Biomarker-based phenotyping of myocardial fibrosis identifies patients with heart failure with preserved ejection fraction resistant to the beneficial effects of spironolactone: results from the Aldo-DHF trial.

Susana Ravassa[1*], Tobias Trippel[2], Doris Bach[2], Diana Bachran[2], Arantxa González[1], Begoña López[1], Rolf Wachter[3], Gerd Hasenfuss[3], Christian Delles[4], Anna Dominiczak[4], Burkert Pieske[2,5,6†], Javier Díez[1,7†], Frank Edelmann[2,3,5†]

[1]University of Navarra, CIMA, Program of Cardiovascular Diseases, Pamplona, Spain; IdiSNA, Navarra Institute for Health Research, Pamplona, Spain; CIBERCV, Carlos III Institute of Health, Madrid, Spain; [2]Institute for Internal Medicine and Cardiology, Charité Universitätsmedizin Berlin, Berlin, Germany; DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Berlin, Germany; [3]Department of Cardiology and Pneumology University of Göttingen Medical Centre Göttingen Germany; DZHK (German Centre for Cardiovascular Research), Partner Site Göttingen, Göttingen, Germany; [4]Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK; [5]Berlin Institute of Health (BIH), Berlin, Germany; [6]Deutsches Herzzentrum Berlin (DHZB), Department of Cardiology, Berlin, Germany; [7]University of Navarra Clinic, Department of Cardiology and Cardiac Surgery, Pamplona, Spain;

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†These authors contributed equally to last authorship

*Corresponding author: Program of Cardiovascular Diseases, Centre for Applied Medical Research, University of Navarra, Pamplona, Spain. Tel: +34-948-194700, Fax:+34-948-194716, Email: sravassa@unav.es

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ABSTRACT

Background. Myocardial fibrosis is characterized by excessive cross-linking (CCL) and deposition of collagen type I (CD) and is involved in the left ventricular stiffening and left ventricular diastolic dysfunction (LVDD). We investigated whether the effect of spironolactone on LVDD in patients with heart failure with preserved ejection fraction (HFpEF) depends on its effects on CCL and/or CD.

Methods and Results. We investigated n=381 HFpEF patients from the multicenter, randomized and controlled Aldo-DHF trial with measures of the E:e' ratio. The ratio of serum carboxy-terminal telopeptide of collagen type I to serum matrix metalloproteinase-1 (CITP:MMP-1, an inverse index of myocardial CCL), and serum carboxy-terminal propeptide of procollagen type I (PICP, a direct index of myocardial CD), were determined at baseline and after 1-year treatment with spironolactone 25mg once-daily or placebo. Patients were classified by CITP:MMP-1 and PICP tertiles at baseline. Whereas CITP:MMP-1 tertiles at baseline interacted ($P<0.05$) with spironolactone effect on E:e', PICP tertiles did not. In fact, whereas spironolactone treatment did not modify E:e' in patients with lower CITP:MMP-1 levels, this ratio was significantly reduced in the remaining spironolactone-treated patients. Of interest, PICP was unchanged in patients with lower CITP:MMP-1 levels but was reduced in the remaining spironolactone-treated patients.

Conclusions. A biochemical phenotype of high CCL identifies HFpEF patients resistant to the beneficial effects of spironolactone on LVDD. It is suggested that excessive CCL, which stabilizes collagen type I fibres, diminishes the ability of spironolactone to reduce collagen type I deposition in these patients.

Key words: Heart failure with preserved ejection fraction, spironolactone, left ventricular diastolic function, biomarkers of myocardial fibrosis, carboxy-terminal propeptide of procollagen type I, carboxy-terminal telopeptide of collagen type I, metalloproteinase-1.

Heart failure (HF) with preserved ejection fraction (HFpEF) is currently considered an epidemic, accounting for up to 50% of all HF cases, showing a consistent trend of increasing prevalence over the next years, with significant morbidity and mortality rates.[1,2] Compared to HF with reduced ejection fraction, progress in the treatment of HF has been much less pronounced in patients with HFpEF, in which personalization of pharmacotherapy regimens remains poorly defined.[3-5]

Myocardial fibrosis contributes to ventricular stiffness and impaired relaxation in patients with HFpEF.[6-8] Specifically, excessive myocardial collagen cross-linking, a process catalyzed by the enzyme lysyl oxidase (LOX) which increases the insolubility, stiffness and resistance to degradation of the collagen fibre (mainly type-I),[9] and increased deposition of collagen type I, are related to elevated filling pressures in patients with HFpEF.[10] Therefore, the functional impact of myocardial fibrosis in HFpEF patients is not just a matter of the quantity (i.e., severity of deposition) but also of the quality (i.e. degree of cross-linking among collagen fibrils) of the collagen type I fibres.

The carboxy-terminal propeptide of procollagen type I (PICP) is generated during the extracellular conversion of procollagen type I into collagen type I by the enzyme procollagen carboxy-terminal proteinase.[11] A net release from the heart into the circulation has been reported in HF,[12] suggesting a cardiac origin for systemic PICP in this syndrome. Serum PICP levels correlate with myocardial collagen and collagen type I deposition.[12,13] On the other hand, as collagen cross-linking determines the resistance of the collagen fibre to metalloproteinase (MMP) degradation, the higher the cross-linking of collagen type I fibres, the lower the cleavage of the carboxy-terminal telopeptide of collagen type I (CITP) by the enzyme MMP-1. Thus, serum CITP:MMP-1 ratio has been shown to be inversely

correlated with myocardial collagen cross-linking.[14] Interestingly, the CITP:MMP-1 ratio is independently associated with the risk of HF hospitalization in HF patients.[14]

The Aldo-DHF study, a multicenter, prospective, randomized, double-blind, placebo-controlled trial performed in n=422 ambulatory patients with HFpEF showed that 1-year treatment with spironolactone was associated with a reduction in left ventricular (LV) filling pressures, LV mass and circulating amino-terminal pro-brain natriuretic peptide (NT-proBNP) levels without changes in maximal exercise capacity.[15] It has been suggested that spironolactone-induced improvement of left ventricular diastolic dysfunction (LVDD) may be related to its anti-fibrotic actions.[16-18] However, it is unknown whether spironolactone is able to affect the degree of myocardial collagen type I content, stiffness and resistance to degradation in patients with HFpEF. In addition, it is not known whether these characteristics of the collagen fibre may impact on the spironolactone's capacity to improve LVDD in HFpEF patients. Therefore, the aim of the current analysis was 2-fold: i) assess the impact of spironolactone treatment on biomarkers of myocardial fibrosis, not only in terms of collagen deposition (as assessed by serum PICP) but also in terms of collagen cross-linking (as assessed by serum levels of the CITP:MMP-1 ratio), and ii) investigate whether the characteristics of myocardial fibrosis at baseline interact with spironolactone effects on LVDD.

