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**A NEW ROLE FOR THE ALDOSTERONE/MINERALOCORTICOID
RECEPTOR PATHWAY IN THE DEVELOPMENT OF MITRAL VALVE
PROLAPSE**

Jaime Ibarrola¹, Amaia Garcia-Peña¹, Lara Matilla¹, Benjamin Bonnard², Rafael Sádaba¹, Vanessa Arrieta¹, Virginia Alvarez¹, Amaya Fernández-Celis¹, Alicia Gainza¹, Adela Navarro¹, Diego Alvarez de la Rosa³, Patrick Rossignol⁴, Frederic Jaisser^{2,4}, and Natalia López-Andrés^{1,4}

¹Cardiovascular Translational Research. Navarrabiomed (Miguel Servet Foundation), Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona. Spain; ²Centre de Recherche des Cordeliers, INSERM, Sorbonne Université, USPC, Université Paris Descartes, Université Paris Diderot, Paris, France; ³Department of Physiology, Institute of Biomedical Technology, University of Laguna, E-38071 La Laguna, Spain. ⁴Université de Lorraine, INSERM, Centre d'Investigations Cliniques-Plurithématique 1433, UMR 1116, CHRU de Nancy, French-Clinical Research Infrastructure Network (F-CRIN) INI-CRCT (Cardiovascular and Renal Clinical Trialists), Nancy, France.

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Corresponding Author: Natalia López-Andrés. Cardiovascular Translational Research. Navarrabiomed (Miguel Servet Foundation). C/Irunlarrea 3, 31008 Pamplona, Spain. Telephone: 34-848428539. Fax: 34-848422300 E-mail: natalia.lopez.andres@navarra.es

ABSTRACT

RATIONALE: Mitral valve prolapse (MVP) is one of the most common valvular disorders. However, the molecular and cellular mechanisms involved in fibromyxomatous changes in the mitral leaflet tissue have not been elucidated. Aldosterone (Aldo) promotes fibrosis in myocardium and mineralocorticoid receptor antagonists (MRAs) improve cardiac function by decreasing cardiac fibrosis.

OBJECTIVE: We investigated the role of the Aldo/MR in the fibromyxomatous modifications associated with MVP.

METHODS AND RESULTS: Aldo enhanced valvular interstitial cell (VIC) activation markers and induced endothelial-mesenchymal transition (EndMT) in valvular endothelial cells (VECs), resulting in increased proteoglycan secretion. MRA blocked all the above effects. Cytokine arrays showed cardiotrophin-1 (CT-1) to be a mediator of Aldo-induced VIC activation and proteoglycan secretion and CD14 to be a mediator of Aldo-induced EndMT and proteoglycan secretion in VECs. In an experimental model of MVP generated by Nordexfenfluramine (NDF) administration, MRA treatment reduced mitral-valve thickness and proteoglycan content. Endothelial-specific MR deletion prevented fibromyxomatous changes induced by NDF administration. Moreover, proteoglycan expression was slightly lower in the mitral valves of MVP patients treated with MRA.

CONCLUSION: These findings demonstrate, for the first time, that the Aldo/MR pathway regulates the phenotypic, molecular, and histological changes of VICs and VECs associated with MVP development. MRA treatment appears to be a promising option to reduce fibromyxomatous alterations in MVP.

KEYWORDS

mitral valve prolapse, aldosterone, mineralocorticoid receptor

TRANSLATIONAL PERSPECTIVE

Mitral valve prolapse (MVP) is the leading cause of isolated mitral regurgitation, requiring surgery. The pathophysiology underlying MVP is still incompletely understood and the discovery of new druggable targets is an unmet clinical need. The novel findings presented herein, obtained using diseased mitral valves, primary human mitral valvular interstitial and endothelial cells, and a pre-clinical model of MVP, suggest that the aldosterone/mineralocorticoid receptor pathway may be a new potential biotarget for mitral valve fibromyxomatous alterations associated with MVP.

