Antiphospholipid Antibodies in Patients with Calcific Aortic Valve Stenosis

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Key words

Antiphospholipid antibodies, Systemic lupus erythematosus, Calcific aortic valve stenosis, Antiphospholipid syndrome, Transcriptomics, Heart valve disease

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

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- The aPL-associated aortic valve transcriptome signature correlates with local disease progression

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 systemic lupus erythematosus (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: 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-β₂Glycoprotein-I of IgG/M/A isotypes. Resilient, thickened, and calcified aortic valve (AV) tissue derived from 5 aPL positive and 5 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 signaling. 100 differentially expressed genes could predict local AV CAVS progression with supervised machine learning algorithms.

Conclusions: aPL IgG was more common in CAVS patients compared to 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.
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), 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 have been linked to CAVS in studies ranging from in vitro to genome wide association studies (5, 7-9). Also phospholipids may serve as source of phosphate to support calcification (10).

The antiphospholipid syndrome (APS) is serologically characterized by anti-phospholipid antibodies (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 systemic lupus erythematosus (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). 233 consecutive patients enrolled between 2013 – 2019 with CAVS undergoing surgical aortic valve replacement with verified tricuspid aortic valves were included after exclusion of 2 patients with congenital or rheumatic aortic valve stenosis (Supplementary Figure 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 ≥68 years (n=52) and males ≥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 ≤76 years (n=48) and males ≤77 years (n=126) from DAVAACA (median age 71.5, IQR 7), as depicted in Supplementary Figure S1. 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 β2-glycoprotein I (anti-β2GPI) of IgG/IgM/IgA isotypes and ENA were analysed using multiplexed beads (BioPlex 2200 Multiplex Testing, Bio-Rad). Positivity for aPL (here defined as anti-CL and/or anti- anti-β2GPI, 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.

74 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, 5 patients were positive for aPL IgG/IgM, and these were pair wise matched for age, sex, and concomitant CAD to 5 aPL negative CAVS patients.
(Supplementary Table S1, available at *Rheumatology* online). The matched groups were compared with mean (SD), 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 (Thermo 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.

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). 12-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 were 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, 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 (Figure 1A and 1B). The higher prevalence of CAD in aPL positive compared with negative subjects was not related to the degree of CAD assessed by VD (Figure 1C). The same results were observed, only including patients positive for aPL IgG/IgM (Supplementary Figure 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 (Figure 1D).

When comparing CAVS patients with age- and sex matched controls (Supplementary Figure S1), 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 (Figure 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) signaling and antigen processing (Table 3).

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. 41 downregulated DEGs were also downregulated in calcified compared with non-calcified aortic valve tissue and represented enriched pathways of IFN signaling (Supplementary Table S5, available at Rheumatology online). 33 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 7 genes that were significantly downregulated (Q-value <0.1) in calcified compared with resilient tissue and in tissue from aPL IgG/IgM positive compared to 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% (Figure 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 Figure 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 to the proposed high titre definition using ELISA which cannot be directly compared to a multiplex flow immunoassay (25). These results are comparable to a meta-analysis of 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 to 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 to 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 between IFN-α levels and aPL in SLE patients (29). Also, in the context of CAVS, it is possible that a down-regulation 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 signaling (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 presence aPL IgG/IgM positivity confer 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), induce endothelial dysfunction by inhibiting endothelial nitric oxide synthase (41) which leads to upregulation of adhesion molecules and promote 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 extracted aPL.
to healthy aPL carriers. In addition, it is believed that not only 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, 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 5 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 decrease the sensitivity and dynamic 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 is a good surrogate for validating the models since 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 signaling, 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 also is 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.

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Conflict of interest

The authors have declared no conflicts of interest.

