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► **To cite this version:**

Hawraa Issa, Lucie Hénaut, Jeanne Bou Abdallah, Cédric Boudot, Gaëlle Lenglet, et al.. Activation of the calcium-sensing receptor in human valvular interstitial cells promotes calcification. *Journal of Molecular and Cellular Cardiology*, 2019, 129, pp.2 - 12. 10.1016/j.yjmcc.2019.01.021 . hal-03485693

HAL Id: hal-03485693

<https://hal.science/hal-03485693v1>

Submitted on 20 Dec 2021

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1 **Activation of the calcium-sensing receptor in human**
2 **valvular interstitial cells promotes calcification**

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16
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26

27 Word count: 6596 (without references)

28

29 **Total number of figures and tables: 5**

30

31 **Glossary:**

32 α SMA: alpha-smooth muscle actin

33 Bmp2: bone morphogenetic protein 2

34 CAVD: calcific aortic valve disease

35 CaSR: calcium-sensing receptor

36 HVIC: human aortic valve interstitial cell

37 Opn: osteopontin

38 Osx: osterix

39 Runx2: runt-related transcription factor 2

40 VSMC: vascular smooth muscle cell

41

42

43 **Highlights:**

- 44 • Human valvular interstitial cells (hVICs) express the calcium-sensing receptor
45 (CaSR)
- 46 • CaSR activation promoted the mineralization and osteogenic transition of
47 hVICs.
- 48 • CaSR activation reduced the expression of the calcification inhibitor
49 osteopontin
- 50 • In hVICs, the CaSR might be a key promoter of calcific aortic valve disease
51 progression.

52

53

54 **Abstract**

55 **Introduction and aims.** Calcific aortic valve disease (CAVD) is the most common
56 heart valve disease in western countries. It has been reported that activation of the
57 calcium-sensing receptor (CaSR) expressed by vascular smooth muscle cells
58 prevents vascular calcification. However, to date, the CaSR's expression and
59 function in cardiac valves have not been studied. The present study sought to
60 evaluate the presence of the CaSR within human valvular interstitial cells (hVICs),
61 assess the CaSR's functionality, and ascertain its involvement in hVIC calcification.

62 **Methods and results.** Data from Western blot, flow cytometry and
63 immunocytochemistry experiments demonstrated that primary hVICs express the
64 CaSR. The receptor was functional, since the incubation of hVICs with the
65 calcimimetic R-568 significantly increased Ca²⁺-induced ERK1/2 phosphorylation,
66 and exposure to the calcilytic NPS2143 reduced ERK1/2 activation. A reduction in
67 endogenous CaSR expression by hVICs (using siRNA) was associated with
68 significantly lower levels of Ca²⁺-induced mineralization (quantified using Alizarin Red
69 staining). Similar data were obtained after the pharmacological inhibition of CaSR
70 activity by the calcilytic NPS2143. In contrast, overexpression of a functional CaSR
71 amplified Ca²⁺-induced calcification. Pharmacological activation of the CaSR with the
72 calcimimetic R-568 showed similar effects. CaSR's procalcific properties are
73 associated with increased osteogenic transition (as characterized by elevated mRNA
74 expression of bone morphogenetic protein 2 and osterix), and reduced the
75 expression of the calcification inhibitor osteopontin. Histological analysis of 12 human
76 aortic tricuspid valves showed that CaSR expression was greater in calcified areas
77 than in non-calcified areas. These data were confirmed by Western blots.

78 **Conclusions.** To the best of our knowledge, this study is the first to have
79 demonstrated that hVICs express a functional CaSR. Taken as a whole, our data
80 suggest that activation of the CaSR expressed by hVICs might be a key promoter of
81 CAVD progression.

82

83 **Keywords:** calcium-sensing receptor, cardiac valve calcification, valvular interstitial
84 cell.

85

86

87

88 **1. Introduction**

89 Heart valve disease is a major public health problem in industrialized countries [1].
90 Indeed, calcific aortic valve disease (CAVD), which affects a relatively high proportion
91 of older adults, is the most common heart valve disease in the western world [2,3].
92 This disease shares a number of risk factors with vascular atherosclerosis, and the
93 very high associated hospitalization rates have prompted the description of CAVD as
94 the “next cardiac epidemic” [4]. Calcific aortic valve disease consists primarily of
95 aortic valve sclerosis, which progresses to end-stage calcific aortic stenosis and is
96 accompanied by the progressive thickening and calcification of the valve leaflets [5].
97 Systemic cardiovascular effects may then occur, since the progressive leaflet fibrosis
98 and calcification interfere with valve cusp opening, and thus impede left ventricular
99 outflow [6]. Potential CAVD promoters include male sex, smoking, high body mass
100 index, hypertension, elevated blood lipids, a bicuspid aortic valve, type II diabetes
101 mellitus, and end-stage renal disease [7]. There are no pharmacologic treatment
102 options at present, and so the only solution is valve replacement [8]. However, valve
103 replacement is risky in elderly adults, and calcification can develop on the
104 bioprosthetic valves - leading to more severe complications. A better understanding
105 of the pathophysiology of CAVD is thus required for the development of novel
106 therapeutic strategies [9] that would slow the disease progression.

107 Calcific aortic valve disease was long considered to be a passive degenerative
108 condition. However, it is now conceptualized as a more complex, actively regulated
109 disease process [10] that results from a phenotypic switch of quiescent valvular
110 interstitial cells to a myofibroblast-like phenotype and then an osteoblast-like
111 phenotype [11]. Heart valves contain a heterogeneous population of valvular
112 endothelial cells and valvular interstitial cells (VICs), which maintain valve

113 homeostasis and leaflet integrity. Valvular interstitial cells (the most abundant cell
114 type in the heart valves) have a key role in CAVD progression. Under physiological
115 conditions, most VICs have a fibroblastic phenotype, produce extracellular matrix
116 proteins, and release the growth factors and cytokines needed to maintain the valve's
117 structure [12]. In calcified aortic valves, however, most of the VICs display a
118 myofibroblast-like phenotype characterized by the expression of high levels of α -
119 smooth muscle actin (α -SMA) and bone morphogenetic protein 2 (Bmp2) [12].
120 Elevated proliferative activity and the overproduction of extracellular matrix by these
121 VICs lead to aortic valve fibrosis and valvular thickening. It is noteworthy that VICs in
122 calcified valves may also display an osteogenic phenotype, with enhanced
123 expression of osteoblast markers like runt-related transcription factor 2 (Runx2),
124 osterix (Osx), Bmp2, and osteopontin (Opn) [12,13]. The osteoblast-like activity of
125 these VICs is thought to be responsible for the mineralization process associated
126 with CAVD [11,14]. However, the exact cellular and molecular pathways of CAVD
127 initiation and progression remain poorly understood.

128 Extracellular calcium (Ca^{2+}) is a key inducer of the calcification process [15] and is
129 also an important messenger in the cardiovascular system - particularly via its effects
130 on the calcium-sensing receptor (CaSR) [16]. The CaSR is a member of the G-
131 protein-coupled receptor family; it was initially cloned in parathyroid gland cells,
132 where the receptor is involved in the regulation of parathyroid hormone synthesis and
133 secretion in response to changes in the extracellular ionized calcium concentration
134 [17]. Within the vasculature, the CaSR is known to be expressed by cardiomyocytes
135 [18,19], endothelial cells [20] and vascular smooth muscle cells (VSMCs) [21–23].
136 Our group recently reported that activation of the CaSRs expressed by VSMCs
137 reduces the development of calcification [24,25]. However, the expression and

138 function of the CaSR in the cardiac valves have not previously been studied. The
139 present study was designed to evaluate the presence of the CaSR within human
140 valvular interstitial cells, (hVICs), assess the receptor's functionality, and to ascertain
141 its involvement in hVIC calcification.

142

143 **2. Materials and Methods**

144

145 **2.1. Culture of hVICs**

146 Human aortic tricuspid valves were collected anonymously from patients with CAVD
147 undergoing valve replacement surgery at Amiens Picardie University Hospital
148 (Amiens, France). **In accordance with French legislation, the patients gave their**
149 **informed consent to participation. The study was approved by the local ethics**
150 **committee (study number: 2009-19).** The hVICs were isolated from non-calcified
151 areas of the valves, and were characterized as described previously [26,27].
152 Dulbecco's Modified Eagle's Medium (DMEM) D6546 (Sigma Aldrich) supplemented
153 with 10% fetal bovine serum (FBS) was used for cell culture. Experiments were
154 performed on cells from passages 2 to 6.

