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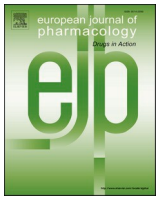

Effects of sacubitril-valsartan on aging-related cardiac dysfunction

Marialucia Telesca [a,1], Antonella De Angelis [a,1], Maria Donniacuo [b], Gabriella Bellocchio [a], Maria Antonietta Riemma [a], Elena Mele [a], Francesco Canonico [c], Eleonora Cianflone [d], Daniele Torella [e], Domenico D'Amario [f,**], Giuseppe Patti [f], Antonella Liantonio [g], Paola Imbrici [g], Annamaria De Luca [g], Giuseppe Castaldo [h,i], Francesco Rossi [a], Donato Cappetta [b,*], Konrad Urbanek [h,i,1], Liberato Berrino [a,1]

[a] Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Via Costantinopoli 16, 80138, Naples, Italy
[b] Department of Experimental Medicine, University of Salento, Via Lecce-Monteroni, 73047, Lecce, Italy
[c] Department of Cardiovascular Sciences, Fondazione Policlinico Universitario A. Gemelli IRCCS, Largo A. Gemelli 8, 00168, Rome, Italy
[d] Department of Medical and Surgical Sciences, Magna Graecia University, Viale Europa, 88100, Catanzaro, Italy
[e] Department of Experimental and Clinical Medicine, Magna Graecia University, 88100, Catanzaro, Italy
[f] Department of Translational Medicine, Università del Piemonte Orientale, via Solaroli, 17, 28100, Novara, Italy
[g] Department of Pharmacy-Drug Sciences, University of Bari "Aldo Moro", Via Orabona 4, 70125, Bari, Italy
[h] Department of Molecular Medicine and Medical Biotechnologies, University of Naples "Federico II", Via A. Pansini 5, 80131, Naples, Italy
[i] CEINGE-Advanced Biotechnologies, Via G. Salvatore 486, 80131, Naples, Italy

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ABSTRACT

Heart failure (HF) remains a huge medical burden worldwide, with aging representing a major risk factor. Here, we report the effects of sacubitril/valsartan, an approved drug for HF with reduced EF, in an experimental model of aging-related HF with preserved ejection fraction (HFpEF).

Eighteen-month-old female Fisher 344 rats were treated for 12 weeks with sacubitril/valsartan (60 mg/kg/day) or with valsartan (30 mg/kg/day). Three-month-old rats were used as control.

No differential action of sacubitril/valsartan *versus* valsartan alone, either positive or negative, was observed. The positive effects of both sacubitril/valsartan and valsartan on cardiac hypertrophy was evidenced by a significant reduction of wall thickness and myocyte cross-sectional area. Contrarily, myocardial fibrosis in aging heart was not reduced by any treatment. Doppler echocardiography and left ventricular catheterization evidenced diastolic dysfunction in untreated and treated old rats. In aging rats, both classical and non-classical renin-angiotensin-aldosterone system (RAAS) were modulated. In particular, with respect to untreated animals, both sacubitril/valsartan and valsartan showed a partial restoration of cardioprotective non-classical RAAS.

In conclusion, this study evidenced the favorable effects, by both treatments, on age-related cardiac hypertrophy. The attenuation of cardiomyocyte size and hypertrophic response may be linked to a shift towards cardioprotective RAAS signaling. However, diastolic dysfunction and cardiac fibrosis persisted despite of treatment and were accompanied by myocardial inflammation, endothelial activation, and oxidative stress.

1. Introduction

Aging represents a major risk factor for heart failure (HF) and other cardiovascular diseases, which according to the 2019 report from the European Society of Cardiology account for more than 6 million new cases every year, with almost 50 million people living with the disease, and the yearly EU cost of €210 billion (Timmis et al., 2020). In the aging myocardium, left ventricle (LV) thickens and stiffens, showing progressive fibrosis and hypertrophy (Dai et al., 2012). These myocardial changes correlate with the impairment of diastolic function, a major

* Corresponding author.
** Corresponding author.
E-mail address: donato.cappetta@unisalento.it (D. Cappetta).
[1] With equal contribution.

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pathophysiological feature of HF with preserved ejection fraction (HFpEF), the most common form of HF in older adults (Borlaug et al., 2010; Pandey et al., 2020). Although the increasing prevalence due to the growing rate of elderly in the population, several therapies have failed to reduce mortality and morbidity in HFpEF patients (McDonagh et al., 2021).

Sacubitril/valsartan, combining a neprilysin inhibitor with an angiotensin II receptor blocker, is approved for HF with reduced EF (Mascolo et al., 2022; McDonagh et al., 2021). The inhibition of neprilysin increases the levels of natriuretic peptides (Vasquez et al., 2020) causes diuresis, natriuresis, and extracellular volume reduction, thus potentiating the effects of renin-angiotensin-aldosterone system (RAAS) blockade on reducing pathological fibrosis and myocardial hypertrophy and improving cardiac function (Paz et al., 2020). When tested in HFpEF patients, sacubitril/valsartan left uncertainty regarding its efficacy (Solomon et al., 2019). Follow-up analysis indicated a clinical response in relation to time from last HF hospitalization or in specific subgroups with lower EF ranges or women (Basile et al., 2023; Solomon et al., 2020; Vaduganathan et al., 2020). Although the modulation of pathophysiological mechanisms underlying HFpEF has been largely investigated (Aroor et al., 2021; Nordén et al., 2021; Shi et al., 2023; Zhang et al., 2021), the effects of sacubiril/valsartan on aging-related HFpEF remain unclear. Epidemiological studies have consistently indicated that women constitute the majority of patients with HFpEF (Beale et al., 2018). Furthermore, cardiac aging has been demonstrated to worsen LV remodeling, diastolic dysfunction and vascular stiffness more in women than in men (Redfield et al., 2005). Therefore, in this study we explored the effects of sacubitril/valsartan in an experimental model of aging-related HFpEF using female Fisher 344 rats that develop chronic and progressive LV diastolic dysfunction (Valero-Muñoz et al., 2017).

2. Materials and methods

2.1. Animal model and drug treatment

Female Fisher 344 rats (Charles River Laboratories) were maintained in a germ-free, controlled temperature, and humidity environment. All animal experimental protocols were in accordance with National and International guidelines (Italian Ministry of Health; D.L.vo 26, March 4, 2014), were approved by the Italian Ministry of Care and Use of Laboratory Animals (protocol n. 275/2013-B), and were conformed to ARRIVE guidelines.

Eighteen-month-old rats were randomized into three experimental groups: 1) SAC/VAL (n = 10), treated for 12 weeks by oral gavage with sacubitril/valsartan, 60 mg/kg/day (1:1 *ratio*); 2) VAL (n = 10), treated with valsartan, 30 mg/kg/day; 3) old (n = 10), treated with vehicle. Three-month-old rats (young, n = 10) served as additional control group. Systolic and diastolic pressure was measured by the tail-cuff method (BP-2000; Visitech Systems).