NON-STANDARD ABBREVIATIONS AND ACRONYMS

MVP: mitral valve prolapse

aVIC: activated valvular interstitial cell

qVIC: quiescent valvular interstitial cell

VEC: valvular endothelial cell

EndMT: endothelial mesenchymal transition

Aldo: aldosterone

MR: mineralocorticoid receptor

MRA: mineralocorticoid receptor antagonist

Spiro: spironolactone

CT-1: cardiotrophin-1

CD14: cluster of differentiation 14

MMP: matrix metalloproteinase

CD31: cluster of differentiation 31

α -SMA: alpha smooth muscle actin

vWF: Von Willebrand factor

VCAM-1: vascular cell adhesion protein 1

NDF: nordexfenfluramine

INTRODUCTION

Mitral valve prolapse (MVP) is estimated to affect 2 to 3% of the general population and over 144 million individuals worldwide ¹ and is the most common cause of mitral valve surgery. MVP is characterized by progressive increases in the area and length of the mitral leaflet tissue, causing leaflets to billow backwards during ventricular contraction. This causes mitral regurgitation, which may progress and cause heart failure (HF).¹

The most common underlying pathology in the case of MVP is myxomatous degeneration, which is defined by the accumulation of mucopolysaccharides, proteoglycans, and other extracellular matrix (ECM) components, responsible for the floppy valves of MVP.² Several studies have shown up-regulated expression of proteoglycans, such as versican, decorin, and biglycan, in the spongiosa layer in MVP.^{3,4} Valve organization, formed by collagen and elastin, is also highly disrupted.⁵ Two cell populations have been identified within the leaflet tissue that are responsible for the maintenance of homeostasis of the valve, valvular interstitial cells (VICs) and valvular endothelial cells (VECs).^{2,6} MVP is characterized by two regulatory phenomena: i) aberrant activation and differentiation of the VEC monolayer to the activated VIC phenotype through the endothelial to mesenchymal transition (EndMT) and ii) high proliferation and activation of VICs into activated VICs (aVICs).^{7,8} A shift to the aVIC phenotype is a key component that mediates the synthesis of matrix metalloproteinases (MMPs), inflammatory cytokines, proteoglycans, and other extracellular components in the leaflet ^{2,8,9}, reducing structural integrity.⁵ Understanding the early molecular mechanisms that regulate the phenotypic changes of VICs and their role in remodeling and disease progression is challenging and could prevent and/or reverse MVP alterations.

Aldosterone (Aldo) is a mineralocorticoid hormone produced by the adrenal gland.¹⁰ It is the agonist of the mineralocorticoid receptor (MR) and a potent mediator of cardiac remodeling and left ventricular dysfunction.^{11,12} The Aldo/MR pathway is involved in cardiac fibroblast activation,¹³ as well as in endothelial cell differentiation via EndMT.¹⁴ Thus, we hypothesized that the Aldo/MR pathway may play a role in the development of MVP, modulating VIC activation and EndMT and increasing proteoglycan synthesis. Furthermore, the Aldo/MR pathway could be a new biotarget in MVP and its blockade with a MR antagonist (MRA) could prevent the fibromyxomatous mitral valve alterations associated with MVP. We tested this hypothesis using human mitral valve samples, human VICs and VECs, and dedicated animal models of MVP.

METHODS

Detailed methods are available in the supplemental material.

RESULTS

Aldosterone induced VIC activation, VEC differentiation, and proteoglycan secretion

MR was expressed by mitral tissue, VICs and VECs (Supplemental Figure 1). Aldo treatment of VICs decreased the mRNA levels of the quiescent VIC (qVIC) marker chondromodulin-1 (Chm-1) (Supplemental Figure 2A) and induced the expression of the VIC activation markers α -SMA, vimentin, and MMP-2 ($p < 0.05$) at the mRNA (Supplemental Figure 2A) and protein levels (Figure 1A) relative to control conditions. Aldo treatment also increased the mRNA levels of proteoglycans (decorin, biglycan, lumican, aggrecan, syndecan-1, hyaluronan, and versican) (Supplemental Figure 2B) and their secretion (Figure 1B) ($p < 0.05$). All the above effects were blocked by treating the cells with spironolactone (Figures 1A-B) (Supplemental Figure 2A-B).

