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Finerenone Reduces Intrinsic Arterial Stiffness in Munich Wistar Frömter Rats, a Genetic Model of Chronic Kidney Disease

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Keywords

Albuminuria · Intrinsic arterial stiffness · Mineralocorticoid receptor antagonists · Mesenteric arteries · Chronic kidney disease · Metalloproteinases

Abstract

Background: Development of albuminuria and arterial stiffness in Munich Wistar Frömter (MWF) rats, a model of chronic kidney disease, is related to alterations in extracellular matrix, increased oxidative stress, and endothelial dysfunction. Finerenone (FIN), a novel, nonsteroidal, potent, and selective mineralocorticoid receptor antagonist, improves endothelial dysfunction through enhancing nitric oxide (NO) bioavailability and decreasing superoxide anion levels due to an upregulation in vascular and renal superoxide dismutase activity. We hypothesize that FIN reduces arterial stiffness in this model associated to the reduction in albuminuria and matrix metalloproteinase (MMP)-2/9 activity. ***Methods:*** Twelve-week-old MWF rats with established albuminuria and age-matched normoalbuminuric Wistar (W) rats were treated with FIN (10 mg/kg/day, once-daily oral gavage) or with vehicle (control, C) for 4 weeks. ***Results:*** Arterial stiffness was significantly higher in mesenteric arteries (MA) of MWF-C as compared to W-C. FIN treatment significantly lowered β-index, a measure of intrinsic stiffness independent of geometry, in MWF ($\beta_{MWF\text{-}FIN} = 7.7 \pm 0.4$ vs. $\beta_{MWF\text{-}C} = 9.2 \pm 0.5$, $p < 0.05$) positively correlating with urinary albumin excretion. Elastin fenestrae area in the internal elastic lamina of MA from MWF-FIN was significantly larger (+377%, $p < 0.05$). FIN increased plasma pro-MMP-2 and decreased plasma MMP-2 and MMP-9 activities, correlating with reductions in β-index. MA from MWF-FIN exhibited higher NO bioavailability and reduced superoxide anion levels compared to MWF-C. ***Conclusion:*** FIN treatment reduces intrinsic arterial stiffness in MA from MWF rats associated with changes in elastin organization, normalization of MMP-2 and MMP-9 ac-

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tivities, and reduction of oxidative stress. Moreover, reduction of arterial stiffness correlates with reduction in albuminuria.

Introduction

Arterial stiffness is an independent predictor of cardiovascular morbidity and mortality [1–4] and is also associated to the progression of chronic kidney disease (CKD) [5–10]. Albuminuria represents an early marker of both renal and vascular damage [11–13]. Epidemiologic data indicate an independent association between arterial stiffness and albuminuria in hypertensive [14, 15] and diabetic patients [16–19], as well as in the general population [20, 21]. This association supports the hypothesis of a generalized vascular dysfunction due to similar pathophysiologic mechanisms as a common pathway linking the cardiovascular-renal axis in patients with albuminuria [22–25].

The Munich Wistar Frömter (MWF) rat is a genetic model of spontaneous nondiabetic albuminuria development that mirrors several features observed in patients with albuminuria and CKD [24, 26]. It develops progressive albuminuria, mild hypertension and renal injury with age [24, 26–31]. In this model, we showed a genetic link between albuminuria development and increased arterial stiffness due to alterations in elastin organization, increased oxidative stress and matrix metalloproteinase (MMP)-9 activity, and endothelial dysfunction [32, 33].

Steroidal mineralocorticoid receptor antagonists (MRA), that is, spironolactone, canrenone, and eplerenone, reduce vascular stiffness [34, 35]. However, their use is not approved in CKD or diabetic kidney disease. Finerenone (FIN; BAY 94-8862) is a novel, nonsteroidal, potent, and selective MRA, which combines the potency of spironolactone with the selectivity of eplerenone. Its structure confers a different binding mode within the MR as well as different physicochemical properties (lipophilicity and polarity) which have an impact on tissue penetration and distribution [36, 37]. Quantitative whole-body autoradiography with FIN revealed a balanced cardiac versus renal distribution ratio in rodents [36, 37]. FIN reduced cardiac hypertrophy, pro-B-type natriuretic peptide, and proteinuria more efficiently than eplerenone when directly comparing equinatriuretic doses in a rat model of hypertensive cardiorenal end-organ damage [36].

