BMP-7 attenuates left ventricular remodelling under pressure overload and facilitates reverse remodelling and functional recovery

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Aims
TGF-β regulates tissue fibrosis: TGF-β promotes fibrosis, whereas bone morphogenetic protein (BMP)-7 is antifibrotic. To demonstrate that (i) left ventricular (LV) remodelling after pressure overload is associated with disequilibrium in the signalling mediated by these cytokines, and (ii) BMP-7 exerts beneficial effects on LV remodelling and reverse remodelling.

Methods and results
We studied patients with aortic stenosis (AS) and mice subjected to transverse aortic constriction (TAC) and TAC release (de-TAC). LV morphology and function were assessed by echocardiography. LV biopsies were analysed by qPCR, immunoblotting, and histology. Pressure overload reduced BMP-7 and pSmad1/5/8 and increased TGF-β and pSmad2/3 in AS patients and TAC mice. BMP-7 correlated inversely with collagen, fibronectin, and β-MHC expressions, and with hypertrophy and diastolic dysfunction, and directly with the systolic function. Multiple linear regression disclosed BMP-7 and TGF-β as hypertrophy predictors, negative and positive, respectively. BMP-7 prevented TGF-β-elicited hypertrophic program in cardiomyocytes, and Col1A1 promoter activity in NIH-3T3 fibroblasts. The treatment of TAC mice with rBMP-7 attenuated the development of structural damage and dysfunction, and halted ongoing remodelling. The reverse remodelling after pressure overload release was facilitated by rBMP-7, and hampered by disrupting BMP-7 function using a neutralizing antibody or genetic deletion.

Conclusion
The disequilibrium between BMP-7 and TGF-β signals plays a relevant role in the LV remodelling response to haemodynamic stress in TAC mice and AS patients. Our observations may provide new important insights aimed at developing novel therapies designed to prevent, halt, or reverse LV pathological remodelling in pressure overload cardiomyopathy.

Keywords
Aortic stenosis • Pressure overload • Myocardial remodelling • Reverse remodelling • BMP-7 • TGF-β

1. Introduction
Aortic stenosis (AS) is an age-related valve disorder; it constitutes the most common adult heart valve disease that requires surgery in the Western world, and it will keep gaining importance due to the progressive increase in life expectancy in our societies.1 Sustained pressure overload (PO) stress can elicit in the LV from AS patients a harmful remodelling, characterized by concentric hypertrophy and interstitial and perivascular fibrosis,2 which constitutes a major independent risk factor for heart failure and mortality.3 Nowadays, the only effective therapy for symptomatic AS patients is the aortic valve replacement. After releasing the biomechanical stress, the LV undergoes a process of reverse remodelling.1,3,5 However, when the LV structural damage is severe, the remodelling process becomes irreversible after surgery, which results in unfavourable short- and long-term outcome of AS patients.3,6,7 The lack of preventive therapies of myocardial remodelling in AS patients highlights need for new effective drugs to delay the progression of LV structural damage before surgery and to improve and accelerate the reverse remodelling after releasing the haemodynamic stress.
The TGF-β superfamily of cytokines is composed, among others, of the prototypic TGF-βs and bone morphogenetic proteins (BMPs). TGF-β and BMP signalling is transmitted by heteromeric complexes of type I [also termed activin-like kinase (ALK)] and type II membrane receptors, with serine/threonine kinase activity. Upon receptor activation, the canonical intracellular signals propagate downstream through the phosphorylation of receptor-activated Smads; p-Smads form complexes with the common partner, Smad4, which translocates to the nucleus to regulate the transcription of target genes. The TGF-β subfamily signals through pSmad2/3, whereas the BMP family signals through pSmad1/5/8 proteins. These signals can be controlled by negative feedback mechanisms via inhibitory Smads.9,10

Overproduction of TGF-β contributes to cardiomyocyte hypertrophy and aberrant synthesis and deposition of extracellular matrix (ECM) which characterizes the pathological remodelling of the LV under pressure overload in animal models and in patients suffering from AS or systemic hypertension.11–17 TGF-β promote resident fibroblast proliferation and activation, and stimulate endothelial-to-mesenchymal transition (EMT), increasing the pool of cardiac myofibroblasts.11,14 On the other hand, BMP-7 signalling counteracts TGF-β1-induced accumulation of myofibroblasts and ECM production in experimental models of progressive interstitial fibrosis affecting several organs, including the heart.16,18 In the present study, we investigated the pathophysiological relevance of an imbalance between TGF-β and BMP-7 signalling in the LV remodelling response in patients suffering from severe AS and in a mouse model of transverse aortic constriction (TAC). The potential for BMP-7 to prevent, slow, or reverse the LV structural damage induced by PO, and to improve the LV reverse remodelling after releasing the haemodynamic stress was assessed in mice.

2. Methods

2.1 Pressure overload studies in mice

The experiments were performed in 12- to 16-week-old littermate female wild-type (C57BL/6) and heterozygous BMP-7-deficient mice (BMP-7+/−) in a C57BL/6 genetic background.20 The study was approved by the University of Cantabria Institutional Laboratory Animal Care and Use Committee (reference IP0415) and conducted in accordance with the guidelines from directive 2010/63/EU of the European Parliament. All animals received humane care, and all efforts were made to minimize animal suffering.

2.1.1 TAC and release (de-TAC)

Mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) and subjected to TAC for 4 weeks.16 In a series of mice, the aortic arch was re-approached, and the constriction was released (de-TAC mice); de-TAC mice were followed up for 1 or 4 weeks. Mice were sacrificed by decapitation under anaesthesia (100 mg/kg ketamine and 5 mg/kg xylazine, i.p.).