2.2. Echocardiography and hemodynamics

Young and aged rats were monitored by echocardiography, before starting and at the end of drug treatments, for the assessment of systolic and diastolic heart function (Vevo770, VisualSonics). Rats were anesthetized with inhaled isoflurane (2–3%), and the animal's body temperature was maintained at 37 °C with a heating pad. Echocardiographic parameters, obtained in B-mode, M-mode, and by pulsed wave and tissue Doppler, were used to assess global LV function (Piegari et al., 2020). EF and fractional shortening (FS) were measured as indices of systolic function, while E wave/A wave (E/A) *ratio*, E deceleration time, isovolumetric relaxation time, and E/e' *ratio* as indices of diastolic function. Wall thickness was measured as well. Prior to euthanasia of the animals, hemodynamic analysis was performed as previously described (Millar Instruments) (De Angelis et al., 2016).

2.3. Tissue harvesting for ex vivo analysis

The animals were euthanized by diaphragm dissection under isoflurane anesthesia. Hearts and lungs were excised and weighed. For the preparation of frozen sections, tissue was placed in a mold and covered with OCT cryo-inclusion medium, preventing the formation of air bubbles. Tissue sections were generated by a Leica CM3050 S cryostat (Leica Microsystems) with a thickness of 10 μm. For molecular biology analysis, the heart samples were frozen in liquid nitrogen and stored at −80 °C (Cianflone et al., 2020).

2.4. Protein extraction and Western blot analysis

The full list of primary antibodies used for Western blot analysis is available as supplementary material (Supplementary Table 1).

Proteins were extracted from cardiac tissue, which were homogenized in 500 μl ice-cold RIPA Lysis Buffer System with protease and phosphatase inhibitors (Santa Cruz Biotechnology). The debris was removed by centrifugation at 13000 rpm for 10 min at 4 °C, and protein concentration was assessed by the Bradford protein assay (Bio-Rad Laboratories). Protein extracts were loaded and separated on 8–12% SDS-PAGE, and transferred onto PVDF membrane (Telesca et al., 2023). The membranes were probed with specific primary antibodies in the appropriate dilution, as reported in Table 1. Loading conditions were determined with GAPDH. Peroxidase-conjugated secondary antibodies were employed to detect primary antibodies (Bethyl Laboratories). The peroxidase activity was measured with Immobilon Western, Chemiluminescent HPR Substrate (Millipore). Optical density of the immunoreactive bands was analyzed and quantified with ImageJ 1.53k software.

2.5. Zymography

Metalloproteinase (MMP) 2 and MMP9 gelatinolytic activity was performed as previously described (Urbanek et al., 2023). Frozen tissue was mechanically homogenized. Equal amounts of proteins were separated by electrophoresis, and gels were incubated in Renaturating Buffer and Developing Buffer according to manufacturer's instructions (Life Technologies). Staining with Coomassie Blue indicated the zone of gelatinolitic-activity as white bands on a uniform blue background. Images were obtained by Gel Doc EZ Imager (Bio-Rad Laboratories).

Table 1
List of Western blot primary antibodies.

Antibody	Species	Diluition	Company (cat. N)
Anti-ACE	Rabbit	1:1000	ThermoFisher Scientific PA5-78711
Anti-ACE2	Rabbit	1:1000	Abcam ab108252
Anti-AT1R	Rabbit	1:1000	ThermoFisher Scientific PA5-20812
Anti-AT2R	Rabbit	1:1000	ThermoFisher Scientific PA5-20813
Anti-catalase	Mouse	1:1000	Sigma-Aldrich C0979
Anti-collagen I	Rabbit	1:1000	Novus Biologicals NB600-408
Anti-endothelin-1	Mouse	1:500	Abcam ab2786
Anti-e-selectin	Rabbit	1:1000	ThermoFisher Scientific PA5-106911
Anti-GAPDH	Mouse	1:20000	Sigma-Aldrich G8795
Anti-IL-1β	Rabbit	1:1000	Abcam ab205924
Anti-IL-6	Mouse	1:1000	Abcam ab9324
Anti-Mas1	Rabbit	1:500	ThermoFisher Scientific PA5-77282
Anti-MCP1	Rabbit	1.500	ThermoFisher Scientific PA5-115555
Anti-MMP2	Mouse	1:1000	Abcam ab86607
Anti-MMP9	Mouse	1:1000	Abcam ab58803
Anti-MnSOD	Rabbit	1:1000	Millipore06-984
Anti-NOX2	Rabbit	1:1000	ThermoFisher Scientific MA5-35348
Anti-NOX4	Rabbit	1:1000	Abcam ab133303
Anti-osteopontin	Rabbit	1:1000	ThermoFisher Scientific PA5-34579
Anti-TNFα	Rabbit	1:1000	Abcam ab6671
Anti-VCAM-1	Rabbit	1:1000	Abcam ab13447

2.6. Histology

Tissue sections were fixed in 70% ethanol in cold PBS, washed, and blocked with 10% donkey serum (Sigma-Aldrich) in a humid chamber at room temperature for 30 min. To visualize extracellular matrix accumulation, sections were stained with collagen I (Novus Biological) or with Masson's trichrome staining kit (Sigma-Aldrich). Cytosolic superoxide production was measured using dihydroethidium oxidative fluorescent dye (Sigma-Aldrich), while peroxynitrite-mediated oxidative damage was detected by anti-3-nitrotyrosine antibody (Millipore). Collagen 1, 3-nitrotyrosine and dihydroethidium images were processed and analyzed by ImageJ 1.53k software for the quantification of fluorescence intensity, measured as the sum of pixel values in the channel of interest per unit area (Cappetta et al., 2019). Tissue sections were stained with anti-laminin antibody (Sigma-Aldrich) and cross-sectional area of myocytes was measured using ImageJ 1.53k software (Torcinaro et al., 2022). For myocyte size distribution, a 100-μm interval was considered to generate a distribution curve. Fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch) was used. The nuclei were counterstained with DAPI. Samples were analyzed with a Leica DM5000B microscope (Leica Microsystems) and a Zeiss LSM700 confocal microscope (Zeiss).

2.7. Statistical analysis

Results were presented as fold of control mean ± standard error of the mean. All data were analyzed with GraphPad Prism 8. Significance between two comparisons was determined by Student's t-test. Multiple comparisons were tested by one-way ANOVA and Tukey's post-test. P values were two-sided and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Functional and structural changes in the aging heart

Prior to starting drug treatments, eighteen-month-old animals were monitored by echocardiography for the assessment of systolic and diastolic performance. Systolic function resulted unchanged, with comparable values of EF and FS between young (three-month-old) and aging rats (Fig. 1A). Contrarily, diastolic parameters were compromised; lower E/A *ratio* and longer E deceleration time and isovolumetric relaxation time were observed in eighteen-month-old animals in comparison to young rats. E/e' *ratio*, although not significant, showed a trend towards reduction in old animals (Fig. 1B). Echocardiography evidenced also a significant increase of diastolic and systolic posterior wall thickness (Fig. 1C). Taken together, our findings revealed an incipient but evident cardiac deterioration by means of a worsening of diastolic function along with a LV hypertrophy in aging rats.

