RHEUMATOLOGY

Rheumatology 2023;62:1187–1196
https://doi.org/10.1093/rheumatology/keac466
Advance access publication 12 August 2022

Original article

Antiphospholipid antibodies in patients with calcific aortic valve stenosis

Oscar Plunde [1,2], Elisabet Svenungsson [1,3], Giulia Ferrannini[1,2], Anders Franco-Cereceda[2,4,*] and Magnus Bäck[1,2,*]

Abstract

Objectives. The antiphospholipid syndrome is defined by antiphospholipid antibodies (aPL) together with arterial and/or venous thromboembolism and/or obstetric morbidities. aPL are overrepresented in SLE and acute myocardial infarction, but it is unknown whether aPL are associated with calcific aortic valve stenosis (CAVS) in the general population. The prevalence of aPL and other SLE-associated autoantibodies and their impact on aortic valve transcriptomics were therefore determined.

Methods. A total of 233 tricuspid CAVS cases (median age 74, 69% male) and an age- and sex-matched control population were included. aPL were measured as anti-cardiolipin and anti-β_2Glycoprotein-I of IgG/M/A isotypes. Resilient, thickened and calcified aortic valve (AV) tissue derived from five aPL positive and five matched aPL negative CAVS patients undergoing surgical aortic valve replacement were analysed by microarrays.

Results. The prevalence of positivity for any aPL (IgG/M/A) in patients with CAVS was 6.4% (95% CI 3.6% – 10.4%: $n = 233$). aPL IgG was significantly more prevalent in CAVS cases *vs* controls (4.6% *vs* 0.6%, $P = 0.04$). AV tissue from aPL IgG/IgM-positive patients was negatively enriched in pathways related to interferon signalling. One hundred differentially expressed genes could predict local AV CAVS progression with supervised machine learning algorithms.

Conclusions. aPL IgG was more common in CAVS patients compared with matched controls and aPL positivity was associated with altered AV transcriptomics related to local disease progression and interferon pathways. Further studies should aim to establish aPL as a possible risk marker and/or causal factor for CAVS and could offer new precision therapeutic targets.

Key words: aPL, SLE, calcific aortic valve stenosis, antiphospholipid syndrome, transcriptomics, heart valve disease

CLINICAL SCIENCE

Rheumatology key messages

- Positivity for aPL IgG is associated with calcific aortic valve stenosis in the general population.
- aPL positivity is associated with aortic valve transcriptome alterations.
- The aPL-associated aortic valve transcriptome signature correlates with local disease progression.

Introduction

Calcific aortic valve stenosis (CAVS) is a slow progressive disease within the aortic valve, characterized by leaflet thickening, fibrosis and severe calcification [1]. In the final stage of the disease, severe left ventricular outflow obstruction ultimately leads to morbidity, impaired left ventricular function and high risk of mortality if left untreated. Only interventional valve replacement may halt the clinical progression as no medical treatment is available and the active processes promoting fulminant CAVS remains to be fully understood. In the early stage, inflammation and lipids are denoted important factors [2] although statins have failed to halt disease progression in clinical trials. A common conception is that an atherosclerotic-like process takes place in the aortic valve, which is supported by common histopathological features of lipid deposition and inflammatory cells [3],

[1]Department of Medicine Solna, Karolinska Institutet, [2]Theme Heart and Vessels, [3]Division of Rheumatology, Department of Medicine Solna, Karolinska Institutet, Karolinska University Hospital and [4]Cardiothoracic Surgery Unit, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

Submitted 21 April 2022; accepted 9 August 2022

Correspondence to: Oscar Plunde, Department of Medicine, Karolinska Institutet, Translational Cardiology, Neo Research Building, Blickagången 16, 14157 Stockholm, Sweden. E-mail: oscar.persson@ki.se

*Anders Franco-Cereceda and Magnus Bäck contributed equally to this study.

shared risk factors [4] and shared genetics [5, 6]. Phospholipid pathways are emerging as causal factors for CAVS including, but not restricted to, Lp(a) which is involved in transport of oxidized phospholipids and has been linked to CAVS in mechanistic and genome-wide association studies [5, 7–9]. Also, phospholipids may contribute to calcification by providing a source of phosphate [10].

The APS is serologically characterized by aPL including anti-cardiolipin (anti-CL) and/or anti-β2-glycoprotein-I (anti-β2GPI) of IgG or IgM isotypes and/or positivity in the functional lupus anticoagulant test, and clinically by arterial, venous, microvascular thrombosis and/or obstetric morbidities [11, 12]. APS was first described in SLE patients, in which aPL are associated with an increased risk of valvular heart disease (VHD), sometimes presenting as Libman–Sacks endocarditis [13]. Importantly, aPL positivity also occurs in the general population and with higher prevalence of aPL IgG in patients with acute myocardial infarction (MI) [14, 15]. aPL have also been associated with subclinical atherosclerosis and higher rates of cardiovascular events [16]. However, the role of aPL in CAVS has not previously been investigated, nor has the SLE-associated extractable anti-nuclear antigens (ENA). The aims of this study were therefore to determine the prevalence of aPL and of autoantibodies targeting ENA in CAVS and their relation to concomitant coronary artery disease (CAD) and association to aortic valve transcriptomics.

Methods

Study cohorts

The current study was an observational cross-sectional study including patients from the ongoing, single-centre study DAVAACA (Disease of the Aortic Valve Ascending Aorta and Coronary Arteries). DAVAACA includes patients referred for elective open-heart surgery for aortic aneurysm and/or aortic valve stenosis with or without coronary artery bypass grafting [17]. A total of 233 consecutive patients enrolled between 2013–2019 with CAVS undergoing surgical aortic valve replacement with verified tricuspid aortic valves were included after exclusion of two patients with congenital or rheumatic aortic valve stenosis (Supplementary Fig. S1, available at *Rheumatology* online). Importantly, no included patient had a documented history of APS or SLE. CAD was defined by at least one of the following criteria: significant coronary artery stenosis subject to concomitant CABG, previous acute coronary syndrome, or previous percutaneous coronary intervention (PCI). CAD was further characterized by vessel disease (VD) defined as number of significantly stenotic coronary artery territories on the pre-operative coronary angiogram. All patients underwent coronary angiograms as part of the preoperative assessment and not due to symptoms related to myocardial infarction. Information on preoperative transvalvular peak aortic valve jet velocity (Vmax) determined with echocardiography, previous medical history and CAD status was obtained from electronic medical records or questioners. Estimated glomerular filtration rate (eGFR) was calculated by the local hospital laboratory using the revised Lund–Malmö formula [18].

