Adenosine derived from ecto-nucleotidases in calcific aortic valve disease promotes mineralization through A2a adenosine receptor

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Aims
In this study, we sought to determine the role of ecto-nucleotidases and adenosine receptors in calcific aortic valve disease (CAVD). The expression of ecto-nucleotidases, which modify the levels of extracellular nucleotides/nucleosides, may control the mineralization of valve interstitial cells (VICs). We hypothesized that expression of ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1), which generates AMP, and 5′-nucleotidase (CD73), an enzyme using AMP as a substrate to produce adenosine, may co-regulate the mineralization of the aortic valve.

Methods and results
We have investigated the expression of NPP1 and 5′-nucleotidase in CAVD tissues and determined the role of these ecto-nucleotidases on the mineralization of isolated VICs. In CAVD tissues (stenotic and sclerotic), we documented that NPP1 and 5′-nucleotidase were overexpressed by VICs. In isolated VICs, we found that mineralization induced by adenosine triphosphate was decreased by silencing NPP1 and 5′-nucleotidase, suggesting a role for adenosine. Adenosine and specific A2a adenosine receptor (A2aR) agonist increased the mineralization of VICs. Silencing of A2aR in human VICs and the use of A2aR−/− mouse VICs confirmed that A2aR promotes the mineralization of cells. Also, A2aR-mediated mineralization was negated by the transfection of a mutant dominant-negative Gαs vector. Through several lines of evidence, we next documented that adenosine stimulated the mineralization of VICs through a cAMP/protein kinase A (PKA)/cAMP response element-binding protein (CREB) pathway, and found that CREB positively regulated the expression of NPP1 in a positive feedback loop by physically interacting with the promoter.

Conclusion
Expression of NPP1 and 5′-nucleotidase by VICs promotes the mineralization of the aortic valve through A2aR and a cAMP/PKA/CREB pathway.

Keywords
Calcific aortic valve disease • Calcific aortic valve stenosis • Aortic stenosis • Biomineralization • NPP1 • ENPP1 • 5′-nucleotidase • NT5E • CD73 • Ectopic mineralization • PKA • CREB • Adenosine • A2a receptor

1. Introduction
Calcific aortic valve disease (CAVD) is the most common heart valve disorder.1 A progressive mineralization of the aortic valve is the major culprit in CAVD.2 Nucleotides are secreted by valve interstitial cells (VICs), the main cellular component of the aortic valve, and exert an important control over the mineralization process.3 The ectonucleotidase is a group of membrane-bound enzymes, which uses nucleotides as a substrate and produces different nucleotides and nucleosides.4 Therefore, the ecto-nucleotidases have an important role in controlling purinergic signalling, which, in turn, may impact on the mineralization of VICs.

Cote et al.3 previously showed that single-nucleotide polymorphisms for the gene encoding ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) were associated with CAVD and a higher level of NPP1 in mineralized aortic valves. NPP1 uses adenosine triphosphate (ATP) as a substrate, and produces pyrophosphate (Pi) and adenosine monophosphate (AMP). Pi is a strong inhibitor of mineralization and NPP1 knockout mice develop ectopic mineralization of tendons.5 On the other hand, overexpression of NPP1 in VICs and chondrocytes...
increases mineralization substantially.\textsuperscript{3,6,7} The pro-mineralizing effect of NPP1 is possibly explained by different mechanisms. NPP1 generates Pi, which can be metabolized by alkaline phosphatase (ALP) into phosphate (Pi), which is a strong promoter of mineralization.\textsuperscript{8,9} Also, it is possible that, by producing AMP, NPP1 modulates the signaling through adenosine receptors.\textsuperscript{10} To this effect, 5′-nucleotidase (CD73) is using AMP as a substrate to produce adenosine, which could exert a control over the mineralization of the aortic valve. In this work, we hypothesized that the activity of NPP1 could be coupled with 5′-nucleotidase to produce adenosine. In turn, adenosine could act upon adenosine receptors (P1) and modulates the cAMP/PKA pathway, which has been shown to play an important role during the mineralization of the aortic valve.\textsuperscript{11} As such, the co-expression of NPP1 and 5′-nucleotidase in CAVD could exert a crucial control on adenosine receptors. In atherosclerosis, adenosine receptors have been shown to play an important role.\textsuperscript{12} However, the function of adenosine receptors in CAVD is still unknown.