Aldo treatment of VECs significantly decreased the mRNA (Supplemental Figure 2C) and protein levels (Figure 1C) of the VEC markers CD31, VE-cadherin, and vWF ($p < 0.05$) relative to controls. In addition, Aldo treatment of VECs increased the mRNA (Supplemental Figure 2D) and protein levels (Figure 1C) of the myofibroblast activation markers α -SMA, vimentin, and MMP-2 ($p < 0.05$). Aldo treatment of VECs also increased proteoglycan mRNA (biglycan, lumican, syndecan-1, hyaluronan and versican) (Supplemental Figure 2E) and protein levels (decorin, biglycan and syndecan-1) (Figure 1D) ($p < 0.05$). All these effects were blocked by spironolactone treatment, highlighting the role of the MR.

Soluble factors produced by Aldo-treated VICs or VECs act in a paracrine manner

We examined whether mitral VECs produce factors or signals that can induce VIC activation and proteoglycan secretion by incubating mitral VICs with conditioned media from the culture of mitral VECs previously treated with Aldo. The expression of VIC activation markers was higher ($p < 0.05$) in cells incubated with the conditioned medium from Aldo-treated VECs than VICs treated with conditioned medium from control VECs (Supplemental Figure 3A). Conditioned medium from VECs treated with Aldo significantly enhanced ($p < 0.05$) the expression and secretion of proteoglycans in VICs (Supplemental Figure 3B). Spironolactone blocked these effects.

We also examined whether mitral VICs produce factors or signals that can induce EndMT and proteoglycan secretion by incubating primary mitral VECs with conditioned media from mitral VICs previously treated with Aldo. VECs incubated with Aldo-treated VICs supernatant showed lower expression of VEC markers ($p < 0.05$) (Supplemental Figure 3C) and higher expression of VICs activation markers (Supplemental Figure 3D) than control cells. In addition, the expression and secretion of proteoglycans was also significantly higher ($p < 0.05$) in VECs treated with conditioned medium from Aldo-stimulated VICs than in control cells (Supplemental Figure 3E). Spironolactone blocked all the above effects.

Cardiotrophin-1 is a mediator of Aldo effects in VICs

We analyzed the mechanisms underlying the effects of Aldo on VICs using a cytokine array. Cardiotrophin-1 (CT-1), a cytokine previously shown to be up-regulated by Aldo in other cell types,^{15,16} was one of the main up-regulated cytokines, showing a 35% increase in its expression in Aldo-treated VICs relative to controls (Supplemental Figure 4A-B). CT-1 increase induced by Aldo was blocked by Spironolactone (Supplemental

Figure 4B). We next investigated the effects of CT-1 on VICs. Recombinant CT-1 decreased Chm-1 ($p < 0.05$) and increased VIC activation marker levels ($p < 0.05$) (Supplemental Figure 4C-D). The expression and secretion of proteoglycans was also significantly increased ($p < 0.05$) by CT-1 (Supplemental Figure 4E-F).

We studied whether CT-1 can mediate the effects of Aldo in VICs by CT-1 expression knock-down. Aldo did not decrease Chm-1 nor induce VIC activation markers in CT-1 silenced VICs (Figure 2A, Supplemental Figure 4G). Moreover, Aldo was unable to increase the expression and secretion of proteoglycans in CT-1 knock-down cells (Figure 2B, Supplemental Figure 4H). A scrambled siRNA was used as a control.

CD14 is a mediator of the effects of Aldo in VECs

The cytokine array revealed that Aldo up-regulated CD14 (40%), a ligand of Toll Like Receptor-4 (TLR4), which has been shown to mediate the effects of mineralocorticoids in cardiomyocytes¹⁷ (Supplemental Figure 5A-B). CD14 increase induced by Aldo was blocked by Spironolactone (Supplemental Figure 5B). Aldo did not decrease VE-cadherin levels in CD14 knock-down VECs (Figure 2C, Supplemental Figure 5C). However, Aldo diminished the expression of CD31 (Supplemental Figure 5C). Aldo treatment also did not modify VIC activation marker levels ($p < 0.05$) in CD14 knock-down cells (Figure 2C, Supplemental Figure 5D). VECs silenced for CD14 treated with Aldo also showed lower proteoglycan levels (Figure 2D, Supplemental Figure 5E). A scrambled siRNA was used as a control.

