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Title: A role for matrix metalloproteinase-10 in calcific aortic valve stenosis

Running title: MMP-10 in calcific aortic stenosis

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Abstract

Objective: Aortic valve (AV) calcification plays an important role in the progression of aortic stenosis (AS). Matrix metalloproteinase-10 (MMP-10 or stromelysin-2) is involved in vascular calcification in atherosclerosis. We hypothesize that MMP-10 may play a pathophysiological role in calcific AS.

Approach and Results: Blood samples (n=112 AS and n=349 controls), and AVs (n=88) from patients undergoing valve replacement were analyzed. Circulating MMP-10 was higher in AS patients compared to controls (p<0.001), and correlated with TNF α ($r_s=0.451$, p<0.0001). MMP-10 was detected by immunohistochemistry in AVs from AS patients co-localized with aortic valve interstitial cells (AVICs) markers α -smooth muscle actin and vimentin and with calcification markers Runx2 and Sox-9. MMP-10 expression in AVs was further confirmed by RT-qPCR and western blot. *Ex-vivo*, MMP-10 was elevated in the conditioned media of AVs from AS patients, and associated with IL-1 β ($r_s=0.5045$, p<0.001) and bone morphogenetic protein (BMP)-2 ($r_s=0.5003$, p<0.01). *In vitro*, recombinant human MMP-10 induced the overexpression of inflammatory, fibrotic and osteogenic markers (IL-1 β , α -SMA, vimentin, collagen, BMP-4, Sox9, osteopontin, BMP-9 and Smad 1/5/8, p<0.05) and cell mineralization in AVICs isolated from human AVs, in a mechanism involving Akt phosphorylation. These effects were prevented by TIMP-1, a physiological MMP inhibitor, or specifically by an anti-MMP-10 antibody.

Conclusions: MMP-10, which is overexpressed in AV from AS patients, appears to play a central role in calcification in AS through Akt phosphorylation. MMP-10 could be a new therapeutic target for delaying the progression of AV calcification in AS.

NONSTANDARD ABBREVIATIONS AND ACRONYMS

AV	Aortic valve
AS	Aortic stenosis
MMPs	Metalloproteinases
AVICs	Aortic valve interstitial cells
BMP	Bone morphogenetic protein
CAVD	Calcific aortic valve disease
proMMP-10	Recombinant human pro-metalloproteinase 10
aMMP-10	Active metalloproteinase 10
ODM	Osteogenic differentiation media
MMP-10ab	Antibody against MMP-10
OPG	Osteoprotegerin
TIMP-1	Tissue inhibitor of metalloproteinases type 1
OPN	Osteopontin
ALP	Alkaline phosphatase
Runx2	Runt-related transcription factor 2;
Sox-9	SRY (sex-determining region Y)-box 9
α -SMA	Alpha smooth muscle actin;
Col1a1	Collagen Type I Alpha 1 Chain
IL	Interleukin
Akt	Protein kinase B
NF κ B	Nuclear factor kappa B
ERK1/2	Extracellular signal-regulated kinase 1/2

Introduction

Aortic stenosis (AS) is the most common valvular heart disease and the prevalence increases with age^{1,2}. There are currently no effective medical treatments capable of altering its course, so it is of foremost importance to delineate and understand the key basic underlying mechanisms³. Calcific aortic valve disease (CAVD) is characterized by lipid infiltration, inflammation, neoangiogenesis, calcification and endothelial dysfunction⁴, features shared with atherosclerosis. It has been recently reported the importance of lipoprotein(a) (Lp(a)) and bioactive phospholipids as specific signalling molecules in AS, providing insights in how atherogenic risk factors may affect valve calcification⁵⁻⁷. Although retrospective studies had suggested that statins could delay the hemodynamic progression rate of AS^{8,9}, randomized controlled studies reported that a lipid-lowering strategy neither resulted in lower aortic valve-related events nor in a slower progression rate of stenosis^{10,11}.

Matrix metalloproteinases (MMPs) are matrix-degrading proteases that play pivotal roles in cardiovascular pathophysiology by degrading matrix or nonmatrix substrates. While MMPs have been extensively investigated in vascular pathologies associated to arterial remodeling, such as atherosclerosis, aortic aneurysm and valvular remodeling, little is known about their role in valvular calcification. Several MMPs have been involved in aortic valve (AV) tissue remodelling and MMP-1, MMP-9 and MMP-12 were found to be among the most highly upregulated genes in calcific aortic valve stenosis³. Moreover, MMP activation has been shown to be increased in a murine model of aortic valve disease, associated to valvular inflammation¹².