3.2. Drug treatments and blood pressure

After 3-month treatments with either sacubitril/valsartan or valsartan alone, functional, molecular and histological analyses were carried out. Although Fisher 344 rats do not experience severe hypertension, a significant elevation of arterial pressure, both in systole and diastole, was detected in twenty one-month-old animals with respect to young rats. With respect to old untreated animals, both sacubitril/valsartan and valsartan treatments induced a significant decrease of blood pressure with comparable intensity (Fig. 2).

3.3. Effects of drug treatments on myocardial hypertrophy

Myocardial hypertrophy was assessed to characterize its contribution in the aging myocardium (Yan et al., 2021). Echocardiographic

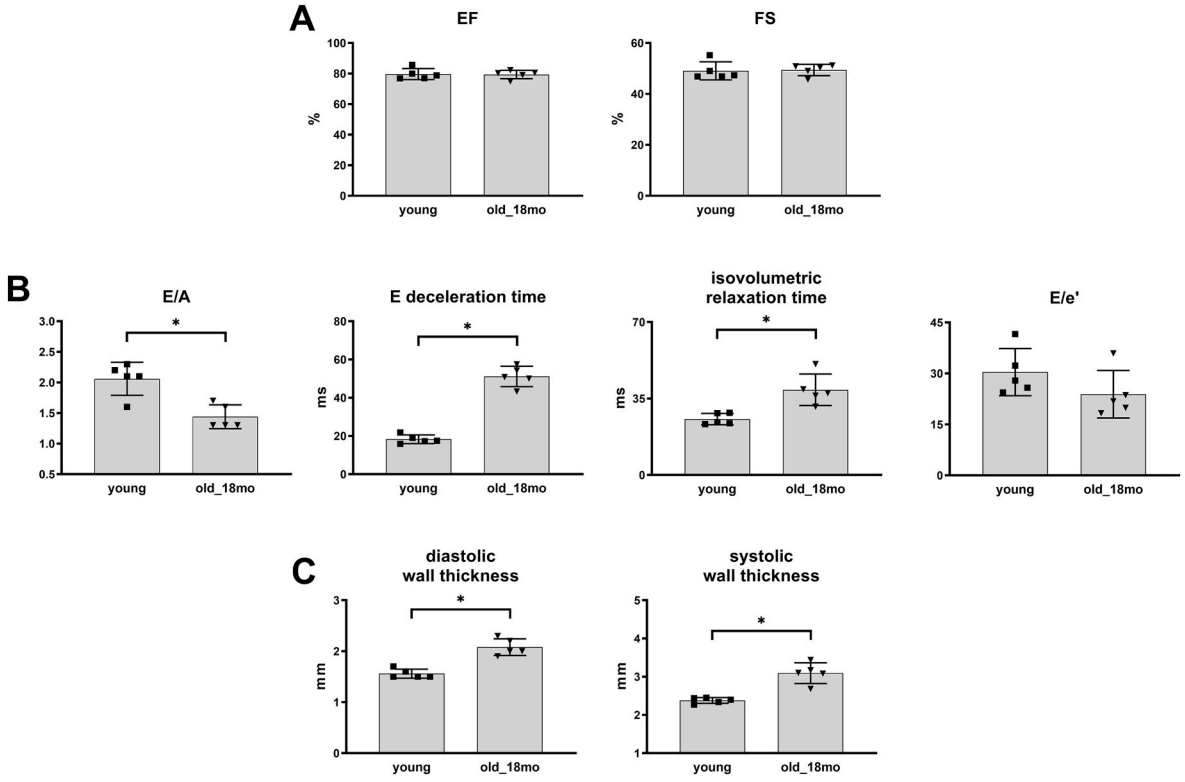

Fig. 1. *Functional analysis in the aging heart prior to starting drug treatments*. (A) Echocardiographic systolic indices EF and FS. (B) Pulsed wave and tissue Doppler showing peak velocity of the E wave, A wave, E/A *ratio*, E deceleration time, isovolumetric relaxation time and E/e' *ratio*. (C) Echocardiographic parameters of diastolic and systolic wall thickness. *$P < 0.05$.

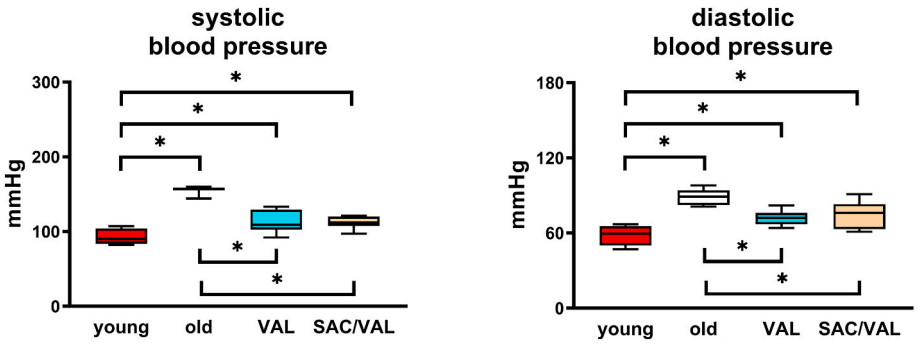

Fig. 2. *Drug treatments and blood pressure.* Mean blood pressure in systole and diastole. *P < 0.05.

analysis revealed a thickening of LV wall in systole and diastole in the old group and both sacubitril/valsartan and valsartan significantly reduced wall thickness to a similar extent (Fig. 3A). As defined by laminin outlining, myocyte cross-sectional area was increased in old animals in comparison to young rats, while drug treatments comparably reduced myocyte size (Fig. 3B–D). Consistently, an increased heart weight, normalized to tibia length, observed in untreated old rats, was significantly reduced by both drug treatments (Fig. 3E).

Overall, both sacubitril/valsartan and valsartan showed a comparable positive anti-hypertrophic effect on the aging heart.

3.4. Effects of drug treatments on left ventricle fibrotic remodeling

Myocardial fibrosis is another hallmark of cardiac aging (Dai et al., 2012). Indeed, histological examination by Masson's trichrome staining and collagen I immunofluorescence showed a greater agglomerate of extracellular matrix in old rat hearts. However, neither sacubitril/valsartan nor valsartan were able to reduce extracellular matrix build-up in the old LV (Fig. 4A–C). Also at isolated protein level, the increase of collagen I observed in the old group was not changed (Fig. 4D and E). Overexpression of MMP2 and MMP9, detected in the old hearts, was not reduced as well (Fig. 4F). Consistently, MMP2 and MMP9 gelatinolytic activity in myocardial tissue that resulted increased in