155

156 **2.2. The hVIC mineralization assay**

157 Mineralization of hVICs (10000 cells per well, in 48-well plates) was induced by 14
158 days of exposure to 1%-FBS-supplemented DMEM containing either 5.0 mmol/l
159 calcium (the pro-calcific condition) or 1.8 mmol/l calcium (the non-calcific condition).
160 To evaluate the impact of CaSR activity on calcium-induced mineralization, a **positive**
161 **allosteric modulator of the CaSR** (the calcimimetic R-568; 0.1 $\mu\text{mol.L}$; Sigma-Aldrich,
162 St. Louis, MO) **or a CaSR inhibitor** (the calcilytic NPS2143; 0.1 $\mu\text{mol.L}$; Tocris
163 Biosciences) was added to the culture medium. Half of the mineralization medium
164 was renewed every two days. Mineral deposition was detected and quantified using
165 Alizarin Red staining. Briefly, samples were fixed (with 50% ethanol for 5 min, and
166 the 95% ethanol for 5 min), stained for 5 min with Alizarin Red S (40 mmol/l, pH 4.2),
167 rinsed with 50% ethanol, and photographed. Alizarin Red was then solubilized by

168 incubation in buffer containing 10 mmol/l NaH₂PO₃ and 10% hexadecylpyridinium
169 chloride monohydrate, prior to reading at 570 nm.

170

171 **2.3. Cell viability assay**

172 A methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO)
173 assay was used to evaluate the viability of hVICs in response to high Ca²⁺
174 concentrations and/or CaSR pharmacological modulators. Briefly, hVICs (5000 cells
175 per well in 96-well plates) were cultured for 14 days under non-calcific conditions or
176 pro-calcific conditions in the presence or absence of 0.1 μmol//L of the calcimimetic
177 R-568 or the calcilytic NPS2143. After stimulation, a MTT solution was added to each
178 well (final MTT concentration: 500 μg/ml) and the plates were incubated for 1 h.
179 Formazan crystals formed by living cells were then dissolved in DMSO, and the
180 absorbance was measured at 570 nm.

181

182 **2.4. RNA extraction and real-time PCR**

183 After experimental treatments, mRNA was extracted from hVICs (200000 cells per
184 well in 6-well plates) using an RNeasy® Mini Kit, according to the manufacturer's
185 specifications (Qiagen). For gene expression analysis, reverse transcription was
186 performed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen).
187 Real-time PCR was performed with the Power SYBR® Green PCR Master Mix
188 (Fisher Scientific) and the following primer sequences: for CaSR (F) 5'-
189 AGGAAAGGGATCATTGAGGG-3' and (R) 5'-ATGCAGGAGGTGTGGTTCTC-3'; for
190 Bmp2 (F) 5'-CGGACTGCGGTCTCCTAA-3' and (R) 5'-
191 GGAAGCAGCAACGCTAGAAG-3'; for Runx2 (F) 5'-
192 GGCCCACAAATCTCAGATCGTT-3' and (R) 5'-CACTGGCGCTGCAACAAGAC-3';

193 for Opn (F) 5'-ATCACCTGTGCCATACCAGTTAAAC-3' and (R) 5'-
194 CCACAGCATCTGGGTATTTG-3'; for Osx (F) 5'-CAAGGTGTATGGCAAGGCTTC-3'
195 & (R) 5'-AGCTCATCCGAACGAGTGAAC-3'; for α SMA (F) 5'-
196 CCAGCAGATGTGGATCAGCA-3' & (R) 5'-AAGCATTTGCGGTGGACAAT-3'; the
197 primer sequences for GAPDH were (F) 5'-TCGGAGTCAACGGATTTGG-3' & (R) 5'-
198 GCAACAATATCCACTTTACCAGAGTTAA-3'. Transcript levels were quantified using
199 CFX ManagerTM software (version 3.1, Bio-Rad) and normalized against *GAPDH*
200 expression.

201

202 **2.5. Western blotting**

203 After experimental treatments, hVICs (200000 cells per well, in 6-well plates) were
204 washed with PBS and then homogenized in lysis buffer (25 mM Tris-HCl, 250 mM
205 NaCl, 5 mM EDTA, and 1% SDS, pH 7.4) prepared with phosphatase and protease
206 inhibitor cocktails. The samples were agitated for 30 min and then centrifuged to
207 collect the supernatant. Proteins were assayed using the DCTM Protein Assay Kit
208 (Bio-Rad) and stored at -80°C for later analysis. For the extraction of proteins from
209 valvular tissues, samples were transferred into liquid nitrogen and pulverized with a
210 homogenizer. Protein samples from hVICs and valve tissues were separated using
211 8% SDS-PAGE under reducing conditions. After electrophoresis, samples were
212 transferred to nitrocellulose membranes (reference 10600002, Amersham,). For the
213 Western blots of ERK1/2, samples were blocked with 5% milk prepared in TBS 0.1%
214 Tween for 1 h. For Western blots of CaSR, the blocking solution consisted of 5% milk
215 prepared in PBS 1% Triton X-100. Incubation with rabbit polyclonal anti-
216 phosphorylated ERK (Cell Signaling, Danvers, MA; #9101, 1:1000 dilution), mouse
217 monoclonal anti-CaSR (Novus Biologicals, #NB120-19347, 1:500 dilution) or mouse

218 monoclonal anti- β -actin (Sigma Aldrich, #A1978, 1:5000 dilution) antibodies was
219 performed overnight at 4 °C. A solution of 5% milk prepared in TBS 0.1% Tween was
220 used to dilute the anti-ERK and -actin primary antibodies, while a solution of 0.5%
221 milk prepared in PBS 1% Triton X-100 was used to dilute the anti-CaSR primary
222 antibody. Blots were washed either with TBS/Tween (for ERK1/2 and β -actin) or PBS
223 1% Triton X-100 containing 0.5% milk (for CaSR), prior to incubation with an
224 appropriate horseradish-peroxidase-conjugated secondary antibody (Santa Cruz
225 Biotechnology, #sc-2004, #sc-2005, 1:5000). After washing, the blots were
226 developed with chemiluminescence reagents (Bio-Rad). The blots were viewed using
227 the Chemidoc Imaging System (Bio-Rad), and quantified using Image J software.
228 The expression levels were corrected for minor loading differences by normalization
229 against β -actin.

230

231 **2.6. Transfection with siRNAs**

232 Human VICs were transfected with siRNA diluted in OptiMEM (Gibco, Carlsbad, CA),
233 using siPORTNeoFX (Invitrogen). SiPORTNeoFX was chosen because it allowed to
234 transfect hVICs with siRNAs several times over the calcification process without
235 cytotoxicity. A siRNA targeting the CaSR (Invitrogen) was used at a concentration of
236 30 nM (sense sequence: CCAUUGGAUUCGCUCUGAAAtt / antisense sequence:
237 UUCAGAGCGAAUCCAAUGGtg). Scrambled siRNA (Silencer Select Negative
238 Control #2 siRNA, cat # 4390847, Ambion, Foster City, CA) served as a negative
239 control. For mineralization experiments on transfected hVICs, cells were re-
240 transfected every 4 days during the mineralization test, in order to ensure optimal
241 siRNA activity.

242

243 **2.7. Plasmid transfection**

244 Wild-type CaSR cDNA (described previously) was used for the transient transfection
245 of hVICs [28]. The coding regions of the Wild-type CaSR gene were amplified by
246 PCR and subcloned into the HindIII/XbaI sites of the pcDNA3.1/Zeo(+) vector (V860-
247 20, Invitrogen). Primary hVICs were transiently transfected with pcDNA3.1/Zeo(+)
248 vector containing CaSR-WT cDNA using Lipofectamine 2000 (Invitrogen, #11668-
249 027). **The ratio of DNA (in µg) to Lipofectamine Tm 2000 (in µl) used to prepare the**
250 **complexes was 1:2.** Human VICs transfected with the empty vector (EV) were used
251 as controls. Cells were then routinely treated in DMEM supplemented with 1% FBS
252 and 1% GlutaMax but without penicillin-streptomycin. To evaluate the impact of
253 CaSR activity on the expression of the early markers, the cells were transfected for 3
254 days only in the presence of 1.8 or 5.0 mmol/L Ca²⁺. **Human VICs couldn't handle**
255 **several plasmids transfections with Lipofectamine 2000 without increasing**
256 **cytotoxicity. Therefore, to avoid excessive death during the 14 days of the**
257 **calcification process, hVICs were transfected only once, at the beginning of the**
258 **experiment. The overexpression of the WT CaSR during the first 3 days of the**
259 **calcification process was sufficient to modify hVICs phenotype and to obtain a**
260 **significant difference in terms of calcification after 14 days of culture.**

261

262 **2.8. CaSR flow cytometry**

263 After experimental treatments, hVICs were detached and permeabilized using the
264 Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit (BD Biosciences, 554 715),
265 according to the manufacturer's instructions. Cells were then incubated for 1 h at 4°C
266 with primary antibody [mouse monoclonal anti-CaSR antibody (5C10, ADD), Abcam
267 ab19 347; 1:40 dilution of the supplied solution], washed in PBS containing 0.5%

268 bovine serum albumin (BSA), and labelled using polyclonal goat anti-mouse
269 immunoglobulins/RPE (Dako-Cytomation R0480, 1:50 dilution). Protein expression
270 was assessed by flow cytometry using a FACS Canto II system (BD Biosciences).
271 The results are presented as the percentage of the control condition (1.8 mmol/L
272 Ca^{2+}).