Data availability

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


### Table 1 – Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Any aPL positive (n=15, 6.4 %)</th>
<th>Any aPL negative (n=218, 93.6 %)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Sex, n (%)</td>
<td>12 (80 %)</td>
<td>148 (68 %)</td>
<td>0.42</td>
</tr>
<tr>
<td>Age</td>
<td>74.3 (4.4)</td>
<td>72.8 (5.8)</td>
<td>0.32</td>
</tr>
<tr>
<td>CAD, n (%)</td>
<td>14 (93.3 %)</td>
<td>102 (46.8 %)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Vmax, n=232</td>
<td>4.4 (0.5)</td>
<td>4.4 (0.5)</td>
<td>0.96</td>
</tr>
<tr>
<td>DVT/PE, n (%)</td>
<td>2 (13.3 %)</td>
<td>17 (7.8 %)</td>
<td>0.35</td>
</tr>
<tr>
<td>Diabetes Mellitus, n (%)</td>
<td>5 (33 %)</td>
<td>44 (20 %)</td>
<td>0.32</td>
</tr>
<tr>
<td>Aortic Aneurysm, n (%)</td>
<td>1 (6.7 %)</td>
<td>4 (1.8 %)</td>
<td>0.29</td>
</tr>
<tr>
<td>Hypertension, n=228, n (%)</td>
<td>10 (67 %)</td>
<td>148 (67 %)</td>
<td>0.78</td>
</tr>
<tr>
<td>CID, n=232, n (%)</td>
<td>0 (0%)</td>
<td>18 (8.3 %)</td>
<td>0.61</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>0 (0 %)</td>
<td>11 (5 %)</td>
<td>1</td>
</tr>
<tr>
<td>Anticoagulant, n (%)</td>
<td>1 (6.7 %)</td>
<td>31 (14.2 %)</td>
<td>0.70</td>
</tr>
<tr>
<td>BMI (kg/m²), n=232</td>
<td>25.9 (14.6)</td>
<td>27.1 (5.4)</td>
<td>0.99</td>
</tr>
<tr>
<td>Leukocytes (x10⁹/L)</td>
<td>6.4 (2.6)</td>
<td>6.2 (2.4)</td>
<td>0.63</td>
</tr>
<tr>
<td>Erythrocytes* (x10¹²/L), n=229</td>
<td>4.2 (0.7)</td>
<td>4.3 (0.6)</td>
<td>0.47</td>
</tr>
<tr>
<td>Hemoglobin* (g/L)</td>
<td>128 (19)</td>
<td>131 (18)</td>
<td>0.58</td>
</tr>
<tr>
<td>Platelets (x10⁹/ L), n=232</td>
<td>184 (60)</td>
<td>208 (76)</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Patient characteristics stratified on presence of antibodies against cardiolipin and/or β2-glycoprotein I (aPL positive). Continuous variables are presented as median (IQR) if non-normally distributed data and mean (SD) if normally distributed data (*). Vmax=Transvalvular peak aortic jet velocity, DVT/PE = Deep vein thrombosis/Pulmonary embolism, CID=chronic inflammatory disease, Anticoagulant = Vit-K antagonist or direct acting oral anticoagulants, BMI = body mass index, eGFR = estimated glomerular filtration rate, hsCRP = high sensitive C-reactive protein. P-values for Fishers 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.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Median (IQR)</th>
<th>Mean (SD)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>fP-glucose (mmol/L), n=224</td>
<td>6.5 (1.4)</td>
<td>6.3 (2.0)</td>
<td>0.57</td>
</tr>
<tr>
<td>HbA1c (IFCC)</td>
<td>40 (14)</td>
<td>39 (9)</td>
<td>0.47</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>70 (11)</td>
<td>66 (17)</td>
<td>0.035</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>1.2 (1.4)</td>
<td>1.6 (3.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>Cholesterol (mmol/L), n=232</td>
<td>3.6 (1.4)</td>
<td>4.2 (1.9)</td>
<td>0.035</td>
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</table>
Table 2 – Antiphospholipid antibodies in CAVS and matched control cohorts

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>CAVS (n=233)</th>
<th>Controls (n=176)</th>
<th>CAVS (n=174)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%), [median, IQRs or titers]</td>
<td>No. (%), [median, IQRs or titers]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β2GPI IgG</td>
<td>7 (3.0 %) [10]</td>
<td>1 (0.6 %) [23, 18 – 27]</td>
<td>7 (4.0 %)</td>
<td>0.067</td>
</tr>
<tr>
<td>β2GPI IgM</td>
<td>5 (2.1 %) [46, 39 – 78]</td>
<td>4 (2.3 %) [112, 77 – 141]</td>
<td>4 (2.3 %)</td>
<td>1.000</td>
</tr>
<tr>
<td>β2GPI IgA</td>
<td>5 (2.1 %) [79, 49 – 101]</td>
<td>4 (2.3 %) [35, 58]</td>
<td>2 (1.1 %)</td>
<td>0.685</td>
</tr>
<tr>
<td>Cardiolipin IgG</td>
<td>8 (3.4 %) [12]</td>
<td>1 (0.6 %) [27, 15 – 33]</td>
<td>7 (4.0 %)</td>
<td>0.067</td>
</tr>
<tr>
<td>Cardiolipin IgM</td>
<td>6 (2.3 %) [54, 47 – 84]</td>
<td>4 (2.3 %) [51, 38 – 124]</td>
<td>5 (2.9 %)</td>
<td>0.750</td>
</tr>
<tr>
<td>Cardiolipin IgA</td>
<td>5 (2.1 %) [78, 44 – 113]</td>
<td>4 (2.3 %) [26, 27, 84]</td>
<td>3 (1.7 %)</td>
<td>1.000</td>
</tr>
<tr>
<td>aPL IgG</td>
<td>9 (3.9 %) [20]</td>
<td>1 (0.6 %) [23, 18 – 27]</td>
<td>8 (4.6 %)</td>
<td>0.037</td>
</tr>
<tr>
<td>aPL IgM</td>
<td>7 (3.0 %) [35, 58]</td>
<td>5 (2.9 %) [51, 38 – 124]</td>
<td>6 (3.4 %)</td>
<td>0.770</td>
</tr>
</tbody>
</table>
Results from measurements of antiphospholipid antibodies (aPL), here defined as Cardiolipin (CL) and/or $\beta_2$-glycoprotein I ($\beta_2$GPI) 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.

