shown to act as a source of osteogenic predictors [43]. In podoplanin deficient mice, EMT correlated with down-regulation of RhoA [38], a small GTPase protein that is associated with increased nodule formation in VIC cultures and the differentiation of VICs into a myofibroblastic and osteoblastic phenotype [44]. Collectively, our results with the previous data underline that podoplanin may play a role in CAVD.

Podoplanin has only one known receptor, CLEC-2, and this is mainly expressed in platelets. We found no changes in CLEC-2's mRNA levels between control and calcified valves (data not shown). However, an injury on the valvular surface endothelium could reveal podoplanin-positive cells allowing them to interact with platelets possibly triggering platelet aggregation and thrombosis formation [45], complicating the clinical manifestation of CAVD.

A limitation of our study is that EDTA was used for decalcification, which prevents staining for e.g. calcium phosphate or calcium oxalate that are lost in the process. This means that we could not use these special stainings to detect calcifications smaller than 10  $\mu\text{m}$  in diameter, so called microcalcifications in the context of atherosclerosis studies [46,47]. More research is needed to show if there is a relationship between microcalcifications and podoplanin expression. However, EDTA is needed to get good tissue sections from paraffin-embedded hard material, which is in turn needed for immunohistochemical stainings to work reliably. Most of the calcified valves in our study were decalcified. In our experience, if EDTA would cause changes in the immunohistochemical stainings, it would lead to a weaker antigenicity, i.e. the resulting stainings would be of lower intensity. As we found more podoplanin positivity in calcified valves, this does not seem to be an issue in our study.

We have shown that podoplanin is expressed in CAVD not only in the endothelium of lymphatic vessels, but also in chondrocytes, osteoblasts, macrophages and interstitial spindle cells, which often are associated with calcifications. Calcification-associated and calcification edge-associated podoplanin were differentially expressed between the tricuspid and bi/unicuspid valves. Our findings support the hypothesis that myofibroblasts had differentiated into cells with an osteoblastic phenotype; these are the source of calcification and furthermore the elevated podoplanin expression may play a role in this process.

### Acknowledgements

We thank Erja Tomperi and Manu Tuovinen for expert technical assistance. The study was funded by the Academy of Finland (grants 276747, 284504 and 312611), the Finnish Foundation for Cardiovascular Research and the Orion Research Foundation.

### Author contributions

Study design: JN, JR. Performed the surgery: PT. Performed the experiments: JN, JW, JR. Analyzed the data: JN, POht, JW, JR. Contributed reagents/materials/analysis tools: JR, TV. Wrote the paper: JN, POht, POhu, JW, TV, TP, PT, JR

### Abbreviations

$\alpha$ -SMA	$\alpha$ -smooth muscle actin
AR	Aortic regurgitation
AR + f	Aortic regurgitation and fibrosis
AS	Aortic stenosis
CAVD	Calcific aortic valve disease
CLEC-2	C-type lectin-like receptor 2

MSLCs	Mesenchymal stem-like cells
VECs	Valvular endothelial cells
VICs	Valvular interstitial cells

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### Figure legends

Figure 1: Lymph vessel count and podoplanin expression in aortic valves. A. Lymph vessel count in individual cases. B. Podoplanin mRNA levels. C. Immunohistochemically quantified podoplanin expression in the different groups. Horizontal lines in B and C depict the 25<sup>th</sup> percentile, median (wide line) and the 75<sup>th</sup> percentile.

Figure 2: Podoplanin expression in the aortic valves (podoplanin stainings in A-G). A. Lymphatic vessel endothelium stains positively. B. Scattered positively stained spindle cells in spongiosa layer (AR group). C. A clinically normal valve shows podoplanin-positive cells surrounding clefts with cholesterol and lipid accumulations. D. Positive staining surrounding a degenerative area marked with arrows (AR+f group). E. Positively stained spindle cells in the spongiosa layer (left side of

picture) and surrounding calcifications (on the right, AR+f group). The inset is Masson's trichrome staining of the area marked with the rectangle, visualizing the calcifications as bright red globuli against the fibrotic blue background. F. Chondrocytes are of the hypertrophic phenotype and stain positively (AS group). G. Osteoblasts at the edge of mature bone and the osteocytes inside the mature bone are podoplanin-positive. There are a few scattered positively stained cells in the fibrotic area as well. H. The cells in the fibrotic area are also CD68-positive, representing macrophages. Pictures G and H are adjacent sections from the same area (AS group). Scale bars depict 200  $\mu\text{m}$ .