273

274 **2.9. CaSR immunostaining**

275 We measured the expression of CaSR on hVICs and human valve samples. For the
276 histological analysis of human tissue, aortic valve samples were isolated from 12
277 different patients. Non-calcified and calcified areas were separated, included in OCT,
278 and cut into 7 μm sections with a cryostat.

279 To perform CaSR immunostaining, samples were fixed with 2% ice-cold
280 paraformaldehyde for 5 min at room temperature (RT), and permeabilized for 10 min
281 at RT with 0.1% Triton X-100 in PBS containing 1% BSA. Samples were then either
282 quenched in 50 mmol/L NH_4Cl /PBS for an additional 10 min (for CaSR fluorescent
283 detection) or exposed to 0.3% H_2O_2 in PBS 1% BSA for 10 min at RT (to block
284 endogenous peroxidase activity when detecting CaSR with peroxidase reagent).
285 Non-specific antibody binding was blocked by incubation in a blocking solution (1%
286 BSA in PBS) for 30 min at RT. Samples were then incubated overnight at 4°C with
287 the primary anti-CaSR antibody (catalog NB 120-19347, Novus Biotechnology, 1:50
288 dilution) or the control isotype (catalog X0936, Dako, 1:50 dilution) in PBS with 0.5%
289 BSA. Samples were then rinsed 3 times in PBS with 1% BSA, and incubated for 1 h
290 at RT with the secondary antibody (catalog #A11004, Invitrogen, 1:500 dilution for
291 fluorescent detection or catalog #sc-2005, Santa Cruz Biotechnology, Inc., 1:500
292 dilution for peroxidase detection). The coverslips were then thoroughly washed in

293 PBS. For peroxidase detection, samples were incubated with substrate kit for
294 peroxidase (Vector® NovaRED™ SK-4800) at RT until suitable staining developed.
295 Finally, the coverslips were counterstained with hematoxylin solution or Hoechst
296 solution, and mounted on glass microscope slides using VectaMount™ mounting
297 medium (Vector® H-5000) for peroxidase detection or Mowiol solution
298 (Calbiochem®, Mowiol® 4-88) for fluorescent detection). Sections were examined
299 with the Zeiss Axiolmager D2 laser fluorescence microscope (Microvision).

300 **2.10. Alizarin Red staining on tissue sections**

301 Briefly, tissue sections were incubated in Alizarin Red S solution (40 mmol/l, pH 4.2)
302 for 2 min. Excess dye was removed, and the sections were dehydrated in 100%
303 acetone for 1 min and then in acetone-toluene solutions for 1 min. Lastly, sections
304 were cleared for 5 min in toluene and mounted using DPX Mountant for histology
305 (Sigma-Aldrich).

306

307 **2.11. Statistical analysis**

308 The results represent at least three independent experiments (i.e. using cells isolated
309 from three different donors) performed in triplicate. The data were quoted as the
310 mean ± standard error of the mean (SEM). Statistical analysis was performed using
311 GraphPad Prism software (version 6.01, GraphPad Software Inc., San Diego, CA).
312 Differences between two groups were assessed with an unpaired Student's t-test,
313 and differences between three or more groups were assessed using a one-way
314 analysis of variance with Tukey's post-test. The threshold for statistical significance
315 was set to $p < 0.05$.

316

317 **3. Results**

318 **3.1. Functional calcium-sensing receptor is expressed by human VICs**

319 Data from our immunocytochemistry and flow cytometry experiments
320 demonstrated that the CaSR is expressed in primary hVICs isolated from human
321 aortic tricuspid valves (Figure 1.A and B). Western blot analysis confirmed the
322 presence of the CaSR in those cells, with one band at approximately 120 kDa
323 (corresponding to the non-glycosylated form) and two bands at approximately 130-
324 140 kDa and 150-160 kDa (corresponding to the immature and mature glycosylated
325 forms of the CaSR, respectively) (Figure 1.C) [29]. In various cell types (including
326 VSMCs, cardiomyocytes and embryonic kidney cells), stimulation of the CaSR results
327 in activation of the ERK1/2 pathway [19,22,30,31]. We therefore investigated the
328 CaSR's functionality in hVICs and the receptor's involvement in Ca²⁺-induced
329 ERK1/2 phosphorylation. In the hVIC model, exposure to 5 mmol/L of Ca²⁺ was
330 associated with a significant increase in ERK1/2 phosphorylation, which peaked after
331 15 to 30 min (Supplementary Figure 1). Blockade of CaSR activation by the calcilytic
332 NPS2143 decreased Ca²⁺-induced ERK1/2 phosphorylation by around a fifth (Figure
333 1.D). In contrast, stimulation of hVICs with the CaSR co-agonist R-568 significantly
334 increased (by a fifth) Ca²⁺-induced ERK1/2 phosphorylation (Figure 1.E).

335 **3.2. High Ca²⁺ promotes the osteogenic transition and calcification of**
336 **hVICs**

337 Experiments with Alizarin Red S staining showed that in our hVIC model, 14
338 days of exposure to increasing Ca²⁺ concentrations (1.8, 3.0 and 5.0 mmol.L)
339 promoted cell calcification in a concentration-dependent manner (Figure 2.A). To
340 better understand the mechanisms by which high Ca²⁺ induced hVIC calcification, we
341 investigated the medium's impact on the osteoblastic differentiation of hVICs. The

342 results of qRT-PCR assays showed that one day of exposure to 5 mmol/L Ca²⁺
343 induced a significant increase in the expression of the osteogenic markers Bmp2,
344 Runx2 and Opn (Figure 2. B, C. and D), which therefore appear to be early markers
345 of the Ca²⁺-induced osteogenic transition of hVICs. The effect of calcium on Bmp2
346 and Runx2 was significant throughout the 14 days of the calcification process (Figure
347 2. B. and C.), whereas Opn expression levels had returned to much the same values
348 as control cells after 14 days of exposure to Ca²⁺ (Figure 2.D). After 14 days, we also
349 observed a significant increase in levels of the osteogenic marker Osx (Figure 2.E),
350 and a significant decrease in expression of the myofibroblast marker α -SMA (Figure
351 2.F). Since these two transcripts were not modulated after a single day of exposure
352 to high-Ca²⁺, they appear to be late markers of the Ca²⁺-induced osteogenic transition
353 in our hVIC model.

354

355 **3.3. Overexpression of a functional wild-type CaSR amplifies Ca²⁺-** 356 **induced hVIC calcification**

357 To investigate the CaSR's role in Ca²⁺-induced hVIC calcification, cells were
358 transiently transfected with either plasmids containing a full-length wild-type CaSR
359 (CaSR-WT) or empty pcDNATM3.1/Zeo(+) plasmids (Empty Vector (EV)). **The**
360 **transfection efficiency was confirmed by flow cytometry and immunocytochemistry**
361 **after transfecting hVICs with a CaSR-WT-GFP plasmid (Supplementary Figure 2.A**
362 **and B).** Western blot analyses with an anti-CaSR antibody demonstrated that CaSR-
363 WT-transfected hVICs expressed greater levels of CaSR (by 50%) than EV-
364 transfected cells did (Supplementary Figure 2.C). This effect **was confirmed by the**
365 **immunocytochemical analysis of CaSR expression (Supplementary Figure 2.D).**
366 Under pro-calcific conditions, mineral deposition was significantly greater in CaSR-

367 WT-transfected hVICs than in EV-transfected cells (Figure 3.A). The latter effect was
368 associated with a significant increase in the expression of the osteogenic marker
369 Bmp2 and lower expression of Opn - a factor known to inhibit calcification (Figure 3.B
370 and C.).