MMP-10 (stromelysin-2) takes part in physiological processes like bone growth¹³ and wound healing¹⁴, while it has been shown to be overexpressed in various tumors¹⁵, and it has been suggested to be involved in vascular pathology¹⁶. In spite of being undetectable in most healthy tissues, MMP-10 is readily induced in response to injury or inflammatory stimuli¹⁷⁻²⁰. It is present in atherosclerotic lesions at rupture-prone sites and increased serum of recombinant human pro-metalloproteinase 10 (proMMP-10) has been associated with clinical and subclinical atherosclerosis^{17,21,22}. Moreover, we have shown that MMP-10 deficiency is associated with delayed plaque progression and reduced calcification in the atherosclerosis-prone apolipoprotein E-deficient background²³. Similarities with initial stages of atherosclerosis, and common risk factors, led us to hypothesize that MMP-10 may play a pathophysiological role in calcific aortic valve stenosis. More precisely, we aimed to study MMP-10 expression in serum and AVs from AS patients and its association with parameters assessing inflammation and calcification. In addition, we aimed to evaluate the effects of recombinant MMP-10 on inflammation and calcification in aortic valvular interstitial cells (AVICs) isolated from aortic valves of AS patients.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patients

This prospective, observational study included a total of 112 consecutive patients with severe AS (AV area $\leq 1 \text{ cm}^2$ [and/or] transaortic mean pressure gradient $>40 \text{ mm Hg}$), referred to Complejo Hospitalario de Navarra for AV replacement from June 2013 to June 2016. Exclusion criteria were moderate or severe concomitant valvular disease, malignant tumor, and chronic inflammatory diseases. All patients were evaluated by echocardiography. Venous blood was drawn for measurement of brain natriuretic peptide (BNP) and other routine laboratory parameters on admission for surgery. Human AVs were obtained after surgery from a subgroup of 88 patients with severe AS. As controls, non-calcified human AVs were obtained at autopsy (control, $n=11$) and were rapidly cut in 2 pieces. One piece was fixed in 4% formaldehyde and embedded in paraffin, and the other was frozen in liquid nitrogen.

Blood samples were also collected from a control group of 349 subjects free from clinically manifested atherosclerotic vascular disease who attended the outpatient service of the Department of Internal Medicine at the Clínica Universidad de Navarra for a general checkup.

Informed consent was obtained from each patient and control, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a previous approval by the institution's human research committee (Comité Ético de Experimentación Clínica. Gobierno de Navarra, Departamento de Salud; 2015/26; Comité Ético de Experimentación Clínica. Clínica Universidad de Navarra, 047/2010).

Cell culture

AVICs were obtained from 10 AS patients undergoing surgical AV replacement. Each independent AVICs culture was obtained by pooling cells obtained from 2-3 different donors, and used between passages 2-5. AVICs were isolated using sequential collagenase digestion to eliminate endothelial cells, and then interstitial cells were collected by centrifugation. AVICs were cultured in DMEM F-12 medium (Gibco) supplemented with 10% FBS (Gibco), 1% Penicilin/Streptomycin (Lonza) at 37°C and 5% CO_2 in a saturation humidified incubator (Panasonic) until stimulation begins, where FBS was reduced to 1%. Before performing further experiments AVICs were cultured on Lab-Tek II Chamber Slides (Nalge Nunc International) and tested positive by immunohistochemistry for alpha smooth muscle actin (α -SMA, Sigma Aldrich) and vimentin (Santa Cruz Biotechnology), and negative for CD31 (Abcam). Cells were treated with recombinant human pro-matrilysin 10 (proMMP-10; 10^{-9} M) or active matrilysin 10 (aMMP-10; 10^{-9} M) for 6, 12, 24 and 48 hours to assess their effect on AVICs activation, fibrotic, inflammatory and calcification markers, and for 14 days to identify calcium deposition. Human proMMP-10 was produced as previously described and activated by 24 hours incubation with 10 mM CaCl_2 at 37°C ²⁴. Cells were also cultured with osteogenic differentiation media [ODM: DMEM 1X (Thermo Fisher), 20% FBS, 1% Penicilin/Streptomycin, $5 \mu\text{g/ml}$ insulin (Sigma Aldrich); 10 ng/ml FGF-2 (Novus biological), $0.2 \text{ M Na}_2\text{HPO}_4$ (Sigma Aldrich) and $0.2 \text{ M NaH}_2\text{PO}_4$ (Sigma Aldrich)], in the absence or presence of a neutralizing antibody against MMP-10 [10^{-9} M , MMP-10ab (R&D Systems)] or osteoprotegerin [10^{-9} M , OPG (Preprotech)] during 7 days. In other set of

experiments cells were incubated with aMMP-10 and tissue inhibitor of metalloproteinases type 1 [10^{-9} M, TIMP-1 (Preprotech)] for 24 hours.

For the intracellular pathways study, cells were treated with proMMP-10 (10^{-9} M) or aMMP-10 (10^{-9} M) for 5, 10, 15 and 30 minutes. The following chemical inhibitors (10^{-6} M) were added 30 minutes before stimulation with proMMP-10 or aMMP-10: the ERK1/2 inhibitor PD98059 (Sigma Aldrich), the NF κ B inhibitor BAY 11-7082 (Sigma Aldrich) and the Akt inhibitor Wortmannin (Sigma-Aldrich).