2.1.2 Treatments

The Protocols and number of mice per group are shown in the Supplementary material online, Figure S1. Recombinant murine BMP-7 (rBMP-7, R&D Systems) was administered at the dose of 10 μg/kg/week using osmotic mini-pumps (Alzet 1002) during (i) the complete 4-week TAC period; (ii) the 3rd and 4th weeks following TAC; or (iii) the first week after de-TAC. Minipump implantation was performed during either TAC or de-TAC surgery under ketamine (100 mg/kg) and xylazine (5 mg/kg) anaesthesia or during the echocardiography procedure under anaesthesia with isoflurane (4%). In a series of mice, a monoclonal anti-BMP-7 antibody (clone 164313, R&D Systems) was administered daily (12 μg/day, i.p.) for 7 days starting at the de-TAC surgery.

2.1.3 Echocardiography

Transthoracic echocardiography was performed under sedation with isoflurane (2.5%), with an ultrasound equipment (Veo-770, VisualSonics, Toronto, ON, Canada) using a high-resolution transducer centred at 30 MHz. The operator was blinded to the study groups. Transcatheter pressure gradients were measured using pulsed-wave Doppler analysis at the distal arch, LV end-diastolic (LVEDd) and end-systolic (LVESd) internal diameters, interventricular septum (IVST), and LV posterior wall (PWT) thicknesses were measured following the recommendations of the American Society of Echocardiography. The degree of geometric concentricity of the remodelled LV was assessed by the relative PWT (rPWT) calculated as: rPWT = PWT/(LVEDd/2). cardiac mass was estimated using the Devereux’s formula. The mitral annular plane systolic excursion (MAPSE) measurements were obtained from four-chamber views using M-mode imaging. The LV ejection fraction (LVEF) and MAPSE were used as surrogates of short axis and longitudinal systolic functions, respectively. Parameters of diastolic LV function (E′/e′) were obtained by pulsed-wave mitral inflow analysis and tissue Doppler imaging to obtain the ratio of peak early transmural flow velocity (E) to peak early myocardial tissue velocity (e′).

2.2 Pressure overload studies in patients

The study followed the Declaration of Helsinki guidelines for biomedical research involving human subjects. The institutional ethics and clinical research committee approved the study, and all patients gave written informed consent. The clinical and demographic characteristics of the AS and control groups are shown in Table 1. The study was performed using LV myocardial intraoperative biopsies obtained from a cohort of 38 patients diagnosed with isolated severe AS and undergoing aortic valve replacement surgery in the University Hospital Marqués de Valdecilla in Santander, Spain. Patients with aortic or mitral regurgitation greater than mild or with major coronary stenosis >50%, previous cardiac operations, malignancies, or poor renal or hepatic function were deemed ineligible for the study. The control group was a cohort of 26 surgical patients with pathologies (atrial septal defect: n = 13, aortic aneurysm: n = 7, mitral stenosis: n = 4, left atrial myxoma: n = 2) that did not associate LV pressure or volume overload, coronary heart disease or cardiomyopathies. Subepicardial biopsies (4–10 mg) were taken from the LV lateral wall with a Tru-cut needle during the surgical procedure. Samples were all harvested by the same surgeon in a protocolized manner and always from the same location in the margo obtusus of the heart.

2.3 Studies in cultured cells

2.3.1 Rat neonatal cardiomyocytes

Cardiomyocytes were obtained from Wistar rats sacrificed by decapitation under sedation with isoflurane (4%) at postnatal day 2–3. The hearts were removed and kept in Ca2+/Mg2+-free HBBS (Hank’s Balanced Salt Solution) medium at 4°C. The tissues were minced using a sterile scalp blade and transferred to a T25 flask containing trypsin (1 × Sigma), type IV collagenase (200 U/mL Sigma), type I collagenase (0.025 mg/mL Sigma), and DNase I (0.2 U/mL Sigma). The flask was settled at 37°C for 15 min, and the supernatant was then collected, mixed with HBBS medium, and centrifuged (5 min, 1500 rpm). The cell pellet was re-suspended in DMEM supplemented with 5% FBS and kept at 37°C. The harvested cells were plated and incubated for 2 h to allow the attachment and removal of fibroblasts. The cardiomyocytes were plated (300 000 cells per well) in M12 multi-well plates pre-coated with 1% gelatin and cultured in DMEM supplemented with 10% FBS for 48 h. The culture medium was then replaced with Opti-MEM containing TGF-β1 (5 ng/mL), BMP-7 (50 ng/mL), or TGF-β1 (5 ng/mL) plus BMP-7 (50 ng/mL) and incubated for 24 h. The cells were collected and processed for mRNA isolation. Five independent experiments were performed.

2.3.2 Rat H9C2 cardiomyocytes

The H9C2 has been reported to show similar hypertrophic responses than primary neonatal cardiomyocytes.20 H9C2 cardiomyocytes (ATCC, USA)
were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin-streptomycin, at 37 °C in 5% CO₂. Cells seeded in 12-well plates (10⁵/well) were cultured for 24 h in Opti-MEM medium and then incubated for 24 h with Opti-MEM containing TGF-β1 (5 ng/mL), BMP-7 (50 nmol/mL), TGF-β1 (5 ng/mL) plus BMP-7 (50 ng/mL), and TGF-β1 plus BMP-7 in combination with the neutralizing antibody anti-BMP-7 (100 ng/mL). The cells were collected and processed for mRNA isolation. Two independent experiments were run in triplicate.