2. Methods

2.1 Procurement of tissues for analyses
We examined stenotic aortic valves (n = 55) and sclerotic aortic valves (n = 18) that were explanted from patients during surgeries. Control non-calciﬁed aortic valves (n = 24) with normal echocardiographic analyses were obtained during heart transplant procedures. Patients with a history of rheumatic disease, endocarditis, and inﬂammatory diseases were excluded. Valves with moderate to severe aortic valve regurgitation (grade > 2) were excluded. Patients with reduced left ventricular ejection fraction (40%) were excluded. All patients underwent a comprehensive Doppler echocardiographic examination preoperatively. Doppler echocardiographic measurements were performed including the left ventricular stroke volume and transvalvular gradients using the modiﬁed Bernoulli equation. The protocol was approved by the local ethical committee, and informed consent was obtained from the subjects. The study conforms to the Declaration of Helsinki.

2.2 Tissue processing and experiments with isolated VICs
VICs were isolated as previously described\textsuperscript{13} and explained in Supplementary material online, Appendix. VICs were isolated from CAVD tissues (n = 10) and control non-mineralized aortic valves (n = 10). Experiments were performed in triplicate in isolated VICs and n represent the number of experiments performed in different donors. See Methods in Supplementary material online, Appendix.

3. Results

3.1 NPP1 and 5′-nucleotidase mediates the mineralization of VICs induced by ATP
We previously showed that VICs liberate ATP in the extracellular space where it controls the mineralization of cell culture.\textsuperscript{3} The expression of ecto-nucleotidases may modulate this response by metabolizing ATP in different products. We isolated human VICs (HVICs) from non-mineralized aortic valves obtained during heart transplantation and from stenotic mineralized aortic valves obtained from surgery for aortic valve replacement (HVICs were used between passages 3–7). In isolated HVICs, we documented by using confocal microscopy and fluorescent wheat germ agglutinin (WGA), a membrane marker, that NPP1 and 5′-nucleotidase were expressed at the cell membrane of cells (Figure 1A). Quantitative analyses (the number of membrane-associated foci) of IF studies indicated that the membrane expression of NPP1 and 5′-nucleotidase was higher in HVICs isolated from mineralized aortic valves when compared with HVICs obtained from control non-mineralized aortic valves (Figure 1B). To further corroborate this finding, we next measured the level of ATP in the supernatants of HVICs as a measure of ecto-nucleotidase activity. When compared with control HVICs isolated from non-mineralized valves, the extracellular level of ATP was signiﬁcantly reduced in the supernatant of HVICs isolated from stenotic mineralized aortic valves (Figure 1C). We next measured tissue NPP and 5′-nucleotidase activities in 59 aortic valves, both control and stenotic. Bicuspid aortic valves (BAV) and tricuspid aortic valves (TAV) were matched for the histologic remodelling score (score 3 from Warren and Yong\textsuperscript{14} Table 1). We found a signiﬁcant increase in NPP and 5′-nucleotidase activities in both bicuspid and tricuspid mineralized aortic valves when compared with control non-mineralized valves (Figure 1D). There was no difference between bicuspid and tricuspid mineralized aortic valves. Considering the age differences between the groups, we next measured the NPP and 5′-nucleotidase enzyme-speciﬁc activities in 20 patients including human control non-mineralized aortic valves and stenotic aortic valves, which were matched for age and gender (Table 2). In the age-matched groups, NPP activity was increased by 1.9-fold in CAVD when compared with control non-mineralized aortic valves (Figure 1E). Also, 5′-nucleotidase enzyme activity was increased by 1.8-fold in CAVD compared with control valves (Figure 1E). There was a positive and signiﬁcant correlation between NPP and 5′-nucleotidase activities (r = 0.69; P = 0.0006; Figure 1F). We next measured NPP1 and 5′-nucleotidase enzyme activities in sclerotic aortic valves (n = 18; thickened and mildly calciﬁed tissues with remodelling score of 1 or 2 from Warren and Yong\textsuperscript{14}, clinical characteristics are presented in Supplementary material online, Table S1. We found that both NPP1 and 5′-nucleotidase activities were signiﬁcantly elevated in sclerotic aortic valves when compared with control non-mineralized aortic valves, indicating that early during the disease process the ecto-nucleotidase enzymes are overexpressed (see Supplementary material online, Figure S1). By using IF studies, we next showed on tissue sections that ﬂuorescence signals for NPP1 and 5′-nucleotidase were mostly located in the ﬁbrosa–spongiosa layers and were higher in mineralized aortic valves, both stenotic (Figure 