METHODS

Aldo-DHF was a prospective, randomized, placebo-controlled, double-blind multicentre study. The trial design and primary results of Aldo-DHF have been previously published.[15] Briefly, eligible patients were enrolled and randomized (1:1 ratio) to spironolactone 25mg once daily or matching placebo and followed over 12 months. Patients with stable HFpEF defined as NYHA class II or III heart failure symptoms, LVEF≥50% at rest,

echocardiographic evidence of grade ≥ I diastolic dysfunction or atrial fibrillation (AF), and peak VO_2≤25 mL/kg/min were eligible for participation. Major exclusion criteria included: prior documented LVEF≤40%, significant coronary artery disease, myocardial infarction or coronary artery bypass graft surgery within 3 months, definitive or probable pulmonary disease [vital capacity <80% or forced expiratory volume in 1s (FEV1)<80% or reference values on spirometry], body mass index ≥36 kg/m^2, or serum creatinine > 1.8mg/dL. The study protocol was reviewed and approved by the institutional review board of each participating center, and all patients provided written informed consent prior to enrolment. Aldo-DHF was conducted in accordance with national laws, guidelines for good clinical practice, and the Declaration of Helsinki.

Circulating biomarkers

Biomarker assessments were performed in the serum collected at baseline and at study end 12 months after randomization. Analysis of CITP was performed using an enzyme-linked immunosorbent assay (ELISA) developed by USCN Life Science. The inter-assay and intra-assay coefficients of variation were 10.5% and 11.6%, respectively. The lower limit of detection was 44.3 pg/ml. Total serum MMP-1 was measured by an ELISA method (GE Healthcare). The inter-assay and intra-assay coefficients of variation were 12.4% and 7.0%, respectively. The lower limit of detection was 1.7 ng/ml. CITP and MMP-1 were expressed in molarity and the CITP:MMP-1 ratio was calculated in each patient as previously reported.[14] Serum PICP was measured by using the EIA MicroVue CICP (Quidel Corporation). The inter-assay and intra-assay coefficients of variation were 12.0% and 8.1%, respectively. The lower limit of detection was 0.2 ng/ml.

Statistical analysis

The study population was categorized into tertiles of the serum CITP:MMP-1 ratio and PICP levels at baseline. Linear test for trend were used to assess any tendency across the different subgroups. The distributions of all analyzed biomarkers were normalized by a logarithmic transformation.

Associations between variables at baseline or between changes in variables over 12 months after treatment were assessed using a bivariate correlation (model 1), and were correlated as partial correlation coefficients corrected for sex and age only (model 2), and as partial correlation coefficients corrected for sex, age, AF, mean arterial pressure (MAP), estimated glomerular filtration rate (eGFR), hemoglobin, and NT-proBNP (model 3).[19] The Benjamini and Hochberg multiple-test correction (false discovery rate of 10%) was applied.

Testing for interaction between the effect of treatment (spironolactone or placebo) on LVDD and non-invasively assessed myocardial fibrosis was conducted in patients categorized into tertiles according to serum CITP:MMP-1 ratio or PICP levels by analysis of covariance with the follow-up value of the E:e' ratio as the dependent variable, the baseline E:e' ratio as covariate, and treatment, subgroup variable, and their respective interaction terms as factor variables. Analyses over 12 months of follow-up to evaluate the effects of spironolactone therapy vs placebo were performed by analysis of covariance, binary, or ordinal logistic regression with the follow-up value as the dependent variable, treatment as a factor, and the baseline value as the continuous or categorical covariate, as appropriate for quantitative, binary, or ordinal categorical variables, on each subgroup of patients. Between groups comparisons are presented as mean differences or odds ratios. Biomarkers were analyzed on the logarithmic scale, and the results were transformed back by the exponential function, leading to a geometric mean instead of a mean difference. *P*-values for the interaction term of treatment with E:e' and PICP were calculated in each

subgroup of patients according to tertiles of the serum levels of CITP:MMP-1 ratio using a univariate analysis of variance (ANOVA) model with repeated measurements.

Values are expressed as mean±SD and median (interquartile range [IQR]) for continuous variables, and as numbers and percentages for categorical variables. A *P* value ≤0.05 was considered statistically significant, and all statistical tests were two-sided. The statistical analyses were performed by using SPSS (15.0 version) and STATA (12.1 version).

RESULTS

A total of n=422 patients were enrolled in the Aldo-DHF trial from March 2007 to April 2011. Of these, n=415 had available blood samples and n=381 had values of CITP, MMP-1 and PICP at baseline (Fig. S1, baseline sample set [BSS]). In addition, values of PICP and of CITP and MMP-1 were available at 12 months in n=370 patients (Fig. S1, follow-up sample set [FSS]).

Analyses at baseline

In the BSS, the distribution of serum CITP:MMP-1, as a marker of collagen cross-linking, departed from normality and was positively skewed (P<0.0001). The median of serum CITP:MMP-1 was 3.91 (IQR: 1.86-6.68). Patients were categorized at baseline according to tertiles of increasing serum CITP:MMP-1 ratio levels. Diastolic and mean blood pressure increased across CITP:MMP-1 tertiles probably due to a decreasing percentage of patients treated with angiotensin converting enzyme inhibitors or angiotensin II type-1 receptor blockers along the tertiles (Table 1). Of interest, patients showed better diastolic function (i.e. lower E:e' ratio) and improved parameters of exercise capacity (i.e. lower VE/VCO2 slope and higher maximum exercise duration and Borg score) with increasing CITP:MMP-1 values (Table 2). Although weak, a continuous inverse association was found between

CITP:MMP-1 ratio and the E:e' ratio, not reaching statistical significance after adjustment for confounders (Table S1). In addition, a direct continuous significant correlation was found between CITP:MMP-1 ratio and the Borg score, independently of confounders (Table S1).

In all patients, the distribution of serum PICP, as a marker of collagen deposition, departed from normality and was positively skewed (P<0.0001). The median of serum PICP was 111 ng/ml (IQR: 92.0-136). Patients were categorized at baseline according to tertiles of increasing PICP serum levels (Tables S2 and S3). Sodium levels were greater with higher PICP values (Table S2). Left atrial (LA) volume index, E-wave velocity and NT-proBNP increased across tertiles of PICP (Table S3). Continuous direct associations were found between PICP and LA volume index and NT-proBNP, independently of confounders in models 2 and 3 (Table S1).

Analyses after twelve months of treatment

Whole cohort

Over the course of 12 months, treatment with spironolactone showed similar effects in the subgroup of patients included in the FSS, as in those reported in the original Aldo-DHF study[15] (Table S4). Levels of serum PICP decreased (*P*=0.050) after 12 months of treatment with spironolactone compared with placebo (Table S4, Fig S2A). The serum CITP:MMP-1 ratio remained unchanged after 12 months of treatment (Table S4, Fig S2B). A weak correlation was found between changes in the E:e' ratio and changes in serum PICP in all patients in the FSS set (r=0.111, *P*=0.033) (Fig S2C) that remained statistically significant after adjustment for confounders in model 2 (*P*=0.028) but did not reach statistical significance after full adjustment in model 3 (*P*=0.065). In addition, changes in serum PICP

were directly correlated with changes in serum NT-proBNP (r=0.146, *P*=0.005) (Fig S2D) independently of confounders in models 2 and 3 ($P \leq 0.022$).