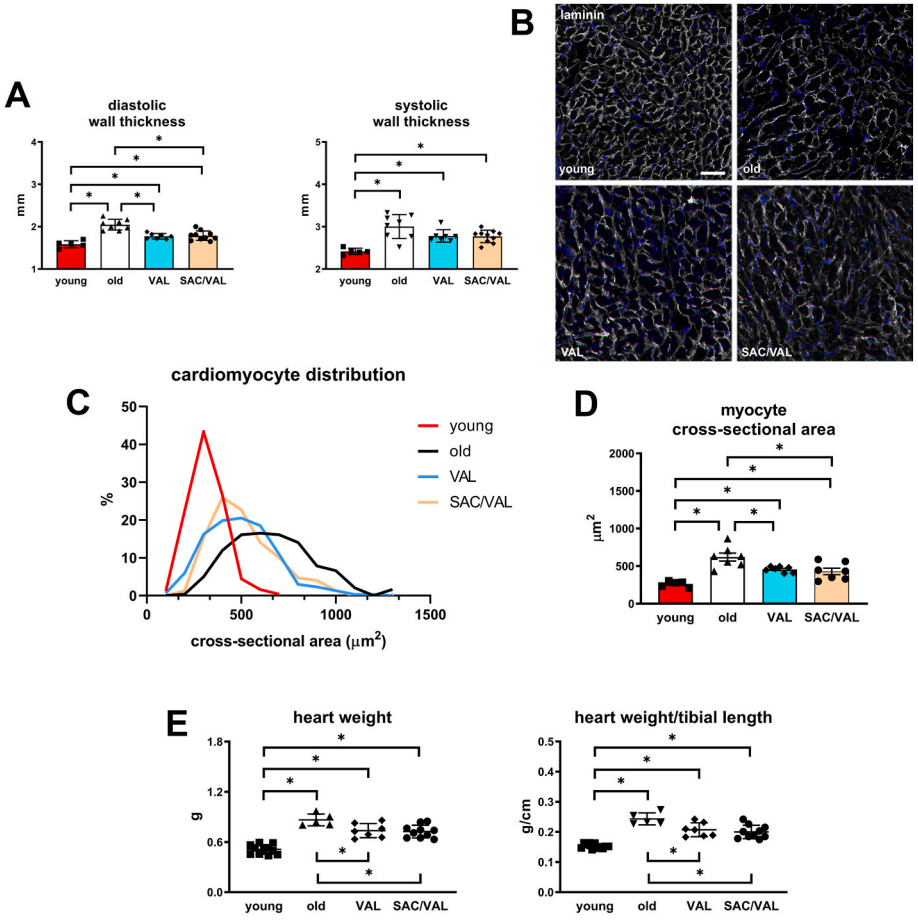

Fig. 3. *Drug treatments and myocardial hypertrophy.* (A) Echocardiographic parameters of diastolic and systolic wall thickness. (B) Representative immunofluorescent images of LV sections stained with anti-laminin antibody (white). Nuclei were counterstained with DAPI (blue). Scale bar: 25 μm. (C) Measurements of cardiomyocyte cross-sectional area. (D) Frequency histogram showing the distribution of cardiomyocyte cross-sectional area. (E) Heart weight and heart weight/tibial length. *P < 0.05.

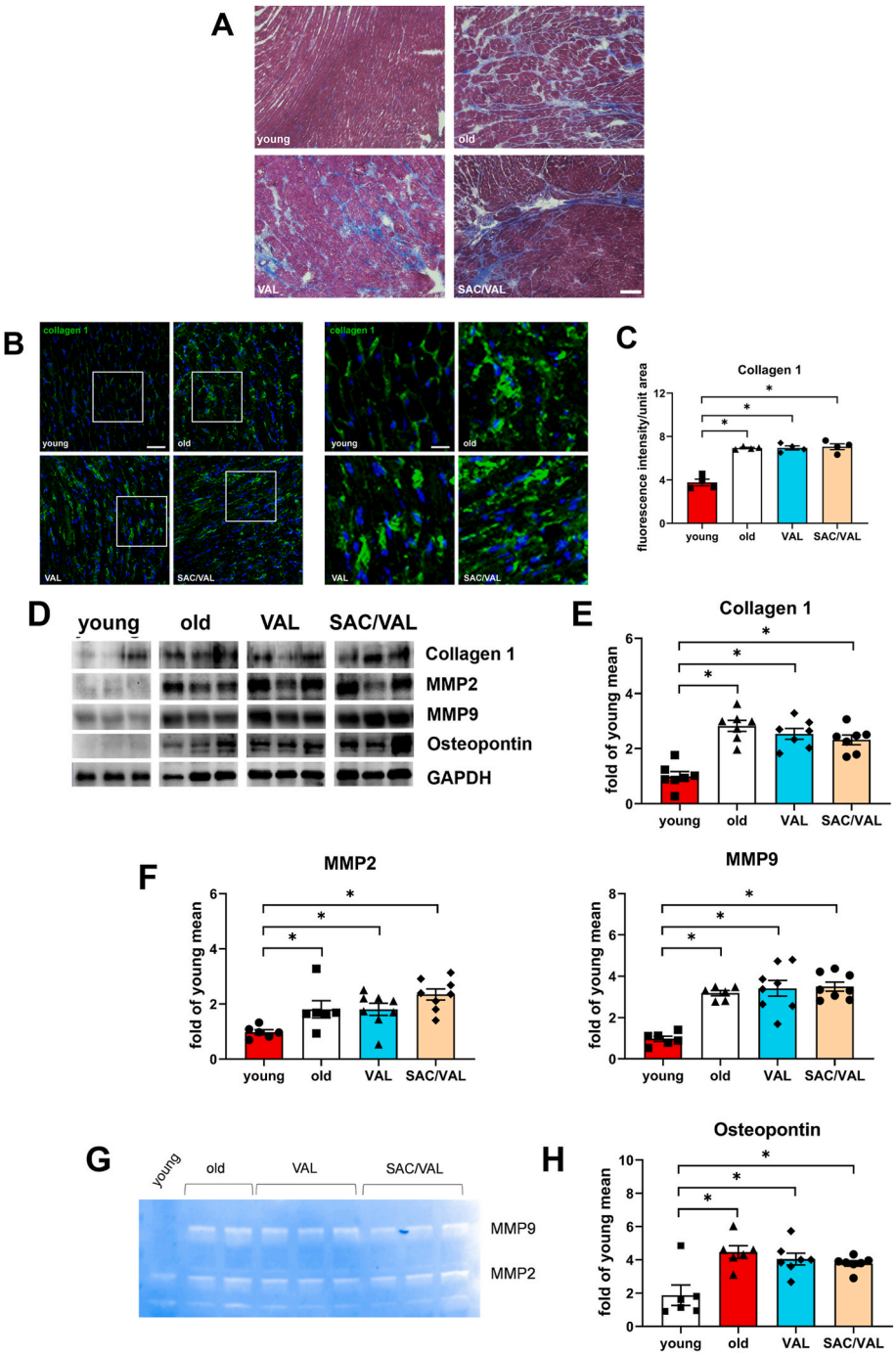

Fig. 4. *Drug treatments and myocardial fibrosis.* (A) Representative images of LV sections with Masson's trichrome staining showing extracellular matrix deposition. Scale bar: 200 μm. (B) Representative immunofluorescent LV images showing collagen I (green) at lower (left panels) and higher (right panels) magnification. Nuclei were counterstained with DAPI (blue). Scale bars: 40 μm (left panels), 15 μm (right panels). (C) Quantification of fluorescence intensity of Collagen 1. (D) Western blotting gel bands of Collagen 1, metalloproteinase (MMP) 2, MMP9 and Osteopontin. (E, F) Bar graphs showing protein expression of Collagen 1, MMP2 and MMP9. (G) MMP2 and MMP9 gelatinase activity shown by gel zymography assay. (H) Bar graphs showing Osteopontin protein expression. *P < 0.05.

aging animals was not reduced by sacubitril/valsartan or valsartan (Fig. 4G). Furthermore, the expression of osteopontin, a matricellular protein strongly correlated with severe cardiovascular outcomes, was significantly increased in the aging myocardium and was not modulated following drug treatment (Fig. 4H). These findings indicated a lack of protection of sacubitril/valsartan or valsartan against cardiac fibrosis in the aging heart.