Paracrine interactions between VICs and VECs

In order to clarify whether CT-1 or CD14 are involved in a potential paracrine effect, VECs have been treated with conditioned media obtained from VICs after CT-1 knock-down. VECs treated with conditioned media from CT-1-silenced VICs in the presence

of Aldo presented similar phenotypic characteristics (EndMT markers) and proteoglycans expression and secretion than control VECs (Supplemental Figure 6A). Secondly, VICs have been treated with conditioned media obtained from VECs after CD14 knock-down. VICs treated with conditioned media from CD14-silenced VECs in the presence of Aldo presented similar levels of vimentin and proteoglycans expression and secretion than control VICs (Supplemental Figure 6B).

Effects of spironolactone in a MVP mouse model

We next analyzed the effect of the MRA spironolactone in a mouse model in which MVP is induced by nordexfenfluramine (NDF)¹⁸. In valves from NDF-treated mice, collagen in the fibrosa is fragmented and appeared less dense than in control valves. Moreover, NDF-treated mice showed higher staining for total proteoglycan content than controls. Some of the collagen and the elastic fibers were disoriented and disrupted. Treatment with Spironolactone blocked NDF-induced collagen and elastin alterations as well as proteoglycans accumulation (Figure 3A).

The increase in mitral valve area and thickness ($p < 0.05$) in NDF mice was fully prevented by Spironolactone treatment (Figures 3B-C). Spironolactone treatment prevented the effects of NDF on mitral valve remodeling (Figure 3A).

NDF-treated mice exhibited higher levels of decorin, biglycan, lumican and aggrecan as compared to Controls (Figure 3D). In Spironolactone-treated mice, proteoglycans immunostainings were normalized as compared with NDF-treated mice (Figure 3D).

Moreover, CT-1 and CD14 immunostainings were higher in mitral valves from NDF-treated mice as compared to controls (Supplemental Figure 7A). Spironolactone treatment decreased CT-1 and CD14 expression in mitral valves from NDF-treated mice (Supplemental Figure 7A).

Effects on mitral valve morphology and proteoglycan content in conditional MR-KO mice treated with NDF.

Both mitral valve area and thickness were significantly higher in NDF-treated control mice than in non-treated mice ($p < 0.05$). Endothelial-specific MR deletion (Vecadh-MR-KO) protected the mice from the deleterious effects of NDF (Figure 4A-C). High magnification images showed that collagen fibers were oriented parallel to each other. Moreover, NDF-treated Vecadh-MR-KO mice did not exhibit any differences in proteoglycan levels relative to controls (Figure 4D). Nevertheless, NDF-treated SMA specific MR KO mice (SMA-MR-KO) showed similar mitral valve area and thickness as control mice (Supplemental Figure 8A-B) whereas, NDF-treated SMA-MR-KO exhibited higher proteoglycan content (Supplemental Figure 8C).

Mitral valve remodeling in MV prolapse patients

The baseline characteristics of MVP patients are shown in Table 1. The mean age was 68 ± 10 years and 68% were male, consistent with the typical characteristics of patients with MVP. A significant proportion suffered from concomitant coronary artery disease, hypertension, diabetes mellitus, and hyperlipidemia. Echocardiographic variables were those expected in patients with severe mitral regurgitation.