Analyses by tertiles

Tests for interaction to globally assess the heterogeneity of treatment effects on the E:e' ratio among tertiles of the baseline variables CITP:MMP-1 and PICP have been performed. A significant interaction was detected between CITP:MMP-1 tertiles and the effect of spironolactone on E:e' (*P*=0.014). Further analyses adjusting by age and prevalence of coronary artery disease at baseline confirmed this interaction. No significant interaction was detected when the categorization was based on PICP tertiles.

Once the heterogeneity of treatment was confirmed for the CITP:MMP-1-based categorization, we analyzed treatment effects within each one of the CITP:MMP-1 tertiles. At baseline, no differences were found in any of the demographic, clinical and biochemical parameters between patients treated with spironolactone or placebo, in any of the CITP:MMP-1 tertile-based subgroups (Table S5 and S6). After 12 months of treatment, spironolactone did not modify the E:e' ratio in patients with low CITP:MMP-1 ratio levels (1st tertile, Fig. 1A) (Table 3), but was associated with a significant decrease in the E:e' ratio in patients with medium or high CITP:MMP-1 ratio values (2nd and 3rd tertiles, Fig 1B and 1C, respectively) as compared with placebo. In patients with low levels of the CITP:MMP-1 ratio, the circulating levels of PICP were unchanged with spironolactone (Fig 2A), whereas in patients with medium or high CITP:MMP-1 ratio values, the decline in E:e' was accompanied by a reduction in the circulating levels of PICP as compared with baseline (2nd and 3rd tertiles, Fig 2B and 2C, respectively). Of interest, only spironolactone-treated patients with the highest CITP:MMP-1 ratio values showed reduction in NT-proBNP circulating levels with treatment, whereas placebo-treated patients did show

similar peptide levels at the end of treatment as compared with baseline (Fig S3). No effects of treatment on NT-proBNP circulating levels were observed in the remaining patients (Fig S3).

In Aldo-DHF, a slight reduction in 6 minute walk distance after 1 year spironolactone versus placebo was observed.[15] Interestingly, this decline in exercise capacity was detectable in those patients with a low CITP:MMP-1 ratio (Table 3).

DISCUSSION

The main findings of this study are the following: 1) in HFpEF patients, spironolactone therapy reduces serum levels of the biomarker of collagen deposition, PICP, but does not modify the serum levels of the biomarker of collagen cross-linking, the CITP:MMP-1 ratio; 2) spironolactone did not improve diastolic function in the subset of patients with lower CITP:MMP-1 ratio (i.e., a higher degree of collagen cross-linking) and 3) spironolactone does not reduce serum levels of PICP in the subset of patients with lower CITP:MMP-1 ratio. To our knowledge, this is the first study analyzing the effects of spironolactone on the quantity and the quality of the myocardial collagen network, as assessed non-invasively by using circulating biomarkers that exhibit histologically proven associations with these characteristics of the collagen tissue, in patients with HFpEF. In addition, to the best of our knowledge, this is the first time that the impact of the characteristics of myocardial fibrosis on the effects of spironolactone on LV diastolic function has been evaluated.

We have observed that, at baseline, HFpEF patients with low serum levels of the CITP:MMP-1 ratio exhibit higher E:e' ratio. These findings support previous clinical evidences of an association between myocardial collagen cross-linking and increased LV filling pressures in patients with HF[20] and specifically in patients with HFpEF.[10] In addition, we describe for the first time that low levels of the CITP:MMP-1 ratio associate

with parameters reflecting worse cardiopulmonary exercise performance. On the other hand, we found that PICP directly associates with NT-proBNP and LA volume index, in HFpEF patients at baseline. In this regard, associations of PICP with NT-proBNP and LA size have been previously reported in HF patients with reduced EF.[21,22] Overall, the observations at baseline suggest that myocardial fibrosis, considering its qualitative and quantitative aspects, is related to LVDD and lower exercise performance in patients with HFpEF.

Previous clinical studies demonstrate that spironolactone reduces the circulating serum levels of PICP in asymptomatic patients with LVDD.[23] This study reports for the first time that spironolactone treatment is associated with a reduction in serum PICP in patients with HFpEF. In addition, we have observed that treatment with spironolactone is not able to modify the serum levels of CITP:MMP-1 in these patients. Therefore, spironolactone seems capable of affecting the myocardial collagen content but unable to modify the characteristics of the collagen fibre that determine its insolubility, stiffness and resistance to degradation. Experimental studies demonstrate that aldosterone increases the expression of LOX[24-26] (the myocardial enzyme responsible for collagen cross-linking), and the tissue inhibitor of metalloproteinases-1 (TIMP-1),[27] indicating that this hormone may also contribute to myocardial fibrosis by incrementing the stability of the collagen fibre. In this regard, it is plausible that incomplete mineralocorticoid receptor blockade with spironolactone in the myocardium might still allow for aldosterone- or another pro-fibrotic molecules-induced collagen cross-linking and deposition. It is uncertain whether agents with greater mineralocorticoid receptor blockage at the myocardial level may be able to inhibit aldosterone actions with sufficient efficacy to influence on the quantitative and

qualitative characteristics of the myocardial collagen network. These possibilities are speculative and further studies are necessary to fully clarify these aspects.

Interestingly, we have observed that spironolactone did not improve LV diastolic function and failed to reduce collagen deposition (as assessed by serum PICP) in patients exhibiting lower CITP:MMP-1 serum levels, or in other words, higher collagen cross-linking, at baseline (see Figure 3). Given that the degree of cross-linking is associated with high filling pressures in HFpEF patients,[10] it is tempting to speculate that the incapacity of spironolactone to improve LV diastolic function in patients with high cross-linking may be related to its inability to affect the stability of the collagen fibre. Therefore, these results suggest that spironolactone could be a more successful treatment in HFpEF patients with a phenotype indicative of a non-excessive degree of collagen cross-linking, in which a higher efficacy to modulate collagen metabolism and improve LV function may be achieved.

In the Aldo-DHF original study,[15] spironolactone was associated with a subtle reduction in submaximal exercise capacity. Interestingly, the current subanalysis reveals that only in patients with high collagen cross-linking, exhibiting symptoms of altered cardiopulmonary exercise performance at baseline, spironolactone treatment is associated with a reduction of the 6-minute walk distance. In this regard, we may speculate that those patients with high collagen cross-linking exhibiting resistance to spironolactone-induced improvement on LV diastolic function may be more susceptible to the treatment side-effects of the drug on submaximal exercise capacity.