Ex vivo experiments

The aortic valves were transversely cut in three pieces of equal size. One of the pieces was weighted and incubated with DMEM F-12 medium supplemented with 10% FBS overnight at 37°C and 5% CO₂, in a saturation humidified incubator. Conditioned media was collected at 24 hours.

Real-time reverse transcription PCR

Total RNA from cells and AVs was extracted with Trizol Reagent (Euromedex) and purified using the RNeasy kit, according to the manufacturer's instructions (Qiagen). First strand cDNA was synthesized according to the manufacturer's instructions (Bio-Rad). Quantitative PCR analysis was then performed with SYBR green PCR technology (Bio-Rad) (Supplemental table I). Relative quantification was achieved with MyiQ (Bio-Rad) software according to the manufacturer's instructions. Data were normalized by HPRT, β -actin and GAPDH levels, and expressed as fold change relative to controls. All PCRs were performed at least in triplicate for each experimental condition.

Western blot analysis

Aliquots of 20 μ g of total proteins were prepared and electrophoresed on SDS polyacrylamide gels (4-15% polyacrylamide, Mini-PROTEAN TGX Stain-Free, BioRad) and transferred to Hybond-C Extra nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were incubated with primary antibodies for: MMP-10 (R&D Systems), α -SMA (Sigma Aldrich), vimentin (Santa Cruz), Runx2 (Sigma Aldrich), Sox-9 (Sigma Aldrich), osteopontin (OPN, Santa Cruz), Periostin (Santa Cruz), bone morphogenetic protein (BMP)-2 (Abcam), BMP-4 (Abcam), BMP-9 (Santa Cruz), Smad 1/5/8 (Sigma Aldrich), phosphorylated (Ser473) and total AKT (Cell Signaling), phosphorylated (Tyr576/577) and total extracellular signal regulated kinases 1/2 (ERK1/2; Cell signaling), phosphorylated (Ser536) and total NF κ B (Cell Signaling); and with secondary antibodies for mouse and rabbit (GE Healthcare). Western blot data were obtained by normalizing bands to total protein in each lane. Peroxidase activity was detected with a chemiluminiscent substrate (ECL, Amersham Biosciences) and images acquired with Chemidoc MP Imaging system (Bio-Rad).

Immunohistological evaluation

Tissue staining was performed on transversal sections of human AV leaflets. All grossly calcified valves were decalcified in 10% formic acid solution (Sigma Aldrich) for 24 hours. Samples were dehydrated, embedded in paraffin, and cut in 5- μ m-thick sections. Slides were treated with H₂O₂ for 10 minutes to block endogenous peroxidase activity. All sections were blocked with 5% normal goat serum in PBS for 1 hour and incubated overnight with MMP-10 (R&D Systems), Vimentin (Santa Cruz), α -SMA (Sigma Aldrich), Runx2 (Sigma Aldrich) and Sox-9 (Sigma Aldrich) and washed 3 times, and then incubated for 30 minutes with the HRP-

labeled polymer conjugated to secondary antibodies (Dako Cytomation). The signal was revealed by using a DAB Substrate Kit (Leica) for MMP-10 and Nuclear Fast Red (Leica) for the other antibodies. Reference samples of all stains before and after decalcification were compared, thereby ensuring that the process of decalcification did not interfere with the staining and hence the result/interpretation of the staining. For the haematoxylin and eosin staining, the slides were hydrated and incubated for 2 h with haematoxylin and eosin staining. After incubation, samples were washed three times and dehydrated. Pictures were taken with a microscope (Leica CMS GmbH).

ELISA

IL-6, Col-1, OPN, BMP-4 and IL-1 β were measured in cell supernatants following the manufacturer's instructions (R&D Systems). MMP-10 was measured in homogenates and conditioned media of aortic valves following manufacturer's instructions (R&D Systems). In serum from AS patients, MMP-10, TNF α and TIMP-1 were quantified following the manufacturer's instructions (R&D Systems).

Alkaline Phosphatase and Alizarin Red Staining

AVICs were cultured in Lab-Tek II Chamber Slides (Nalge Nunc International). After 14 days of treatment with proMMP-10 (10^{-9} M) or aMMP-10 (10^{-9} M), cells were fixed with 4% formaldehyde (pH 7.2) for 20 minutes at RT. Cells were washed 3 times for 5 minutes and incubated with alkaline phosphatase solution [0.6 mg/ml Blue BB salt + Tris 0.1 M, 0.1 mg/ml naphthol, 0.5% N,N-dimethylformamide, 2 mM MgCl₂, pH 8.5 (Sigma Aldrich)] for 30 minutes. After incubation, cells were washed 3 times for 5 minutes.

For alizarin red staining, after formaldehyde fixation, cells were incubated with alizarin red solution [2% alizarin red S, pH 4.1-4.3 (Sigma Aldrich)] for 30 minutes. Cells were then washed 3 times for 5 minutes and images were taken at a Leica DM IL LED microscope.