371

372 **3.4. Downregulation of endogenous CaSR expression decreases hVIC** 373 **calcification**

374 To better characterize the CaSR's involvement in hVIC mineralization, and to
375 discriminate between CaSR-mediated and CaSR-independent Ca²⁺ effects (e.g.
376 those occurring through ion channels), CaSR expression was targeted using a siRNA
377 approach. Three days after transfection, endogenous CaSR expression was 40%
378 lower in hVICs transfected with a siRNA targeting the CaSR (CaSR-siRNA) than in
379 scrambled-transfected cells (Supplementary Figure 3 A. and B). In our model, hVICs
380 were transfected every three days in order to maintain a low level of CaSR
381 expression throughout the mineralization process (Supplementary Figure 3.C, D and
382 E). Alizarin Red staining experiments demonstrated that transfection with the CaSR-
383 siRNA significantly decreased Ca²⁺-induced mineral deposition in hVICs (about 3-
384 fold), relative to scrambled siRNA-transfected cells (Figure 3.D). **A second siRNA**
385 **targeting CaSR expression was used to confirm these data (Supplementary Figure**
386 **4).** This effect was associated with a significant increase in Opn expression in CaSR-
387 siRNA-transfected hVICs, relative to scrambled-transfected cells (Figure 3.F). Under
388 these culture conditions, CaSR downregulation did not modulate Bmp2 expression
389 (Figure 3.E).

390

391 **3.5. Pharmacological modulation of the endogenous CaSR in hVICs**
392 **dramatically impacts high-Ca²⁺-induced calcification.**

393 To confirm the data obtained after up- or down-regulation of the CaSR, we
394 next sought to evaluate whether pharmacological modulation of the CaSR by its co-
395 agonists (calcimimetics) or antagonists (calcilytics) could affect the calcification
396 process. **To this end**, hVICs were incubated for 14 days in pro-calcific condition (5
397 mmol/L) alone or **supplemented with increasing concentrations of either the**
398 **calcimimetic R-568 (0.1, 0.5 and 1 μmol/L) or the calcilytic NPS2143 (0.001, 0.01 or**
399 **0.1 μmol/L). Exposure to the calcimimetic R-568 was associated with a**
400 **concentration-dependent increase in Ca²⁺-induced hVIC mineralization (Figure 4.A).**
401 **It is noteworthy that the lowest concentration of R-568 (0.1 μmol/L) promoted a 3-fold**
402 **increase in Ca²⁺-induced hVIC mineralization. Hence, this concentration was used in**
403 **all subsequent experiments involving R-568. An early decrease in Opn expression**
404 **was observed (using qRT-PCR) one day after exposure to 0.1 μmol/L R-568 in pro-**
405 **calcific condition (Figure 4.C). The expression of the early osteogenic marker Bmp2**
406 **was not modulated by the calcimimetic under these culture conditions (Figure 4.B).**
407 However, in hVICs exposed to pro-calcific conditions for 14 days, the expression of
408 Osx (identified as a late osteogenic marker in this model) was significantly increased
409 in response to the calcimimetic (Supplementary Figure 5.A).
410 In contrast, exposure to **0.1 μm of the calcilytic NPS2143** induced a significant, 2.5-
411 fold decrease in hVIC mineralization (Figure 4.D). This effect was associated with an
412 early decrease in Bmp2 expression (Figure 4.E). Confirming this observation, Osx
413 levels were significantly lower in hVICs exposed to the calcilytic in pro-calcific
414 conditions for 14 days (Supplementary Figure 5.B). The expression of Opn was not

415 modulated by the calcilytic (Figure 4.F). It should be noted that none of these
416 treatments affected cell viability (Supplementary Figure 6).

417

418 **3.6. CaSR expression is elevated in calcified human aortic tricuspid**
419 **valves.**

420 To better assess the CaSR's role in CAVD, expression of the receptor was
421 measured in 12 human aortic tricuspid valves. A histological analysis demonstrated
422 that in a given patient, CaSR expression was higher in calcified areas of the valve
423 leaflets than in non-calcified areas ("C" and "NC", respectively, in Figure 5.A). This
424 observation was confirmed by a Western blot analysis, which showed a significant
425 more intense CaSR bands in protein samples extracted from calcified aortic tricuspid
426 valves than in samples from non-calcified aortic tricuspid valves (Figure 5.B and C).

427

428 **4. Discussion**

429 Since the presence of the CaSR within the cardiovascular system was
430 demonstrated, several investigators have suggested that local activation of this
431 receptor might modulate the development and progression of cardiovascular disease.
432 In the heart, the CaSR has been reported to be expressed in cardiac fibroblasts and
433 myocytes, where it contributes to normal cardiac function and composition of
434 extracellular matrix [18] . Within the vasculature, the CaSR expressed by endothelial
435 cells participates in vasorelaxation by increasing nitric oxide production [20,32]. It is
436 noteworthy that the CaSR is also expressed in VSMCs [21–23,33]. Indeed, CaSR
437 activation reportedly delay the mineralization process in the latter cells [22,24]. The
438 present study is, we believe, the first to have demonstrated the expression of a
439 functional CaSR in hVICs. We found that the siRNA-induced reduction in
440 endogenous CaSR expression in hVICs was associated with significantly less high-
441 Ca²⁺-induced osteogenic transition and calcification. Similar data were obtained
442 following the pharmacological inhibition of CaSR activity with the calcilytic NPS2143.
443 In contrast, overexpression of a functional CaSR amplified Ca²⁺-induced osteogenic
444 transition and calcification. Again, similar data were obtained by the pharmacological
445 activation of the CaSR with the calcimimetic R-568. Taken as a whole, our data
446 suggest that activation of the CaSR in hVICs may be a key driver of CAVD.

447 In our hVIC model, Bmp2, Runx2, and Opn appeared to be early markers of
448 Ca²⁺-induced cell calcification, while Osx and α -SMA appeared to be late markers.
449 Overexpression of the CaSR-WT in hVICs almost doubled Bmp2 expression
450 (confirming the importance of CaSR activation in the Ca²⁺-induced hVIC osteogenic
451 transition), while the blockade of CaSR activity with the calcilytic was associated with
452 a statistically significant, three-fold decrease in the expression of Bmp2. In support of

453 this observation, CaSR activation with the calcimimetic R-568 significantly increased
454 the expression of the osteoblast-specific transcription factor Osx [34,35], while CaSR
455 inhibition with the calcilytic NPS2143 decreased Osx expression by a factor of two. In
456 our model, neither siRNA targeting of CaSR nor exposure to the calcimimetic R-568
457 modulated Bmp2 levels. It is known that activation of the CaSR by different ligands
458 may stabilize the receptor in unique activation states, allowing the preferential
459 stimulation of different signaling pathways. This phenomenon is common in G-
460 protein-coupled receptors, and is referred to as “ligand-biased signaling” [36]. In this
461 context, our group reported that Ca²⁺-induced CaSR activation promoted osteoclasts
462 apoptosis following phospholipase C activation and NF-κB translocation, whereas
463 strontium-induced CaSR activation promoted osteoclast apoptosis after protein
464 kinase C activation [37]. Orthosteric CaSR modulators (such as Ca²⁺) bind to the
465 CaSR’s orthosteric fixation site, which is located in the extracellular domain. In
466 contrast, the allosteric modulators (like calcimimetics) bind outside the orthosteric site
467 [38]. Their binding changes the CaSR’s three-dimensional conformation, which in
468 turn modulates the receptor’s affinity for orthosteric CaSR modulators. **Interestingly,**
469 **R-568 and NPS2143 are both known to promote biased signaling at the CaSR**
470 **[39,40]. In this context, we** cannot rule out the possibility that binding of the
471 calcimimetic R-568 to the CaSR expressed by hVICs in our study may **have induced**
472 **a ligand-biased signaling by stabilizing** the receptor in a conformation that promoted
473 the osteogenic transition of hVICs through a Bmp2-independent pathway. However,
474 we do not know why Bmp2 was not modulated in CaSR-siRNA transfected cells.