1G) and sclerotic (see Supplementary material online, Figure S2), when compared with control valves. In mineralized aortic valves, confocal studies showed that NPP1 and 5′-nucleotidase were co-expressed with vimentin, a marker of VICs\textsuperscript{15} (Figure 1H and see Supplementary material online, Figure S3). Some of the VICs that expressed NPP1 and 5′-nucleotidase in sclerotic valves also co-expressed alpha actin, indicating that VICs are activated with a secretory phenotype\textsuperscript{16} early during the mineralization process of the aortic valve (see Supplementary material online, Figure S4). These ﬁndings suggested that NPP1 and 5′-nucleotidase, which are expressed by HVICs in CAVD tissues, may co-regulate the mineralization of cells. To document whether NPP1 and 5′-nucleotidase have functional relationships with the mineralization process, we investigated the responses of isolated HVICs (from non-mineralized aortic valves) to different interventions. First, we compared the effects of ATP and ATP\textsubscript{γS}, a non-hydrolyzable ATP analogue, on the mineralization of isolated HVICs. We previously reported that the non-hydrolyzable ATP prevented the mineralization of HVICs by acting on P2Y2 receptor.\textsuperscript{3} Cells were treated with the mineralizing medium (phosphate-containing medium) for 7 days and supplemented with ATP or ATP\textsubscript{γS}. We hypothesized that ATP, which could be hydrolyzed...
Figure 1  Levels of NPP1 and 5′-nucleotidase in CAVD. (A) Confocal images of NPP1 and 5′-nucleotidase in cultured HVICs (n = 10 for each condition). In red: WGA, a membrane marker; in green: NPP1 or 5′-nucleotidase. One confocal plane, scale bar 20 μm. (B) Quantification of confocal analyses for membrane expression of NPP1 and 5′-nucleotidase (n = 10 for each condition). (C) Level of ATP in the supernatant of HVIC cultures (n = 3). (D and E) NPP1 and 5′-nucleotidase enzymatic activity measurements in aortic valve tissues in BAV–TAV-matched tissues (n = 59) (D) and age-matched tissues (n = 20) (E). (F) Correlation between NPP1 and 5′-nucleotidase activities (r = 0.69; P = 0.0006). (G) H&E is shown to depict the orientation of tissues (scale bar 250 μm); epifluorescence images of NPP1 and 5′-nucleotidase expressions in control (CTL) and CAVD, pictures were taken with similar acquisition conditions (scale bar 50 μm; representative section of n = 10). (H) Co-distribution of NPP1 and 5′-nucleotidase with vimentin in CAVD tissues. One confocal plane, scale bar 25 μm. Cells that co-express ecto-nucleotidases and vimentin appear as yellow in the merge. Data are mean ± SEM. *P < 0.05 compared with CTL; F: fibrosa; Ao: aortic side; LV: left ventricular side.
by a concerted action of NPP1 and 5'-nucleotidase, would possibly modulate the mineralization process by being metabolized into adenosine. Whereas ATPγS prevented the mineralization of HVIC cultures, ATP increased the mineralization of cell cultures by 2.7-fold, suggesting that it was potentially transformed into metabolites with osteogenic properties (Figure 2A). We next performed siRNAs in HVICs for NPP1 and 5'-nucleotidase, which reduced significantly mRNA and enzyme-specific activities (Figure 2B and C). Silencing of NPP1 and 5'-nucleotidase reduced significantly the mineralization of HVIC cultures induced by ATP and the mineralizing medium (Figure 2D). It should be pointed out that NPP1 produces AMP, which is metabolized into adenosine by 5'-nucleotidase. Hence, it is likely that ATP-mediated mineralization of VICs relied on adenosine receptors.

### 3.2 Role of adenosine in promoting the mineralization of VICs

We next treated HVICs with adenosine and the mineralizing medium. After 7 days, we found that adenosine increased the mineralization of HVIC cultures (Figure 3A). The response to adenosine was similar in HVICs isolated from mineralized aortic valves (n = 4) when compared with HVICs isolated from control non-mineralized valves (n = 4; Figure 3A). All experiments were next carried out with HVICs isolated from control non-mineralized aortic valves. After 6 h, adenosine significantly increased the expression of ALP, osteocalcin, osteonectin, and runx2, a transcription factor involved in osteogenesis (Figure 3B). Adenosine is metabolized by adenosine deaminase into inosine. We thus treated HVICs with erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), an inhibitor of adenosine deaminase, and documented that it significantly increased adenosine-induced mineralization (Figure 3C). Taken together, these data suggested that adenosine exerts a pro-mineralizing effect on VICs. In HVICs, we next used confocal microscopy and fluorescent WGA, a membrane marker, to document the expression of adenosine receptors. We found that HVICs expressed both A1R and A2a adenosine receptor (A2aR), which formed clusters at the cell membrane (Figure 3D). The A2aR is coupled with G\textsubscript{qa},