In order to compare CAVS cases to controls, an age- and sex-matched control group was established by including females $\geq$68 years ($n = 52$) and males $\geq$69 years ($n = 124$), (median age 71.0, IQR 3) from the PAROKRANK (Periodontitis and Its Relation to Coronary Artery Disease) study [14] and females $\leq$76 years ($n = 48$) and males $\leq$77 years ($n = 126$) from DAVAACA (median age 71.5, IQR 7), as depicted in Supplementary Fig. S1, available at *Rheumatology* online. The cut-off values were chosen blinded to autoantibody results, to achieve a control cohort as representative as possible to the whole CAVS cohort with respect to sex, sample sizes and to minimize the age difference between DAVAACA patients and PAROKRANK controls. All subjects gave informed written consent, and the studies were approved by the local ethics committee (2012/1633–31/4, DAVAACA and 2008/152–31/2, PAROKRANK) and conducted in accordance with the declaration of Helsinki. Autoantibodies targeting cardiolipin (anti-CL) and β_2-glycoprotein I (anti-β_2GPI) of IgG/IgM/IgA isotypes and ENA were analysed using multiplexed beads (BioPlex 2200 Multiplex Testing, Bio-Rad, Hercules, CA, USA). Positivity for aPL (here defined as anti-CL and/or anti-anti-β_2GPI, IgG/IgM/IgA) was set at 99% of local controls [12] (>10U/ml IgG, >20U/ml IgA, >30U/ml IgM) and for ENA (centromere B, chromatin, dsDNA, Jo-1, ribosomal P, RNP 68, RNP A, Scl-70, Sm, Sm RNP, SSA 52, SSA 60, SSB) according to manufacturer's instructions.

Seventy-four of the 233 patients in the current study have previously been characterized with aortic valve transcriptomics in an established biobank [19] from the DAVAACA study. Among the patients in the biobank cohort, five patients were positive for aPL IgG/IgM, and these were pair-wise matched for age, sex and concomitant CAD to five aPL-negative CAVS patients (Supplementary Table S1, available at *Rheumatology* online). The matched groups were compared with mean (S.D.), median (IQR) and standardized mean difference.

Aortic valve transcriptomics and genotyping

Aortic valve tissue representing three stages of the continuum of CAVS were dissected from each aortic valve following SAVR as previously described [19]. Briefly, resilient tissue was transparent and pliable, thickened tissue was stiffened non-transparent and calcified tissue was severely solidified and calcified. After dissection, RNA was isolated using TissueLyzer and RNeasy Tissue Mini Kit (Qiagen, Hilden, Germany). NanoDrop (ThermoFisher Scientific, Waltham, MA, USA) was used to assure sufficient RNA concentration and 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) to control RNA integrity. Human Transcriptome Arrays 2.0 (Affymetrix, Santa Clara, CA, USA) via the bioinformatics core facility at Karolinska Institutet were used to obtain

gene expression data. Normalization by signal space transformation robust multiple-array average (SST-RMA) was carried out with the Transcriptome Analysis Console Software 4.0.2 (ThermoFisher Scientific) which was also used for quality and batch control. The processed log2 gene expression data was imported into Qlucore Omics Explorer 3.7 (Lund, Sweden) and annotated non-coding probes and genes below median expression (if true in >50% of the samples) were removed, yielding 12 593 genes. When aPL IgG/IgM positive *vs* negative patients were compared, genes with a Q-value (Benjamini–Hochberg adjusted *P*-value) <0.1 and a fold change <0.8 or >1.2 were considered differentially expressed (DEGs) unless otherwise stated. To limit over-interpretation of single gene hits and to gain biologically relevant data, gene set enrichment analysis (GSEA) was carried out. GSEA was performed within QOE with default settings (Match Set Size 15–500, 1000 permutations) and variable imputation. To rank the genes, a paired *t* test adjusted for tissue type was used and the complete list of Gene Ontology biological processes was used. Pathway enrichment analysis for individual DEGs was carried out with string functional enrichment in Cytoscape (3.8.2) [20] using default settings.

A total of 179 patients in the whole cohort were genotyped with Illumina Human 610-Quad BeadChip and Infinium global Screening Arrays as previously described [21]. A single nucleotide polymorphism (SNP) within STAT4 (rs10181656) was interrogated for its known association with APS and SLE [22, 23].

Supervised machine learning

Supervised machine learning algorithms were built in R 4.0.5 with the caret package using the models 'rf' (random forest), 'lda' (linear discriminant analysis), 'svmRadial' (support-vector machines) and 'gbm_h2o' (gradient boosted machine). Twelve-fold cross validation was used to measure the performance of the models. Briefly, the data was randomly partitioned into 12 equal parts in which the model was trained on 11 and validated on the held-out part. This process was then repeated so that each partition was only used as validation one time and hence, an average estimate of the performance on the held-out samples was provided. The default hyperparameter settings were used in all models. Aortic valve gene expression from 64 patients (192 samples) not included in the aPL comparison were included and the DEGs identified in the aPL comparison were used as features (predictors).

General statistics

Categorical data was compared with Fisher's exact test and Cramér's V when a correlation was determined. Normality was tested with the Shapiro-Wilk test and if significant, the Mann–Whitney *U* test was used. Independent *t* test was used on normally distributed data. Qlucore Omics explorer 3.7 (Lund, Sweden) and R 4.0.5 were used for statistical analyses.

Results

Prevalence of aPL in CAVS

The prevalence of any aPL positivity was 6.4% (95% CI 3.6% – 10.4%: $n = 233$) (Table 1). All aPL positive patients were screened for previous rheumatologic visits and signs of APS which were absent in all cases. Subjects positive for any aPL had a higher proportion of CAD, higher eGFR, and lower total cholesterol levels compared with aPL negative subjects (Table 1). Positivity for aPL was equally distributed over age and sex strata (Fig. 1A and B). The higher prevalence of CAD in aPL-positive compared with negative subjects was not related to the degree of CAD assessed by VD (Fig. 1C). The same results were observed, only including patients positive for aPL IgG/IgM (Supplementary Fig. S2 and Supplementary Table S2, available at *Rheumatology* online).