H9C2 cardiomyocytes seeded on glass coverslips were incubated for 24 h with Opti-MEM containing TGF-β1 (3 ng/mL), BMP-7 (50 ng/mL), or TGF-β1 (5 ng/mL) plus BMP-7 (50 ng/mL). After fixation in PFA (3.7% in PBS), cellular actin was stained with FITC-conjugated phalloidin (1% in PBS) for 30 min and with a primary Ab to β-MHC for 60 min. Slides were mounted in Vectashield with DAPI (Vector Labs). A Zeiss Axioplan II microscope was used to acquire the images, and cell area was measured using ImageJ software. Results are reported as the average area of 75 cells per treatment, measured in two different experiments.

2.6 Western blot
Thirty micrograms of protein lysates were electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad, CA, USA). The primary antibodies were goat polyclonal to p-Smad2/3 (Santa Cruz Biotechnology, sc-11769), rabbit polyclonal to p-Smad1/5/8 (cell signalling 95115); rabbit monoclonal to Smad7 (Abcam, ab90086); mouse monoclonal to BMP7 (R&D, MAB71405); mouse monoclonal to GAPDH (Santa Cruz, sc-32233); and mouse monoclonal to tubulin (Sigma-Aldrich, TS168). After incubation with the appropriate secondary antibodies, proteins were immunodetected with ECL Advance Western Blotting Detection Kit (GE Healthcare) or with infrared fluorescence (Odyssey imager). The results were expressed as optical density of the sample dots normalized to that obtained for GAPDH. Samples from 3–6 subjects per group were tested in two independent experiments.

2.7 Statistics
Data were assessed for normality with the Kolmogorov–Smirnov test. Values were reported as means ± S.E.M. Continuous variables were compared using two-tailed Student’s t-test or Mann–Whitney U test. Categorical variables were compared with the Fisher’s exact test. The influence of genotype, drug treatments, and pressure overload on gene expression was assessed by a two-way ANOVA and on echocardiographic parameters by repeated-measures two-way ANOVA. Bonferroni post-hoc test was used when appropriate. Correlations between mRNA expression levels were performed using Pearson’s correlation analysis. Multiple linear regression analysis was used to identify predictors of LV hypertrophy. Post-hoc

### Table 1: Clinical and demographic characteristics of aortic stenosis and control patients

<table>
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<td>Statins (%)</td>
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ACE, angiotensin-converting enzyme; AT-II, angiotensin II; ARBs, AT-II receptor blockers. Statistical analysis: continuous variables, t-test; categorical variables, Fisher’s exact test.
assessment of the regression model in patients was performed with the bootstrapping method with 2000 iterations. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001. Statistical packages: GraphPad Prism 5.03 and PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 LV remodelling involves unbalance between TGF-β1- and BMP-7-mediated signalling in a mouse model of pressure overload

TAC caused a stable transcoarctational pressure gradient of ≈50 mmHg during the 4-week follow-up period (Figure 1A). Pressure overload resulted in the development of a rapid progressive LV hypertrophy with concentric geometry (increased LVPWT/LVEDd), which was accompanied by LV systolic functional deterioration both in the radial (LVEF) and in the longitudinal axes (MAPSE), and diastolic dysfunction reflected by the rise in the LV filling pressure (E/e'). Four weeks after TAC, mice were subjected to de-TAC surgery, and the morpho-functional echocardiographic evolution was followed for four further weeks (Figure 1A). The release of overload stress activated the reverse remodelling process; as a result, LV mass decreased and LVEF, MAPSE, and E/e' improved significantly within the first week after de-TAC. Then, recovery continued at a more gradual rhythm and at 4 weeks, even though LVEF and E/e' had already normalized their values, LV mass and MAPSE had not yet returned to baseline figures (Figure 1A).

LV expression of BMP-7 was significantly reduced in mice subjected to TAC, whereas the expression levels of TGF-β1 and TGF-β2 were up-regulated (Figure 1B and C); consequently, the ratios TGF-β1/BMP-7 increased significantly. The mRNA and protein levels of the inhibitory Smad7 were significantly reduced. Protein levels of p-Smad2/3 increased while those of p-Smad1/5 and Smad7 diminished (Figure 1D). Four weeks after releasing the overload stress, the expression levels of BMP-7, the ratios TGF-β1/2 to BMP-7, and the expressions of p-Smad2/3, p-Smad1/5/8, and Smad7 recovered the control values (Figure 1B, C, and D). No differences between groups were observed in TGF-β3 at gene (control: 0.82 ± 0.13; TAC: 0.85 ± 0.11; de-TAC: 1.02 ± 0.10) and protein level (control: 0.88 ± 0.06; TAC: 1.1 ± 0.16; de-TAC: 1.0 ± 0.15).

Linear regression and correlation analysis, performed in the cohorts of TAC and de-TAC mice, show that BMP-7 in the LV correlated significantly and inversely with the mRNA levels of TGF-β1 and TGF-β2 and directly with those of the inhibitory Smad7 (Figure 2A). Additionally, the transcript levels of BMP-7 correlated significantly and inversely with the expression of genes encoding ECM elements (Col I, Col III, and FN-1) (Figure 2B), consistent with the antifibrogenic properties of this cytokine.