### Table 1 Clinical characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>Control valves (n = 14)</th>
<th>CAVD TAV (n = 24)</th>
<th>CAVD BAV (n = 21)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>58 ± 1</td>
<td>73 ± 1</td>
<td>65 ± 2</td>
<td>&lt;0.00001</td>
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<tr>
<td>Male (%)</td>
<td>50</td>
<td>63</td>
<td>48</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>0</td>
<td>4</td>
<td>19</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>43</td>
<td>83</td>
<td>52</td>
<td>0.01</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>33</td>
<td>33</td>
<td>24</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>27.7 ± 1.4</td>
<td>28.4 ± 0.9</td>
<td>27.9 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>102.7 ± 10</td>
<td>106.5 ± 3</td>
<td>98.4 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>50</td>
<td>87</td>
<td>67</td>
<td>NS</td>
</tr>
<tr>
<td>ARBs (%)</td>
<td>14</td>
<td>46</td>
<td>14</td>
<td>0.02</td>
</tr>
<tr>
<td>Aortic valve area (cm\textsuperscript{2})</td>
<td>–</td>
<td>0.78 ± 0.04</td>
<td>0.64 ± 0.04</td>
<td>0.008</td>
</tr>
<tr>
<td>Aortic peak gradient (mmHg)</td>
<td>–</td>
<td>61.8 ± 3.4</td>
<td>78.7 ± 5.6</td>
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</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.81 ± 0.43</td>
<td>1.45 ± 0.10</td>
<td>1.44 ± 0.12</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.65 ± 0.39</td>
<td>2.02 ± 0.09</td>
<td>2.47 ± 0.19</td>
<td>0.08</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.13 ± 0.08</td>
<td>1.34 ± 0.07</td>
<td>1.37 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>121.5 ± 15</td>
<td>94.6 ± 6</td>
<td>82.3 ± 4</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Values are denoted as mean ± SEM or %.
BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TAV: tricuspid aortic valves; BAV: bicuspid aortic valves; ARBs: angiotensin II receptor blockers. NS: not significant.

### Table 2 Clinical characteristics of patients for the age-matched group

<table>
<thead>
<tr>
<th></th>
<th>Control valves (n = 10)</th>
<th>CAVD (n = 10)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>58 ± 2</td>
<td>59 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Male (%)</td>
<td>50</td>
<td>50</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>0</td>
<td>30</td>
<td>0.03</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>50</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>40</td>
<td>30</td>
<td>NS</td>
</tr>
<tr>
<td>Bicuspid aortic valves (%)</td>
<td>0</td>
<td>60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>28.8 ± 1.9</td>
<td>28.8 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>105.7 ± 16</td>
<td>100 ± 5.01</td>
<td>NS</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>50</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>ARBs (%)</td>
<td>20</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>Aortic valve area (cm\textsuperscript{2})</td>
<td>–</td>
<td>0.78 ± 0.07</td>
<td>–</td>
</tr>
<tr>
<td>Aortic peak gradient (mmHg)</td>
<td>–</td>
<td>63.3 ± 8</td>
<td>–</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.41 ± 0.06</td>
<td>1.49 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.54 ± 0.44</td>
<td>1.94 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.13 ± 0.06</td>
<td>1.27 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>116.6 ± 19.7</td>
<td>78.8 ± 6.4</td>
<td>0.048</td>
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</tbody>
</table>

Values are denoted as mean ± SEM or %.
BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; ARBs: angiotensin II receptor blockers. NS: not significant.
whereas A1R has been shown to be coupled with Gαi. In HVICs, a silencing of A2aR (Figure 3E) reduced significantly adenosine-induced mineralization (Figure 3F), whereas a silencing for A1R (Figure 3E) exacerbated the mineralization of cell cultures (Figure 3F). We next treated HVIC cultures for 7 days with CV1808, an A2aR agonist. CV1808 increased the mineralization of HVICs (Figure 3G). To further corroborate the role of A2aR in the mineralization of VICs, we next isolated wild-type mouse VICs (MVICs) and documented their response to adenosine. Similar to HVICs, the wild-type MVICs responded to adenosine by increasing the mineral content of cell cultures (Figure 3H). However, in A2aR−/− MVICs, adenosine had the opposite effect and decreased the mineralization process when compared with the phosphate-containing medium (Figure 3H). These data suggested that A2aR has pro-mineralizing activity, whereas A1R promotes the opposite effect. To verify if a Gαs-coupled receptor was involved in the pro-mineralizing activity, we next treated HVICs with the triple mutant dominant-negative Gαs (a3β5/G226A/A366S) vector. In cells treated with a dominant-negative Gαs (a3β5/G226A/A366S) encoding vector, we found that mineralization induced by adenosine was significantly reduced (Figure 3I).