Of interest, some evidence suggests that other therapeutic options are able to target the excess of collagen cross-linking in the myocardium. For instance, the loop diuretic torasemide is associated with significant reductions in both the severity of the deposition of collagen type I fibres and the degree of myocardial cross-linking, as well as with significant

improvement of LV function and NYHA functional class in patients with HF.[13,28] None of these effects were observed in furosemide-treated HF patients.[13,28] In addition, the losartan metabolite EXP3179 prevents excessive myocardial collagen cross-linking, among other anti-fibrotic actions, in a model of experimental hypertension.[29] Collectively, these findings allow us to suggest further clinical designs to investigate whether these compounds may be optional drugs to complement therapeutic strategies for the personalized treatment of HFpEF patients with a biochemical phenotype indicative of excessive collagen cross-linking.

Some limitations need to be acknowledged. First, this is a post-hoc analysis re-evaluating the Aldo-DHF clinical trial by subgroups. Clinical trial subanalyses are prone to false negative results since they may be inadequately powered to uncover treatment effect differences, even in the presence of true treatment effect modification. Nonetheless, these analyses revealed significant treatment effect differences in subgroups exhibiting similar sample size. Second, the reduction in sample size due to CITP:MMP-1 tertile categorization may have affected the statistical power to detect significant differences in between-subject analyses comparing spironolactone vs placebo effects on the biomarkers PICP and NT-proBNP. Nonetheless, the less conservative within-subject comparisons did detect significant effects of spironolactone on these biomarkers. Third, covariates unaccounted for in the analyses and potential problems related with multiplicity could have influenced the findings obtained. Fourth, these findings have not been validated in an independent cohort. Therefore, we believe that the results showing the impact of collagen cross-linking on the spironolactone effects on LV dysfunction and collagen deposition should be hypothesis-generating and need to be confirmed in larger prospectively designed studies.

In conclusion, this study shows for the first time that spironolactone is unable to modify the circulating levels of a biomarker of myocardial collagen cross-linking, the CITP:MMP-1 ratio, in HFpEF patients. In addition, we describe for the first time that patients with low serum levels of this biomarker before treatment, indicative of a high degree of myocardial collagen cross-linking, do not respond to spironolactone in terms of LV diastolic function improvement and reduction of the myocardial collagen content (see figure 3). On the whole, these data suggest that the ability of spironolactone to reduce myocardial fibrosis and improve LV diastolic function is determined by the stability of the collagen fibre in HFpEF patients. These findings support the notion that a precise biomarker-based phenotyping of patients with HFpEF is critical to advance in the field of HFpEF therapy.

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LEGENDS

Figure 1. Peak early transmitral ventricular filling velocity to early diastolic tissue Doppler velocity (E:e') according to study treatment in patients with low (*A*), medium (*B*) and high (*C*) serum carboxy-terminal telopeptide of collagen type I to matrix metalloproteinase-1 ratio levels as classified by tertiles. Data are expressed as mean values and 95% confidence interval at baseline and at 12 months in patients treated with placebo (○) or with spironolactone (●). *P<0.05 spironolactone at 12 month vs baseline, **P<0.01 spironolactone at 12 month vs baseline.

Figure 2. Serum carboxy-terminal propeptide of procollagen type I (PICP) levels (log) according to study treatment in patients with low (*A*), medium (*B*) and high (*C*) serum carboxy-terminal telopeptide of collagen type I to matrix metalloproteinase-1 ratio levels as classified by tertiles. Data are expressed as mean values and 95% confidence interval at baseline and at 12 months in patients treated with placebo (○) or with spironolactone (●). Medians (interquartile range) for values at baseline and at 12 months are shown below each graph *P<0.01 spironolactone at 12 month vs baseline.

Figure 3. Diagrammatic representation of the spironolactone effects in patients with low or high levels of myocardial collagen cross-linking, as assessed by high or low circulating values of the carboxy-terminal telopeptide of collagen type I to the metalloproteinase-1 (CITP:MMP-1) ratio, respectively. In the process of degradation of the collagen type I fibre within the myocardial interstitum, MMP-1 cleaves a small C-terminal telopeptide or CITP. A higher degree of cross-linking between collagen type I fibrils increases their resistance to degradation by MMP-1, generating a lower amount of CITP. Both MMP-1 and CITP reach

the systemic circulating and thus can be measured by specific enzyme-linked immunosorbent assay methods. Only in patients with a high CITP:MMP-1 ratio, indicative of a low collagen-cross-linking and therefore a lower resistance of the collagen type I fibre to degradation, treatment with spironolactone is able to reduce myocardial collagen content (as assessed by the biomarker of myocardial collagen content carboxy-terminal propeptide of procollagen type I or PICP), and improve diastolic function (as assessed by the E/e' ratio).

Table 1. Demographic, clinical and biochemical characteristics at baseline in all patients and in patients classified according to CITP:MMP1 ratio tertiles

	Total (n=381)	CITP:MMP-1 ratio tertiles			*P* for trend
		1st (n=127)	2nd (n=127)	3rd (n=127)	
CITP:MMP-1 ratio		<2.50	2.50-5.49	>5.49	
Demographics					
Age, years	67.1 ± 7.6	68.0 ± 7.5	67.1 ± 7.8	66.1 ± 7.6	0.052
Male gender, n (%)	182 (47.8)	61 (48.0)	61 (48.0)	60 (47.2)	0.90
Medical History, n (%)					
Hospitalized within 12 months	143 (37.5)	48 (37.8)	48 (37.8)	47 (37.0)	0.90
Coronary artery disease	152 (39.9)	59 (46.5)	50 (39.4)	43 (33.9)	0.041
History of myocardial infarction	63 (16.5)	29 (22.8)	16 (12.6)	18 (14.2)	0.06
Atrial fibrillation	63 (16.5)	25 (19.7)	20 (15.7)	18 (14.2)	0.24
Hypertension	349 (91.6)	118 (92.9)	119 (93.7)	112 (88.2)	0.18
Hyperlipidaemia	225 (59.1)	72 (56.7)	77 (60.6)	76 (59.8)	0.61
Diabetes mellitus	62 (16.3)	22 (17.3)	20 (15.7)	20 (15.7)	0.73
Cerebrovascular diseases	40 (10.5)	16 (12.6)	14 (11.0)	10 (7.9)	0.22
Peripheral arterial occlusive disease	15 (3.9)	2 (1.6)	9 (7.1)	4 (3.1)	0.52
COPD	13 (3.4)	4 (3.1)	5 (3.9)	4 (3.1)	0.99
Physical examination					
BMI, kg/m^2	29.0 ± 3.5	28.6 ± 3.7	29.5 ± 3.4	28.9 ± 3.5	0.43
SBP, mmHg	136 ± 18.3	133 ± 19.0	140 ± 18.1	136 ± 17.5	0.19
DBP, mmHg	79.3 ± 11.0	76.3 ± 10.4	80.4 ± 11.5	81.2 ± 10.4	0.0004
MAP, mmHg	98.2 ± 11.8	95.2 ± 11.5	99.6 ± 11.9	99.6 ± 11.6	0.003
HR, beats/min	66.7 ± 11.6	65.7 ± 10.1	67.0 ± 12.5	67.5 ± 11.9	0.23
Signs and symptoms, n (%)					
NYHA class					
II	326 (85.6)	111 (87.4)	104 (81.9)	111 (87.4)	0.99
III	55 (14.4)	16 (12.6)	23 (18.1)	16 (12.6)	
Peripheral edema	154 (40.4)	52 (40.9)	52 (40.9)	50 (39.4)	0.80
Paroxysmal nocturnal dyspnoea	66 (17.3)	17 (13.4)	23 (18.1)	26 (20.5)	0.14
Nocturnal cough	60 (15.7)	22 (17.3)	19 (15.0)	19 (15.0)	0.50
Laboratory measures					
Hemoglobin, g/dL	13.8 ± 1.2	13.6 ± 1.2	13.8 ± 1.1	13.9 ± 1.2	0.08
Sodium, mmol/L	140 ± 3.0	140 ± 3.2	141 ± 2.7	140 ± 3.0	0.75
Potassium, mmol/L	4.2 ± 0.4	4.2 ± 0.4	4.2 ± 0.4	4.2 ± 0.4	0.49
eGFR, mL/min/1.73 m^2	75.7 ± 19.2	73.2 ± 20.3	76.2 ± 17.4	77.7 ± 19.5	0.06
Baseline medications, n (%)					
ACE inhibitor or ARB	292 (76.6)	107 (84.3)	97 (76.4)	88 (69.3)	0.005
Beta-blocker	275 (72.2)	95 (74.8)	91 (71.7)	89 (70.1)	0.40
Diuretics	202 (53.0)	71 (55.9)	70 (55.1)	61 (48.0)	0.21
Calcium channel blocker	95 (24.9)	26 (20.5)	37 (29.1)	32 (25.2)	0.39
Lipid-lowering drug	208 (54.6)	71 (55.9)	69 (54.3)	68 (53.5)	0.71
Spironolactone	195 (51.2)	68 (53.5)	61 (48.0)	66 (52.0)	0.80