The most prevalent aPL isotype was IgG, with a prevalence of 3.9% (95% CI 1.8% – 7.2%) in the whole CAVS cohort (Table 2). There was a significant overlap between aPL IgG and aPL IgM (Cramér's $V = 0.75$) but not for aPL IgA (Fig. 1D).

When comparing CAVS patients with age- and sex-matched controls (Supplementary Fig. S1, available at *Rheumatology* online), aPL IgG was significantly more prevalent in CAVS cases *vs* controls (4.6% *vs* 0.6%, $P = 0.037$), corresponding to an odds ratio of 8.3 (95% CI 1.03–67.4) for CAVS (Table 2). Neither aPL IgM, IgA nor any antibodies targeting ENA specificities differed between CAVS patients and controls (Table 2 and Supplementary Table S3, available at *Rheumatology* online).

Genetic risk factor for APS and SLE

The risk allele (G) in the STAT4 snp rs10181656 is associated with APS and SLE(23). The proportion of patients ($n = 179$) homozygous for the risk allele (G) was significantly higher in aPL IgG/IgM positive patients (33% *vs* 1.4% *vs* 3.6% in GG, GC and CC carriers, respectively, $P = 0.017$). The distribution according to aPL positivity is shown in Supplementary Table S4, available at *Rheumatology* online.

Aortic valve transcriptomics in relation to aPL IgG/IgM positivity

Aortic valve transcriptomics revealed 100 differentially expressed genes (DEGs) between aPL IgG/IgM positive compared with aPL negative CAVS patients. All of these genes were also differentially expressed when only including IgG positive patients ($n = 4$, data not shown). Of these, 46 genes were upregulated, and 54 genes were downregulated with aPL IgG/IgM positivity (Fig. 2). Among the significantly enriched pathways identified by GSEA, valves derived from aPL IgG/IgM positive CAVS patients exhibited downregulated pathways related to interferon (IFN) signalling and antigen processing (Table 3).

Table 1 Patient characteristics

CAVS ($n = 233$)	Any aPL positive ($n = 15$, 6.4 %)	Any aPL negative ($n = 218$, 93.6 %)	*P*-value
Male Sex, *n* (%)	12 (80%)	148 (68%)	0.42
Age	74.3 (4.4)	72.8 (5.8)	0.32
CAD, *n* (%)	14 (93.3%)	102 (46.8%)	0.0003
Vmax, $n = 232$	4.4 (0.5)	4.4 (0.5)175	0.96
DVT/PE, *n* (%)	2 (13.3%)	17 (7.8%)	0.35
Diabetes mellitus, *n* (%)	5 (33%)	44 (20%)	0.32
Aortic aneurysm, *n* (%)	1 (6.7%)	4 (1.8%)	0.29
Hypertension, $n = 228$, *n* (%)	10 (67%)	148 (67%)	0.78
CID, $n = 232$, *n* (%)	0 (0%)	18 (8.3%)	0.61
Current smoker, *n* (%)	0 (0%)	11 (5 %)	1
Anticoagulant, *n* (%)	1 (6.7%)	31 (14.2%)	0.70
BMI (kg/m^2), $n = 232$	25.9 (14.6)	27.1 (5.4)	0.99
Leukocytes ($\times 10^9$/L)	6.4 (2.6)	6.2 (2.4)	0.63
Erythrocytes[a] ($\times 10^{12}$/L), $n = 229$	4.2 (0.7)	4.3 (0.6)	0.47
Haemoglobin[a] (g/L)	128 (19)	131 (18)	0.58
Platelets ($\times 10^9$/L), $n = 232$	184 (60)	208 (76)	0.12
fP-glucose (mmol/L), $n = 224$	6.5 (1.4)	6.3 (2.0)	0.57
HbA1c (IFCC)	40 (14)	39 (9)	0.47
eGFR (ml/min/1,73 m^2)	70 (11)	66 (17)	0.035
hsCRP (mg/L)	1.2 (1.4)	1.6 (3.3)	0.11
Cholesterol (mmol/L), $n = 232$	3.6 (1.4)	4.2 (1.9)	0.035

Patient characteristics stratified on presence of antibodies against cardiolipin and/or β_2-glycoprotein I (aPL positive). Continuous variables are presented as median (IQR) if non-normally distributed data and mean (S.D.) if normally distributed data ([a]). anticoagulant: Vit-K antagonist or direct acting oral anticoagulants; CID: chronic inflammatory disease; DVT/PE: deep vein thrombosis/pulmonary embolism; eGFR: estimated glomerular filtration rate; hsCRP: high sensitive CRP; Vmax: transvalvular peak aortic jet velocity. *P*-values for Fisher's exact test on categorical data and for continuous data, independent sample *t* test for normally distributed data and Mann–Whitney *U* test for non-normally distributed data.

The relation of the identified DEGs in aPL IgG/IgM positivity to CAVS disease progression was assessed by comparisons of different disease stages within the valves. Forty-one downregulated DEGs were also downregulated in calcified compared with non-calcified aortic valve tissue and represented enriched pathways of IFN signalling (Supplementary Table S5, available at *Rheumatology* online). Thirty-three upregulated DEGs were also upregulated in calcified compared with non-calcified aortic valve tissue and constituted genes related to microtubule and dynein pathways (Supplementary Table S6, available at *Rheumatology* online). The top enriched interferon gene list (Table 3) consisted of seven genes that were significantly downregulated (Q-value <0.1) in calcified compared with resilient tissue and in tissue from aPL IgG/IgM positive compared with aPL negative patients (Supplementary Table S7, available at *Rheumatology* online).