Taken together, these findings suggested that A2aR coupled with Gαs and may stimulate the cAMP pathway by which mineralization can be promoted. Of note, cAMP may promote the activation of two signalling pathways: the protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC) cascades each with their own downstream effectors. To document which pathway may impact on the mineralization process, we first treated VIC cultures with 8-CPT cAMP, a cell permeable cAMP analogue, which increased mineralization of cell culture by 3.1-fold (Figure 4A). In contrast, the use of the EPAC-selective cAMP analogue, 8-pCPT-2′-O-Me-cAMP, did not modify the mineralization of HVICs (Figure 4A). These data indicated that the cAMP/PKA pathway is involved in the mineralization of VICs. In this regard, adenosine-mediated mineralization of HVIC cultures was inhibited by the use of SQ22536, an adenosine cyclase inhibitor, and the PKA inhibitor fragment (6–22) amide (Figure 4B).

3.3 Adenosine-mediated mineralization of VICs is dependent on the cAMP/PKA pathway

A2aR is coupled with Gαs and may stimulate the cAMP pathway by which mineralization can be promoted. Of note, cAMP may promote the activation of two signalling pathways: the protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC) cascades each with their own downstream effectors. To document which pathway may impact on the mineralization process, we first treated VIC cultures with 8-CPT cAMP, a cell permeable cAMP analogue, which increased mineralization of cell culture by 3.1-fold (Figure 4A). In contrast, the use of the EPAC-selective cAMP analogue, 8-pCPT-2′-O-Me-cAMP, did not modify the mineralization of HVICs (Figure 4A). These data indicated that the cAMP/PKA pathway is involved in the mineralization of VICs. In this regard, adenosine-mediated mineralization of HVIC cultures was inhibited by the use of SQ22536, an adenosine cyclase inhibitor, and the PKA inhibitor fragment (6–22) amide (Figure 4B).
Figure 3  Adenosine-induced mineralization relies on A2aR signalling through G\(\alpha_s\). (A) Effect of adenosine (Ado; 30 \(\mu\)M) on the mineralization of HVICs (n = 4). (B) Adenosine-induced expression of osteogenic genes (at 6 h; n = 3). (C) Adenosine-induced mineralization of HVICs with adenosine deaminase inhibitor, EHNA (n = 4). (D) Confocal images of A1R and A2aR in cultured HVICs, in red: WGA, a membrane marker; in green: A1R or A2aR (n = 10). One confocal plane, scale bar 20 \(\mu\)m. (E) Q-PCR analyses showing a reduction of A1R and A2aR mRNA levels in response to siRNA treatments (n = 3). (F) Effects of A1R and A2aR silencing on adenosine-induced mineralization (n = 4). (G) Effect of CV1808, an A2aR agonist, on the mineralization of VICs (n = 6). (H) Adenosine-induced mineralization relies on A2aR expression in MVICs (n = 4). (I) \(\alpha\)3\(\beta\)5 dominant-negative mutant (\(\alpha\)3\(\beta\)5/G226A/E268A/A366S) (pCMV G\(\alpha\)s DN) reduced adenosine-induced mineralization (n = 4). (J) Confocal studies showing A2aR and NPP1 expression in stenotic valves (representative section of n = 10 for each condition). (K) Immunohistochemistry for A1R and A2aR (scale bar 250 \(\mu\)m) and also confocal images of A1R and A2aR in CAVD showing a co-distribution of A2aR with vimentin. Co-expression of A2aR with vimentin appears as yellow in the merge. One confocal plane, scale bar 25 \(\mu\)m. Data are mean ± SEM. *P < 0.05 compared with CTL; #P < 0.05 compared with the mineralizing medium (PO4); ‡P < 0.05 compared with PO4 + Ado; (E) \(\beta\)P < 0.05 compared with siA1R (PO4 + Ado). F: fibrosa; OCA: osteocalcin; ONE: osteonectin; ALP: alkaline phosphatase.
We next measured in response to adenosine and CV1808, the levels of phosphorylated cAMP response element-binding protein (CREB; Ser 133), and phosphorylated activating transcription factor 1 (ATF-1), which are downstream to PKA. Both adenosine and CV1808 promoted the phosphorylation of CREB (Ser133) and ATF-1 (Ser63; Figure 4C). The phosphorylation was maximal at 10 min and started to decline progressively at 30 min after treatment with adenosine/CV1808 (Figure 4C). In A2aR<sup>−/−</sup> MVICs, a treatment with adenosine did not increase the phosphorylation of CREB and ATF-1 (Figure 4D). Also, in HVICs treated with the dominant-negative G<sub>as</sub> (α3β5/G226A/E268A/A366S), adenosine-induced phosphorylation of CREB (Ser133) and ATF-1 (Ser63) were decreased (Figure 4E). Hence, these data indicate that adenosine signals in VICs through A2aR coupled with G<sub>as</sub>, which promotes the activation of the cAMP/PKA pathway.
3.4 CREB activates NPP1 promoter activity in a positive feedback loop