Statistical analyses

Continuous variables were expressed as mean \pm standard deviation or median (interquartile range/IQR), unless otherwise stated. Data normality was assessed through Shapiro-Wilk's test. Differences in serum MMP-10 concentration between patients and controls were analyzed by ANCOVA. Association between two variables was assessed by Pearson's correlation test (normal distribution) or Spearman's rank correlation test (non-parametric distribution). Differences between two groups were analyzed by Student's t-test when following normal distribution or Mann-Whitney U test in case of non-parametric distribution. When comparing more than two groups, one factor ANOVA with post hoc test was used, in the case of parametric distribution, or Kruskal Wallis test followed by Mann-Whitney test with Bonferroni correction, in the case of non-parametric. The Pearson's χ^2 - test was used to compare frequency distributions of categorical variables. Stata version 12 was used for the analysis, and statistical significance was established as $p < 0.05$.

Results

MMP-10 levels are elevated in AS patients

The baseline characteristics of the controls and the AS cohorts are summarized in Table 1. In keeping with the typical characteristics of AS patients, mean age was 72 ± 9 years and 60% were male. A significant proportion suffered from concomitant coronary artery disease, hypertension, diabetes and hyperlipidemia. Echocardiographic variables were those expected in patients with severe AS. AS patients were treated according to the guidelines.

The mean circulating level of MMP-10 in AS patients was 717 pg/ml, showing a wide IQR (552–1093 pg/ml, $n=112$), and was higher than in control subjects [593 (452–801) pg/ml; $n=349$, $p<0.001$]. This difference was maintained after multivariate analysis, adjusting for age and sex (ANCOVA $p<0.0001$), since the control group was younger and with a greater proportion of men. Particularly, no differences in MMP-10 were found between male [732 pg/ml, (586–1104)] and female [686 pg/ml, (484–1066)] patients. We could not find any significant effect for β blockers, ACE inhibitors, statins, diuretics, arterial hypertension and NYHA on MMP-10 in the univariate analysis. However, we could observe that MMP-10 in AS patients was associated with TNF α ($r_s=0.451$, $p<0.0001$). This association could also be observed when men ($r_s=0.514$, $p<0.0001$) and women ($r_s=0.394$, $p=0.013$) were analyzed separately.

MMP-10 is expressed in aortic valves from AS patients and co-localizes with calcification markers

Locally, immunohistochemical analysis showed that MMP-10 was spontaneously expressed in the AVs, located principally in the spongiosa layer and in calcium deposits (Figure 1A). AS patients presented a higher expression of MMP-10 in valvular tissue at both mRNA (RT-PCR) and protein level (western blot), as compared with controls (Figure 1B). Likewise, there was a 4-fold increase in MMP-10 in AVs conditioned media (Figure 1B, $p<0.05$). MMP-10 double immunostaining in the aortic valves showed its co-localization with vimentin and α -SMA (Figure 1C and D) and two well-known calcification markers: Runx2 and Sox-9 (Figure 1E and F respectively). MMP-10 is shown in brown and the other markers in red. Interestingly, MMP-10 was also found in the extracellular matrix, reinforcing the idea that it is a secreted protein (Figure 1C-F).

Interestingly, MMP-10 concentration in the conditioned media of cultured AS valves was strongly associated with the proinflammatory cytokine IL-1 β ($r_s=0.5045$, $p<0.001$) and the osteogenic factor BMP-2 ($r_s=0.5003$, $p<0.01$), as shown in Figure 1G- H.

MMP-10 effects on inflammatory and fibrotic markers in human AVICs

Since MMP-10 is overexpressed in diseased human aortic valves and associated with inflammatory and calcification markers, the effects of recombinant human MMP-10 either as a proform (proMMP-10) or in its active conformation (aMMP-10) were assessed on human AVICs. Recombinant aMMP-10 increased IL-6 mRNA expression at 12 and 24 hours of treatment (Figure 2A, $p<0.05$), although no increase at protein level was observed (Figure 2B). In contrast, treatment with aMMP-10 increased IL-1 β gene expression and secretion in a time-dependent manner from 6 to 48 hours (Figures 2A-2B, $p<0.05$). Similar results, although less pronounced, were obtained when AVICs were stimulated with pro MMP-10 (Supplemental figure I A-B).

The expression of fibrotic markers was also determined in AVICs before and after aMMP-10 stimulation. As shown in Figure 2C, treatment with aMMP-10 enhanced vimentin mRNA

levels at 6 hours of incubation ($p < 0.05$) without altering α -SMA or Col1a1 mRNA expressions. At the protein level, AVICs treated with aMMP-10 exhibited increased α -SMA and vimentin expressions at 48 and 24 hours respectively and augmented collagen type I secretion into the culture medium at 48 hours (Figure 2D). No effect of proMMP-10 on fibrotic markers was observed (Supplemental figure I C-D).