We have recently demonstrated the efficacy of FIN to ameliorate albuminuria and normalize endothelial dysfunction in the aorta of MWF rats at a blood pressure-lowering dosage [38]. This was found to be related to an increase in endothelial nitric oxide (NO) availability due to an upregulation in peNOS, Mn-superoxide dismutase (SOD) and Cu, Zn-SOD expression in the vascular wall with a subsequent decrease in superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) levels. In addition, the upregulation of renal total SOD activity after FIN treatment supports a functional link between extrarenal normalization of vascular dysfunction and improvement of glomerular permeability dysfunction. The hypothesis of this study is that FIN reduces arterial stiffness in the MWF CKD model associated to the reduction of albuminuria and MMP-2/9 activities.

Materials and Methods

Animals and Experimental Protocol

Twelve-week-old male Wistar (W; Charles River, Barcelona, Spain) and MWF rats (Charité – University Medicine Berlin, Germany) were housed in groups of 2 under controlled dark–light cycles (12/12 h), temperature conditions, and with food (A.04, Panlab) and water available *ad libitum*. Animals were randomly grouped to receive FIN (10 mg/kg/day in 10% ethanol, 40% polyethylene glycol 400, 50% water; W-FIN; MWF-FIN; $n = 10$ per group) or vehicle (10% ethanol, 40% polyethylene glycol 400, 50% water; W-C; MWF-C; $n = 10$ per group) for 4 weeks by once-daily oral gavage, as previously described [38]. Systolic blood pressure (SBP) was measured at the end of treatment by the tail-cuff method after a previous adaptation to the cuff. Urinary albumin excretion (UAE) was determined placing the rats in metabolic cages for 24 h after a 1-day adaptation period. UAE was measured by enzyme-linked immunosorbent assay using a rat-specific antibody (ICN Biomedicals, Eschwege, Germany). The Institutional Animal Care and Use Committee approved all experimental procedures according to the guidelines for ethical care of experimental animals of the European Community (PROEX413/15). All efforts were made to avoid animal suffering in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting experiments involving animals [39, 40]. All experimental procedures were blinded.

Structural and Mechanical Properties in Mesenteric Resistance Arteries

Second-order branch of mesenteric resistance arteries (MA) were dissected to determine structural and mechanical properties with a pressure myograph (Model P100, Danish Myo-Tech) as previously described [32, 41, 43] (online suppl. methods; for all online suppl. material, see www.karger.com/doi/10.1159/000506275).

Elastin Content and Organization in Mesenteric Resistance Arteries

Elastin content and organization in the external elastic lamina and internal elastic lamina (IEL) were studied in intact pressure-fixed second branch MA with fluorescent confocal microscopy based on the autofluorescent properties of elastin (excitation 488 nm/emission 500–560 nm) as previously described [32, 41, 43] (online suppl. Methods).

Table 1. Characteristics of the animal model and treatment

	W	W-FIN	MWF	MWF-FIN
Body weight, g	391.8±17.2	392.5±12.7	362.8±12.8	350.3±9.0
Kidney weight, mg/cm tibia	1.1±0.1	1.1±0.04	1.1±0.04	1.0±0.03
Tibial length, cm	4.0±0.1	4.3±0.1	4.2±0.1	4.1±0.1
Urine volume, mL/24 h	11.1±1.3	12.1±1.8	10.1±0.4	10.5±0.2
Urinary albumin excretion, mg/24 h	0.17±0.01	0.22±0.04	64.4±5.6*	37.8±3.8#
SBP, mm Hg	126.9±4	127.5±5	151.5±9*	133.9±3#
HR, bpm	355.3±9.7	351.9±8.9	352.3±7.3	353.7±6.1
PWV, ms^{-1}	5.4±0.5	5.3±0.5	5.6±0.5	5.6±0.5