Both the sarcomeric hypertrophic marker β-MHC (Figure 2B) and LV mass correlated negatively with BMP-7 (Figure 2C), suggesting an antihypertrophy role for BMP-7. Thus, multiple regression analysis (Figure 2D) indicated that the myocardial expression of BMP-7 constituted an independent negative predictor of LV posterior wall thickening after TAC, whereas TGF-β2 was a positive predictor. The regression equation was the following: PWT (mm) = 0.73 – 1.45 × [BMP-7] + 0.2 × [TGF-β2]. The adjusted R² (0.53; P < 0.001) indicates that 53% of the variance in PWT after TAC can be estimated from this model.

Regarding the echocardiographic functional parameters, BMP-7 transcript levels correlated inversely with the mean transcoarctation gradient in TAC mice (R = 0.52**, indicating a relationship between the severity of the constriction and BMP-7 down-regulation. Moreover, the systolic function was positively related to myocardial BMP-7, both in the short-axis (LVEF) and in the long-axis (MAPSE); while the degree of diastolic dysfunction, as reflected by the increase in E/e’, was inversely related to BMP-7 mRNA levels (Figure 2C).

3.2 BMP-7 prevents TGF-β-induced transcriptional activation of the Col1A1 promoter in NIH-3T3 fibroblasts

The stimulation with TGF-β1 (3 ng/mL) of NIH-3T3 fibroblasts, transfected with a full-length promoter of Col1A1-Luc construct, resulted in significant increase of the luciferase activity. BMP-7 (20 ng/mL) significantly repressed transcriptional activation of Col1A1-Pro-luc by TGF-β1 (3 ng/mL) (Figure 3A).

3.3 BMP-7 inhibits TGF-β-induced hypertrophic program in cultured cardiomyocytes

In cultured neonatal rat ventricular cardiomyocytes, the addition of recombinant BMP-7 (50 ng/mL) to the culture medium significantly dampened the overexpression of the hypertrophy markers, ANP, BNP, and β-MHC, induced by TGF-β (5 ng/mL) (Figure 3B). The inhibitory effect of BMP-7 on TGF-β-induced overexpression of hypertrophy-related genes in cultured H9C2 cardiomyocytes (Figure 3C) was prevented by adding BMP-7 neutralizing Ab (100 ng/mL) to the medium.

H9C2 cardiomyocytes seeded on glass coverslips were incubated with TGF-β1, BMP-7, or TGF-β1 plus BMP-7 for 24 h. Cells were stained with FITC-conjugated phalloidin, and cell area was measured using ImageJ. As shown in Figure 3C, TGF-β1 significantly increased the cell area whereas addition of BMP-7 to the medium prevented TGF-β1-induced hypertrophy.

3.4 Sustained treatment with recombinant BMP-7 protects the LV from remodelling under biomechanical stress

The usefulness of rBMP7 as pre-emptive treatment against LV remodelling and to stop the ongoing pathological remodelling response was assessed under the following experimental conditions (see Supplementary material online, Figure S1): (i) mice treated with a 4-week subcutaneous infusion of rBMP7 starting at the moment of TAC surgery (BMP-71–4wk group); (ii) mice treated with a 2-week subcutaneous infusion rBMP7 starting on Day 15 after TAC surgery when LV hypertrophy and functional deterioration were already taking place (BMP-73–4wk group); and (iii) TAC mice treated with a 4-week subcutaneous infusion of saline as control group. The studies in the literature which analyse the effects of recombinant BMP-7 in mouse models of chronic organ fibrosis reported antifibrotic effects of this cytokine at doses ranging from 1 to 1000 μg/kg/week.11,21–24 In a preliminary series, we started treating four TAC mice with 1 μg/kg/week of BMP-7. However, in our hands, the cytokine at such low dose did not induce any significant change in the morpho-functional echocardiographic parameters. Therefore, we increased the dose to 10 μg/kg/week.

TAC caused similar transcoarctation pressure gradients (Figure 4A) in saline and rBMP7-treated mice at any time of the follow-up, indicating similar degrees of constriction in all groups. The administration of rBMP7 during the 4-week TAC follow-up period diminished PO-induced PW and IVS thickening as well as chamber dilation; as a
Aortic constriction in mice induces LV morpho-functional, gene, and protein remodelling changes that regress after deferred constriction release. (A) Echocardiographic morphological and functional changes induced by pressure overload in mice subjected to 4-week TAC (n = 17), and their recovery 4 weeks after releasing the aortic constriction (de-TAC, n = 8). PWT, posterior wall thickness; LVM, LV mass; LVEDd, LV end-diastolic diameter; PWT/LVEDr, posterior wall thickness/LV end-diastolic radius; LVEF, LV ejection fraction; MAPSE, mitral annular plane systolic excursion; E/e', ratio of peak early transmitral flow velocity (E) to peak early myocardial tissue 1 velocity (e'). Data are expressed as mean ± SEM. Repeated-measures ANOVA followed by Bonferroni’s test. (B) Myocardial mRNA levels of BMP-7, TGF-β1, and TGF-β2 and the ratios between TGF-βs and BMP7 in sham (n = 7), TAC (n = 8), and de-TAC mice (n = 7). ANOVA followed by Bonferroni’s test. (C) Myocardial protein levels of BMP-7, TGF-β1, and TGF-β2 determined by western blot in sham, TAC, and de-TAC mice (n = 3–4 per group). Data are expressed as relative optical density (RD) vs. GAPDH. ANOVA followed by Bonferroni’s test. (D) Myocardial protein levels of pSmad1/5/8, pSmad2/3, and Smad7 in sham (n = 4), TAC (n = 5), and de-TAC (n = 4) mice. Data are expressed as relative optical density (RD) vs. GAPDH. Smad7 was determined also by qPCR (sham, n = 6; TAC, n = 6; de-TAC, n = 7). ANOVA followed by Bonferroni’s test. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001. (See detailed statistical analysis in the Supplementary material). RE, relative mRNA expression normalized to 18S.
result, BMP-7\textsuperscript{1–4wk} mice developed a significantly lower degree of LV hypertrophy compared with saline-treated mice. BMP-7 completely prevented TAC-induced systolic dysfunction in the short-axis (LVEF), and the LV systolic function in the long-axis (MAPSE) was significantly less impaired in BMP-7\textsuperscript{1–4wk} mice compared with the saline group. The rise in $E/e'$ induced by TAC was completely prevented by BMP-7\textsuperscript{1–4wk}, which indicates a protection against the development of diastolic dysfunction (Figure 4A).