MMP-10 effects on calcification markers in human AVICs

The effect of MMP-10 on the expression of osteogenic markers was determined in cultured AVICs. The active form induced a significant increase of Sox9, OPN, and BMP-4 at both mRNA and protein levels ($p < 0.05$, Figure 3A-D). MMP-10 induced a 2 fold increase on BMP-2 mRNA levels at 48 h ($p < 0.05$), although no elevation in cell culture supernatant was observed. Secreted protein levels of BMP-9 and Smad 1/5/8 were greatly increased in AVICs 24 and 48 h after aMMP-10 administration ($p < 0.05$, Figure 3E). In stark contrast, proMMP-10 did not induce any changes in the expression of calcification markers both at the mRNA and protein levels (Supplemental figures II A-D).

As shown in Figure 3F, the number of alkaline phosphatase (ALP) positive AVICs was greatly increased after 14 days of incubation in osteogenic medium in the presence of aMMP-10, compared to control cells. Accordingly, aMMP-10 treatment also induced more deposits of mineralized matrix in Alizarin red stained AVICs compared to controls. The effect of proMMP-10 on ALP activity was lower compared to aMMP-10 treated cells (Supplemental Figure II E), and no effect on matrix mineralization was observed (Supplemental Figure II E).

Intracellular pathways involved in MMP-10 effects

The possible intracellular mechanisms by which MMP-10 exerts its pro-inflammatory, pro-fibrotic and pro-osteogenic effects in AVICs were analyzed. Recombinant aMMP-10 induced phosphorylation of Akt (at 5, 10, 15 and 30 minutes of stimulation; Figure 4A), NF κ B (at 10, 15 and 30 minutes of stimulation; Figure 4B) and ERK1/2 (at 5, 10, 15 and 30 minutes of stimulation; Figure 4C). Similar results were obtained when AVICs were stimulated with proMMP-10 (Supplemental figure III A-C).

As shown in Figure 4D-F, wortmannin, the specific inhibitor of Akt, totally blocked aMMP-10-induced upregulation of pro-inflammatory IL-1 β expression (Figure 4D), as well as the pro-fibrotic molecules α -SMA and collagen type I (Figures 4E and F). Moreover, wortmannin blocked the effect of aMMP-10 on the osteogenic markers osteopontin, BMP-4, Runx2, Sox-9, BMP-9 and Smad1/5/8 (Figures 4G-L). A partial reduction of IL-1 β by the specific inhibitors against NF κ B and ERK1/2 pathways was observed in aMMP-10 stimulated AVICs, while no effect on other targets was reported (Figures 4D-L). No impact of molecular pathway inhibitors was observed on inflammatory, fibrotic or osteogenic markers when AVICs were stimulated with proMMP-10 (Supplemental Figure III D-H).

TIMP-1 prevents the increase of inflammatory and osteogenic markers in AVICs treated with aMMP-10

We then performed *in vitro* experiments to address if the observed overexpression of inflammatory and osteogenic markers induced by aMMP-10 could be prevented by its main physiological inhibitor TIMP-1. AVICs were treated with 10^{-9} M aMMP-10 alone or in combination with an equimolar amount of TIMP-1 for 24h to determine the expression of different markers by ELISA or western blot in cell culture supernatants. As shown in Figure 5A-E, TIMP-1 prevented the release of IL-1 β and the osteogenic markers Sox9, BMP-4 and

Smad 1/5/8 induced by aMMP-10 in AVICs. Moreover, AVICs treated with the combination of aMMP-10 and TIMP-1 presented less calcium deposits, measured by Alizarin Red staining than aMMP-10 treated cells (Figure 5F).

Specific inhibition of MMP-10 prevents osteogenic differentiation of AVICs

Finally, experiments were performed in presence of ODM for 7 days to determine if the specific inhibition of MMP-10 with a monoclonal antibody (MMP-10ab) was able to prevent AVICs mineralization, and compared the results with OPG, a known osteogenic inhibitor^{25,26}. As shown in Figure 5G-L, both anti-MMP-10 and OPG reduced protein levels of the osteogenic markers Runx2, Sox9, OPN, BMP-4 and Smad 1/5/8, while no effect on BMP-9 was observed. Furthermore, calcium deposits were more intensely reduced in AVICs treated with the anti-MMP-10 antibody compared to the reduction achieved by the OPG treatment (Figure 5M). A control monoclonal antibody was used as negative control (data not shown).

Discussion

CAVD shares pathophysiological features with atherosclerosis, such as chronic inflammation, increased extracellular matrix (ECM) remodeling, cell proliferation and development of calcific lesions²⁷. The aim of this study was to evaluate the role of MMP-10 in AV calcification in patients with severe CAVD. Our results demonstrate that calcified valves overexpress and secrete MMP-10. Moreover, MMP-10 co-localized with calcification markers in stenotic AVs, suggesting that it may play a role in this process. *In vitro*, MMP-10 increased inflammatory, fibrotic, and calcification markers in AVICs, mainly through the Akt pathway, while MMP-10 inhibition with either TIMP1 or a specific blocking antibody prevented those effects. Taken together, these results suggest that MMP-10 plays a relevant role in AV calcification and progression of AS.