Figure 3: Calcification-associated podoplanin expression in aortic valves. A. Podoplanin is positive in spindle cells, some tightly surrounding small calcifications (arrows) measuring here 10 to 50  $\mu\text{m}$  in diameter, and in the surrounding extracellular matrix. The inset is Masson's trichrome staining of the area marked with the rectangle, visualizing the calcifications as bright red globuli against the fibrotic blue background and some red myofibroblastic spindle-shaped cells. B. The staining pattern of  $\alpha$ -SMA (a myofibroblast marker) is similar to podoplanin. C. CD31 and D. CD34 are localized in the endothelium of the blood vessels. Since CD31 is also a lymphatic endothelium marker, it is concluded that there are no lymph vessels in this view. There is no positivity in spindle cells. All pictures are from the same area and represent adjacent sections (AS group). Scale bars depict 100  $\mu\text{m}$ .

Figure 4: Calcification-associated podoplanin-positivity in aortic valves. A. Total and area-wise podoplanin. B. Calcification-associated podoplanin. C. Calcification edge-associated podoplanin. Horizontal lines depict the 25<sup>th</sup> percentile, median (wide line) and the 75<sup>th</sup> percentile.

Figure 5: Podoplanin expression in bicuspid vs. tricuspid valves A. Calcification-associated podoplanin. B. Calcification edge-associated podoplanin. The bicuspid group contains two unicuspid cases. Horizontal lines depict the 25<sup>th</sup> percentile, median (wide line) and the 75<sup>th</sup> percentile.

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**Conflicts of interest:** none.

**Declaration of interest:** TP is currently an employee of MSD Finland.

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Table 1. Patient characteristics.

Parameter	Control	AR	AR+f	AS
Patients, n	7	8	15	49
Male gender, n (%)	6 (85.7)	6 (75.0)	11 (73.3)	36 (73.5)
Bi/Unicuspid valve, n (%)	0 (0)	5 (62.5)	8 (53.3)	17 (34.7)
Age, years	56 (51–59.5)	38.5 (35–66)	60 (53–71)	69 (63–76)
Comorbidities, n (%)	5 (71.4)	1 (12.5)	9 (60)	34 (69.4)
Statin medication <sup>1</sup> , n (%)	2 (28.6)	1 (12.5)	6 (40)	32 (71.1)
LVEF <sup>2</sup> , %	60 (59–69)	46.5 (45–50.5)	50 (43–60)	60 (50–66.5)

Values are mean  $\pm$  SD. Age and LVEF stated as median (25<sup>th</sup>–75<sup>th</sup> percentile). Comorbidities include: diabetes, coronary artery disease, peripheral atherosclerosis, chronic obstructive pulmonary disease, hyperlipidemia, atrial fibrillation and hypertension. <sup>1</sup>Data was available from 45 patients in the AS group. <sup>2</sup>Data was available from 6 and 48 patients in the control and AS groups, respectively.

**Highlights**

- Podoplanin expression is associated with aortic valve calcification
- Podoplanin expression is often seen tightly surrounding small calcifications
- Tricuspid valves have more calcification-associated podoplanin than bi/unicuspid valves
- Podoplanin may play a role in myofibroblast differentiation into osteoblastic phenotype.