The treatment with rBMP7 during the complete TAC period prevented myocardial overexpression of the remodelling-related genes analysed (TGF-β1, TGF-β2, Col I, Col III, FN1, and β-MHC) (Figure 5A–F) and attenuated the structural remodelling, as indicated milder histological fibrosis and shorter cardiomyocyte diameters displayed by BMP-7\textsuperscript{1–4wk} compared with saline-treated mice (Figure 5G–I).

The administration of rBMP7 during the 3rd and 4th weeks of TAC halted the progression of wall thickening, LV hypertrophy, chamber

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**Figure 2** LV BMP-7 expression correlates with remodelling-related genes and morpho-functional parameters. (A) Linear regression and Pearson’s correlation analyses showing the relationship of BMP-7 mRNA expression with elements of TGF-β signalling, (B) remodelling-related genes (collagen I, collagen III, fibronectin-1, and β-myosin heavy chain), and (C) morpho-functional echocardiographic parameters (LVM, LV mass; LVEF, LV ejection fraction; MAPSE, mitral annular plane systolic excursion; $E/e'$, ratio of peak early transmitral flow velocity ($E$) to peak early myocardial tissue $1$ velocity ($e'$)), in the LV myocardium from TAC ($n=7$) and de-TAC mice ($n=7$). R, Pearson’s correlation coefficient. Significance levels: *$p<0.05$, **$p<0.01$, ***$p<0.001$. (D) Multiple linear regression model for predicting the posterior wall thickness after 4 weeks of TAC ($n=17$). Adjusted $R^2=0.53$ ($p<0.001$). RE, relative mRNA expression normalized to 18S.
dilation, and systolic and diastolic dysfunctions (Figure 4A). The expressions of the remodelling-related genes were lower in BMP-7–4wk than in TAC mice treated with saline (Figure 5A–F). At the structural level, the average cardiomyocyte diameter (Figure 5I) was significantly smaller, and the LV area occupied by histological fibrosis (Figure 5G and H) displayed a decremental trend, but without statistical significance due to the interindividual variability.

Overall, our results indicate that down-regulation of BMP-7 during the haemodynamic stress condition was a relevant maladaptive feature of myocardial remodelling and that sustained administration of recombinant BMP-7 prevented pressure overload-induced myocardial hypertrophy, structural damage, and systolic and diastolic dysfunctions. Moreover, when treatment begins once the pathological myocardial remodelling has been established, BMP-7 can stop the progression of the ongoing structural damage and its deleterious functional consequences.

As an approach to ascertain the BMP-7 receptor type involved in the observed effects on gene remodelling and function, linear regression and correlation analysis were performed in a cohort of mice integrated by sham (n = 5), TAC (n = 7), and TAC-BMP-7–4wkTAC (n = 7). The results in Figure 6 show that the relationship of myocardial ALK3 expression levels with all parameters analysed was similar to that of BMP-7. Thus, ALK3 correlated significantly and inversely with the mRNA levels of TGF-β2, Col I, FN1, and β-MHC and directly with Smad7. Also, the transcript levels of ALK3 (Figure 6C) correlated inversely with the LVM, directly with the systolic function (LVEF and
and inversely with the degree of diastolic dysfunction (E/e'). On the other hand, the expression levels of the other major BMP-7 type I receptor, ALK6, showed no significant relationship with any of the parameters analysed (data not shown).

3.5 BMP-7 deficiency potentiates LV hypertrophy in BMP-7

BMP-7+/− mice were significantly bigger than wild-type mice (body weight, WT: 20.9 ± 0.2 vs. BMP-7+/−: 23.9 ± 0.3, t = 7.8, P < 0.001) (naso-anal body length, WT: 9.4 ± 0.07 vs. BMP-7+/−: 10.1 ± 0.05, t = 8.8, P < 0.001). BMP-7+/− mice exhibited at baseline significantly larger thicknesses of LV walls and LV mass than their WT littermates (Figure 4B). No differences between genotypes were evidenced in chamber dimensions, systolic and diastolic functions at baseline. Following TAC, the two genotypes developed similar transcoarctational gradients (wild type: 46.1 ± 3.1 mmHg; BMP-7+/−: 45.6 ± 2.5 mmHg). However, BMP-7+/− mice developed greater levels of LV hypertrophy, either row or indexed, and a more concentric LV geometry than wild-type mice. The systolic (LVF, MAPSE) and diastolic (E/e') functions displayed a similar deterioration in both genotypes at any time after TAC (Figure 4B). At the structural level (Figure 7B), the average diameter of cardiomyocytes was larger in BMP-7+/−
than in their wild-type littermates both at baseline and after TAC. The degree of myocardial fibrosis developed was higher in BMP-7+/2 than in C57BL/6 mice.