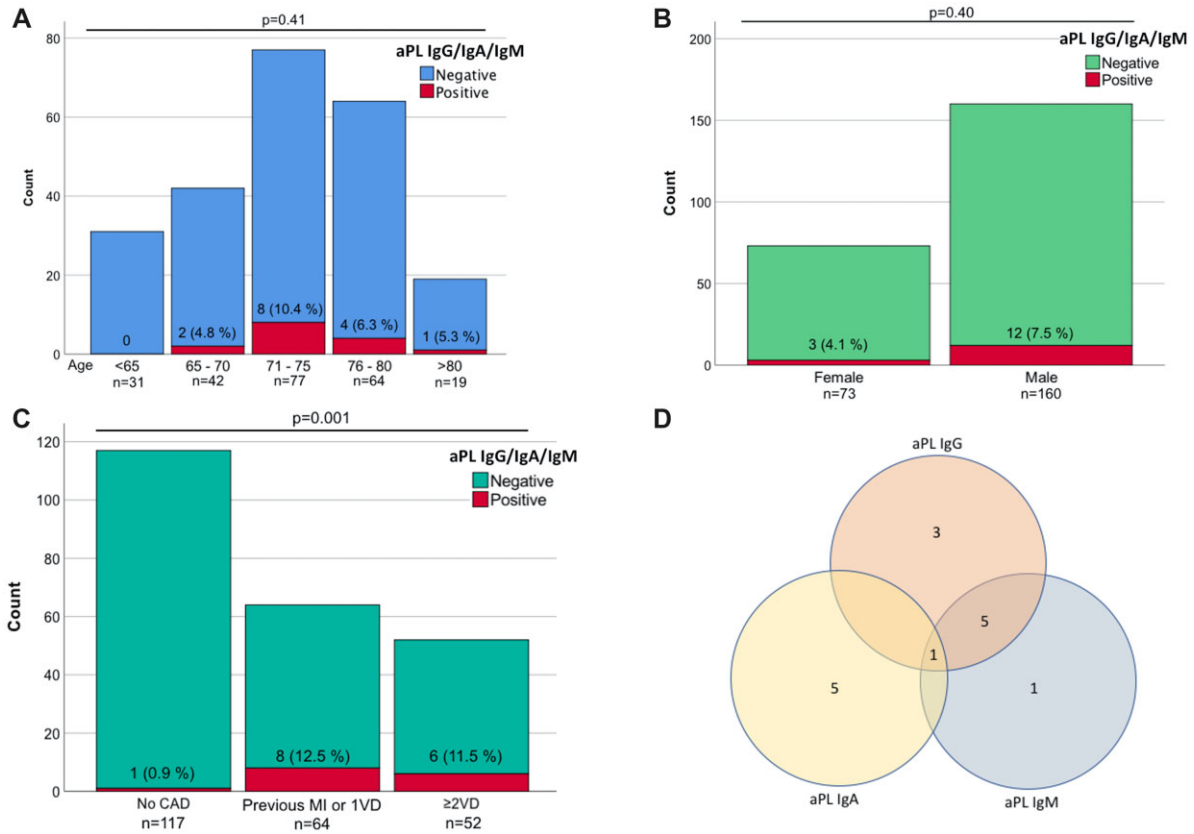
Finally, supervised machine learning using the 100 aPL IgG/IgM DEGs predicted resilient, thickened, and calcified tissue samples from 64 valves derived from patients not included in the aPL comparison. All models performed with an accuracy ranging from 94% – 100% (Fig. 3). All models had 100% sensitivity for calcified tissue, but linear discriminate analysis was also able to predict resilient and thickened tissue at 100% accuracy (Supplementary Table S8, available at *Rheumatology* online). The 20 most important variables from each model (Supplementary Fig. S3, available at *Rheumatology* online) did not show any significantly enriched pathways.

Discussion

The present study is the first to report an increased aPL positivity in CAVS and aPL-associated changes in aortic valve transcriptomics linked to local disease progression. The findings are based on observations in a well-characterized cohort of degenerative tricuspid CAVS cases and a matched control cohort.

aPL positivity in CAVS cases was 1–3 percentage units higher than previously reported in younger control populations [14, 24]. Age and sex were not associated with aPL in the current cohort which contrasts previous findings with an overrepresentation of younger women in APS. In the present study, a comparison with an age- and sex-matched control population revealed an 8-fold higher proportion of aPL IgG positivity in CAVS patients compared with matched controls. Notably, the cut-off for aPL positivity was set at the 99th percentile of the normal population, thus all positive patients met the moderate-to-high titre definition [12]. Nevertheless, the titres were relatively low compared with the proposed high titre definition using ELISA which cannot be directly compared with a multiplex flow immunoassay [25]. These results are comparable to a meta-analysis of