3.5. Drug treatments and heart function

Transthoracic echocardiography evidenced preservation of systolic performance in all the experimental groups, as shown by comparable values of EF and FS (Fig. 5A). At the same time, evident deterioration of diastolic function was not counteracted by any pharmacological treatment. Doppler echocardiography showed that sacubitril/valsartan or valsartan did not affect LV filling pattern as it resulted similar prolongation of isovolumetric relaxation time and transmitral flow velocity (E/

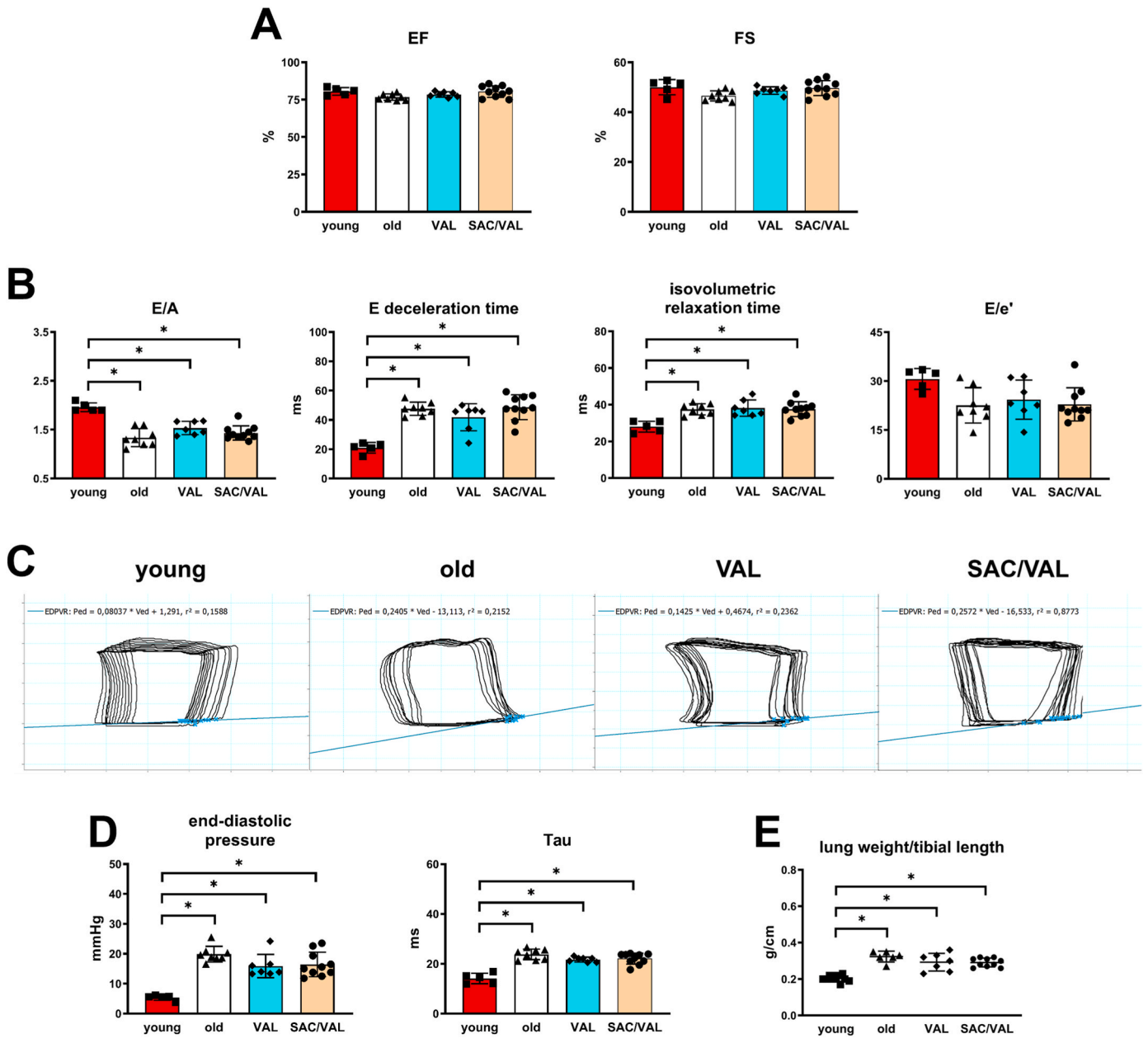

Fig. 5. *Drug treatments and heart function*. (A) Echocardiographic systolic indices EF and FS. (B) Pulsed wave and tissue Doppler showing peak velocity of the E wave/A wave (E/A) *ratio*, E deceleration time, isovolumetric relaxation time and E/e' *ratio*. (C) Representative end-diastolic pressure-volume loops. (D) Hemodynamic parameters of end-diastolic pressure and time constant Tau. (E) Lung weight/tibial length. *P < 0.05.

A *ratio* and E deceleration time) in untreated and treated old rats. E/e' *ratio* was not changed as well (Fig. 5B). This unexpected ineffectiveness of treatments in ameliorating diastolic function was confirmed by hemodynamic analysis. The slope of end-diastolic pressure-volume relationship, indicative of end-diastolic left ventricle stiffness, shifted upward in old rats, and was not lowered by drug treatments (Fig. 5C). Similarly, end-diastolic pressure and time constant Tau, in untreated and treated rats, were comparable (Fig. 5D). Increased lung weight-to-tibial length *ratio* in old rats was not affected by treatments (Fig. 5E).

3.6. Effects of drug treatments on cardiac inflammation and endothelial activation

In the view of the above structural and functional findings, the contribution of principal molecular aspects of age-related cardiomyopathy were determined. Analysis of cardiac inflammation at protein level evidenced an enhanced expression of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin (IL)-6 and IL-1β, in treated and untreated old animals (Fig. 6A and B). Homeostasis of coronary endothelium resulted impaired in aging animals. No drug treatment was able to counteract the increment of vascular cell adhesion protein-1 (VCAM-1), e-selectin and endothelin-1 (Fig. 6C). Similarly, elevation of monocyte chemoattractant protein-1 (MCP-1) was not modulated (Fig. 6D). Endothelin-1 that is associated with long-term mortality in HFpEF patients, may be viewed as a potential biomarker (Bayes-Genis et al., 2022).