Within this patient population, we were able to compare nine patients receiving a MRA with 14 patients with similar left ventricular dysfunction who were not receiving a MRA. There were no significant differences between the two groups in terms of clinical variables, other medications, or echocardiographic parameters, with the exception of systolic pulmonary artery pressure. VEC marker levels were similar in the two subgroups (Figure 5A). Patients treated with MRA showed lower mRNA levels of α -

SMA and MMP-2 ($p < 0.05$) (Figure 5B). The expression of CT-1 and CD14 was also lower ($p < 0.05$) in MRA-treated patients than those not receiving MRA treatment (Figure 5C). Proteoglycan expression was slightly reduced in the mitral valves from MVP patients treated with MRA and lumican expression was significantly lower ($p < 0.05$) (Figure 5D). Representative immunohistological images of human mitral valves from MVP patients showed higher VEC marker levels and lower proteoglycan levels in the spongiosa layer of patients treated with MRA than those who did not receive MRA treatment (Figure 5E).

DISCUSSION

The purpose of this study was to investigate the involvement of the Aldo/MR pathway in the development of mitral valve alterations associated with MVP. Our results show that Aldo induces VIC activation and EndMT, increasing the secretion of proteoglycans and ECM proteins. In VICs, the effects of Aldo are mediated by CT-1, whereas the effects of Aldo are mediated by CD14 in VECs. Furthermore, *in vivo*, MR blockade has beneficial effects by preventing mitral valve remodeling associated with MVP. Specific endothelial MR deletion provided protection from the fibromyxomatous alterations associated with MVP. The remodeling process in human mitral valves from MVP patients treated with a MRA was slightly attenuated relative to mitral valves from MVP patients not receiving MRA treatment.

Aldo has a well-established pathophysiological role in hypertension and cardiovascular disease.¹⁹ In addition to its primary function in regulating blood pressure and electrolyte balance, Aldo directly promotes vascular remodeling and profibrotic changes in blood vessels.²⁰ Although VIC activation is characteristic of several forms of valve disease, the stimulus and function of this process is still poorly understood. We demonstrate, for the first time, that Aldo induces VIC activation and VEC differentiation, resulting in an

increase in the level of proteoglycans and ECM components. Conditioned media experiments showed that soluble factors produced by Aldo-treated VICs or VECs act in a paracrine manner, promoting EndMT and VIC activation and proteoglycan secretion. In this study, CT-1 emerges as a mediator of Aldo-induced VIC activation. Indeed, CT-1 has already been described to mediate the effects of Aldo in other cardiac cells^{15,21,22} and has been shown to stimulate myofibroblast differentiation and ECM production in human cardiac fibroblasts.²³ CT-1 does not appear to mediate the effects of Aldo in VECs (data not shown). Our results show that Aldo induces EndMT in VECs via CD14, a glycoprotein classically expressed by monocytes but also found in endothelial cells.²⁴ Of note, CD14 has been described to be a mediator of the chronotropic effects of the Aldo/MR in cardiomyocytes.¹⁷ Classically, the TLR4 signaling pathway is associated with inflammation, but recent studies have confirmed its role in endothelial cell proliferation and differentiation through EndMT.²⁵ In addition, the inhibition of TLR-4 signaling attenuates Aldo-induced EndMT in a rat hypertension model.²⁶ Our results further support the involvement of CD14 and TLR4 signaling on the effects of Aldo in VECs.

Several drugs, such as anorectic compounds, including NDF,²⁷ have been associated with remodeling of the mitral valve. Microscopically, the affected valve leaflets showed typical myxomatous lesions with plaques of proliferative myofibroblasts in an abundant ECM, composed primarily of glycosaminoglycans.¹⁸ These drugs interact with the serotonergic system by targeting 5-HT2 receptor subtypes.²⁸ Studies using microarray technology highlight the contribution of serotonergic 5-HT2B receptors in human myxomatous mitral valve tissues.²⁹ Thus, the up-regulation of 5-HT2 receptors induces VIC activation and increases the expression of proteoglycans.³⁰ Consistent with this evidence, NDF-treated mice showed an increased area and thickness of the mitral valve

leaflets, as well as enhanced proteoglycan content, confirming previous results from Ayme-Dietrich and coworkers.¹⁸ Importantly, our results show that pharmacological intervention using a MRA can prevent the increase in the area and thickness of mitral valve leaflets, as well as the increased expression of proteoglycans. In accordance with our findings, the addition of spironolactone to conventional therapy has been reported to increase survival compared to treatment with conventional therapy alone in dogs with naturally occurring myxomatous mitral valve disease.³¹