3.6 LV reverse remodelling in mice is hampered by BMP-7 signalling loss of function and improved by recombinant BMP-7

Four weeks after TAC, a series of mice were subjected to de-TAC surgery. Given that the bulk of the remodelling regression had occurred within the first week after de-TAC (Figure 1A), the follow-up of this part of the study was limited to 1 week after de-TAC surgery. We assessed the influence of BMP-7 signalling loss of function on the capability of the heart to reverse the LV remodelling after pressure overload release by de-TAC surgery (Figure 7). The subjects of study (see Supplementary material online, Figure S1) were the following: (i) TAC-BMP-7+/2 mice treated with a subcutaneous saline infusion during 7 days after de-TAC; (ii) TAC-WT mice treated with daily injections of a specific monoclonal neutralizing antibody against BMP-7 (BMP-7-Ab, 500 μg/kg/day, 7 days) starting at the moment of de-TAC surgery; and (iii) TAC-WT mice treated with a subcutaneous saline infusion during 7 days after de-TAC.

The transcoarctational gradient fell significantly after de-TAC surgery with no differences between groups (de-TAC + saline: 16.9 ± 2.3; de-TAC-BMP-7+/2 + saline: 12.5 ± 1.2; de-TAC + BMP-7-Ab: 17.6 ± 1.1). Both, heterozygous deletion of BMP-7 and BMP-7 neutralization with a BMP-7-Ab during the 7-day de-TAC period hampered the LV morpho-functional recovery after releasing the haemodynamic stress. Regression of LV hypertrophy and the recovery of systolic (LVEF and MAPSE) and diastolic (E/e’) functions were significantly worse in both groups of loss-of-BMP-7 function in comparison with C57BL/6 mice treated with saline (Figure 7A). At the structural level (Figure 7B), the remaining fibrosis and the average cardiomyocyte diameter after 1-wk de-TAC were significantly higher in BMP-7+/2 mice and in mice treated with BMP-7-Ab than in wild-type littermates treated with saline.

The effect of BMP-7 gain-of-function on reverse remodelling was assessed in a series of wild-type mice treated with rBMP7 during the 7-day de-TAC period (Figure 7A). The loss of LV mass during the first de-TAC week was significantly higher in rBMP7-treated than in saline-treated mice. Chamber dilation (LVEDd and LVESd; not shown)
decreased and systolic function (LVEF and MAPSE) improved to a significantly greater extent with rBMP7 than with saline treatment during the first week after de-TAC. At the structural level (Figure 7B), both saline- and rBMP7-treated mice reduced the cardiomyocyte diameter and LV fibrosis area to similar extents.

3.7 Translation of the results obtained in the experimental model to the clinical aortic stenosis

The changes induced by pressure overload in the LV expression levels of BMP-7, TGF-β1, and Smad7 were assessed in a subgroup of 26 pairs of controls and AS patients individually matched by age (within 5 year) and sex. The LV myocardium from AS patients exhibited significantly lower BMP-7 and higher TGF-β1 preoperative expression levels (mRNA and protein) compared with surgical controls (Figure 8A). BMP-7 mRNA levels in the AS patients’ heart correlated significantly and inversely with the gene expression of TGF-β1 and directly with SMAD7 (Figure 8B). As observed in TAC mice, the myocardial gene expression of BMP-7 correlated inversely with the expressions of Col1A1 and Col3A3 (Figure 8B), and there was a significant and positive association between BMP-7 expression and the systolic function in the short axis (LVEF). Consistent with an antihypertrophic effect induced by BMP-7, there was an inverse and significant relationship between the cytokine and the LV mass (Figure 8B). Stepwise multiple linear regression analysis (Figure 8C) evidenced that preoperative BMP-7 was a significant negative predictor of the LV mass, whereas TGF-β1 appeared as a significant positive predictor. The regression equation was the following: 

\[
\text{LV mass (g)} = 257.5 - 111.6 \times [\text{BMP-7}] + 21.3 \times [\text{TGF-β1}].
\]

The adjusted \(R^2\) (0.45; \(P < 0.001\)) indicated that 45% of the variance in LV mass can be estimated from this model in AS patients.

4. Discussion

Our findings in patients with severe AS and in a mouse model of reversible pressure overload raise two important notions: (i) an imbalance between BMP-7 and TGF-β signals could play a major pathogenic role in the maladaptive LV remodelling under pressure overload; and (ii) strategies to enhance the activity of BMP-7 signalling may have putative therapeutic value to attenuate ongoing myocardial hypertrophy and to favour the reverse remodelling after releasing the LV from the haemodynamic load.

TGF-β and BMP-7 belong to the same superfamily; however, each of these cytokines exhibit a unique signalling pathway through specific Smad proteins that determine responses that are opposite in each pathway.\(^8,18,19\) During the process of pathological remodelling induced by haemodynamic stress, TGF-β is a primary and potent mediator of myocardial fibrosis and hypertrophy both in mice and in humans.\(^11–17\) On the other hand, BMP-7 acts as an antifibrotic cytokine in experimental models of pathological organ fibrosis.\(^18,19,22–28\) Our current results evidenced that pressure overload resulted in biased cellular signalling towards pro-fibrogenic cytokines of the TGF-β family in detriment of BMP-7-mediated signals, both in TAC mice and in AS patients. We observed LV up-regulation of TGF-βs and down-regulation of
BMP-7 expressions and increased TGF-βs/BMP-7 ratios. Accordingly, the levels of BMP-7 canonical downstream effectors (pSmads1/5/8) were reduced, while those of TGF-β (pSmad2/3) appeared increased. In the group of pressure-overloaded mice, the balance between TGF-β and BMP-7 signalling recovered normal values after releasing the haemodynamic stress by de-TAC. Furthermore, myocardial mRNA levels of BMP-7 and those of TGF-βs correlated inversely both in the cohort of operated mice and in AS patients. These results support the existence of a regulatory mechanism between BMP-7 and TGF-βs in the context of ventricular remodelling.