3.7. Effects of drug treatments on oxidative stress

Increased production of reactive oxygen and nitrogen species was evidenced by dihydroethidium and 3-nitrotyrosine, respectively; amplification of oxidative and nitrosative stress was not

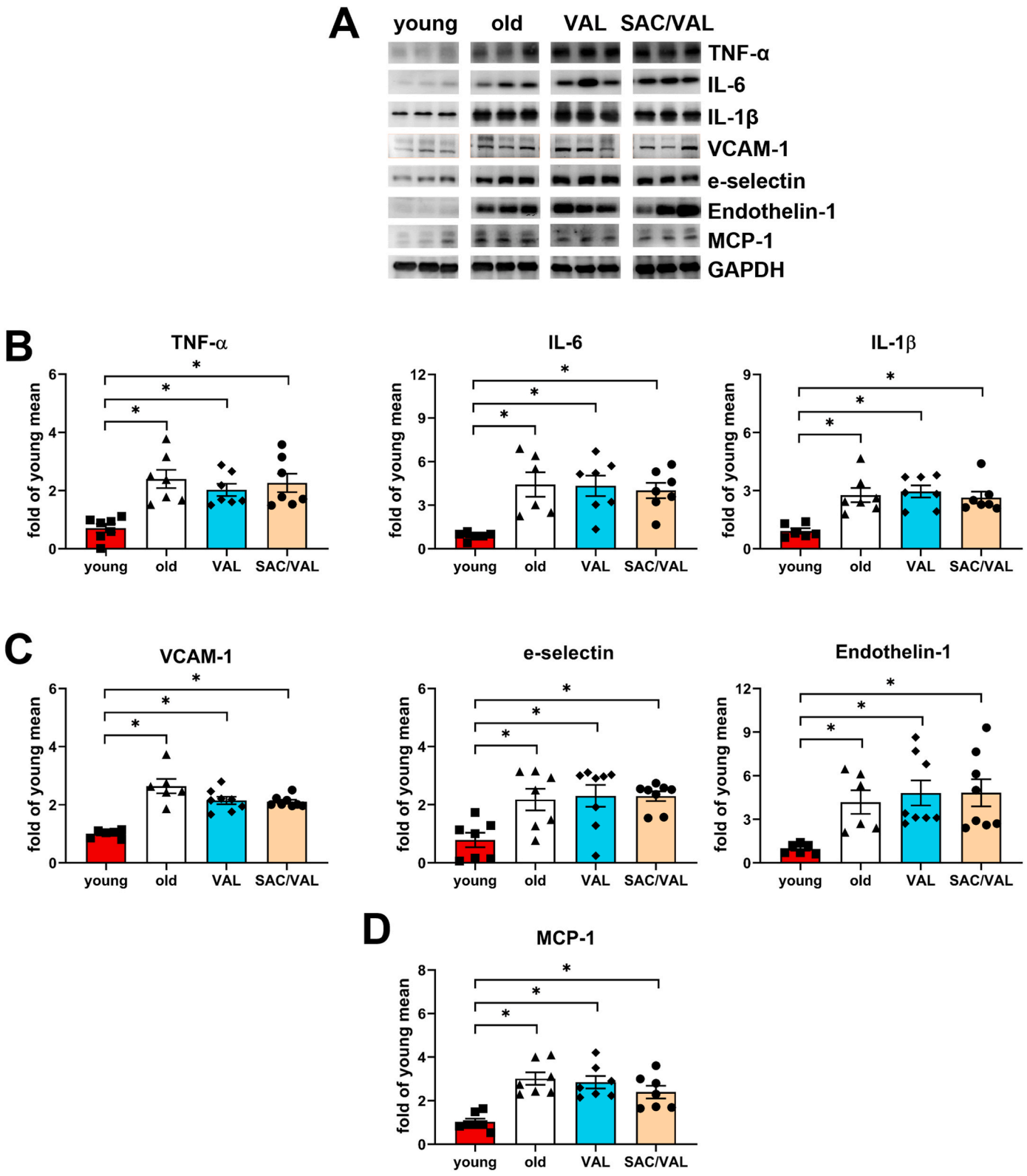

Fig. 6. *Drug treatments, cardiac inflammation and endothelial activation.* (A) Western blotting gel bands and (B–D) bar graphs of tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β, vascular cell adhesion protein-1 (VCAM-1), e-selectin, Endothelin-1 and monocyte chemoattractant protein-1 (MCP-1). *P < 0.05.

counterbalanced by sacubitril/valsartan and valsartan (Fig. 7A–D). Consistently, the expression of NADPH oxidase (NOX) 2 and NOX4, major sources of reactive oxygen species, was significantly elevated in the aging hearts with respect to young myocardium and was not decreased by treatments (Fig. 7E and F). In addition, the antioxidant enzyme manganese superoxide dismutase (MnSOD) remained down-regulated in untreated and treated old rats. The expression of catalase, a second major myocardial scavenger of oxidants, was not modulated by age and treatments (Fig. 7G).