NDF failed to increase mitral valve area, thickness, or proteoglycan content in mice specifically lacking endothelial MR expression. However, no changes were observed in the SMA-MR-KO model. Only aVICs are positive for α -SMA but not qVICs. Thus, it is possible that MR activation in both qVICs and VECs allows NDF to induce proteoglycan synthesis, whereas the absence of the MR in VECs could protect them from EndMT, decreasing the number of aVICs in the mitral leaflets and leading to decreased proteoglycan expression. Thus, the MR in VECs is involved in the development of MVP in the serotonin-induced model. More studies are needed to clarify the possible interactions between the MR and the serotonin pathway.

Our results show that the valves from MVP patients treated with MRA showed a modest but significant reduction in proteoglycan content. Our results also provide evidence for a partially beneficial effect of MRAs on proteoglycan content in the mitral valves of patients with the worst prognoses. This opens the possibility of leaflet-specific therapy to improve mitral valve adaptation and reduce regurgitation. Clinical evidence has been accumulating suggesting that Aldo has direct adverse effects on the heart that are independent of its effects on blood pressure, as shown by Savart and colleagues in one of the largest cohort of patients with primary hyperaldosteronism (PA)³². However,

the prevalence of valvular heart disease in PA has never been investigated and studies on this field are needed to establish the relation between PA and MVP.

In conclusion, Aldo/MR modulates mitral valve remodeling, inducing VIC activation and EndMT and increasing proteoglycan deposition (Figure 6). We showed that MRA reduces mitral valve alterations in an experimental model of MVP, suggesting a novel therapeutic approach to reduce mitral valve remodeling associated with MVP.

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LIMITATIONS

This study had several limitations. First, although VICs and VECs were isolated from human valves from MVP patients of similar age with similar risk factors, the cells were not separated by individual or sex. Second, valve function was not evaluated in the MVP animal models. Third, a relatively small number of patients were treated with a MRA in our clinical study due to MRA is not a conventional treatment for MVP patients. In consequence, our cohort of patients treated with MRA are very limited and accordingly, the statistical power is weak. Fourth, the treatment of patients with MRA depends on the clinician's criteria. Considering that this drug is indicated for more advanced stages of HF, it is possible that there is an indication-based bias, *i.e.* MRA treatment acts as a confounding factor by selecting patients with a higher degree of valvular disease, as suggested by the observed higher pulmonary artery pressure. Nonetheless, we observed a reduction in proteoglycan expression in the patients treated with an MRA. The findings of the clinical study are only observational and future

studies are warranted to test whether MRA reduces mitral-valve alterations as well as myocardial alterations in a larger cohort of patients with MVP.

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DISCLOSURES

None.

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TABLES

Table 1. Baseline characteristics of patients

n	103
Age (years \pm SD)	68 \pm 10
Male (%)	68 (65)
Hypertension (%)	59 (56)
Hypercholesterolemia (%)	48 (46)
Diabetes mellitus (%)	9 (9)
Coronary artery disease (%)	22 (21)
Heart failure	
NYHA functional class	
I	9 (9)
II	51 (49)
III	42 (40)
IV	2 (2)
Admission for heart failure (%)	20 (19)
BNP (pg/ml \pm SD)	266 \pm 342
Medications	
ACE inhibitors (%)	38 (36)
ARB (%)	21 (20)
Spirolactone (%)	6 (6)
Eplerenone (%)	3 (3)
Diuretics (%)	80 (76)
Betablockers (%)	52 (50)
Digoxin (%)	18 (17)
Echocardiographic parameters	
LVEF (mean % \pm SD)	60.6 \pm 10.4
Systolic dysfunction (LVEF <60%) (%)	36 (34)
Severe systolic dysfunction (LVEF <35%) (%)	2 (2)
End diastolic diameter (mm \pm SD)	59 \pm 7
Indexed end diastolic diameter (mm/m ² \pm SD)	33 \pm 4
End systolic diameter (mm \pm SD)	40 \pm 8
Indexed end systolic diameter (mm/m ² \pm SD)	23 \pm 7
Systolic pulmonary artery pressure (mmHg \pm SD)	47 \pm 12
MVP etiologic subgroups	
Barlow disease (%)	70 (67)
Fibroelastic deficiency (%)	30 (30)
Syndromic MVP	1 (1)
Associated to hypertrophic cardiomyopathy	2 (2)