**Figure 7** BMP7 gain and loss of function exerts opposite effects on reverse remodelling after de-TAC in mice. In a series of mice, four weeks after TAC, the aortic constriction was removed (de-TAC) and mice were followed-up for 1 week after de-TAC. C57BL6 mice were treated with saline (n = 8), a BMP7 neutralizing antibody (n = 6, BMP7-Ab) or recombinant BMP7 (n = 5). BMP7+/− mice were treated with saline after de-TAC (n = 7). (A) LV hypertrophy regression after de-TAC. Recovery of systolic function in the short axis (LVEF). Recovery of systolic function in the long axis (MAPSE). Data are expressed as mean ± SEM of the percentage of change vs. 4-week TAC. (B) Reduction of LV loading pressure (E/e’). Data are expressed as mean ± SEM of the percentage of change vs. 4-week TAC. The percentage of fibrosis and the cardiomyocyte diameter were measured in LV sections from 3 to 6 mice per group stained with Masson trichrome. Data are means ± SEM. ANOVA followed by Bonferroni’s test. Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001 (see detailed statistical analysis in the Supplementary material). Representative images of LV sections stained with Masson trichrome showing myocardial fibrosis in blue. Scale bar, 50 μm.
of a reciprocal inhibition between BMP-7 and TGF-β in the heart and suggest its clinical relevance in human cardiac diseases.

BMP-7 prevents and even reverses, in vitro, TGF-β-induced EMT, fibroblast accumulation and transdifferentiation into myofibroblasts, blocks the production of ECM proteins by these cells, and stimulates MMP-2-dependent breakdown of the fibrotic matrix.18,19,29 – 31 Our findings in vivo show that the transcript levels of BMP-7 kept an inverse relationship with the expression of Col I, Col III, and FN1 in the LV from TAC mice. Moreover, the luciferase reporter assays indicate that BMP-7 inhibits Col I promoter transcription activity induced by TGF-β in cultured NIH-3T3 fibroblasts. Interstitial fibrosis is a major cause of LV wall stiffening, diastolic and systolic dysfunction, and progression to heart failure.2,32 Accordingly, BMP-7 expression in TAC mice was related directly to parameters of systolic function (LVEF

Figure 8 Pressure overload in aortic stenosis patients induces opposed LV expression changes of TGF-β and BMP signalling elements. (A) Myocardial mRNA expression of BMP-7, TGF-β1, and Smad7 in the LV from AS and surgical control patients (n = 26 pairs of case and controls matched by age and sex, paired t-test). Protein expression levels determined by western blot and expressed as relative optical density (RD) (n = 3 – 4 patients per group, t-test). (B) Linear regression and Pearson’s correlation analyses showing the relationship of BMP-7 mRNA levels with TGF-β1, Smad7, collagens I and III, LV mass index (LVMI), and LV ejection fraction (LVEF). R, Pearson’s correlation coefficient. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001. (C) Significant preoperative predictors of LV mass in AS patients undergoing aortic valve replacement (n = 38). Adjusted R² = 0.53 (P < 0.001). RE, Relative expression normalized to the ribosomal subunit 18S.
and MAPSE) and inversely to the degree of diastolic dysfunction, reflected by the LV filling pressure (E/e').

TGF-β, not only plays the above-mentioned key role in the pathogenesis of cardiac fibrosis, but also transduces hypertrophic signals in cardiomyocytes. Cardiomyocyte-targeted canonical (Smads) and non-canonical (TAK1) TGF-β signalling plays a central role in the mal-adaptive hypertrophic response to sustained pressure overload. Overexpression of TGF-β1 in transgenic mice results in hypertrophic growth of cardiac myocytes accompanied by interstitial and perivascular fibrosis. Studies in vitro also demonstrate that TGF-β directly causes cardiomyocyte hypertrophy and participates in bi-directional regulatory signalling between fibroblasts and cardiomyocytes.

LV hypertrophy, although long considered beneficial to preserve fibre shortening in the afterload excess condition, is now recognized as an independent predictor of cardiovascular events as well as of global and cardiovascular mortality. Persistence of LV hypertrophy after aortic valve replacement in AS patients stands as a limiting factor for short- and long-term outcome, whereas LV mass normalization constitutes an independent positive predictor of long-term survival in multivariate analysis.