CITP means carboxy-terminal telopeptide of collagen type I; MMP-1, matrix metalloproteinase-1; COPD, chronic obstructive pulmonary disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; NYHA, New York Heart Association; eGFR, estimated glomerular filtration rate; ACE, angiotensin converting enzyme; ARB, angiotensin II type 1 receptor blockers . Values are expressed as mean ± SD and categorical variables as numbers and percentages.

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Table 2. Echocardiography, biomarkers, exercise testing and quality of life at baseline in all patients and in patients categorized according to CITP:MMP1 ratio tertiles

	Total (n=381)	CITP:MMP-1 ratio tertiles			*P for trend*
		1st (n=127)	2nd (n=127)	3rd (n=127)	
CITP:MMP-1 ratio		<2.50	2.50-5.49	>5.49	
Echocardiography					
LVEF, %	67.7 ± 7.9	67.9 ± 8.2	66.5 ± 7.0	68.6 ± 8.3	0.50
LVEDD, mm	46.6 ± 6.3	46.3 ± 6.7	46.7 ± 5.7	46.7 ± 6.5	0.56
LVESD, mm	25.2 ± 6.5	25.4 ± 6.1	25.4 ± 6.7	24.8 ± 6.7	0.51
LVMI, g/m^2	109 ± 28.4	110 ± 33.0	107 ± 24.5	108 ± 27.3	0.60
RWT	0.50 ± 0.09	0.51 ± 0.09	0.50 ± 0.08	0.50 ± 0.09	0.71
Left atrial volume index, mL/m^2	28.3 ± 8.5	29.1 ± 7.9	27.5 ± 8.1	28.3 ± 9.3	0.44
E-wave velocity, cm/s	72.7 ± 17.7	73.1 ± 16.4	73.5 ± 17.0	71.5 ± 19.5	0.45
Medial e' wave velocity, cm/s	7.1 ± 1.5	6.9 ± 1.4	7.2 ± 1.7	7.2 ± 1.5	0.11
E/e' ratio	12.7 ± 3.6	13.1 ± 3.6	12.6 ± 3.7	12.3 ± 3.6	0.049
E:A ratio	0.90 ± 0.32	0.90 ± 0.33	0.87 ± 0.26	0.93 ± 0.36	0.59
IVRT, ms	87.1 ± 25.1	90.0 ± 26.4	86.1 ± 24.6	85.2 ± 24.3	0.13
DT, ms	244 ± 63.2	247 ± 64.1	248 ± 68.3	236 ± 56.3	0.15
Biomarkers, median (IQR)					
NT-proBNP, ng/L	166 (84.3-312)	187 (111-331)	142 (65.9-276)	165 (86.0-332)	0.43
PICP, ng/mL	111 (92.0-136)	111 (88.5-135)	111 (93.2-136)	113 (92.3-138)	0.84
Cardiopulmonary exercise testing					
Maximum exercise duration, s	539 ± 176	522 ± 167	530 ± 175	566 ± 184	0.043
Peak VO_2, mL/min/kg	16.3 ± 3.5	16.3 ± 3.5	16.0 ± 3.4	16.5 ± 3.5	0.56
$ATVO_2$, mL/min/kg	11.5 ± 3.2	11.5 ± 3.2	11.5 ± 3.2	11.5 ± 3.4	0.87
V_E/VCO_2 slope	30.0 ± 4.2	30.8 ± 4.5	29.6 ± 4.1	29.7 ± 4.0	0.027
Borg scale	5.2 ± 1.8	4.9 ± 1.8	5.2 ± 1.8	5.5 ± 1.8	0.005
Six-minute walk test					
Walk distance, m	535 ± 78.2	532 ± 78.8	536 ± 69.9	539 ± 85.8	0.46
Health-related quality of life scores					
SF-36 physical function score	62.7 ± 22.2	62.2 ± 21.1	61.3 ± 23.5	64.7 ± 22.0	0.39
PHQ-Sum	5.7 ± 4.2	5.7 ± 4.2	5.5 ± 4.3	5.8 ± 4.1	0.98

CITP means carboxy-terminal telopeptide of collagen type I; MMP-1, matrix metalloproteinase-1; LVEF, left ventricular (LV) ejection fraction; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVMI, LV mass index; RWT, relative wall thickness; E, peak early transmitral ventricular filling velocity; e', early diastolic tissue Doppler velocity; A, peak atrial transmitral ventricular filling velocity; IVRT, isovolumetric relaxation time; DT, deceleration time; NT-proBNP, amino-terminal pro-brain natriuretic peptide; PICP, carboxy-terminal propeptide of procollagen type I; VO_2, oxygen consumption; $ATVO_2$, oxygen consumption at anaerobic threshold; V_E, expired volume per unit time; VCO_2, volume of expired carbon dioxide; SF-36, short form 36; PHQ-Sum, Patient Health Questionnaire sum score. Higher values indicate better performance for LVEF, medial e' wave velocity, duration of exercise, peak VO_2, $ATVO_2$, Borg scale, walk distance and SF-36 physical function score. Lower values indicate better performance for left atrial volume index, E/e' (medial) velocity ratio, VE/VCO2 slope and PHQ-Sum. Values are expressed as mean ± SD, median and interquartile range (IQR) and categorical variables as numbers and percentages.