Values are mean \pm SD. LVEF = left ventricular ejection fraction; NYHA = New York Heart Association classification of heart failure; ACE= angiotensin converting enzyme; ARB= angiotensin receptor blockers; BNP=brain natriuretic peptide; MVP: mitral valve prolapse

Table 2. Baseline characteristics of patients treated with or without MRA

	MRA	No MRA	p
n	9	14	
Age (years \pm SD)	72 \pm 9	72 \pm 7	1.000
Male (%)	6 (67)	9 (64)	1.000
Hypertension (%)	9 (100)	9 (64)	0.116
Hypercholesterolemia (%)	2 (22)	5 (36)	0.657
Diabetes mellitus (%)	0 (0)	2 (14)	0.502
Coronary artery disease (%)	3 (33)	1 (7)	0.260
Heart failure			
NYHA functional class			
I	0 (0)	0 (0)	1.000
II	3 (33)	8 (57)	0.400
III	5 (55)	6 (43)	0.680
IV	1 (11)	0 (0)	0.391
Admission for heart failure (%)	5 (55)	2 (14)	0.179
BNP (pg/ml \pm SD)	505 \pm 635	320 \pm 433	0.393
Medications			
ACE inhibitors (%)	4 (44)	4 (29)	0.657
ARB (%)	3 (33)	4 (29)	1.000
Spirolactone (%)	7 (78)	0 (0)	n/a
Eplerenone (%)	2 (22)	0 (0)	n/a
Diuretics (%)	9 (100)	10 (71)	0.127
Betablockers (%)	5 (55)	6 (43)	0.680
Digoxin (%)	4 (44)	1 (7)	0.056
Echocardiographic parameters			
LVEF (mean % \pm SD)	55 \pm 16	57 \pm 13	0.734
Systolic dysfunction (LVEF <60%) (%)	5 (55)	7 (50)	1.0
Severe systolic dysfunction (LVEF <35%) (%)	1 (11)	1 (7)	1.0
End diastolic diameter (mm \pm SD)	61 \pm 7	57 \pm 7	0.374
Indexed end diastolic diameter (mm/m ² \pm SD)	33 \pm 4	32 \pm 3	0.697
End systolic diameter (mm \pm SD)	43 \pm 10	39 \pm 10	0.453
Indexed end systolic diameter (mm/m ² \pm SD)	23 \pm 6	22 \pm 3	0.627
Systolic pulmonary artery pressure (mmHg \pm SD)	53 \pm 11	44 \pm 8	0.033

Values are mean \pm SD. LVEF = left ventricular ejection fraction; NYHA = New York Heart Association classification of heart failure; ACE= angiotensin converting enzyme; ARB= angiotensin receptor blockers; BNP=brain natriuretic peptide; MRA=mineralocorticoid receptor antagonist; MVP: mitral valve prolapse; n/a= not applicable

FIGURE LEGENDS

Figure 1. Effects of Aldo and spironolactone on human mitral VICs and VECs.

(A) Quantification of VIC activation marker proteins (α -SMA, vimentin, and MMP-2) in VICs. (B) Quantification of proteoglycan proteins (decorin, biglycan, lumican, syndecan-1, hyaluronan, and versican) in VICs. (C) Quantification of VEC marker proteins (CD31, VE-cadherin and vWF) and VIC activation marker proteins (α -SMA, vimentin and MMP-2) in VECs. (D) Quantification of proteoglycan proteins (decorin, biglycan, lumican, syndecan-1 and hyaluronan) in VECs. The bars of the histograms represent the mean \pm SEM of each group (n=6-12) in arbitrary units (AU) normalized to the signal of stain-free protein gels. *p<0.05 vs. control. [§]p<0.05 vs. Aldo.