Several MMPs have been shown to mediate the extensive tissue remodeling characteristic of CAVD³. For example, increases in MMP-9 and MMP-3 in AS have been associated to inflammatory state, whereas MMP-2 activity is similar in control and pathological valves²⁸. In agreement with previous studies in subclinical²⁹ and clinical atherosclerosis²², we found that MMP-10 was increased in the blood of patients with AS, compared to controls, and locally expressed in stenotic AV, where high expression of inflammatory markers (IL-6, IL-1 β and TNF α) has been reported³⁰. Moreover, similar to stroke patients³¹, a correlation between circulating levels of MMP-10 and TNF α was observed in AS patients and, locally, stenotic AVs secreted both MMP-10 and IL-1 β . These results suggest a possible feedback loop between MMP-10 and chronic inflammation, which is enforced by the fact that TNF α and IL-1 β are potent inducers of MMP-10 *in vitro*^{31,32} and, conversely, MMP-10 promoted IL-1 β secretion in cultured AVICs. Inflammatory cells can also contribute to cytokine and protease release into the diseased valves. Infiltrating monocytes differentiate to macrophages³³ and secrete inflammatory mediators such as TNF- α , IL-1 β or MMPs, leading to cardiac valve inflammation and remodeling³⁴. It has been reported that naive macrophages do not express MMP-10, but they are able to do so upon M0 differentiation and M1 polarization²⁰. Moreover, M1 macrophage polarization mRNA profile has been reported in calcific aortic valves obtained from patients with aortic stenosis³³ so that, among inflammatory cells, this cell population could be at least one of the sources of MMP-10 in the diseased valve.

Interestingly, MMP-10 co-localized with calcification markers Runx2 and Sox-9 in human AS valves. *In vitro*, AVICs treated with aMMP-10 exhibited enhanced expression of calcification markers, together with increased ALP activity and mineralized matrix deposits. Our results suggest a role for MMP-10 in the calcification process, supported by previous results from our group in bone repair. Likewise, the administration of MMP-10 enhanced BMP2-mediated bone repair, and accelerated the mineralization rate in a murine model of calvarial critical-size defect³⁵. Moreover, MMP-10^{-/-} mice presented delayed plaque progression and reduced calcification in apolipoprotein E-deficient background²³. Other proteases such as MMP12 have been shown to induce osteogenesis in human AVICs³⁶. Moreover, aMMP-10 also increased IL-1 β and TNF- α , two mediators that could accelerate the calcification process^{37,38}. Although inflammation precedes ECM remodeling and calcification, inflammation over the course of the disease has not been fully explored yet, and MMP-10 could emerge as an interesting target of both processes, inflammation and calcification.

MMPs are synthesized and secreted in latent form, as proenzymes, and require extracellular activation by proteolysis of the propeptide, thereby blocking the active site from being fully functional. This proteolytic activation can be performed by furin, plasmin, MMPs or other proteases³⁹. Specifically, it has been reported that several serin proteases, such as kallikrein,

trypsin, tryptase, cathepsin G, neutrophil elastase and chymase, can activate MMP-10 zymogen in a dose-dependent manner⁴⁰. In addition, our group demonstrated that plasmin can also activate human MMP-10²⁴. Although the expression of most of these activators has been detected in human diseased aortic valves (GSE12644) and AVICs (GSE88803), the incubation time in the *in vitro* experiments is probably too short to produce enough protein, and only incomplete activation of pro-MMP-10 is achieved. Accordingly, we observed no effect, or a very subtle one, in the expression of inflammatory, fibrotic and calcific markers when AVICs were stimulated with proMMP-10, while aMMP-10 greatly increased the levels of those proteins. The fact that TIMP1, an endogenous MMP inhibitor, blocked the increase of inflammatory and osteogenic markers in AVICs treated with aMMP-10 strongly suggests that these effects are mediated by the catalytic activity of MMP-10. However, since TIMP-1 inhibits most MMPs, the participation of other MMPs present in the valve could not be ruled out. We have tested an antibody that blocks MMP-10 activity to overcome this limitation and demonstrated that specific MMP-10 inhibition prevented most of aMMP-10 effects and the osteogenic differentiation of AVICs, further supporting a relevant role for MMP-10 in calcific aortic valve stenosis.