Considering that the cAMP/PKA pathway mediated the mineralization induced by adenosine, we hypothesized that this pathway may control the expression of ecto-nucleotidase in a positive feedback loop. To test this hypothesis, we next treated HVICs with different agonists of the cAMP pathway and we measured the NPP1 promoter activity in luciferase assay. After 6 h of treatment, forskolin and the cAMP analogue, 8-CPT-cAMP, increased significantly the NPP1 promoter activity, whereas the EPAC-selective cAMP analogue, 8-pCPT-2′-O-Me-cAMP, did not modify the promoter activity (Figure 5A). Also, a treatment of HVICs with forskolin, which stimulates adenylate cyclase, increased the mRNA expression of NPP1 measured at 24 h (Figure 5B). Hence, the cAMP/PKA pathway activated the transcription of NPP1. We next evaluated the role of adenosine on NPP1 expression. In promoter luciferase assay, adenosine increased NPP1 promoter activity significantly at 6 h (Figure 5C). Adenosine increased the level of mRNA encoding for NPP1, whereas the PKA inhibitor fragment (6–22) amide prevented this rise (measured at 24 h; Figure 5D). These data thus suggested that A2aR promoted the mineralization of HVICs in a positive feedback loop with NPP1. To test this hypothesis, we treated HVICs with the A2aR agonist CV1808 and documented that ARL67156, an ecto-nucleotidase inhibitor, prevented the mineralization of cell cultures (Figure 5E). We next silenced NPP1 in adenosine-treated HVICs and measured the mineralization of cell cultures. In this experiment, a siRNA targeting NPP1 prevented adenosine-induced mineralization of HVIC cultures (Figure 5F). Hence, these data confirmed that adenosine promoted the expression of NPP1 by HVICs in a positive feedback loop. By using the transcription factor SEARCH software,17 we documented putative CREB and ATF-1 response sequences within the NPP1 promoter region (Figure 6A). By using chromatin immunoprecipitation (ChIP) assay, we next assessed whether CREB or ATF-1 could bind to the promoter region of NPP1 by using site-specific PCR. In this experiment, we stimulated VICs with forskolin for 6 h and then proceeded with the ChIP. Immunoprecipitation of ATF-1 did not yield positive PCR amplification. However, immunoprecipitation of CREB was followed by a positive amplification by PCR at 2853–2520 Kb upstream from the translation initiation site of NPP1, indicating that CREB physically interacted with the promoter region of NPP1 (Figure 6B). We next transfected a dominant mutant-active vector encoding for CREB, CREBDIEDML, in HEK293T cells, and we documented the activity of NPP1 promoter in luciferase assay. The transfection of a dominant-active CREBDIEDML vector increased the activity of NPP1 promoter by...
6.5-fold (Figure 6C). Hence, these data indicate that CREB physically interacts with the promoter region of NPP1 and is a positive regulator of its function.

4. Discussion

This work identified that (i) NPP1 and 5′-nucleotidase are coupled in promoting the mineralization of VICs through the production of adenosine; (ii) adenosine is promoting the mineralization of VIC cultures through A2aR coupled with Gs and a cAMP/PKA/CREB pathway; and (iii) CREB physically interacts with the NPP1 promoter and positively regulates its function in a positive feedback loop (Figure 6D). Hence, this study showed that the regulation of nucleotide/nucleoside levels exerted by ecto-nucleotidases is one important mechanism that controls ectopic aortic valve mineralization.