475

476 Osteopontin is best known as an inhibitor of vascular calcification [41,42].
477 Levels of the dephosphorylated, inactive form of Opn are correlated with more severe

478 calcification in patients with CAVD [43]. In the present work, Opn expression was 4-
479 fold lower in response to either CaSR overexpression or CaSR activation by the
480 calcimimetic R-568. This finding was confirmed by the statistically significant doubling
481 of Opn expression upon transfection of hVICs with a siRNA targeting the CaSR.
482 Taken as a whole, these data suggest that CaSR activation may indirectly favor hVIC
483 calcification by inhibiting the release of the calcification inhibitor Opn. **However, the**
484 **exposure of hVICs to the calcilytic NPS2143 in our study did not modulate Opn**
485 **expression. Again, it is possible that NPS2143's binding to the CaSR may have**
486 **induced a ligand-biased signaling by stabilizing the receptor in a conformation that**
487 **inhibited hVIC calcification through an Opn-independent pathway.**

488

489 **As explained above in the Methods section, the hVICs used in this study were**
490 **isolated from the non-calcified pieces of human aortic tricuspid valves that had been**
491 **collected during valve replacement surgery. Since these cells were extracted from a**
492 **generally disease valve, most of them displayed a myofibroblast-like phenotype at**
493 **the start of the cell culture, as demonstrated by their high baseline level of α SMA**
494 **expression (Supplementary Figure 7). This constituted a study limitation, and made it**
495 **impossible for us to assess the CaSR's role in the early transformation of hVICs into**
496 **myofibroblasts. For this reason, we focused on the impact of CaSR activation on the**
497 **hVICs' phenotypic transition from myofibroblast-like cells to osteoblast-like cells.**
498 **Future studies of hVICs isolated from normal/healthy valves will have to address the**
499 **effect of CaSR activation on the transformation of hVICs into myofibroblast-like cells.**

500

501 Most of the *in vitro* and *in vivo* literature data have evidenced CaSR's
502 protective effect against vascular calcification. Indeed, calcimimetic stimulation of the

503 CaSR in VSMCs delayed both Pi- and Ca²⁺-induced mineralization processes in *in*
504 *vitro* studies [22,24,44]. In particular, CaSR activation protects VSMCs from the
505 osteogenic transition and collagen secretion, and can favor synthesis of the matrix
506 Gla protein [24,45]. In animal models, calcimimetics were even able to prevent
507 vascular calcification in the absence of changes in the serum parathyroid hormone
508 level [46]. Given the calcimimetics' systemic effects on the reduction in the Ca x P
509 product and their local inhibition of VSMC mineralization, it was predicted that the
510 clinical use of these compounds would slow the progression of vascular calcification
511 in patients suffering from chronic kidney disease. To address this hypothesis, the
512 ADVANCE study assessed the progression of vascular and cardiac valve
513 calcifications in hemodialysis patients with secondary hyperparathyroidism in
514 response to the calcimimetic cinacalcet-HCl. Although the ADVANCE trial's results
515 tended to show that cinacalcet slowed down the progression of valve calcification
516 [47–49], an unadjusted intention-to-treat analysis of the EVOLVE study data showed
517 that treatment with cinacalcet did not significantly reduce the risk of death or major
518 cardiovascular events in dialyzed patients with moderate-to-severe secondary
519 hyperparathyroidism [50,51]. In the present study, we demonstrated that activation of
520 the CaSR expressed by hVICs directly promotes the calcification process. It is
521 therefore plausible that because cinacalcet regulates the Ca x Pi product by
522 increasing the CaSR's sensitivity to Ca²⁺ in parathyroid tissue, the calcimimetic might
523 have activated the CaSR expressed by hVICs and thus promoted their calcification.

524

525 The behavior of valvular cells has often been compared to that of vascular
526 cells, in view of the anatomic proximity of these two physiological systems [52].
527 However, recent studies have suggested that valvular cells have more complex,

528 unique behaviors than vascular cells [53–56]. In line with this hypothesis, human
529 VSMCs exposed to an osteogenic environment expressed higher levels of
530 osteogenic markers and displayed greater matrix remodeling than hVICs from same
531 patient [52]. These data suggest the existence of cell-mediated differences between
532 vascular and valvular calcification processes, which may account for the opposite
533 calcification responses of hVICs and VSMCs following CaSR activation.

534

535 Previous studies of VSMCs have reported that the development of *in vitro*
536 calcification is associated with a significant decrease in CaSR expression [22,24].
537 Furthermore, CaSR expression is lower in the aortic tissue of patients with chronic
538 kidney disease (a population prone to arterial calcification) than in healthy subjects
539 [23]. Given the local protective effects reported after activation of the CaSR
540 expressed by VSMCs, it was initially suggested that the reduction in CaSR
541 expression in calcified vessels accounted for the development of vascular
542 calcification. In contrast, CaSR expression in the present work was seen to be higher
543 in the calcified areas of aortic valves than in the non-calcified areas. It remains to be
544 seen whether this elevated CaSR expression results from hydroxyapatite deposition
545 or, conversely, represents an early calcification-promoting event.

546

547 **5. Conclusions**

548 The present study is - to the best of our knowledge – the first to have demonstrated
549 that hVICs express a functional CaSR, the activation of which leads to an accelerated
550 Ca²⁺-induced osteogenic transition and subsequent calcification. The inhibition of the
551 calcification inhibitor Opn observed after CaSR activation may account for these pro-
552 calcific properties. In this context, targeting hVICs' CaSR activity with

553 pharmacological inhibitors (such as calcilytics) might be a promising strategy for
554 preventing the development of CAVD in patients with normal renal function.

555

556 **Acknowledgments**

557 We thank the staff at the Plateforme d'imagerie cellulaire et d'analyse des proteines
558 (ICAP, University of Picardie Jules Verne, Amiens, France) for performing the flow
559 cytometry analyses. **We also thank Sabrina Poirot-Leclercq for technical assistance**
560 **during the reviewing process.**

561

562 **Sources of funding**

563 This research was funded by the Fédérations Hospitalo-Universitaire Amiens Caen
564 Rouen. HI received a doctoral fellowship from the Lebanese "*Association de*
565 *Spécialisation et d'Orientation Scientifique*" (ASOS). This work was also funded by
566 the French National Research Agency as part of the French government's
567 "Investissements d'Avenir" program (reference: ANR-16-RHUS-0003_STOP-AS).

568

569 **Disclosure**

570 The authors declare no conflicts of interest regarding the present work.

571

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790

791 **Figure legends**

792 **Figure 1. Expression and functionality of the CaSR in hVICs. (A and B)**
793 Evaluation of CaSR protein expression in hVICs, using immunocytochemistry **(A)** and
794 flow cytometry **(B)**. A control isotype was used as a negative control in both
795 experiments. In the immunocytochemistry results, the scale bars represent 100 μm .
796 **C.** Assessment of CaSR expression by Western blotting. The experiment was
797 performed on protein extracts of hVICs isolated from five different patients. **(D and E)**
798 Western blot assessment of ERK1/2 phosphorylation in hVICs exposed for 15 min to
799 5 mmol/L Ca^{2+} in the presence of either the calcilytic NPS2143 **(D)** or the calcimimetic
800 R-568 **(E)**. The results correspond to four independent experiments. Error bars
801 correspond to the SEM. *** $p < 0.001$ vs. hVICs exposed to 1.8 mmol/L Ca^{2+} . \$ $p < 0.05$
802 vs. hVICs exposed to 5.0 mmol/L Ca^{2+} .

803 **Figure 2. Effects of Ca^{2+} on hVIC calcification and the osteogenic transition. (A)**
804 Evaluation (using Alizarin Red staining) of the impact of Ca^{2+} on the development of
805 hVIC mineralization. Alizarin Red staining was performed after 14 days of culture in
806 medium containing 1% FBS and various Ca^{2+} concentrations (1.8, 3.0, 5.0 mmol/L).
807 The results correspond to five independent experiments performed in triplicate. Scale
808 bars: 500 μm . The error bars correspond to the SEM. *** $p < 0.001$ vs. hVICs exposed
809 to 1.8 mmol/L Ca^{2+} . **(B.-F)** Impact of Ca^{2+} (5 mmol/L) on mRNA expression of Bmp2
810 **(B)**, Runx2 **(C)**, Opn **(D)**, Osx **(E)** and αSMA **(F)**, assessed by qRT-PCR following 1
811 day (D1), 7 days (D7) and 14 days (D14) of treatment. The results correspond to
812 eight independent experiments performed in duplicate. The error bars correspond to
813 the SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. hVICs exposed to 1.8 mmol/L Ca^{2+}
814 on the same day.