The effect of MMP-10 seems to be mediated by several pathways, since we could show early activation of Akt, NFkB and ERK1/2 in AVICs. However, wortmannin, the specific inhibitor of Akt, was the most efficient blocker of the inflammatory, fibrotic and osteogenic response of AVICs to MMP-10, suggesting a particular relevance for this pathway, in agreement with previous reports⁴¹⁻⁴³. It has been recently reported the importance of lipoprotein (a) (Lp(a)) and bioactive lipids as specific signaling molecules in AS, providing insights in how atherogenic risk factors may affect valve calcification⁵⁻⁷. Lp(a)/apo(a) interferes with the plasminogen/MMP-9 activation pathway and macrophage recruitment in a murine model of abdominal aortic aneurysm⁴⁴. Since plasmin is one of the main MMP activators, it could be hypothesized that Lp(a)/apo(a) could modulate MMP-10 activation in the aortic valve.

Our results could be in apparent contradiction with recent reports showing that doxycycline, a nonselective MMP inhibitor, had no effect on CAVD progression in an experimental model performed in apoE^{-/-} mice⁴⁵. Since individual MMPs can regulate specific (patho)physiological processes in different stages of the valvular disease, we could hypothesize that a more targeted inhibition in a well-defined time frame would be required to achieve a significant effect.

Overall, this study demonstrates that MMP-10 is expressed in stenotic valves, where it can play a relevant role by favoring the proinflammatory, profibrotic, and pro-osteogenic response. Available evidence from diseased AVs and human AVICs open the possibility that MMP-10 may be a potential target for delaying AV calcification and CAVD prevention.

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Highlights

- MMP-10 is overexpressed in calcified human aortic valves and is increased in the blood of patients with calcific aortic stenosis.
- Active MMP-10 (aMMP-10) induces the expression of inflammatory markers (TNF α [tumor necrosis factor- α] and IL-1 β [interleukin-1 β]) in valvular interstitial cells derived from human calcified stenotic aortic valves.
- aMMP-10 promotes the mineralization and osteoblastic differentiation of human valvular interstitial cells.
- The effects of aMMP-10 on inflammatory, fibrotic and osteogenic markers in AVICs can be prevented through Akt pathway inhibition.

Figure legends

Figure 1. MMP-10 expression and localization in aortic valves from AS patients.

Representative micrographs of aortic valve sections from AS patients stained for haematoxylin and eosin (on the left) and MMP-10 (on the right) (A). MMP-10 mRNA and protein expressions in tissue homogenates and conditioned media from aortic valves of control and AS patients (B). Histogram bars represent the mean \pm SEM of each group of subjects (Control n=11 and AS patients, n=76) in arbitrary units (AU). Gene expression data were normalized to HPRT, β -actin and GADPH. MMP-10 protein levels were normalized to total protein. *p<0.05 vs Control. Representative micrographs of aortic valve sections from AS patients double immunostained for MMP-10 in brown and; vimentin (C), α -SMA (D) Runx2 (E) and Sox-9 (F) in red. Arrows show nuclear staining (50 μ m). Scattergrams showing the association between MMP-10 and IL-1 β (G) and BMP-2 (H) in the conditioned media from calcified aortic valves. AS: aortic stenosis; MMP-10: metalloproteinase-10; Runx2: Runt-related transcription factor 2; Sox-9: SRY (sex-determining region Y)-box 9.

Figure 2. aMMP-10 induces inflammatory and fibrotic markers expression in AVICs.

mRNA (A) and secreted protein levels (B) of the inflammatory markers IL-6 and IL-1 β in AVICs after aMMP-10 stimulation. α -SMA, vimentin and Col1a1 mRNA (C) and protein (D) levels in AVICs treated with 10⁻⁹ M aMMP-10. Representative blots for α -SMA and vimentin are presented at the top of panel D. All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to HPRT, β -actin and GADPH for cDNA and to stain free for protein. *p<0.05 vs Control. aMMP-10: active metalloproteinase 10; IL-6: interleukin 6; IL-1 β : interleukin 1 beta; α -SMA: alpha smooth muscle actin; Col1a1: collagen Type I Alpha 1 Chain.

Figure 3. aMMP-10 induces osteogenesis in AVICs.

mRNA expression (A) and secreted levels (B) of Runx2, Sox-9 and osteopontin in AVICs treated with 10⁻⁹ M aMMP-10. Gene expression (C) and protein levels (D) of BMP-2 and BMP-4 in AVICs before and after aMMP-10 stimulation. BMP-9 and Smad 1/5/8 protein levels in AVICs (E). At the top of panels B, D and E representative western blots for the studied proteins are presented. Alkaline phosphatase (ALP) (on the left) and alizarin red staining (on the right) of AVICs cultivated with aMMP-10 at day 14 (100 μ m) (F). All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to HPRT, β -actin and GADPH for cDNA and to stain free for protein. *p<0.05 vs Control. aMMP-10: active metalloproteinase-10; Runx2: runt-related transcription factor 2; Sox-9: SRY (sex-determining region Y)-box 9; OPN: osteopontin; BMP: bone morphogenetic protein.

Figure 4. Intracellular pathways involved on inflammatory, fibrotic and osteogenic effects of aMMP-10 in AVICs.