BMP-7 has been reported to be involved in cardiac myogenesis in the chick embryo, and BMP type I and type II receptors and signalling pathways are functional in cardiac myocytes from humans, mice, and rats. However, to our knowledge, there are no studies addressing a possible role for BMP-7 in the regulation of cardiomyocyte function and pathophysiology. Our data strongly suggest that BMP-7 exerts an antihypertrophic effect. Such statement is based upon the following findings: (i) In TAC mice, there was an inverse relationship between BMP-7 gene expression and LV mass, which is concordant with the negative correlation between the expressions of BMP-7 and the hypertrophy marker, β-MHC. (ii) The treatment with recombinant BMP-7 after TAC attenuated significantly the degree of LV hypertrophy developed. (iii) BMP-7+/− mice, compared with wild type, exhibited a higher LV wall thickness and LV mass at baseline and, under pressure overload, developed a greater degree of LV hypertrophy with a more concentric geometry. (iv) Regression of LV hypertrophy after de-TAC was improved by recombinant BMP-7, whereas it was hampered by BMP-7 loss of function. (v) Multiple regression analysis in TAC mice showed that myocardial transcript levels of BMP-7 and TGF-β2 constituted significant independent predictors, negative and positive, respectively, which can explain as much as 53% of the variance in PW thickness after 4 weeks of TAC. (vi) rBMP-7 inhibited the capability of TGF-β signalling, more specifically low. Indeed, the regulation of the trophic state of the heart is orchestrated in a complex biological scenario, pro- and anti-remodelling effectors act in an orchestrated manner and none of them isolatedly explains fully the variance in structural, genetic, and functional LV outcomes.

Overall, our findings strongly suggest that, as observed in TAC mice, during the myocardial remodelling process in humans, lower myocardial expression levels of BMP-7 associate to more severe structural damage and greater functional echocardiographic abnormalities.

The putative benefit of the activation of BMP-7 signalling to resolve established organ damage has been investigated in rodent models of renal, hepatic, and pulmonary progressive fibrosis. Most of the results obtained in the experimental model can be translated into a prevalent clinical condition of pressure overload such as aortic valve stenosis. Thus, the LV from severe AS patients exhibited a lower BMP-7 expression and a higher TGF-β to BMP-7 ratio compared with surgical controls and both transcripts correlated inversely. As observed in TAC mice, in the LV from AS patients, BMP-7 mRNA expression levels correlated inversely with the indexed LV mass, and with Col I and Col III. Also, the systolic function in the short axis (LVEF) was directly related with BMP-7 expression. In fact, the correlation coefficients for patients, with the exception of LV mass, were generally low. Indeed, the regulation of the trophic state of the heart involves multiple, co-ordinated, and redundant signalling circuits that are modified during pathological myocardial remodelling. In this complex biological scenario, pro- and anti-remodelling effectors act in an orchestrated manner and none of them isolatedly explains fully the variance in structural, genetic, and functional LV outcomes.
the normal myocardial structure and function after de-TAC. On the contrary, exogenously administered rBMP7 improved some features of the reverse remodelling. Particularly, hypertrophy, chamber dilation, and radial systolic function were significantly improved during the de-TAC recovery period by this treatment.

Reduction of cardiomyocyte volume and LV fibrosis is a usual finding after de-TAC in experimental models of pressure overload. The recovery of the balance between TGF-β- and BMP-7-mediated signals after cessation of haemodynamic overloading could contribute to the reverse remodelling. The correlations between the expression of genes encoding BMP-7 and both sarcomeric and ECM elements support their link with the LV structural changes observed in mice after de-TAC. In line with our present data, Gao et al. in their seminal study described that pressure overload release triggers the reverse remodelling process early after TAC, although various structural, functional, and genetic components display distinct time courses in their reversal towards the baseline levels. Moreover, the time required for recovery of LV structure and function after de-TAC is conditioned by the duration of the preceding pressure overload. Full functional recovery is reached within 6 weeks of de-TAC when TAC is maintained for 4 weeks; on the other hand, after 8 weeks of TAC, restoration of LV structure (particularly the ECM content and organization), chamber geometry, and systolic function require a longer de-TAC period and is less complete. In our study, 4 weeks of de-TAC were not sufficient for the LV to fully reverse hypertrophy, concentric geometry, and MAPSE, a functional parameter strongly linked to myocardial fibrosis.

Clinical evidences support the potential for reverse remodelling in patients with chronic heart failure, even rather severe, who have received medical, device-based, or surgical interventions. In AS patients, Krayenbuehl et al. reported that LV fibrous content decreases significantly later than the regression of myocardial cellular hypertrophy after successful valve replacement. Even 6–7 years after valve replacement, LV structural abnormalities compared with the normal myocardium still persist. Such slow myocardial recovery, after releasing the presumably long-standing injury imposed by pressure overload, emphasizes the need for new therapies that improve reversion towards a normal myocardial phenotype.

There are a number of limitations in this study. Although TAC in mice is the most widely used experimental model of pressure overload hypertrophy, there are several differences with the clinical scenario (age and sex of the affected subjects, cardiovascular risk factors, pharmacological treatments, coronary artery disease, anatomical level, and time course of the stenosis) that prompt to caution when tempted to directly extrapolate experimental results into clinical practice. On the other hand, pressure overload hypertrophy in humans is subjected to idiosyncratic variation between individuals and influenced by various known factors including age, sex, systemic hypertension, coronary artery disease, diabetes, metabolic syndrome, and obesity. As an example, diabetes mellitus, which affects 22% of our cohort, is an acknowledged confounder in the clinical studies in this field. The diabetic heart undergoes peculiar structural changes that are independently linked to heart failure progression and probably underlie an increased susceptibility to pressure overload. Therefore, the impact of this disease and other confounders in our observations is unavoidable.

In summary, our findings support that the imbalance between BMP-7 and TGF-β opposing signals may play an important role in the remodelling response of the heart to the haemodynamic stress. The value of BMP-7 signalling as therapeutic target for the palliative treatment of LV hypertrophy or to improve the reverse remodelling after valve replacement in AS patients deserves further attention.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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