815

816 **Figure 3. The genetic modulation of CaSR expression dramatically affects Ca²⁺-**
817 **induced hVIC mineralization and the osteogenic transition. (A-C)** Impact of the
818 overexpression of a functional CaSR (CaSR-WT) on the mineralization and
819 osteogenic transition of hVICs. **(A)** Alizarin Red S staining of hVICs transfected with
820 either EV or a plasmid containing the CaSR-WT and then exposed for 14 days to 1.8
821 or 5.0 mmol/L Ca²⁺ in medium containing 1% FBS. The results correspond to six
822 independent experiments performed in triplicate. Scale bars: 500 μm. The error bars
823 correspond to the SEM. ***p<0.001 vs. hVICs transfected with the same plasmid and
824 exposed to 1.8 mmol/L Ca²⁺. \$\$p<0.01 vs. EV-transfected hVICs exposed to 5.0
825 mmol/L Ca²⁺. **(B and C)** The impact of Ca²⁺ (5 mmol/L) on Bmp2 **(B)** and Opn **(C)**
826 mRNA expression in EV- or CaSR-WT-transfected cells, assessed by qRT-PCR
827 following 3 days (D3) of treatment. The results correspond to four independent
828 experiments performed in duplicate. The error bars correspond to the SEM.
829 ***p<0.001 vs. hVICs transfected with the same plasmid and exposed to 1.8 mmol/L
830 Ca²⁺. \$p<0.05 and \$\$p<0.01 vs. EV-transfected hVICs exposed to 5.0 mmol/L Ca²⁺.
831 **(D-F)** The impact of CaSR downregulation on the mineralization and osteogenic
832 transition of hVICs. **(D)** Alizarin Red S staining performed on hVICs transfected with
833 either a scrambled (scr) siRNA or the CaSR-siRNA and exposed for 14 days to 1.8 or
834 5.0 mmol/L of Ca²⁺ in medium containing 1% FBS. The results correspond to eight
835 independent experiments performed in triplicate. Scale bars: 500 μm. The error bars
836 correspond to the SEM. *p<0.05, ***p<0.001 vs. hVICs transfected with the same
837 **siRNA** and exposed to 1.8 mmol/L Ca²⁺. \$\$\$p<0.001 vs. hVICs transfected with the
838 **scrambled** siRNA and exposed to 5.0 mmol/L Ca²⁺. **(E and F)** Impact of Ca²⁺ (5
839 mmol/L) on Bmp2 **(E)** and Opn **(F)** mRNA expression in scrambled (scr) or CaSR-
840 siRNA transfected cells, assessed by qRT-PCR following 3 days (D3) of treatment.

841 The results correspond to four independent experiments performed in duplicate. The
842 error bars correspond to the SEM. * $p < 0.05$ and *** $p < 0.001$ vs. hVICs transfected with
843 the same siRNA and exposed to 1.8 mmol/L Ca^{2+} . \$ $p < 0.05$ vs. scrambled-
844 transfected hVICs exposed to 5.0 mmol/L Ca^{2+} .

845

846 **Figure 4. Pharmacological modulation of CaSR activity dramatically affects**
847 **Ca^{2+} -induced hVIC mineralization and osteogenic transition. (A-C)** Impact of the
848 calcimimetic R-568 on the mineralization and osteogenic transition of hVICs. **(A)**
849 Alizarin Red S staining performed on hVICs exposed for 14 days to 1.8 or 5.0 mmol/L
850 of Ca^{2+} in the presence or **absence of increasing concentrations of R-568 (0.1, 0.5**
851 **and 1 $\mu\text{mol/L}$)** in medium containing 1% FBS. The results correspond to five
852 independent experiments performed in triplicate. Scale bars: 500 μm . The error bars
853 correspond to the SEM. *** $p < 0.001$ vs. hVICs exposed to 1.8 mmol/L Ca^{2+} . \$ $p < 0.05$
854 **and \$\$\$ $p < 0.01$** vs. hVICs exposed to 5.0 mmol/L Ca^{2+} in the absence of R-568. **(B**
855 **and C)** Impact of Ca^{2+} (5 mmol/L) on Bmp2 **(B)** and Opn **(C)** mRNA expression in
856 cells exposed or not to 0.1 $\mu\text{mol/L}$ of R-568, assessed by qRT-PCR **following 1 day**
857 **(D1) of treatment**. The results correspond to eight independent experiments
858 performed in duplicate. The error bars correspond to the SEM. *** $p < 0.001$ vs. hVICs
859 exposed to 1.8 mmol/L Ca^{2+} . \$\$\$ $p < 0.01$ vs. hVICs exposed to 5.0 mmol/L Ca^{2+} in the
860 absence of R-568. **(D-F)** Impact of the calcilytic NPS2143 on the mineralization and
861 osteogenic transition of hVICs. **(D)** Alizarin Red S staining of hVICs exposed for 14
862 days to 1.8 or 5.0 mmol/L of Ca^{2+} in the presence **or absence of increasing**
863 **concentrations of NPS2143 (0.001, 0.01, and 0.1 $\mu\text{mol/L}$)** in medium containing 1%
864 FBS. The results correspond to nine independent experiments performed in triplicate.
865 Scale bars: 500 μm . The error bars correspond to the SEM. *** $p < 0.001$ vs. hVICs

866 exposed to 1.8 mmol/L Ca²⁺. $$$$p<0.01$ vs. hVICs exposed to 5.0 mmol/L Ca²⁺ in the
867 absence of NPS2143. **(E and F)** Impact of Ca²⁺ (5 mmol/L) on Bmp2 **(E)** and Opn **(F)**
868 mRNA expression in cells exposed or not to 0.1 μmol/L of NPS2143, as assessed
869 using qRT-PCR **after one day (D1)** of treatment. The results correspond to eight
870 independent experiments performed in duplicate. The error bars correspond to the
871 SEM. $***p<0.001$ vs. hVICs exposed to 1.8 mmol/L Ca²⁺. $$$$p<0.01$ vs. hVICs
872 exposed to 5.0 mmol/L Ca²⁺ in the absence of NPS2143.

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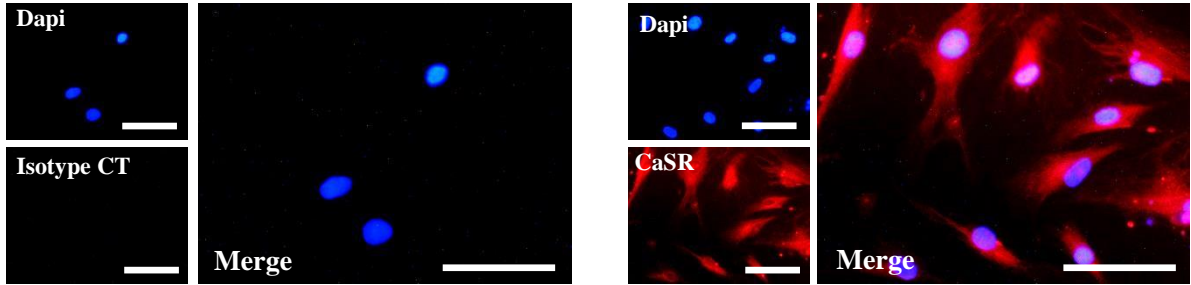
874 **Figure 5. CaSR expression is elevated in calcified human aortic tricuspid**
875 **valves. (A)** Immunohistologic evaluation of CaSR expression in calcified (C) and
876 non-calcified (NC) human aortic tricuspid valves. **Calcification was developed by**
877 **Alizarin Red staining. A control IgG was used to assess the specificity of the CaSR**
878 **antibody. Representative images were obtained from four patients. Scale bars: 200**
879 **μm. (B and C)** Western blot evaluation of CaSR expression within calcified (C) and
880 non-calcified (NC) human aortic tricuspid valves. A representative Western blot of
881 samples from four patients is presented in **(B)**. The Western blot data obtained from
882 calcified and non-calcified pieces of valve from 12 different donors are quantified in
883 **(C)**. The results correspond to 12 independent experiments. The error bars
884 correspond to the SEM. $**p<0.01$ vs. non-calcified pieces of aortic tricuspid valves.