Overall, our results demonstrated a persistent and uncontrolled

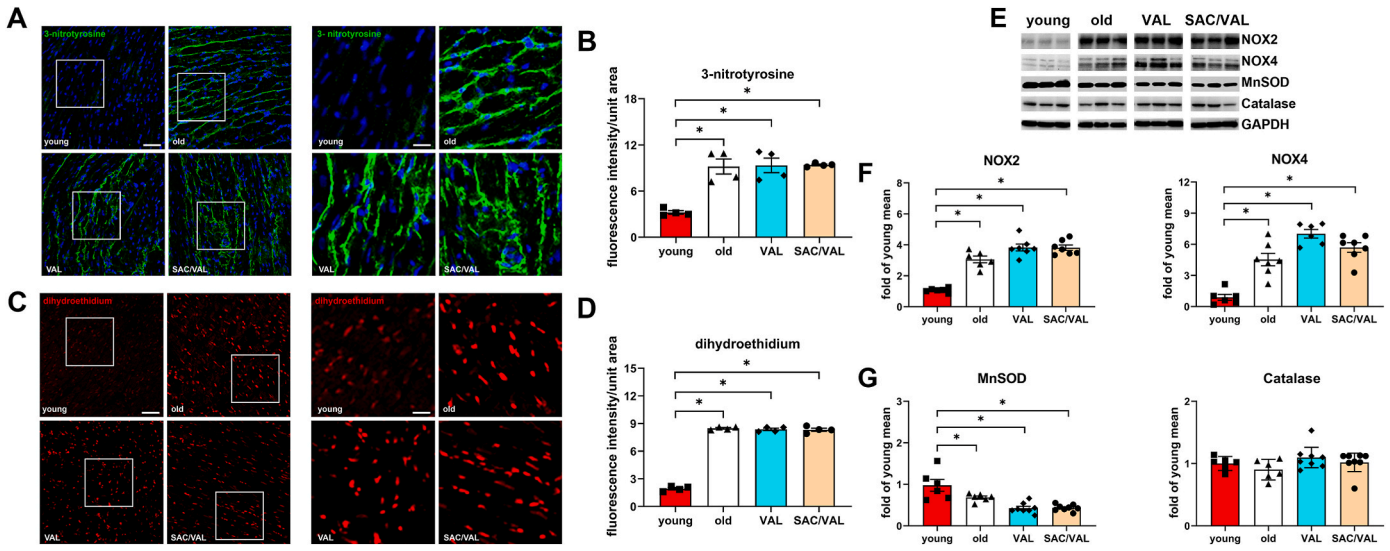

Fig. 7. *Drug treatments and oxidative stress.* (A) Representative immunofluorescent images of LV sections stained with 3-nitrotyrosine (green) at lower (left panels) and higher (right panels) magnification. Nuclei were counterstained with DAPI (blue). Scale bars: 40 μm (left panels), 15 μm (right panels). (B) Quantification of fluorescence intensity of 3-nitrotyrosine. (C) Representative immunofluorescent images of LV sections stained with dihydroethidium (red) at lower (left panels) and higher (right panels) magnification. Scale bars: 40 μm (left panels), 15 μm (right panels). (D) Quantification of fluorescence intensity of dihydroethidium. (E) Western blotting gel bands of NADPH oxidase (NOX) 2, NOX4, manganese superoxide dismutase (MnSOD) and Catalase. (F) Bar graphs showing protein expression of NOX2 and NOX4. (G) Bar graphs showing protein expression of MnSOD and Catalase. *P < 0.05.

increase of cardiac inflammation and oxidative stress as significant pathophysiological elements driving aging heart injury.

3.8. Effects of drug treatments on local RAAS

There is evidence that cardiac aging *per se* is associated with local cardiac RAAS activation, which has critical implications with respect to pathogenesis and treatment of HFpEF (Carter and Groban, 2008). Our findings indicated a modulation of both classical and non-classical RAAS pathways in aging rats. Specifically, protein expression of angiotensin converting enzyme (ACE) and type 1 angiotensin II receptor (AT1R) resulted elevated in the old group and was not affected by drug treatments (Fig. 8A and B). Interestingly, in aging rats ACE2, AT2R and Mas1, were downregulated. With respect to old untreated animals, both sacubitril/valsartan and valsartan significantly re-established AT2R and Mas1 expression and showed a trend towards restoration of ACE2 (Fig. 8C). These results evidence the anti-hypertrophic properties of sacubitril/valsartan and valsartan that positively affect non-canonical components of RAAS.

4. Discussion

This study reports the effects of sacubitril/valsartan (and valsartan alone) in a model of age-related HFpEF. The absence of a differential

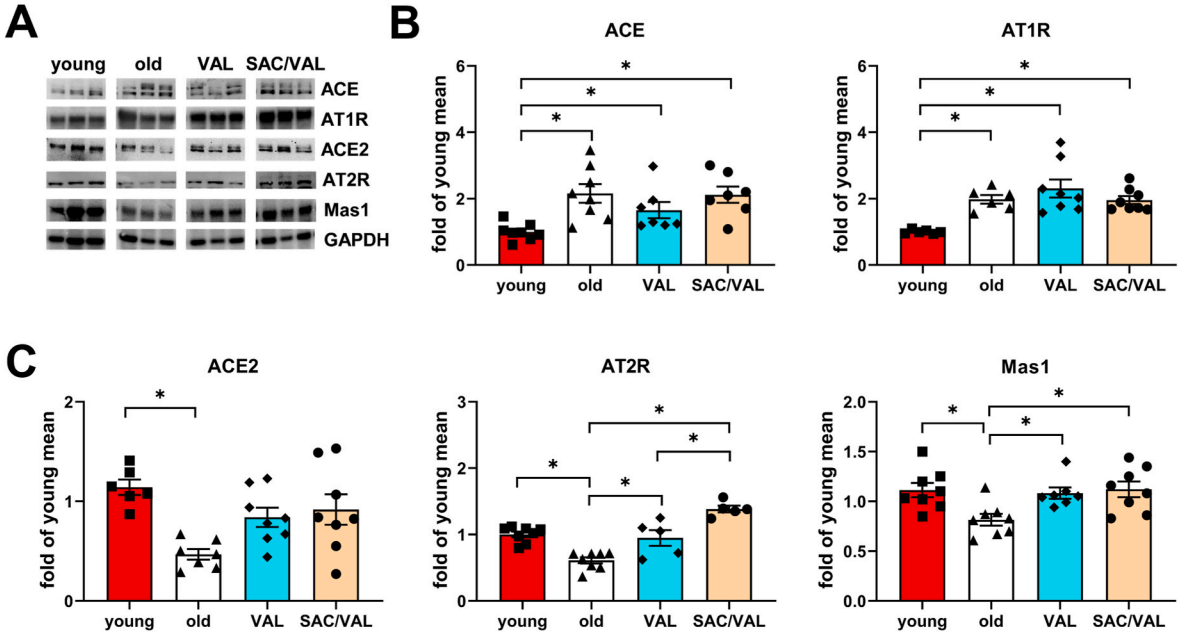

Fig. 8. *Drug treatments and local RAAS.* (A) Western blotting gel bands and (B, C) bar graphs of angiotensin converting enzyme (ACE), type 1 angiotensin II receptor (AT1R), ACE2, AT2R and Mas1. *P < 0.05.

action of sacubitril/valsartan *versus* valsartan, either positive or negative, suggested that the addition of neprilysin inhibition to angiotensin II receptor blockade did not significantly contribute to functional benefits in our experimental model. Both treatments showed a similar effect in attenuating cardiac hypertrophy while myocardial fibrosis, accentuated in aging rats, was not modulated. This provides the insights into relative contribution of two major drivers of myocardium dysfunction in aging: hypertrophy and fibrosis (Croteau et al., 2020; Díez-Villanueva et al., 2021). Aged Fisher 344 rats are highly recommended for age-related studies, as they show a progressive decline in LV function and structure that is consistent with clinical data regarding age-related early and late LV diastolic filling abnormalities (Boluyt et al., 2004; Carrick--Ranson et al., 2012). In the present study, echocardiography and hemodynamic analysis highlighted the impairment of diastolic function with increased passive stiffness and longer active relaxation. The ineffectiveness of pharmacological treatments in counteracting the decline of diastolic function reflects clinical outcomes of patients with HFpEF in whom sacubitril/valsartan is safe and well tolerated, but although capable of reducing the levels of N-terminal prohormone brain natriuretic peptide, has no significant effect on cardiovascular outcomes and mortality (Redfield & Borlaug, 2021; Solomon et al., 2019; Tomasoni et al., 2020).

A major finding of our study is the differential response of hypertrophy and fibrosis to drug therapy. Both treatments attenuated heart weight, LV wall thickness and cardiomyocyte size, indices of hypertrophy. In contrast, the excessive accumulation of extracellular matrix was not affected. Despite the benefit on hypertrophy, the ineffectiveness on fibrosis, which prevents the heart from relaxing, may explain the persistence of diastolic dysfunction. Possibly, the severity of myocardial fibrosis is the signature underlying functional unresponsiveness to pharmacotherapy, thus representing a stronger pathological hallmark (more than LV hypertrophy) that correlates aging with diastolic dysfunction (Burlew and Weber, 2002; Moreo et al., 2009; Ribeiro Vitorino et al., 2023). Although hypertrophy and fibrosis are strictly interconnected, the mechanisms that regulate the two processes differ and are diversely modulated by pharmacotherapy.