Figure 2. The effects of Aldo on VICs are mediated by cardiotrophin-1 and the those on VECs by CD14.

siRNA for CT-1 knock down was used in VICs. (A) The effects of Aldo on VIC activation marker protein levels (α -SMA, vimentin and MMP-2) in CT-1 knockdown cells. (B) The effect of Aldo on proteoglycan protein levels (decorin, biglycan, lumican, syndecan-1 and hyaluronan) in CT-1 knockdown cells. siRNA for CD14 knock down was used in VECs. (C) The effects of Aldo on VEC marker (CD31, VE-cadherin and vWF) and VIC activation marker protein levels (vimentin and MMP-2) in CD14 knockdown cells. (D) The effects of Aldo on proteoglycan protein levels (decorin, biglycan, lumican, and hyaluronan) in CD14 knockdown cells. The bars in the histograms represent the mean \pm SEM of each group (n=6-12) in arbitrary units (AU) normalized to the signal of stain-free protein gels. *p<0.05 vs. control. [§]p<0.05 vs. Aldo.

Figure 3. Effects of spironolactone in a MVP mouse model.

Representative microphotographs of mouse mitral valve sections with Sirius red, Sirius red/Alcian blue

and elastin staining (A). Individual datapoints are shown as circles (control group), squares (NDF group), and triangles (NDF + Spiro group) to represent the mitral valve area (B) and thickness (C). Representative microphotographs of mouse mitral valve sections stained for decorin, biglycan, lumican, and aggrecan (D). Box plots show the individual datapoints and the horizontal bars indicate the mean and SEM in arbitrary units *versus* the control group (n=5-7). Magnifications 10x and 100x (A) and 40x (D). *p<0.05 *vs.* control.

Figure 4. Effects of NDF administration on mitral valve area, thickness, and proteoglycan content in Vecadh-MR-KO mice. Representative microphotographs of mouse mitral valve sections with Sirius red, Sirius red/Alcian blue and elastin stainings (A). Individual datapoints are shown as circles (control group), squares (NDF group), and triangles (Vecadh-MR-KO + NDF group) to represent the mitral valve area (B) and thickness (C). Representative microphotographs of mitral valve sections from Vecadh-MR-KO mice stained for decorin, biglycan, lumican, and aggrecan (D). Box plots show the individual datapoints and horizontal bars indicate the mean and SEM in arbitrary units *versus* the control group (n=5-9). Magnifications 10x and 100x (A) and 40x (D). *p<0.05 *vs.* control.

Figure 5. Differences between MRA-treated/non-treated MVP patients. (A) Quantification of VEC marker mRNA (CD31 and VE-cadherin) in human mitral valves. (B) Quantification of VIC activation marker mRNA (α -SMA, vimentin, and MMP-2) in human mitral valves. (C) Quantification of CT-1 and CD14 mRNA in human mitral valves. (D) Quantification of proteoglycan mRNA (decorin, biglycan, lumican, syndecan-1, hyaluronan, and versican) in human mitral valves. (E) Representative microphotographs of human mitral valve sections stained with Sirius red and Alcian blue and for VCAM-1, CD31, decorin, biglycan, and versican. The bars in the

histogram represent the mean \pm SEM of each group (n=9-14) as a percentage of staining normalized to the signal of stain-free protein gels. Magnification 10x. *p<0.05 vs. without MRA. *Spongiosa layer.

Figure 6. Schematic model of the effects of Aldo in MVP. Proposed scheme in which circulating Aldo induces up-regulation of CD14 in VECs via the MR, leading to activation of TLR-4 signaling, which in turn induces the End-MT to VICs. In VICs, Aldo induces up-regulation of CT-1, causing the activation of qVICs. Moreover, there is a crosstalk between VICs and VECs. The final consequence is increased proliferation of aVICs, with an increase in proteoglycan synthesis that finally leads to the development of MVP. The effects of Aldo can be blocked by MRA.