Fig. 1 aPL associations



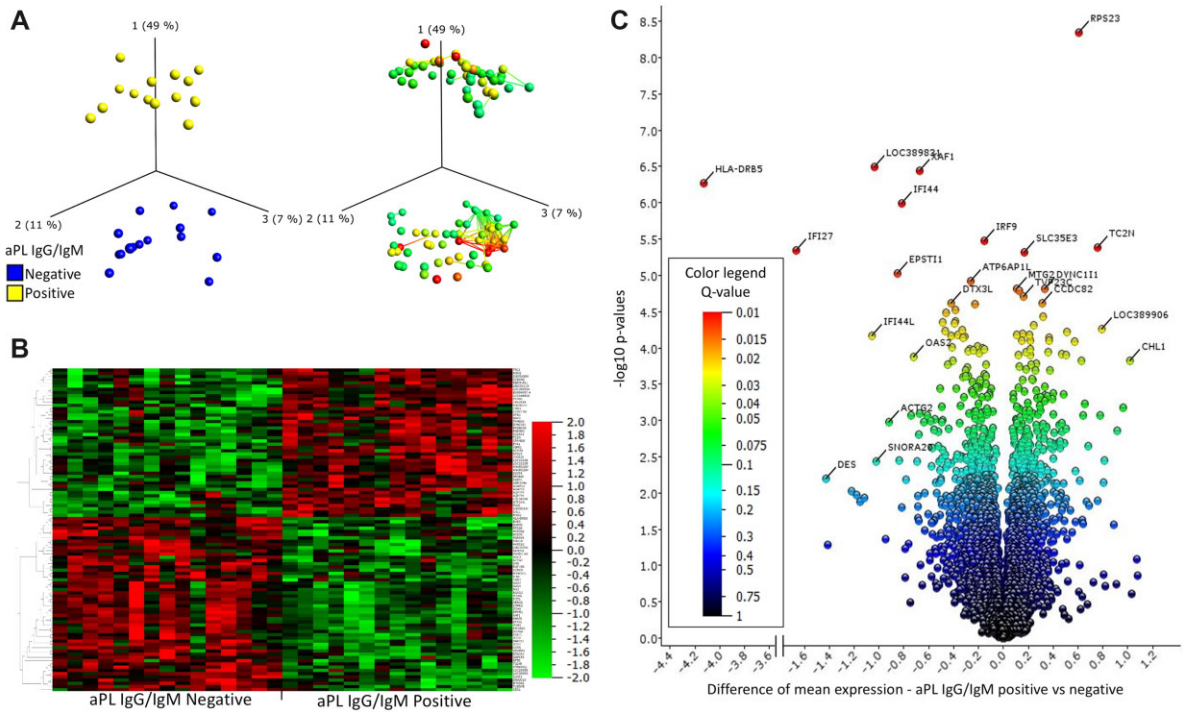
The distribution of (**A**) age groups, (**B**) sex and (**C**) degree of coronary artery disease (CAD) in relation to positivity for any aPL (anti-cardiolipin and/or anti-β_2-glycoprotein I) of IgG, IgA or IgM isotype in the 233 cases with calcific aortic stenosis. (**D**) depicts degree of overlap in aPL-positive patients with respect to isotypes. CAD was graded based on vessel disease (VD) defined as number of significantly stenotic coronary territories on pre-operative coronary angiogram or previous myocardial infarction (MI). Numbers in the bars represent number of positive patients with corresponding percentage within the group. *P*-values stem from Fisher's exact test on any aPL positivity between the groups.

Table 2 Antiphospholipid antibodies in CAVS and matched control cohorts

Autoantibodies targeting	CAVS ($n = 233$)	Age/sex matched cohorts		*P*-value
		Controls ($n = 176$) No. (%), [median, IQRs or titres]	CAVS ($n = 174$) No. (%), [median, IQRs or titres]	
β_2GPI IgG	7 (3.0%)	1 (0.6%) [10]	7 (4.0%) [23, 18–27]	0.067
β_2GPI IgM	5 (2.1%)	4 (2.3%) [46, 39–78]	4 (2.3%) [112, 77–141]	1.000
β_2GPI IgA	5 (2.1%)	4 (2.3%) [79, 49–101]	2 (1.1%) [35, 58]	0.685
Cardiolipin IgG	8 (3.4%)	1 (0.6%) [12]	7 (4.0%) [27, 15–33]	0.067
Cardiolipin IgM	6 (2.3%)	4 (2.3%) [54, 47–84]	5 (2.9%) [51, 38–124]	0.750
Cardiolipin IgA	5 (2.1%)	4 (2.3%) [78, 44–113]	3 (1.7%) [26, 27, 84]	1.000
aPL IgG	9 (3.9%)	1 (0.6%)	8 (4.6%)	0.037
aPL IgM	7 (3.0%)	5 (2.9%)	6 (3.4%)	0.770
aPL IgA	6 (2.6%)	4 (2.3%)	3 (1.7%)	1.000

Results from measurements of aPL, here defined as Cardiolipin (CL) and/or β_2-glycoprotein I (β_2GPI) autoantibodies in a calcific aortic valve stenosis cohort and comparison with an age- and sex-matched control cohort. *P*-values from Fisher's exact test from comparisons between age/sex-matched controls with CAVS cohort.

Fig. 2 aPL IgG/IgM positivity associated change in aortic valve gene expression



Different visualization of 100 genes that had a fold change over 1.2 and q-value <0.1 comparing aortic valve gene expression from patients positive for antiphospholipid antibodies (aPL), here anti-cardiolipin and/or anti-β2-Glycoprotein I of IgG or IgM isotype. In (**A**), a synchronized principal component analysis plot where the left plot includes individual samples and the right individual genes. The genes are approximated to the samples depending on expression, i.e., genes appearing in the top are higher expressed in aPL IgG/IgM positive patients and vice versa. Genes that were highly correlated (Pearson r >0.8) were connected with lines. The cluster of genes to the far right of the graph are interferon-related genes. The genes are also colour coded according to the q-value colour legend in panel C. (**B**) represents a heat map of all genes, sorted by hierarchical clustering and the samples are ordered based on aPL positivity. (**C**) includes a volcano plot of all included genes with x-values >0 represent higher gene expression with aPL positivity and vice versa. The genes are coloured by their q-value.

patients with SLE, reporting that anti-CL IgG positivity confers a 5.6-fold increased risk of VHD [13]. In general, aPL IgG positivity is known to be more strongly associated with risk for thrombosis and clinical manifestations compared with aPL IgM [26]. These previous reports further strengthen the potential clinical importance of the observation that aPL IgG conferred the strongest association with CAVS in the present study.

The observation that aPL positivity was more common in CAVS with concomitant CAD is supported by several recent studies where atherosclerosis was linked to aPL. Two studies of patients with MI report that aPL IgG positivity was present in 11% within 6–12 weeks after MI [14, 15], while the frequency was 6% in MI patients with normal coronary arteries [15]. However, the relation between CAVS and CAD in the context of aPL merits further studies.

The significant association of the STAT4 SNP with aPL IgG/IgM in the present CAVS cohort supports that aPL positivity did not occur because of CAVS. STAT4 SNPs are associated with SLE [22] and the presence of aPL and stroke in SLE patients [23]. The present study thereby extends previous findings in SLE to CAVS patients from the general population and suggests that the significant STAT4 genetic variations is related to aPL status independent of SLE.