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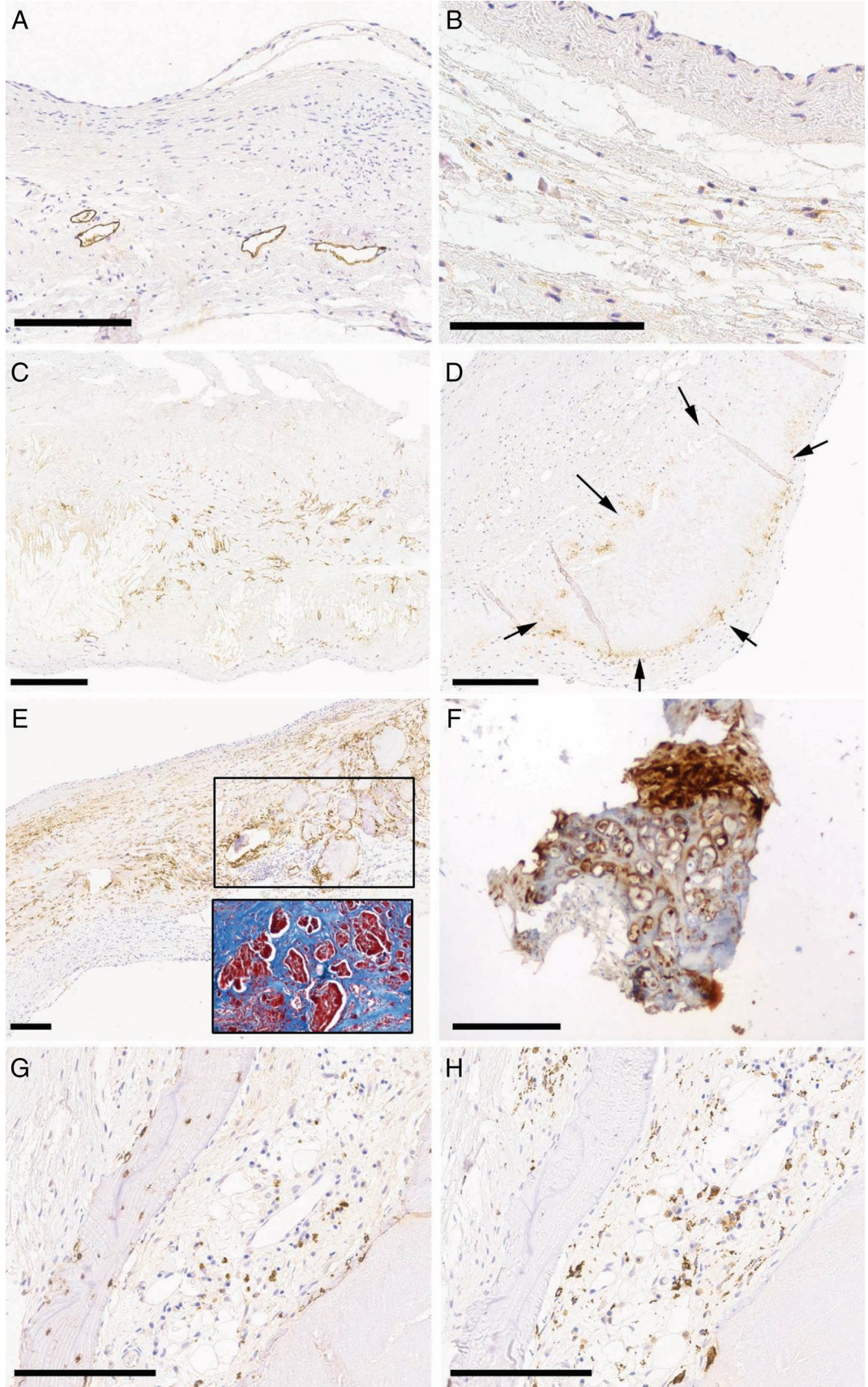