The lack of effect on fibrosis may be related to the inability of sacubitril/valsartan and valsartan alone to mitigate the ongoing endothelial damage, inflammation, and oxidative stress. Low-grade sterile inflammation is an established source of fibrogenic mediators occurring with aging (Wang and Shah, 2015). The recruitment of inflammatory cells by chemotactic cytokines, such as MCP-1, amplifies the fibrogenic potential of macrophages, by enhancing transforming growth factor β and collagen synthesis (Dewald et al., 2005; Sakai et al., 2006). Interestingly, IL-1β and TNF-α enhance the fibrotic process by upregulating AT1R density on cardiac fibroblasts (Gurantz et al., 2005).

Endothelial compartment is a crucial component of cardiac fibrosis (Premer et al., 2019). Adhesion molecules, such as VCAM-1 and e-selectin, by promoting immune cell infiltration into the heart, significantly impact profibrotic response (Wang et al., 2021; Valen et al., 2001). In this scenario, elevated levels of endothelin-1, a fibrogenic mediator acting downstream of transforming growth factor β and angiotensin II, promotes fibrotic cardiac remodeling by favoring fibroblast proliferation and matrix protein synthesis (Mueller et al., 2011; Yamamoto et al., 2000). Moreover, the increased level of endothelin-1 in the aging myocardium is consistent with the emerging consideration of endothelin-1 as potential biomarker and a therapeutic target (Bayes--Genis et al., 2022).

In the current study, expression of the inflammatory molecules TNF-α, IL-6, and MCP-1, along with the markers of endothelial cell activation VCAM-1, e-selectin and endothelin-1, resulted increased in the aging myocardium. These changes were not modulated by any treatment. In addition, osteopontin, an extracellular matrix protein and a cytokine regulating fibroblast growth and survival (Abdelaziz et al., 2019), remained significantly overexpressed.

The implication of oxidative stress in the pathogenesis of age-associated cardiac fibrosis consists in regulating the effects of cytokines and angiotensin II on fibroblasts, as well as the quantity and quality of interstitial extracellular matrix (Kong et al., 2014). Our findings revealed a significant increase of NOX2 and NOX4 expression along with a decrease of MnSOD level in untreated and treated rats, demonstrating a widespread prooxidative state in the aging heart that treatments were unable to address.

On the other hand, the regulation of local RAAS (at cardiac level) responded to treatment. RAAS is a key component of adverse remodeling with aging (Singam et al., 2020). Upregulation of ACE and AT1R is strictly associated to angiotensin II-related negative remodeling, and exacerbates cardiac hypertrophy (Bhullar and Dhalla, 2022; Sadoshima and Izumo, 1993). Contrarily, ACE2/angiotensin 1-7/Mas1 axis operates as negative regulators of RAAS signaling (Oudit et al., 2007; Mascolo et al., 2020). Alteration of ACE2 functioning is linked to the development of pathological myocardial hypertrophy and heart disease in humans (Lieb et al., 2006; Yang et al., 2006). In cardiomyocytes exposed to angiotensin II to induce cell hypertrophy, treatment with sacubitril/valsartan results in the increased expression of ACE2 and in the suppression of hypertrophic factors (Wang et al., 2019). Our findings indicated that sacubitril/valsartan and valsartan alone, by increasing AT2R/ACE2/Mas1 without any effect on AT1R/ACE, shifted the balance of RAAS signaling towards cardioprotective components, and attenuated cardiomyocyte size and LV hypertrophic response. It remains to be tested if the potentiation of a cardioprotective RAAS axis can be beneficial in the longer run or in the presence of additional stress imposed on the aged and already compromised heart. The effect on hypertrophy may derive not only from a direct impact on cardiomyocytes, but also from a systemic action of drugs that reduce blood pressure (Drazner, 2011). In this study, aging animals exhibited mild rise of blood pressure and both treatments, by decreasing blood pressure, may have affected the hypertrophic response.

The opposite response on hypertrophy and fibrosis after treatment may indicate how different cell populations, within the same organ, contribute to pathological signaling and respond to therapy in a different way. In the aging and failing heart, cardiomyocytes increase in size and undergo senescence and apoptosis, whilst fibroblasts increase in number and differentiate into myofibroblasts (Tang et al., 2020). Therefore, the modulation of non-classical RAAS, which may have contributed to the antihypertrophic response observed on cardiomyocytes, did not produced remarkable effects on cardiac fibroblasts that remained unresponsive to drugs. This possibility is consistent with a previous research conducted on an angiotensin II-induced hypertensive model, in which sacubitril/valsartan suppresses increment in LV wall thickness and cardiomyocyte size, but does not ameliorate cardiac fibrosis (Tashiro et al., 2020). This suggestive hypothesis awaits more investigations at cellular and molecular level.

The results of the present study are not at variance with the recent scientific statement released by the Heart Failure Association and other ESC bodies, which introduced the concept of a phenotype-based approach for the treatment of patients with HFpEF. Heterogeneous clinical profiles are driven by different pathophysiological mechanisms and therefore the efficacy of certain drug regimens might be dampened within subgroups of patients (D'Amario et al., 2023).

In conclusion, the present study evidenced the favorable effects on age-related cardiac hypertrophy that was comparably reduced by sacubitril/valsartan and valsartan alone. However, diastolic dysfunction and cardiac fibrosis persisted despite of treatment, and were accompanied by continuing myocardial inflammation, oxidative stress and endothelial activation. Although guidelines do not recommend age-related differences in pharmacotherapy, the impact of age on sacubitril/valsartan efficacy is a matter of debate. In general, studies are not specifically designed for the elderly population, and elderly HF patients are underrepresented in randomized clinical trials, despite real-world community-dwelling patients being significantly older. Thus, preclinical studies may be relevant to elucidate the complex pathophysiology of

myocardial aging to validate clinically relevant biomarkers of pathology progression and drug responsiveness, and to study mechanisms by which novel therapies protect the aging heart.

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CRediT authorship contribution statement

Marialucia Telesca: Writing – original draft, Formal analysis, Conceptualization. **Antonella De Angelis:** Writing – original draft, Supervision, Conceptualization. **Maria Donniacuo:** Investigation, Formal analysis. **Gabriella Bellocchio:** Investigation, Formal analysis. **Maria Antonietta Riemma:** Investigation, Formal analysis. **Elena Mele:** Investigation, Formal analysis. **Francesco Canonico:** Investigation, Formal analysis. **Eleonora Cianflone:** Supervision, Formal analysis. **Daniele Torella:** Supervision, Formal analysis. **Domenico D'Amario:** Writing – review & editing, Formal analysis. **Giuseppe Patti:** Supervision. **Antonella Liantonio:** Supervision. **Paola Imbrici:** Supervision. **Annamaria De Luca:** Supervision, Funding acquisition. **Giuseppe Castaldo:** Supervision. **Francesco Rossi:** Supervision. **Donato Cappetta:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Konrad Urbanek:** Writing – review & editing, Writing – original draft, Conceptualization. **Liberato Berrino:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2024.176794.

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