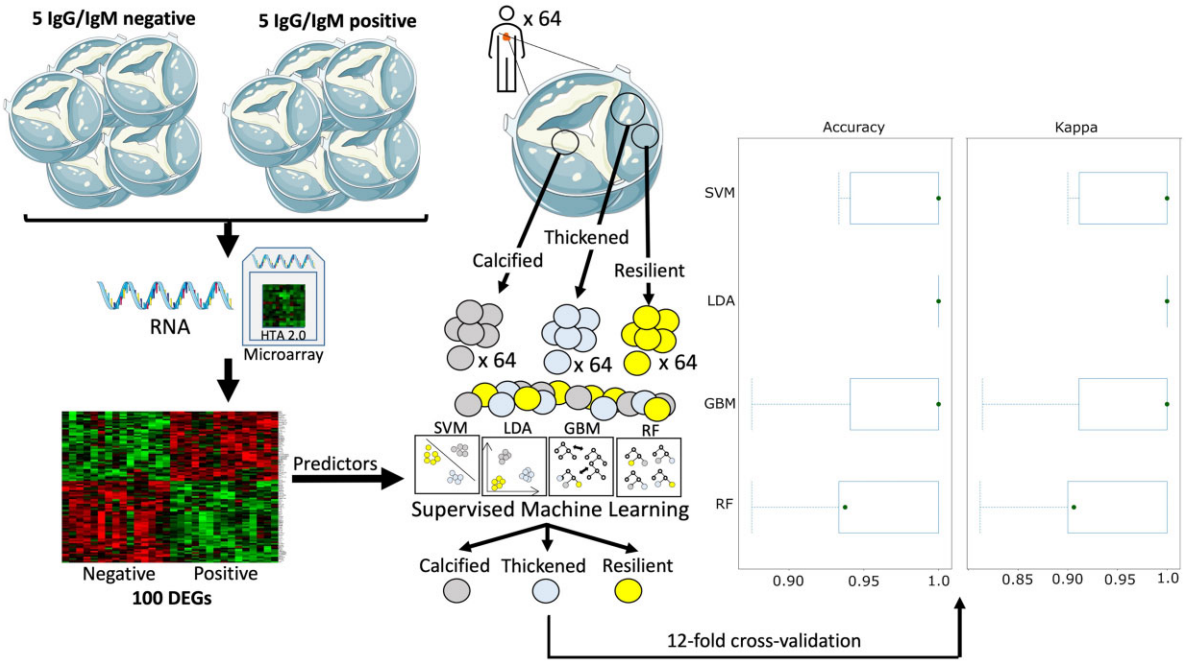
A transcriptome-wide approach identified 100 DEGs when comparing tissue from matched CAVS patients, yielding CAD-independent results. The use of both Q-value and fold change as cut-off was applied to promote biological relevant findings. Interestingly, enrichment analyses identified downregulated IFN pathways in aortic valves from aPL IgG/IgM-positive patients and in calcified compared with resilient valve tissue. IFN-α treatment of valvular interstitial cells induces genes related to calcification and downregulation of calcification inhibitors [27]. Furthermore, an upregulated type I IFN score has been associated with aPL in primary APS and SLE based on gene expression in peripheral blood mononuclear cells [28]. Here, we observed downregulated type I IFNs pathways in aPL IgG/IgM-positive CAVS patients, which is in line with the negative association

TABLE 3 Enriched pathways in aortic valve tissue from aPL IgG/IgM positive patients

Name	Size	Matches	NES	q
GO_RESPONSE_TO_TYPE_I_INTERFERON	96	66	−2,64	0
GO_INTERFERON_GAMMA_MEDIATED_SIGNALING_PATHWAY	90	70	−2,62	0
GO_RESPONSE_TO_INTERFERON_GAMMA	197	141	−2,56	0
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN	188	159	−2,49	0
GO_ANTIGEN_PROCESSING_AND_PRESENTATION	223	179	−2,46	0
GO_DEFENSE_RESPONSE_TO_VIRUS	239	157	−2,44	0
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I	95	89	−2,4	0
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_EXOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I	79	74	−2,35	0
GO_RESPONSE_TO_VIRUS	323	210	−2,31	0
GO_MUSCLE_CONTRACTION	360	181	−2,3	0
GO_NUCLEAR_TRANSCRIBED_MRNA_CATABOLIC_PROCESS	207	184	2,3	0

Top results from gene set enrichment analysis comparing aortic valve tissue from aPL IgG or IgM (antibodies against cardiolipin and/or β_2-glycoprotein I of IgG or IgM isotypes) positive ($n = 5$) and negative ($n = 5$) subjects. All gene lists within Gene Ontology (GO) biological processes were used. NES: normalized enrichment score.

FIG. 3 Supervised machine learning to predict resilient, thickened and calcified aortic valve tissue with aPL-related genes



Gene expression data was obtained from microarray from all samples. Aortic valve tissue from 64 patients were dissected into resilient, thickened and calcified tissue were predicted by four supervised machine learning algorithms (SVM: support vector machine; LDA: linear discriminant analysis; GBM: gradient boosted machine; RF: random forest). The predictors used in the models were obtained from a comparison of tissue from five patients positive for aPL IgG or IgM (presence of antibodies against cardiolipin and/or β_2-glycoprotein I of IgG or IgM isotypes) and five controls pairwise matched for age, sex and coronary artery disease. The performance was evaluated during 12-fold cross-validation and accuracy denotes the average number of correct predictions and Kappa is a more robust form of accuracy taking the possibility of chance into the metric. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

between IFN-α levels and aPL in SLE patients [29]. Also, in the context of CAVS, it is possible that a downregulation of IFN regulatory elements could lead to increased expression of pro-calcifying mediators. For example, the downregulated IFN-induced mRNA encoding a protein with tetratricopeptide repeats-1 (IFIT1) negatively regulates procalcifying TNF-α [30, 31].

The DEGs upregulated in aPL IgG/IgM-positive and calcified samples were involved in intraflagellar transport and dynein complex, which are vital parts of responses to mechanical stimuli affecting intracellular signalling [32] in areas of disturbed shear stress [33], indicating an important mechano-sensing function. Dynein is also important in the breakdown of the nuclear envelope [34] which communicate with the cytoskeleton in response to mechanical stimuli. Recently, the CAVS genetic risk factor PALMD was described to contribute to CAVS through disrupted nuclear envelope function [35]. Of importance, the DEGs identified in the aPL comparison were able to predict the degree of disease progression in valve tissue through supervised machine learning. A similar approach has previously been shown to accurately predict resilient and calcified tissue with sex-specific transcripts [36] and was extended to intermediate disease stages at thickened tissue and to the context of aPL.