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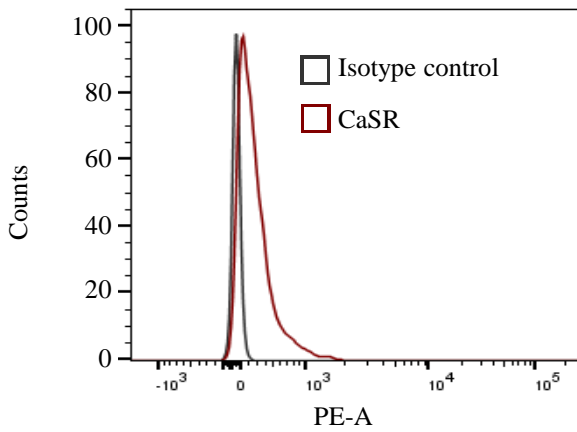
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Figure 1

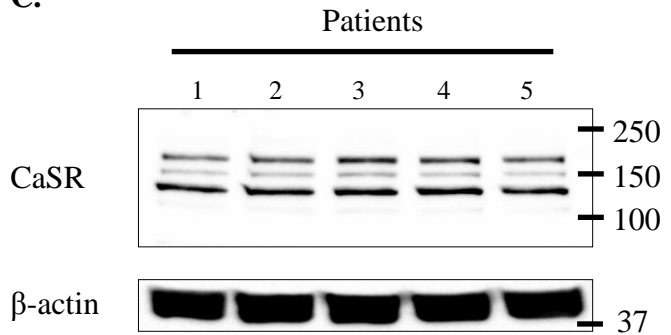
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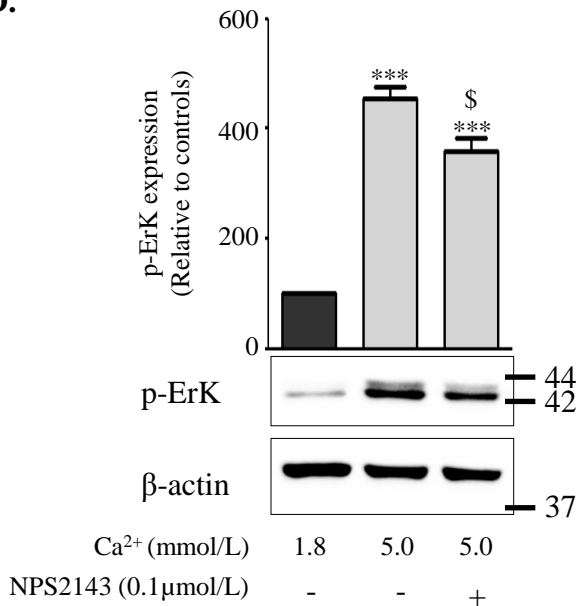
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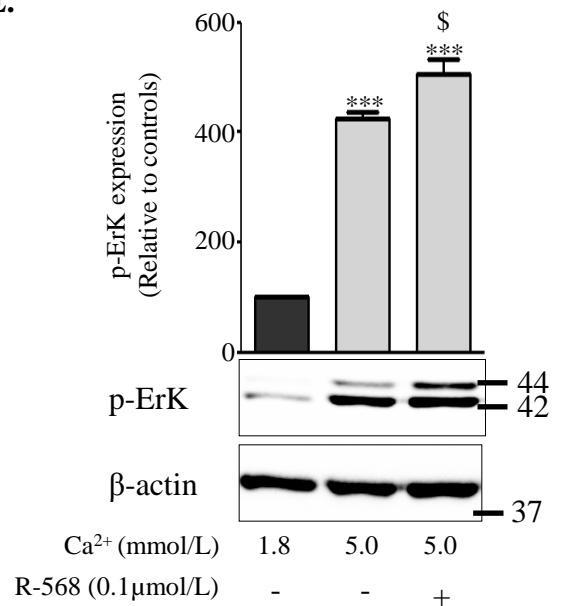


Figure 2

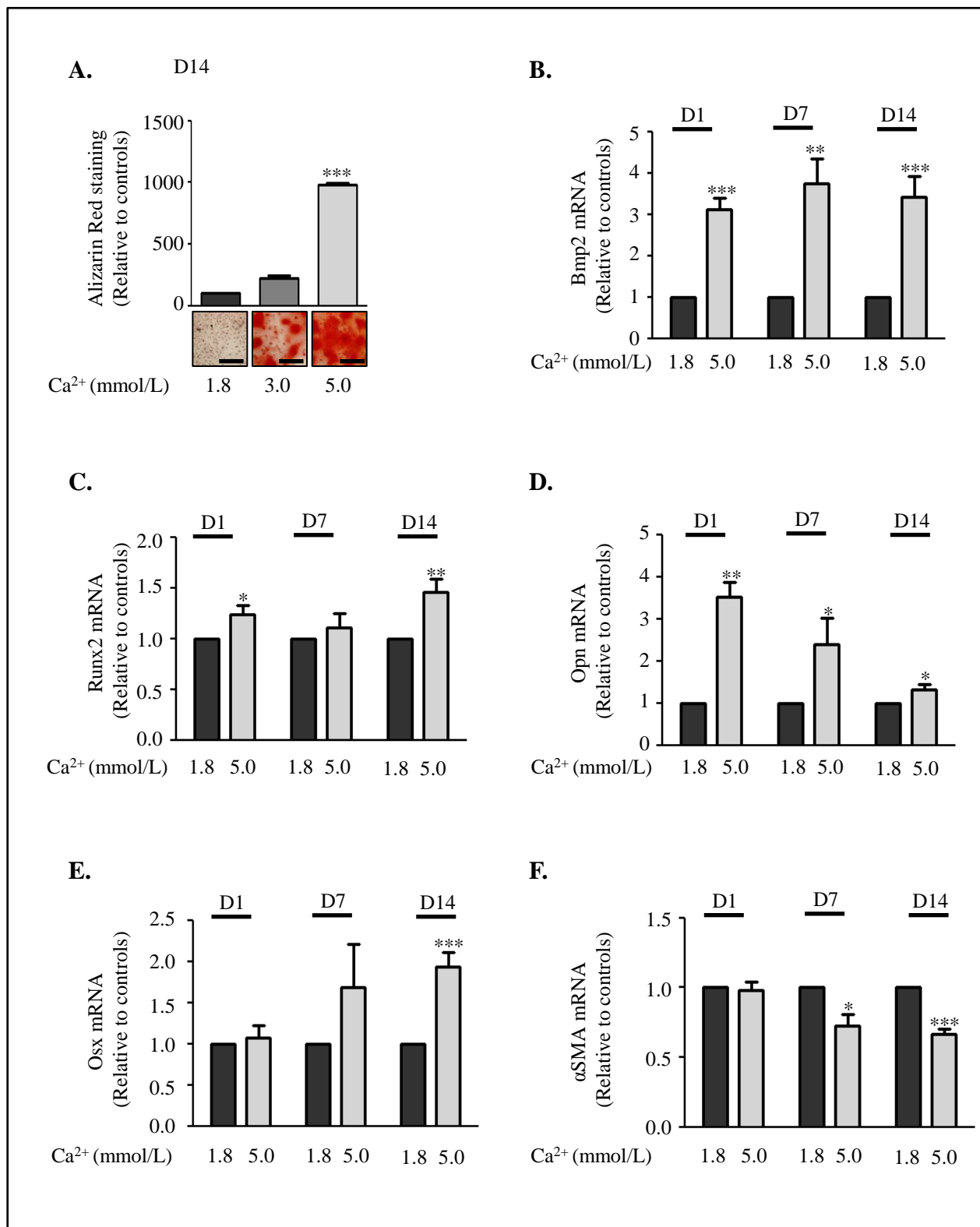


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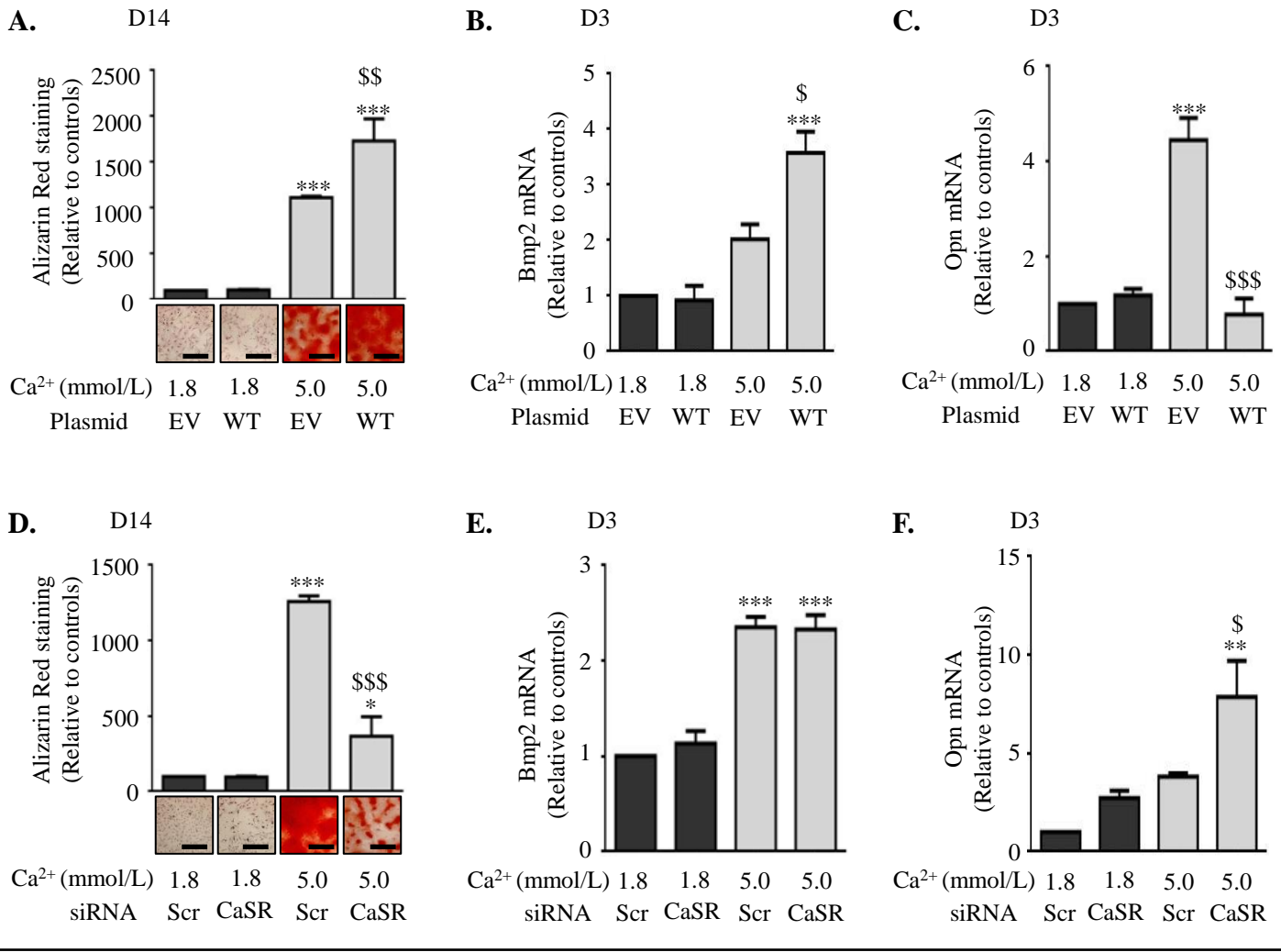