Data are expressed as mean ± SEM, $n = 10$.
* $p < 0.05$ compared to the W-C group.
# $p < 0.05$ compared to the MWF-C group.
W, Wistar; FIN, finerenone; MWF, Munich Wistar Frömter; C, control; MMP, matrix metalloproteinase; SBP, systolic blood pressure.

Detection of Pro-MMP-2, MMP-2, and MMP-9 Activities and Tissue Inhibitors of Metalloproteinases (TIMP-1) Levels

Plasma samples were diluted to load a final amount of 4 and 20 μg of proteins for pro-MMP-2, MMP-2, and MMP-9 activity assays, respectively. Laemmli solution (0.125 mol L^{-1} Tris, 25% glycerol, 20% SDS, and 0.01% bromophenol blue) was added to plasma samples (1:5 dilution) and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gels electrophoresis containing 0.1% gelatin. After being washed with distilled water, gels were incubated for 1 h with the activation buffer (50 mmol L^{-1} Tris-HCl, 6 mmol L^{-1} $CaCl_2$, and 2.5% Triton X-100) and for 24 h at 37 °C with Triton X-100 free activation buffer. Gels were then stained with Coomassie Brilliant Blue (BioRad) for 10 min and destained with a solution containing 40% methanol and 10% acetic acid for 1 min. Thereafter gels were incubated with a stop solution (10% acetic acid) for 24–48 h, as required. Gelatin zymographies were quantified using optical density values by ImageJ software, as described [35]. TIMP-1 plasma levels were determined by rat TIMP-1 Quantikine enzyme-linked immunosorbent assay (RTM100, BoTechne).

NO, Superoxide Anion, and Hydrogen Peroxide Bioavailability

NO bioavailability was calculated by the analysis of the difference in area under the concentration-response curve (ΔAUC) elicited by noradrenaline in MA (online suppl. Methods) in the presence and absence of the NO synthase inhibitor, N omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (10^{-4} mol L^{-1}). Superoxide anion and hydrogen peroxide bioavailability was calculated by the analysis of ΔAUC elicited by noradrenaline in the presence and absence of the inhibitor of superoxide generation, apocynin (10^{-4} mol L^{-1}), and the catalase inhibitor, 3-amino-1,2,4-triazole (5×10^{-3} mol L^{-1}), respectively.

Statistical Analysis

Number of animals per group was $n = 10$ to reach a significance level of 5% ($p < 0.05$), with a required power of 80% and a difference to be detected of 1.6 typical deviation. Student t tests or ANOVA followed by Newman-Keuls post hoc test was used as appropriate. Correlation analysis was performed through linear regression as well as analyzed by Pearson's correlation. AUC was calculated from each individual concentration-response curve plot (GraphPadSoftware). Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Results

FIN Significantly Reduced Albuminuria and SBP in MWF

FIN treatment leads to a significant reduction in UAE and SBP in MWF rats with no effect on the W group (Table 1). No differences were observed in body or kidney weight, urinary volume, and heart rate of pulse wave velocity between strains and treatments (Table 1).

FIN Reduced Arterial Stiffness in MA from MWF

In MA from W-FIN rats, vascular stress (Fig. 1a) and strain (Fig. 1b) were significantly higher compared with the W-C group. No differences were observed in incremental distensibility (Fig. 1c), in the stress/strain relationship, or in β-index (Fig. 1d). Vascular stress (Fig. 1a), strain (Fig. 1b), and incremental distensibility (Fig. 1c) were similar between MWF-C and W-C. However, in MA from MWF-C, the stress/strain relationship was significantly shifted to the left with a significantly larger β-index compared with W-C ($\beta_{W\text{-}C} = 6.4 \pm 0.4$ vs. $\beta_{MWF\text{-}C} = 9.2 \pm 0.5$; Fig. 1d).