Taken together, the transcriptomic results from the present study indicated that the presence of aPL IgG/IgM positivity confers direct effects on aortic valve gene expression, which is linked to local disease progression. However, several additional possible direct effects of aPL can be linked to CAVS pathogenesis. aPL have been shown to activate platelets [37] which are implicated in the role of CAVS by promoting an osteogenic phenotype in valvular interstitial cells [38, 39]. Furthermore, aβ2GPI facilitates foam cell formation [40], induces endothelial dysfunction by inhibiting endothelial nitric oxide synthase [41] which leads to upregulation of adhesion molecules and promotes a pro-inflammatory and thrombotic state [42]. Also, aPL might expose phospholipids, which through enzymatic processing promote calcification by the release of inorganic phosphate [10]. Hence several direct effects of aPL can be linked to CAVS, although some caution is advised extrapolating experimental findings from APS extracting aPL to healthy aPL carriers. In addition, it is believed that not only the presence of aPL but also a secondary hit is necessary to elicit the true effects carried out by aPL, which may be provided by the inflammatory disease that is CAVS [43].

This is the first study to explore autoantibodies in CAVS and their impact on aortic valve transcriptomics using a well-characterized cohort of surgical patients with verified tricuspid aortic valves. There are, however, certain limitations that should be acknowledged. The present study cannot distinguish whether aPL are associated with CAD alone or concomitant CAVS and CAD due to lack of controls with isolated CAD. The lack of citrated plasma prevented us from measuring the functional lupus anticoagulant and only a multiplex flow immunoassay was used to detect aPL, which limits the interpretation of titres. The duration of aPL positivity is unknown and was only measured once, which may increase the risk of finding transient positivity, although the genetic results support long-duration withstanding aPL positivity. Furthermore, samples were taken pre-surgery removing any effect of surgery on the aPL outcomes. Due to the observational and cross-sectional design, causality cannot be addressed. Only five patients positive for aPL IgG/IgM were available for aortic valve transcriptomics and hence some relevant findings may have been lost due to insufficient power. Also, only males were included, limiting the extrapolation of results to women. Furthermore, valves from diagnosed APS patients were not included, and the similarities or differences between APS-related valve disease and aPL-associated CAVS remain to be established. The use of microarray instead of RNA sequencing decreases the sensitivity and dynamics of the gene expression comparison but could reduce the risk of finding false-positive results. We did not use a validation cohort when performing the supervised machine learning but with cross-validation, more cases can be used for training and it is a good surrogate for validating the models because held-out samples are used.

In summary, the present study demonstrates an increased prevalence of aPL in subjects with CAVS. Positivity for the most common aPL isotype (IgG or IgM) was associated with altered aortic valve transcriptomic profile, related to downregulated IFN signalling, upregulated mechanosensing pathways and local disease progression. Further studies are needed to establish aPL as a possible risk marker and/or causal factor for CAVS with CAD. aPL is associated with thrombosis, which is also emerging as a risk factor for CAVS. Future work should aim to decide if aPL-positive CAVS patients could benefit from a precision treatment. Elaborated mechanistic studies are needed to provide insights to precision therapeutical strategies in these patients.

Acknowledgements

O.P. was supported by the Clinical Scientist Training Programme (CSTP) at Karolinska Institutet. A.F.-C. was supported by donations from Mr Fredrik Lundberg and The Schörling Foundation.

Funding: This work was supported by the Swedish Research Council (grant numbers 2019–01486, 2018–02535), the Swedish Heart and Lung Foundation (grant numbers 20180571, 20170257), the King Gustaf V and Queen Victoria Freemason Foundation, and Region Stockholm County Council (grant number 20170365, 20170038), Swedish Society of Medicine (grant number SLS-713911) and the Ingegerd Johansson Donation.

Disclosure statement: The authors have declared no conflicts of interest.

Data availability statement

Individual participant data that underlie the results reported will be shared, after deidentification, with researchers who provide a methodologically sound proposal.

Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1 Lindman BR, Clavel MA, Mathieu P *et al.* Calcific aortic stenosis. Nat Rev Dis Primers 2016;2:16006.

2 New SE, Aikawa E. Molecular imaging insights into early inflammatory stages of arterial and aortic valve calcification. Circ Res 2011;108:1381–91.

3 Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation 1994;90: 844–53.

4 Stewart BF, Siscovick D, Lind BK *et al.* Clinical factors associated with calcific aortic valve disease. Cardiovascular Health Study. J Am Coll Cardiol 1997;29: 630–4.

5 Thanassoulis G, Campbell CY, Owens DS *et al.* Genetic associations with valvular calcification and aortic stenosis. N Engl J Med 2013;368:503–12.

6 Yuan S, Larsson SC. Association of genetic variants related to plasma fatty acids with type 2 diabetes mellitus and glycaemic traits: a Mendelian randomisation study. Diabetologia 2020;63:116–23.

7 Zheng KH, Tsimikas S, Pawade T *et al.* Lipoprotein(a) and oxidized phospholipids promote valve calcification in patients with aortic stenosis. J Am Coll Cardiol 2019;73: 2150–62.

8 Mathieu P, Boulanger MC. Autotaxin and lipoprotein metabolism in calcific aortic valve disease. Front Cardiovasc Med 2019;6:18.

9 Capoulade R, Chan KL, Yeang C *et al.* Oxidized phospholipids, lipoprotein(a), and progression of calcific aortic valve stenosis. J Am Coll Cardiol 2015;66: 1236–46.

10 Bäck M, Michel JB: From organic and inorganic phosphates to valvular and vascular calcifications.Cardiovasc Res 2021;117:2016–29.

11 Schreiber K, Sciascia S, de Groot PG *et al.* Antiphospholipid syndrome. Nat Rev Dis Primers 2018;4: 17103.

12 Miyakis S, Lockshin MD, Atsumi T *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006;4:295–306.

13 Zuily S, Regnault V, Selton-Suty C *et al.* Increased risk for heart valve disease associated with antiphospholipid antibodies in patients with systemic lupus erythematosus: meta-analysis of echocardiographic studies. Circulation 2011;124:215–24.