Figure 4

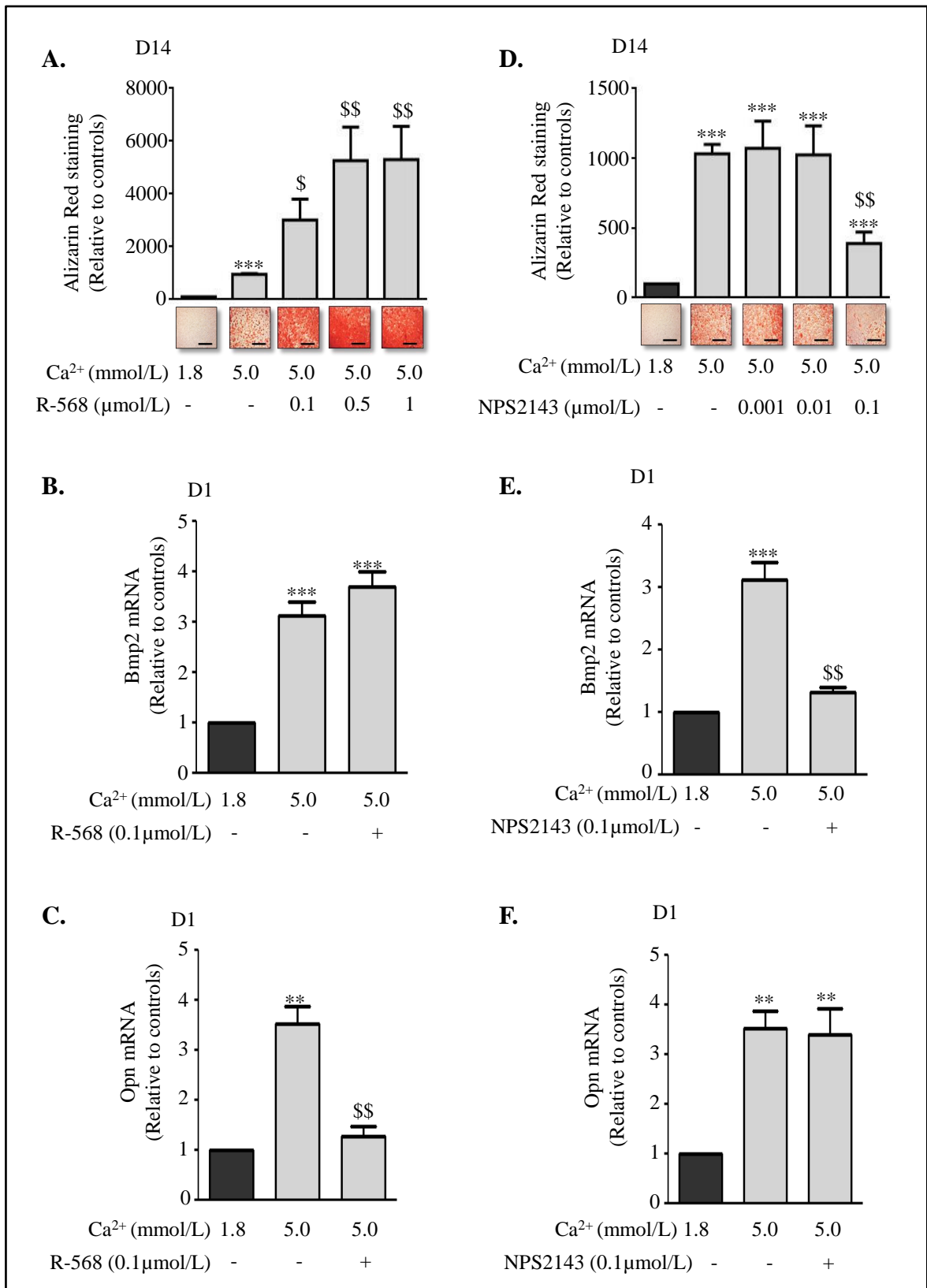
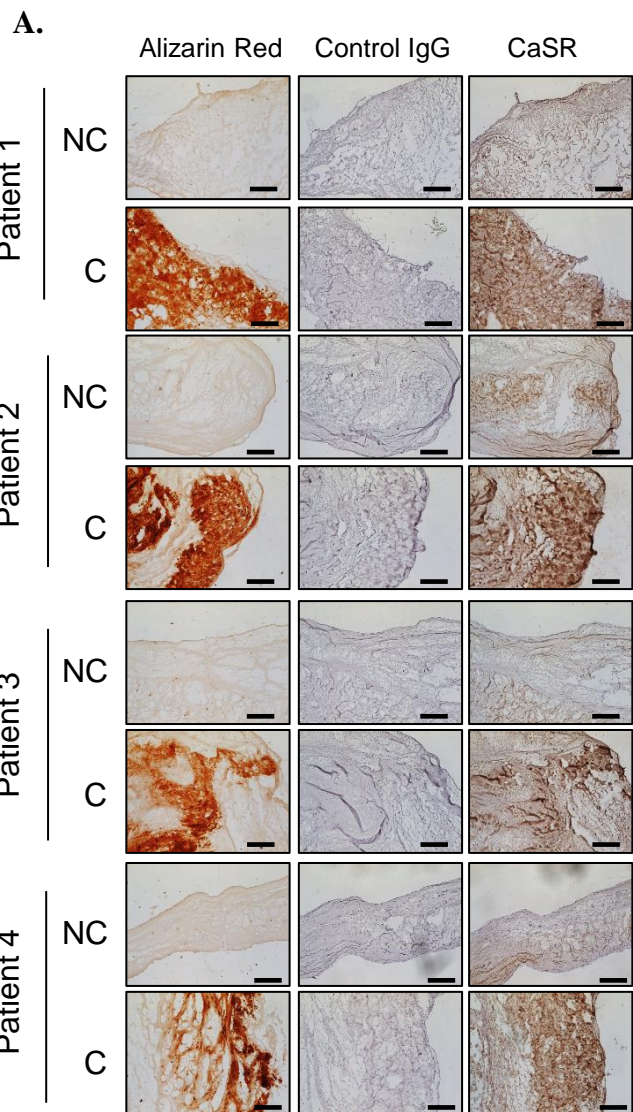
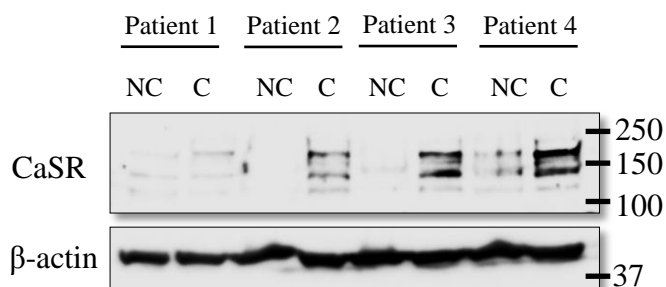


Figure 5



B.



C.