4.1 NPP1 and 5′-nucleotidase work in tandem in VICs to promote mineralization

NPP1 is highly expressed in CAVD. In this work, we showed that expression of NPP1 in mineralized aortic valves was related to the co-expression of 5′-nucleotidase. As such, there is an efficient process whereby ATP, which is secreted by VICs, is transformed into adenosine. By using different mechanisms, such as exocytosis and channel-mediated transport, ATP is released by vascular and valvular cells. In isolated VICs, Osman et al. showed that ATP promoted the expression of ALP. Similarly, we found that ATP but not ATPγS promoted the mineralization of HVICs. Taken together, these findings suggested that ATP is metabolized into nucleosides with pro-mineralizing activity. To this effect, NPP1 generates AMP, which is next hydrolyzed to adenosine by 5′-nucleotidase. It is worth to highlight that expression of NPP1 is crucial in the regulation of ectopic mineralization. Mice with a knockout for NPP1 develop mineralization of soft tissues, which is largely a...
consequence of the absence or the near-absence of PPI, a crucial and important inhibitor of mineralization. A recent report showed that APOE-/-/NPP1-/- and APOE-/-/NPP1 +/- mice develop smaller atherosclerotic lesions, whereas the heterozygous ApoE-/-/NPP1 +/- mice have a similar level of vascular mineralization when compared with ApoE-/-/ mice. These data suggest that NPP1 promotes atherosclerosis, and that a certain amount of NPP1 is required to prevent excessive mineralization. On the other hand, the overexpression of NPP1 in VICs promotes the mineralization of cell cultures. NPP1 and 5'-nucleotidase are overexpressed in mineralized stenotic aortic valves. During the mineralization of VICs, NPP1 produces AMP, which is the substrate for 5'-nucleotidase. Therefore, in conditions where NPP1 and 5'-nucleotidase are co-expressed and up-regulated, it is likely that adenosine will be produced and therefore will modulate the function of adenosine receptors. We found that HVICs expressed a high level of NPP1 and 5'-nucleotidase. Accordingly, a recent report has also documented that porcine VICs express 5'-nucleotidase. Transferring growth factor-β1, a growth factor suspected to play a role in CAVD, is also a positive regulator of NPP1. The Wnt signalling pathway, which is activated during CAVD, regulates the expression of 5'-nucleotidase. Moreover, in bone cells, 5'-nucleotidase-derived adenosine promotes the differentiation of osteoblast. In the present work, we found that VICs expressed a high level of 5'-nucleotidase; furthermore, we documented that adenosine promoted the mineralization of cell cultures and the expression of osteoblastic genes. The mechanisms that promote a high expression of ecto-nucleotidases in CAVD remain to be fully elucidated, including cross-talk between pathways and epigenetic factors, but our work clearly points towards a positive feedback loop mechanism between adenosine, A2aR, and NPP1 as well as the involvement of the cAMP/CREB pathway.

4.2 Adenosine promotes the mineralization of VICs through A2a receptor

The A1R has been described to be coupled with Go, whereas A2aR has been shown to mediate its effect through Gαs. Hence, it is likely that the differential expression of adenosine receptors among different tissues will determine the overall response to adenosine. In this regard, it should be pointed out that Gαs activates the cAMP pathway, whereas Gαi is known to antagonize the cAMP pathway. It then follows that, depending on the relative proportion of receptors, the overall response to adenosine may vary greatly. We determined that adenosine was a strong promoter of mineralization in VIC cultures. Moreover, we documented that silencing of A2aR reduced significantly adenosine-induced mineralization of VICs, whereas silencing of A1R increased the mineralization of VIC cultures. These findings were substantiated in MVICs A2a-/- receptor, which showed a decreased content of minerals following exposure to adenosine. Hence, it suggests that A2a receptor promotes a strong pro-mineralizing response, whereas A1R has the opposite effect. In rare isolated familial cases of lower-extremity vascular mineralization, a mutation of 5'-nucleotidase has been described. In these patients, a defective 5'-nucleotidase promoted the mineralization of isolated fibroblasts, which was partially rescued by adenosine. Though not investigated, it is possible that lower limb blood vessels express a higher level of adenosine receptors coupled with Gαs such as A1R, which could prevent mineralization in this vascular bed. In the present work, by using a dominant-negative approach, we documented that adenosine-mediated mineralization of VICs relied on Gαs-coupled receptor. Taken together, these findings indicate that A2aR coupled with Go, mediates the mineralization of VICs induced by adenosine. This view was reinforced by the use of CV1808, an A2aR agonist, which also increased the mineralization of cell cultures. In the liver, the A2aR antagonists have been shown to prevent fibrosis. On the other hand, the A2aR has been shown to mediate a pro-inflammatory effect in different models. However, when compared with ApoE-/-/ mice, the ApoE-/-/ A2aR-/-/ mice develop less atherosclerosis. The aortic valve phenotype in ApoE-/-/ A2aR-/-/ mice is, to our knowledge, not yet described. Also, A2aR deficiency has been shown to protect against ischaemic brain injury. In the present work, through several lines of evidence, we showed that A2aR promotes the mineralization of the aortic valve through a cAMP/PKA pathway.