FIN treatment significantly shifted the stress/strain relationship curve to the right, reducing β-index in MWF-FIN ($\beta_{MWF\text{-}FIN} = 7.7 \pm 0.4$ vs. $\beta_{MWF\text{-}C} = 9.2 \pm 0.5$, $p < 0.05$; Fig. 1d). There was a significant correlation between UAE and β-index in MWF rats ($r = 0.44$; $p < 0.05$; Fig. 1e).

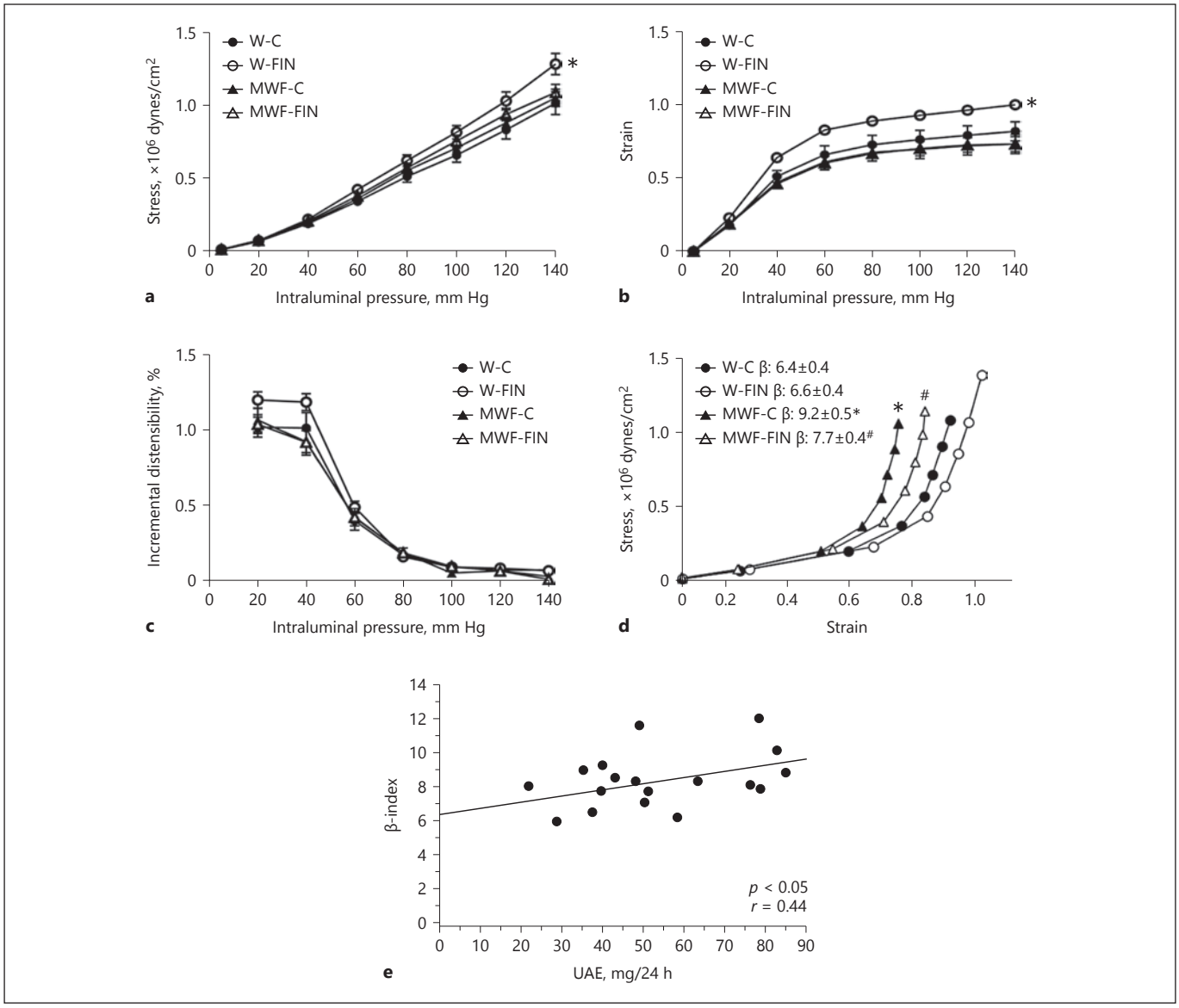

Fig. 1. Characterization of mechanical parameters in second-order mesenteric resistance arteries. **a** Wall stress-pressure. **b** strain-pressure. **c** incremental distensibility-pressure curves and (**d**) stress-strain relationships with β-values obtained from fully relaxed (Ca^{2+}-free PSS) in MA segments from C and FIN-treated W and MWF rats. **e** Correlation between UAE and β-values in MWF rats. Data are expressed as mean ± SEM of $n = 10$. * $p < 0.05$ compared with W. # $p < 0.05$ compared with MWF. MWF, Munich Wistar Frömter; W, Wistar; FIN, finerenone; C, control.

FIN Induced Changes in Elastin Organization in Mesenteric Resistance Arteries from MWF

In order to assess if the improvement in arterial elasticity is related to changes in elastin organization, MA were analyzed by confocal microscopy. Elastin organization in the IEL was altered in MA from MWF-C animals, showing a significant reduction in elastin content (Fig. 2a). FIN significantly increased both elastin content (Fig. 2b) and fenestrae area in MWF-FIN (Fig. 2c) without changes in the total number of fenestrae (Fig. 2d). No differences in elastin content were observed in the external elastic lamina of MA (Fig. 2e).

FIN Reduced Both MMP-2 and MMP-9 Activity in Plasma from MWF

Gelatinase pro-MMP-2, MMP-2, and MMP-9 activities were analyzed by zymography. Pro-MMP-2 (72 kDa) activity was significantly lower in plasma samples from