Akt (A), NF κ B (B) and ERK1/2 (C) phosphorylation was measured by Western Blot in AVICs 5, 10, 15 and 30 minutes after aMMP-10 treatment (10⁻⁹ M). Protein activation is normalized to total Akt, total NF κ B, total ERK1/2 levels. IL-1 β (D), α -SMA (E), Collagen type I (F), OPN (G), BMP-4 (H), Runx2 (I), Sox9 (J), BMP-9 (K) and Smad 1/5/8 (L) protein expressions were measured by Western Blot or ELISA in AVICs. Cells were treated with PD98059, BAY117082 or Wortmannin 30 minutes before the addition of aMMP-10 (10⁻⁹ M). Supernatants were collected 24h after aMMP-10 stimulation. Representative blots are shown at the upper part

of each panel. Protein levels are normalized to total protein. All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) *p<0.05 vs Control; §p<0.05 vs aMMP-10. aMMP-10: active metalloproteinase-10; Akt: protein kinase B; NFκB: nuclear factor kappa B; ERK1/2: extracellular signal-regulated kinase 1/2, IL-1β: interleukin-1 beta; α-SMA: alpha smooth muscle actin; Col1: collagen Type I; OPN: osteopontin; BMP: bone morphogenetic protein; Runx2: runt-related transcription factor 2; Sox9: SRY (sex-determining region Y)-box 9.

Figure 5. Effects of aMMP-10 blockade in AVICs.

IL-1β (A), Sox9 (B), BMP-4 (C), BMP-9 (D) and Smad 1/5/8 (E) protein expression was measured by Western Blot or ELISA in AVICs treated with aMMP-10 (10^{-9} M) alone or in combination with TIMP-1 (10^{-9} M) for 24 h. Alizarin red staining of AVICs treated with aMMP-10 and TIMP-1 (100 μm) (F). Secreted protein levels of Runx2 (G), Sox9 (H), OPN (I), BMP-4 (J), BMP-9 (K) and Smad 1/5/8 (L) were measured by Western Blot or ELISA in AVICs cultured with ODM in presence or absence of MMP-10ab or OPG during 7 days. Alizarin red staining of AVICs cultivated with ODM, ODM+MMP-10ab and ODM+OPG (M). All conditions were performed at least in triplicate. Whisker plots represent median and minimum to maximum range of 6 assays in arbitrary units (AU) normalized to total protein. *p<0.05 vs Control; §p<0.05 vs ODM or aMMP-10, respectively. ODM: osteogenic differentiation media; OPG: osteoprotegerin; aMMP-10: active metalloproteinase-10; TIMP-1: metalloproteinase inhibitor 1; Runx2: runt-related transcription factor 2; Sox9: SRY (sex-determining region Y)-box 9; OPN: osteopontin; BMP: bone morphogenetic protein; IL-1β: interleukin-1 beta.

Table 1. Baseline characteristics of control individuals and AS patients

	Controls	AS patients
n	349	112
Age (years)	54.4 ± 11.4	72±9 ^{***}
Male	272 (78.7%)	67 (60%) ^{**}
Hypertension	70 (20%)	84 (75%)
Diabetes		35 (31%)
Hiperlipidemia		70 (62%)
NYHA		
I		13%
II		50%
III		35%
IV		3%
BNP (pg/mL)		105 (48-251)
NT pro-BNP (pg/mL)		463 (212-1342)
Glucose (mg/dL)		111±27
Creatinine (pg/mL)		0.94±0.27
GFR (mL/min/1,73m ²)		77±26
Medication		
Beta blockers		31 (28%)
ACEi/ARB		61 (54%)
Statins		76 (68%)
Diuretics		72 (64%)
Echocardiography		
LVEF (%)		66±14
End-diastolic diameter (mm)		49±6
End-diastolic volume (mL)		132±45
End-systolic volume (mL)		49±36
Medical history		
CAD disease		41 (36%)
Other CV disease		30 (27%)
Renal disease		10 (9%)
Serum markers		
MMP10 (pg/mL)	593 (452-801)	717 (552–1093) ^{***}
TNFα (pg/mL)		29.7±9.1
TIMP1 (ng/mL)		167±(53-272)

Data are expressed as mean±standard deviation or median (Q1-Q3). AS = aortic stenosis; NYHA = New York Heart Association classification of heart failure; BNP = brain natriuretic

peptides; NT-proBNP = N-terminal fragment brain natriuretic peptides; GFR: glomerular filtration rate calculated by CKD-EPI equation; ACEi/ARB=angiotensin converting enzyme inhibitors/angiotensin receptor blocker; LVEF = left ventricular ejection fraction; CAD = coronary artery disease; CV = cardiovascular; MMP-10= matrix metalloproteinase-10; TNF- α = Tumor necrosis factor alpha; TIMP-1 = tissue inhibitor of metalloproteinases 1. ** p<0.01; *** p<0.001 (controls vs AS patients)