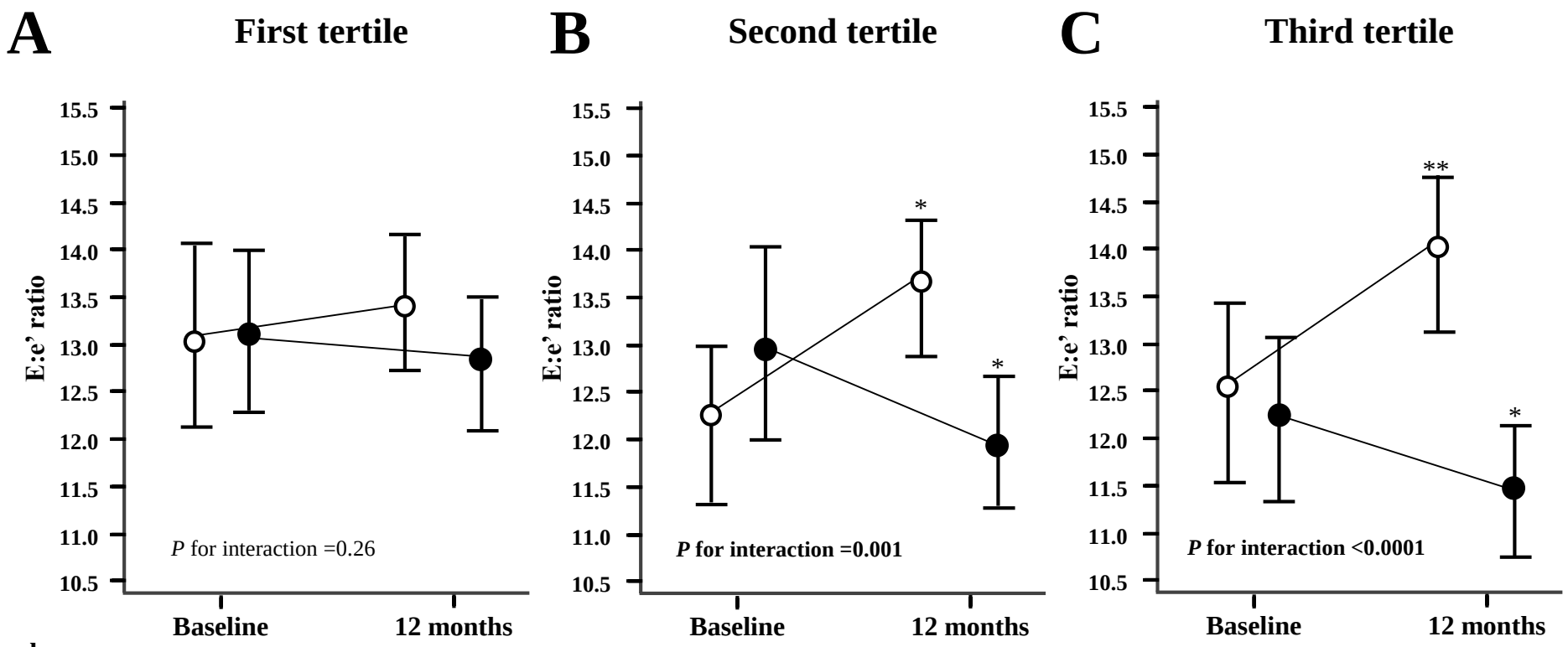
Table 3. Spironolactone versus placebo differences after 12 months of treatment in patients categorized according to tertiles of the CITP:MMP-1 ratio

	1st tertile (n=121)		2nd tertile (n=125)		3er tertile (124)	
	Spironolactone(n=65)-Placebo(n=56)[a]		Spironolactone (n=60)-Placebo (65)[a]		Spironolactone(n=65)-Placebo(n=59)[a]	
	Difference (95% CI)	*P* value	Difference (95% CI)	*P* value	Difference (95% CI)	*P* value
CITP:MMP-1 ratio	<2.50		2.50-5.49		>5.49	
Primary endpoints						
E/e' (medial) velocity ratio	-0.63 (-1.67 to 0.41)	0.23	-1.63 (-2.60 to -0.66)	0.001	-2.46 (-3.48 to -1.44)	<0.0001
Peak VO_2, mL/min/kg	0.19 (-1.05 to 1.43)	0.76	-0.03 (-1.17 to 1.24)	0.96	-0.26 (-1.56 to 1.04)	0.69
Six-minute walk test						
Walk distance, m	-29.2 (-48.2 to -10.3)	0.003	-16.0 (-45.1 to 13.0)	0.28	-8.27 (-25.2 to 8.63)	0.34
Other clinical variables						
NYHA class, n (%)						
I						
II	0.81 (0.25 to 2.64)[b]	0.73	1.70 (0.61 to 4.73)[b]	0.31	1.00 (0.30 to 3.37)[b]	0.99
III						
Peripheral edema, n (%)	0.70 (0.29 to 1.69)[b]	0.43	0.55 (0.23 to 1.32)[b]	0.18	0.92 (0.34 to 2.44)[b]	0.92
SBP, mm Hg	-5.97 (-11.6 to -0.30)	0.039	-9.86 (-15.3 to -4.42)	0.0004	-8.43 (-13.0 to -3.83)	0.0004
DBP, mm Hg	-2.79 (-6.05 to 0.48)	0.09	-3.21 (-6.19 to -0.24)	0.035	-4.28 (-7.59 to -0.97)	0.012
HR, beats/min	0.30 (-3.24 to 3.83)	0.87	1.66 (-1.42 to 4.73)	0.29	1.63 (-1.48 to 4.74)	0.30
Sodium, mmol/L	-0.60 (-1.49 to 0.28)	0.18	-1.32 (-2.25 to -0.39)	0.006	-0.95 (-1.87 to -0.04)	0.041
Potassium, mmol/L	0.24 (0.09 to 0.39)	0.002	0.31 (0.20 to 0.42)	<0.0001	0.18 (0.05 to 0.30)	0.007
Hemoglobin, g/dL	-0.55 (-0.97 to -0.13)	0.011	-0.25 (-0.52 to 0.02)	0.07	-0.13 (-0.38 to 0.12)	0.31
eGFR, mL/min/1.73 m^2	-4.49 (-7.89 to -1.09)	0.010	-6.79 (-10.5 to -3.05)	0.0005	-3.42 (-6.89 to 0.05)	0.053
Other echocardiographic variables						
E-wave velocity, cm/s	-4.39 (-9.33 to 0.55)	0.08	-3.75 (-8.25 to 0.75)	0.10	-4.10 (-8.63 to 0.42)	0.07
Medial e' wave velocity, cm/s	0.33 (-0.12 to 1.77)	0.15	0.16 (-0.24 to 0.56)	0.44	0.63 (0.19 to 1.06)	0.005
E/A velocity ratio	-0.05 (-0.15 to 0.05)	0.29	-0.04 (-0.12 to 0.04)	0.30	-0.03 (-0.12 to 0.06)	0.47
IVRT, ms	7.52 (-2.13 to 17.2)	0.13	-9.11 (-17.5 to -0.76)	0.033	-5.59 (-14.2 to 3.02)	0.20
DT, ms	13.2 (-9.61 to 36.0)	0.25	8.08 (-12.7 to 28.9)	0.44	-7.41 (-28.9 to 14.1)	0.50
LVEF, %	0.76 (-1.94 to 3.45)	0.58	2.48 (-0.27 to 5.24)	0.08	2.10 (-0.77 to 4.98)	0.15
LVMI, g/m^2	-6.93 (-15.1 to 1.21)	0.10	-8.47 (-16.1 to -0.84)	0.030	-3.23 (-12.1 to 5.61)	0.47
Left atrial volume index, mL/m^2	-1.09 (-3.01 to 0.83)	0.26	-0.65 (-2.49 to 1.19)	0.49	0.67 (-1.60 to 2.94)	0.56
LVEDD, mm	-1.49 (-3.64 to 0.67)	0.17	-2.23 (-4.11 to -0.35)	0.021	-0.88 (-2.88 to 1.12)	0.39
LVESD, mm	-0.26 (-2.18 to 1.66)	0.79	-0.66 (-2.70 to -1.39)	0.53	-1.24 (-3.37 to 0.89)	0.25
Biomarkers, median (IQR)						
NT-proBNP, ng/L	0.81 (0.64 to 1.03)[c]	0.08	0.89 (0.68 to 1.16)[c]	0.39	0.84 (0.69 to 1.01)[c]	0.06