4.3 cAMP/PKA/CREB mediates the mineralization of VICs in a positive feedback loop with NPP1

The mineralization of vascular smooth cells has been shown to be dependent on the cAMP/PKA pathway. Recently, Mahmut et al. showed in HVICs that lipid-mediated mineralization of cells relied on the cAMP/PKA pathway and NPP1. In addition, when compared with the non-mineralized aortic valve, the content of cAMP is increased in CAVD tissues, which emphasizes that the cAMP pathway is activated in CAVD. We showed that adenosine promoted the activation of CREB downstream of PKA. Also, we found that stimulation of the cAMP/PKA cascade increased the mineralization of cell cultures. Moreover, we documented that the cAMP/PKA pathway activates the promoter of NPP1. By using ChIP assay, we next documented that CREB physically interacted with the NPP1 promoter. These findings thus indicate that adenosine activates the cAMP/PKA/CREB cascade, which increases the expression of NPP1 in a positive feedback loop.

4.4 Clinical implications

This work has potential important clinical implications. VICs represent a heterogeneous population including a subpopulation with a strong osteogenic potential. It is believed that VICs undergo an osteogenic programme during CAVD, whereby the mineralization of valvular tissue is promoted. In this study, VICs were grown on a rigid polystyrene support, which has been shown to mimic the biomechanical properties found in sclerotic valves. Thus, the present findings suggest that modulation of ecto-nucleotidases/purinergic receptor may affect the biology of VICs early during the course of CAVD. In the same line, we also found an elevated expression and enzyme activities of NPP1 and 5'-nucleotidase in sclerotic aortic valves. Moreover, we found that the overexpression of NPP1/5'-nucleotidase was elevated to similar extent in mineralized tricuspid and bicuspid aortic valves. Taken together, these findings strongly suggest that NPP1/5'-nucleotidase is induced early during the disease process and may participate in the mineralization of both tricuspid and bicuspid aortic valves. There is, so far, no medical treatment for CAVD that could stop or prevent the mineralization of the aortic valve. Thus, the identification of pathways that play a key role in the mineralization of the aortic valve is of prime importance to develop novel therapeutic strategies. We previously showed in a rat model that mineralization of the aortic valve is prevented by the use of ecto-nucleotidases inhibitor. However, for the time being there is no potent and selective inhibitor of ecto-nucleotidases. The development of such inhibitor would represent a breakthrough in order to
probe the role of ecto-nucleotidases in different disorders. In addition, this study suggests that blocking the A2aR might provide benefit in preventing the mineralization of the aortic valve.

4.5 Limitations
In this study, the role of NPP1 and 5′-nucleotidase as well as adenosine receptors was investigated in vitro. Further work is necessary to translate these findings in vivo. However, it is worth to point out that we also documented in human CAVD tissues that NPP1 and 5′-nucleotidase were overexpressed by VICs. Their strong expression by VICs in human mineralized aortic valves gives support to the in vitro data, which indicate a pro-mineralizing role for NPP1/5′-nucleotidase and A2aR.

5. Conclusion
This work underscores the importance of ecto-nucleotidases and adenosine during the development of CAVD. Furthermore, we identified that mineralization induced by adenosine relied on a cAMP/PKA/CREB pathway, which increased the expression of ecto-nucleotidases. Hence, this work exemplified the notion that ecto-nucleotidases/purinergic receptors play an important role in controlling ectopic aortic valve mineralization. Further investigation of this pathway in animal models of CAVD is necessary and may help the development of novel therapeutic avenues.

Authors’ contributions
A.M., M.-C.B., and P.M. conceived and designed experiments. P.M. wrote the manuscript. A.M. and M.-C.B. performed IF and confocal studies. F.H., R.B., and A.M. performed q-PCR analyses. A.M. and M.C.B. performed in vitro experiments including enzyme activities and transfection experiments with the different vectors.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: P.M. has patent applications for the use of ecto-nucleotidase inhibitors and purinergic agonists in the treatment of CAVD.

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