<table>
<thead>
<tr>
<th>aPL IgA</th>
<th>6 (2.6%)</th>
<th>4 (2.3%)</th>
<th>3 (1.7%)</th>
<th>1.000</th>
</tr>
</thead>
</table>

Downloaded from https://academic.oup.com/rheumatology/advance-article/doi/10.1093/rheumatology/keac466/6664008 by guest on 20 August 2022
**Table 3 – Enriched pathways in aortic valve tissue from aPL IgG/IgM positive patients**

<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>Match</th>
<th>NES</th>
<th>q</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO_RESPONSE_TO_TYPE_I_INTERFERON</td>
<td>96</td>
<td>66</td>
<td>-2.64</td>
<td>0</td>
</tr>
<tr>
<td>GO_INTERFERON_GAMMA_MEDIATED_SIG</td>
<td>90</td>
<td>70</td>
<td>-2.62</td>
<td>0</td>
</tr>
<tr>
<td>NALING_PATHWAY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO_RESPONSE_TO_INTERFERON_GAMMA</td>
<td>197</td>
<td>141</td>
<td>-2.56</td>
<td>0</td>
</tr>
<tr>
<td>GO_ANTIGEN_PROCESSING_AND_PRESENT</td>
<td>188</td>
<td>159</td>
<td>-2.49</td>
<td>0</td>
</tr>
<tr>
<td>GO_ANTIGEN_PROCESSING_AND_PRESENT_VIA_MHC_CLASS_I</td>
<td>95</td>
<td>89</td>
<td>-2.4</td>
<td>0</td>
</tr>
<tr>
<td>GO_ANTIGEN_PROCESSING_AND_PRESENT_VIA_MHC_CLASS_I</td>
<td>223</td>
<td>179</td>
<td>-2.46</td>
<td>0</td>
</tr>
<tr>
<td>GO_DEFENSE_RESPONSE_TO_VIRUS</td>
<td>239</td>
<td>157</td>
<td>-2.44</td>
<td>0</td>
</tr>
<tr>
<td>GO_ANTIGEN_PROCESSING_AND_PRESENT_VIA_MHC_CLASS_I</td>
<td>79</td>
<td>74</td>
<td>-2.35</td>
<td>0</td>
</tr>
<tr>
<td>GO_RESPONSE_TO_VIRUS</td>
<td>323</td>
<td>210</td>
<td>-2.31</td>
<td>0</td>
</tr>
<tr>
<td>GO_MUSCLE_CONTRACTION</td>
<td>360</td>
<td>181</td>
<td>-2.3</td>
<td>0</td>
</tr>
</tbody>
</table>
Top results from gene set enrichment analysis comparing aortic valve tissue from aPL IgG or IgM (antibodies against cardiolipin and/or β2-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.

**Figure Legends**

**Figure 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-β2-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.

**Figure 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 panel (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. Panel B represents a heat map of all genes, sorted by hierarchical clustering and the samples are ordered based on aPL positivity. Panel (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.

**Figure 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 4 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 5 patients positive for aPL IgG or IgM (presence of antibodies against cardiolipin and/or β2-glycoprotein I of IgG or IgM isotypes) and 5 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/).
Fig 1

270x188mm (300 x 300 DPI)
Fig 2

316x189mm (300 x 300 DPI)
Fig 3

338x190mm (300 x 300 DPI)