PICP, ng/mL	1.05 (0.96 to 1.14)[c]	0.28	0.88 (0.81 to 0.95)[c]	0.002	0.94 (0.87 to 1.02)[c]	0.15
CITP:MMP-1 ratio	0.94 (0.72 to 1.22)[c]	0.63	0.97 (0.79 to 1.20)[c]	0.81	0.94 (0.76 to 1.16)[c]	0.77
Cardiopulmonary exercise testing						
Maximum exercise duration, s	-6.28 (-44.7 to 32.1)	0.75	28.31 (-11.3 to 67.9)	0.16	-4.47 (-40.9 to 31.9)	0.81
$ATVO_2$, mL/min/kg	0.11 (-1.18 to 1.40)	0.87	-0.74 (-1.75 to 0.27)	0.15	-0.69 (-1.94 to 0.56)	0.28
V_E/VCO_2 slope	0.97 (-0.38 to 2.32)	0.16	0.89 (-0.41 to 2.20)	0.18	0.71 (-0.34 to 1.76)	0.18
Borg scale	-0.06 (-0.72 to 0.61)	0.86	0.13 (-0.40 to 0.67)	0.62	0.12 (-0.72 to 0.96)	0.78
Health-related quality of life scores						
SF-36 physical function score	2.45 (-3.29 to 8.20)	0.40	-5.39 (-11.6 to 0.80)	0.09	6.55 (0.78 to 12.3)	0.026
PHQ-Sum	-0.48 (-1.53 to 0.57)	0.63	0.87 (-0.47 to 2.20)	0.20	-0.82 (-1.79 to 0.15)	0.10

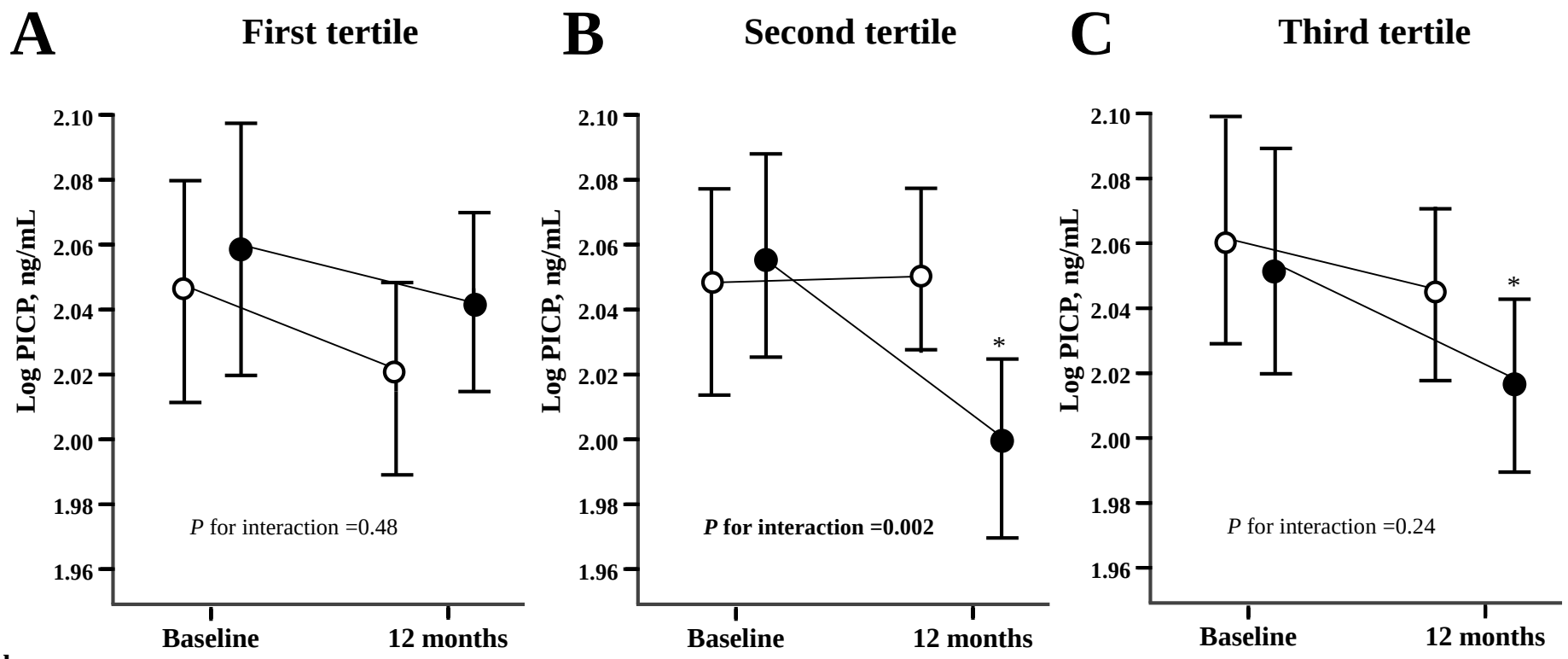
CITP means carboxy-terminal telopeptide of collagen type I; MMP-1, matrix metalloproteinase-1; E, peak early transmitral ventricular filling velocity; e', early diastolic tissue Doppler velocity; VO_2, oxygen consumption; NYHA, New York Heart Association; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; eGFR, estimated glomerular filtration rate; A, peak atrial transmitral ventricular filling velocity; IVRT, isovolumetric relaxation time; DT, deceleration time; LVEF means left ventricular (LV) ejection fraction; LVMI, LV mass index; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; NT-proBNP, amino-terminal pro-brain natriuretic peptide; PICP, carboxy-terminal propeptide of procollagen type I; $ATVO_2$, oxygen consumption at anaerobic threshold; V_E, expired volume per unit time; VCO_2, volume of expired carbon dioxide; SF-36, short form 36; PHQ-Sum, Patient Health Questionnaire sum score; IQR, interquartile range.