Figure 2

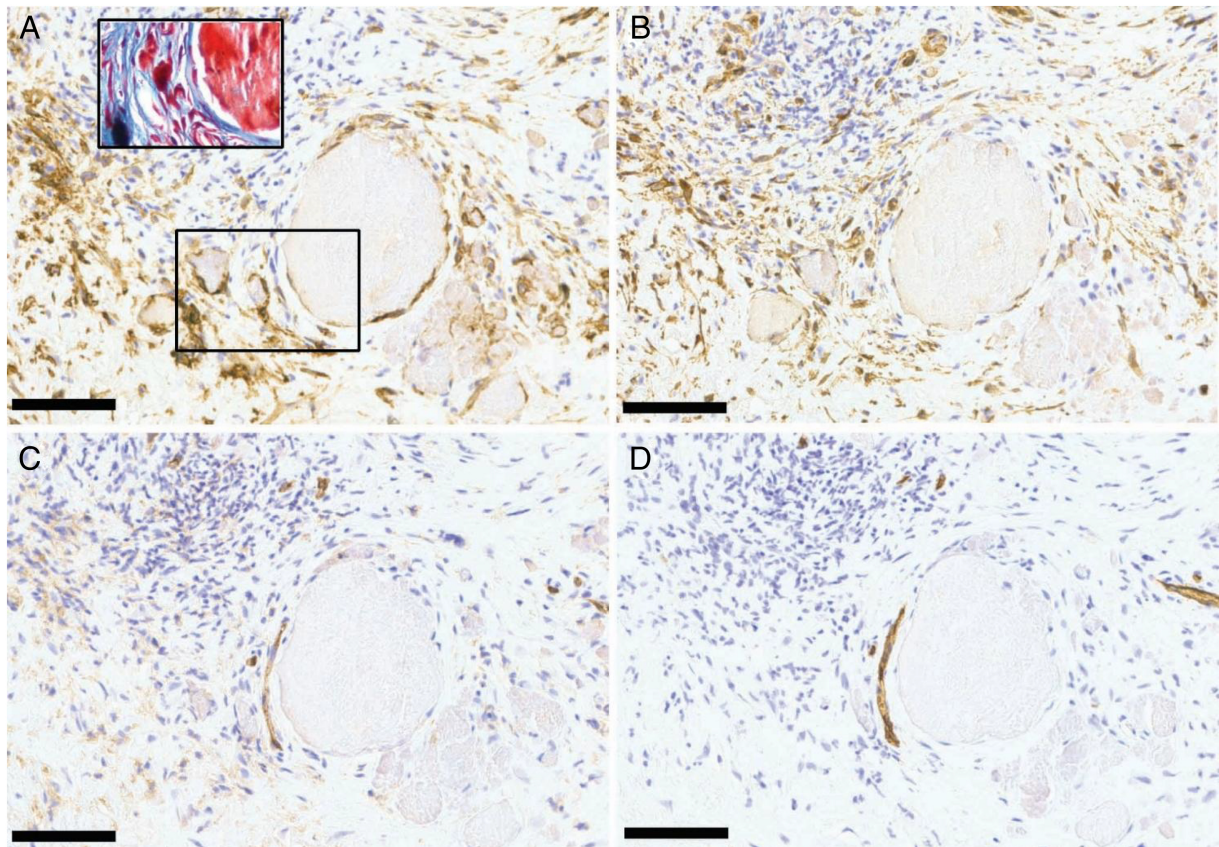


Figure 3

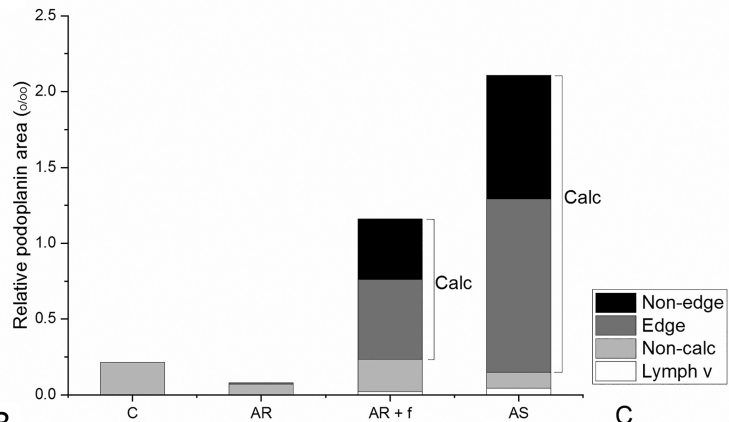
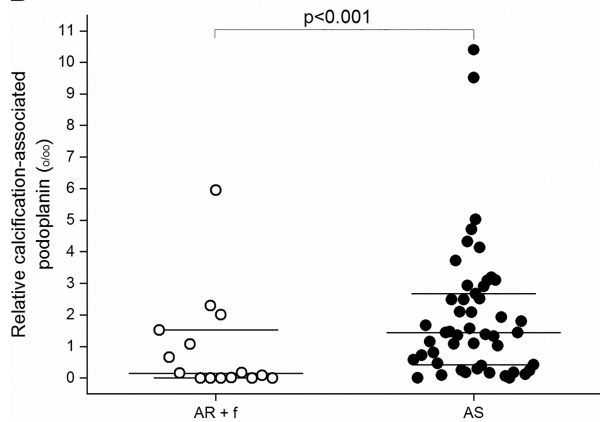
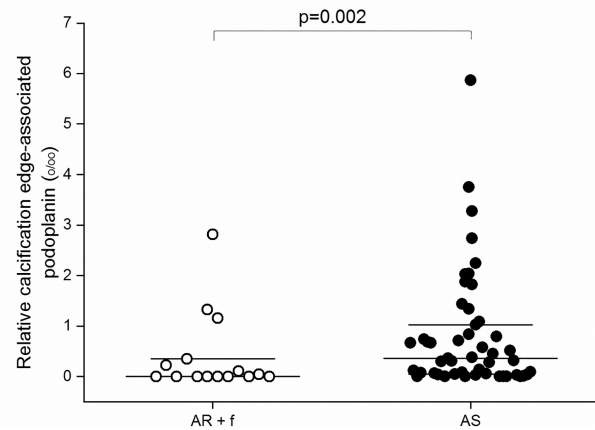
**A****B****C**

Figure 4

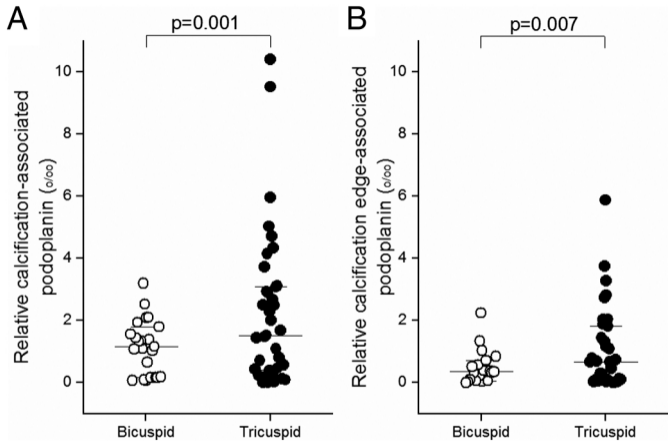


Figure 5