14 Grosso G, Sippl N, Kjellström B *et al.* Antiphospholipid antibodies in patients with myocardial infarction. Ann Intern Med 2019;170:277–80.

15 Svenungsson E, Spaak J, Strandberg K *et al.* Antiphospholipid antibodies in patients with myocardial infarction with and without obstructive coronary arteries. J Intern Med 2022;291:327–37.

16 Selmi C, De Santis M, Battezzati PM *et al.* Antiphospholipid antibody prevalence and association with subclinical atherosclerosis and atherothrombosis in the general population. Int J Cardiol 2020;300:209–13.

17 Glaser N, Jackson V, Eriksson P, Sartipy U, Franco-Cereceda A. Relative survival after aortic valve surgery in patients with bicuspid aortic valves. Heart 2021;107: 1167–72.

18 Nyman U, Grubb A, Larsson A *et al.* The revised Lund-Malmö GFR estimating equation outperforms MDRD and CKD-EPI across GFR, age and BMI intervals in a large Swedish population. Clin Chem Lab Med 2014;52:815–24.

19 Plunde O, Larsson SC, Artiach G *et al.* FADS1 (Fatty Acid Desaturase 1) genotype associates with aortic valve FADS mRNA expression, fatty acid content and calcification. Circ Genom Precis Med 2020;13:e002710.

20 Shannon P, Markiel A, Ozier O *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13: 2498–504.

21 Folkersen L, van't Hooft F, Chernogubova E *et al.* Association of genetic risk variants with expression of proximal genes identifies novel susceptibility genes for cardiovascular disease. Circ Cardiovasc Genet 2010;3: 365–73.

22 Remmers EF, Plenge RM, Lee AT *et al.* STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. New Engl J Med 2007;357:977–86.

23 Svenungsson E, Gustafsson J, Leonard D *et al.* A STAT4 risk allele is associated with ischaemic cerebrovascular events and anti-phospholipid antibodies in systemic lupus erythematosus. Ann Rheum Dis 2010;69: 834–40.

24 Pericleous C, Ferreira I, Borghi O *et al.* Measuring IgA Anti-β2-glycoprotein I and IgG/IgA anti-domain I antibodies adds value to current serological assays for the antiphospholipid syndrome. PLoS one 2016;11:e0156407.

25 Vandevelde A, Chayoua W, de Laat B *et al.* Semiquantitative interpretation of anticardiolipin and anti-β2-glycoprotein I antibodies measured with various analytical platforms: communication from the ISTH SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies. J Thromb Haemost 2022;20:508–24.

26 Kelchtermans H, Pelkmans L, de Laat B, Devreese KM. IgG/IgM antiphospholipid antibodies present in the classification criteria for the antiphospholipid syndrome: a critical review of their association with thrombosis. J Thromb Haemost 2016;14:1530–48.

27 Parra-Izquierdo I, Castaños-Mollor I, López J *et al.* Calcification induced by type I interferon in human aortic valve interstitial cells is larger in males and blunted by a janus kinase inhibitor. Arterioscler Thromb Vasc Biol 2018;38:2148–59.

28 Palli E, Kravvariti E, Tektonidou MG. Type I interferon signature in primary antiphospholipid syndrome: clinical and laboratory associations. Front Immunol 2019;10:487.

29 Oke V, Gunnarsson I, Dorschner J *et al.* High levels of circulating interferons type I, type II and type III associate with distinct clinical features of active systemic lupus erythematosus. Arthritis Res Ther 2019;21:107.

30 John SP, Sun J, Carlson RJ *et al.* IFIT1 exerts opposing regulatory effects on the inflammatory and interferon gene programs in LPS-activated human macrophages. Cell Rep 2018;25:95–106.e6.

31 Yu Z, Seya K, Daitoku K *et al.* Tumor necrosis factor-α accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway. J Pharmacol Exp Ther 2011;337:16–23.

32 AbouAlaiwi WA, Takahashi M, Mell BR, Jones TJ *et al.* Ciliary polycystin-2 is a mechanosensitive calcium channel involved in nitric oxide signaling cascades. Circ Res 2009;104:860–9.

33 Van der Heiden K, Hierck BP, Krams R *et al.* Endothelial primary cilia in areas of disturbed flow are at the base of atherosclerosis. Atherosclerosis 2008;196:542–50.

34 Salina D, Bodoor K, Eckley DM *et al.* Cytoplasmic dynein as a facilitator of nuclear envelope breakdown. Cell 2002; 108:97–107.

35 Sáinz-Jaspeado M, Smith RO, Plunde O *et al.* Palmdelphin regulates nuclear resilience to mechanical stress in the endothelium. Circulation 2021;144:1629–45.

36 Sarajlic P, Plunde O, Franco-Cereceda A, Bäck M. Artificial intelligence models reveal sex-specific gene expression in aortic valve calcification. JACC Basic Transl Sci 2021;6:403–12.

37 Hollerbach A, Müller-Calleja N, Ritter S *et al.* Platelet activation by antiphospholipid antibodies depends on epitope specificity and is prevented by mTOR inhibitors. Thromb Haemost 2019;119:1147–53.

38 Bouchareb R, Boulanger MC, Tastet L *et al.* Activated platelets promote an osteogenic programme and the progression of calcific aortic valve stenosis. Eur Heart J 2019;40:1362–73.

39 Sellers SL, Gulsin GS, Zaminski D *et al.* Platelets: implications in aortic valve stenosis and bioprosthetic valve dysfunction from pathophysiology to clinical care. JACC Basic Transl Sci 2021;6:1007–20.

40 Hasunuma Y, Matsuura E, Makita Z *et al.* Involvement of beta 2-glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. Clin Exp Immunol 1997;107: 569–73.

41 Ramesh S, Morrell CN, Tarango C *et al.* Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via β2GPI and apoER2. J Clin Invest 2011;121:120–31.

42 Corban MT, Duarte-Garcia A, McBane RD *et al.* Antiphospholipid syndrome: role of vascular endothelial cells and implications for risk stratification and targeted therapeutics. J Am Coll Cardiol 2017;69:2317–30.

43 Otto CM, Prendergast B. Aortic-valve stenosis–from patients at risk to severe valve obstruction. N Engl J Med 2014;371:744–56.