[a]Between-group differences are from analysis of covariance, adjusting for baseline. [b]Odds ratio (95% CI). [c]Geometric mean ratio (95% CI).



	First tertile		Second tertile		Third tertile	
Placebo						
Nº of patients	59	56	66	65	61	59
Mean (95% CI)	13.1 (12.1-14.1)	13.4 (12.7-14.2)	12.2 (11.3-13.0)	13.6 (12.9-14.3)	12.5 (11.5-13.5)	14.0 (13.2-14.7)
Spironolactone						
Nº of patients	68	65	61	60	66	65
Mean (95% CI)	13.1 (12.3-14.0)	12.8 (12.1-13.5)	13.0 (12.0-14.1)	11.9 (11.3-12.7)	12.2 (11.3-13.1)	11.5 (10.8-12.2)

Fig. 1



	First tertile		Second tertile		Third tertile	
Placebo						
Nº of patients	59	56	66	65	61	59
Median (IQR), ng/mL	110 (88.5-134)	104 (84.2-126)	111 (91.0-136)	111 (88.4-133)	116 (93.5-138)	112 (88.6-130)
Spironolactone						
Nº of patients	68	65	61	60	66	65
Median (IQR), ng/mL	112 (89.4-140)	111 (87.4-138)	107 (94.4-136)	96.7 (81.3-119)	110 (90.6-138)	104 (78.2-129)

Fig. 2

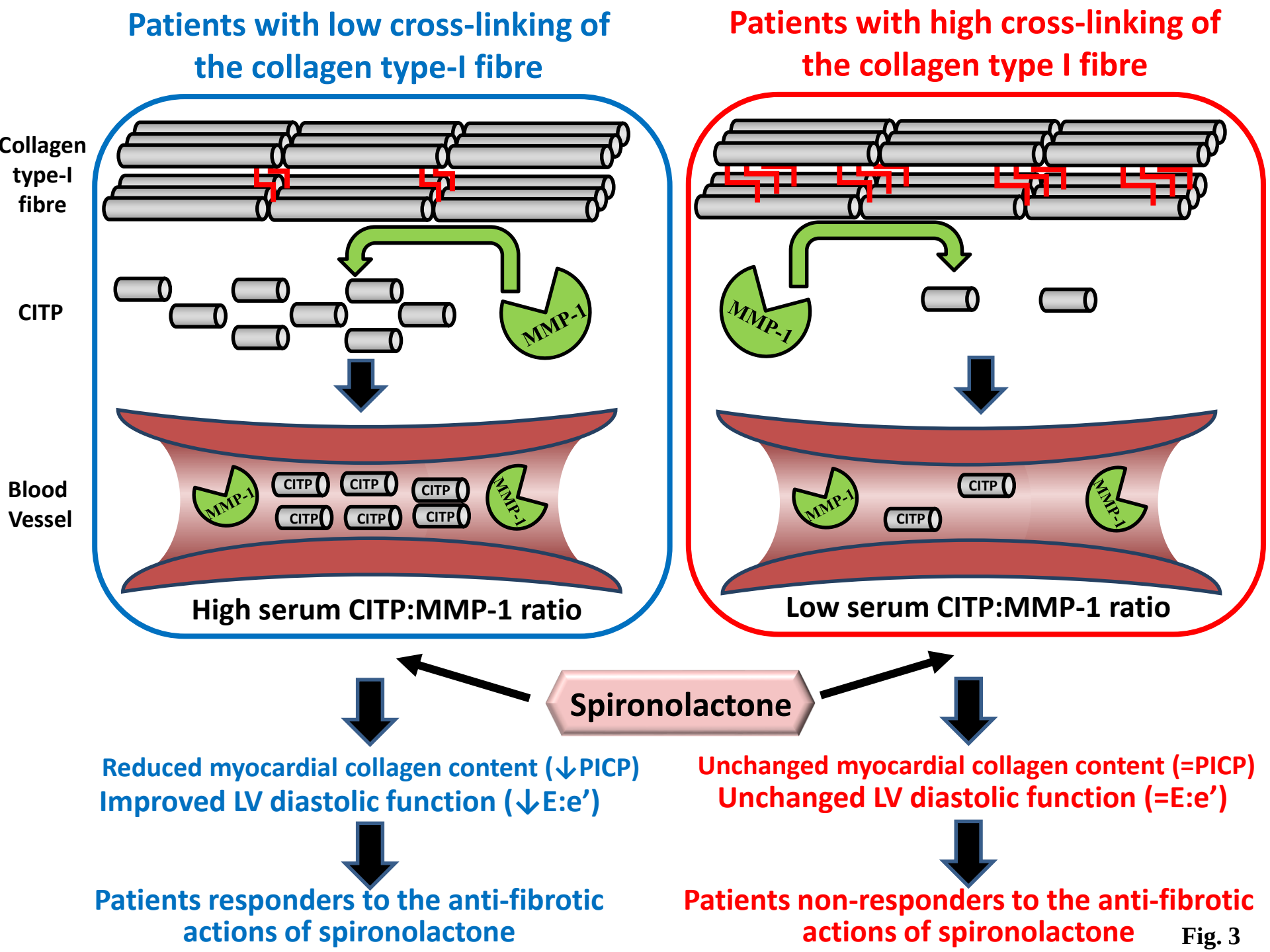

Patients with low cross-linking of the collagen type-I fibre
Patients with high cross-linking of the collagen type I fibre
Collagen type-I fibre
CITP
Blood Vessel
MMP-1
CITP
High serum CITP:MMP-1 ratio
Low serum CITP:MMP-1 ratio
Spironolactone
Reduced myocardial collagen content (↓PICP)
Improved LV diastolic function (↓E:e')
Unchanged myocardial collagen content (=PICP)
Unchanged LV diastolic function (=E:e')
Patients responders to the anti-fibrotic actions of spironolactone
Patients non-responders to the anti-fibrotic actions of spironolactone

Fig. 3