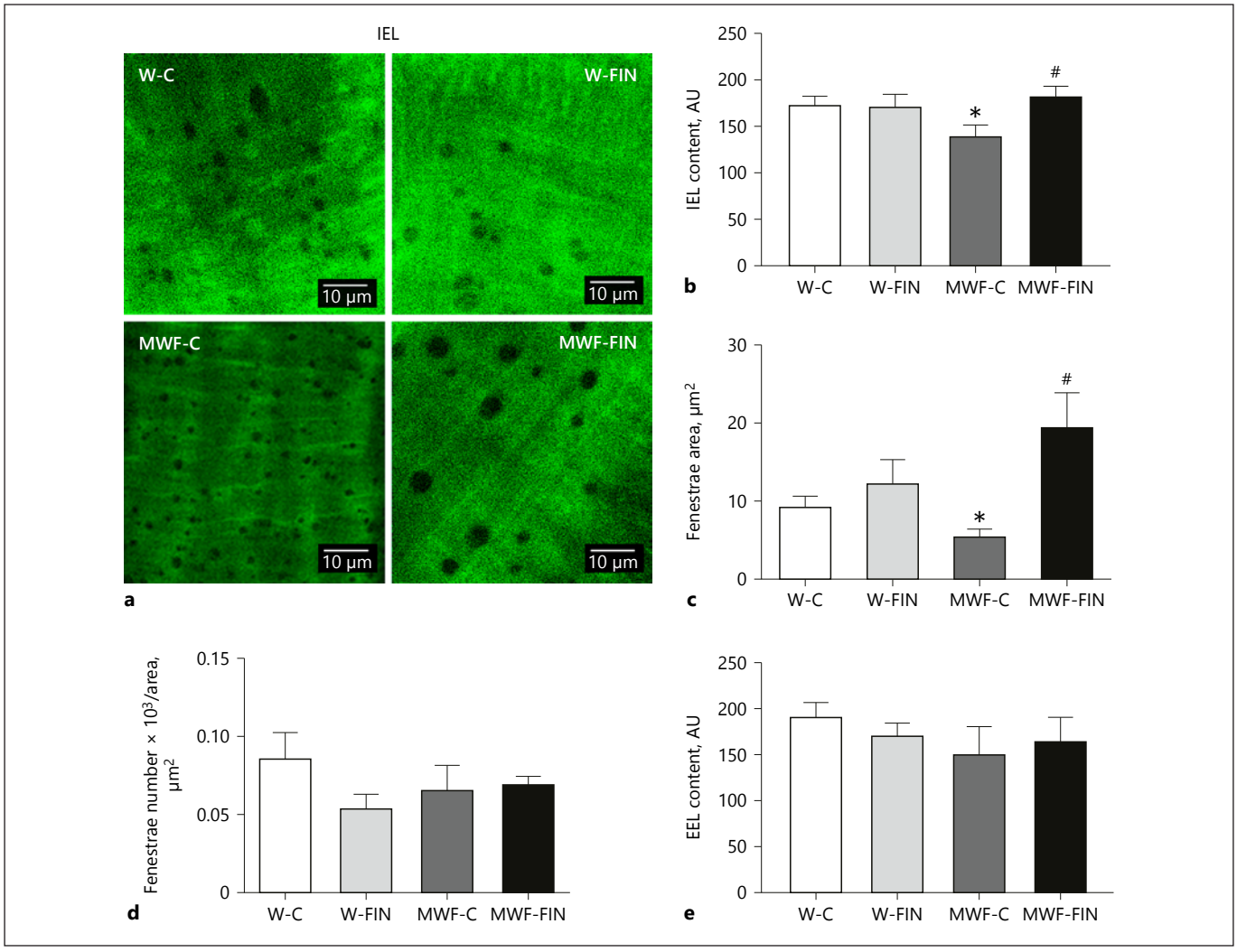

Fig. 2. Elastin content and organization in second-order mesenteric resistance arteries. Representative confocal projections of the IEL (**a**) of MA from C and FIN-treated W and MWF rats. Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×63 oil immersion objective. zoom ×2). Bars show quantification of elastin proportion in IEL (**b**), fenestrae area (**c**), fenestrae number (**d**), and quantification of elastin proportion in EEL (**e**). Results are expressed as mean ± SEM of $n = 5$. * $p < 0.05$ compared with W. # $p < 0.05$ compared with MWF. MWF, Munich Wistar Frömter; W, Wistar; FIN, finerenone; C, control; IEL; internal elastic lamina; EEL, external elastic lamina.

MWF-C rats compared with W-C rats, paralleled by higher levels of active MMP-2 (62 kDa). Active MMP-9 (82 kDa) activity was also higher in MWF-C compared to W-C rats. FIN treatment restored pro-MMP-2, MMP-2, and MMP-9 activities in MWF to control levels (Fig. 3). No differences between groups were observed in TIMP-1 levels (Fig. 3). Pro-MMP-2 showed a negative correlation with β-index, whereas correlation between β-index and MMP-2 or MMP-9 activities was positive (Fig. 3). Moreover, there was a positive correlation between UAE and MMP-2/9 activities in the MWF groups (online suppl. Fig. 1).

FIN Did Not Modify Structural Parameters in MA from MWF

No differences were observed between groups in structural parameters, that is, external diameter (online suppl. Fig. 2a), internal diameter (online suppl. Fig. 2b), wall-to-lumen ratio (online suppl. Fig. 2c), and cross-sectional area (online suppl. Fig. 2d) from MA at all intraluminal pressures tested. Confocal microscopy analyses in pressure-fixed segments at 70 mm Hg showed that adventitial, medial, and wall thicknesses were also similar between groups (online suppl. Table 1).

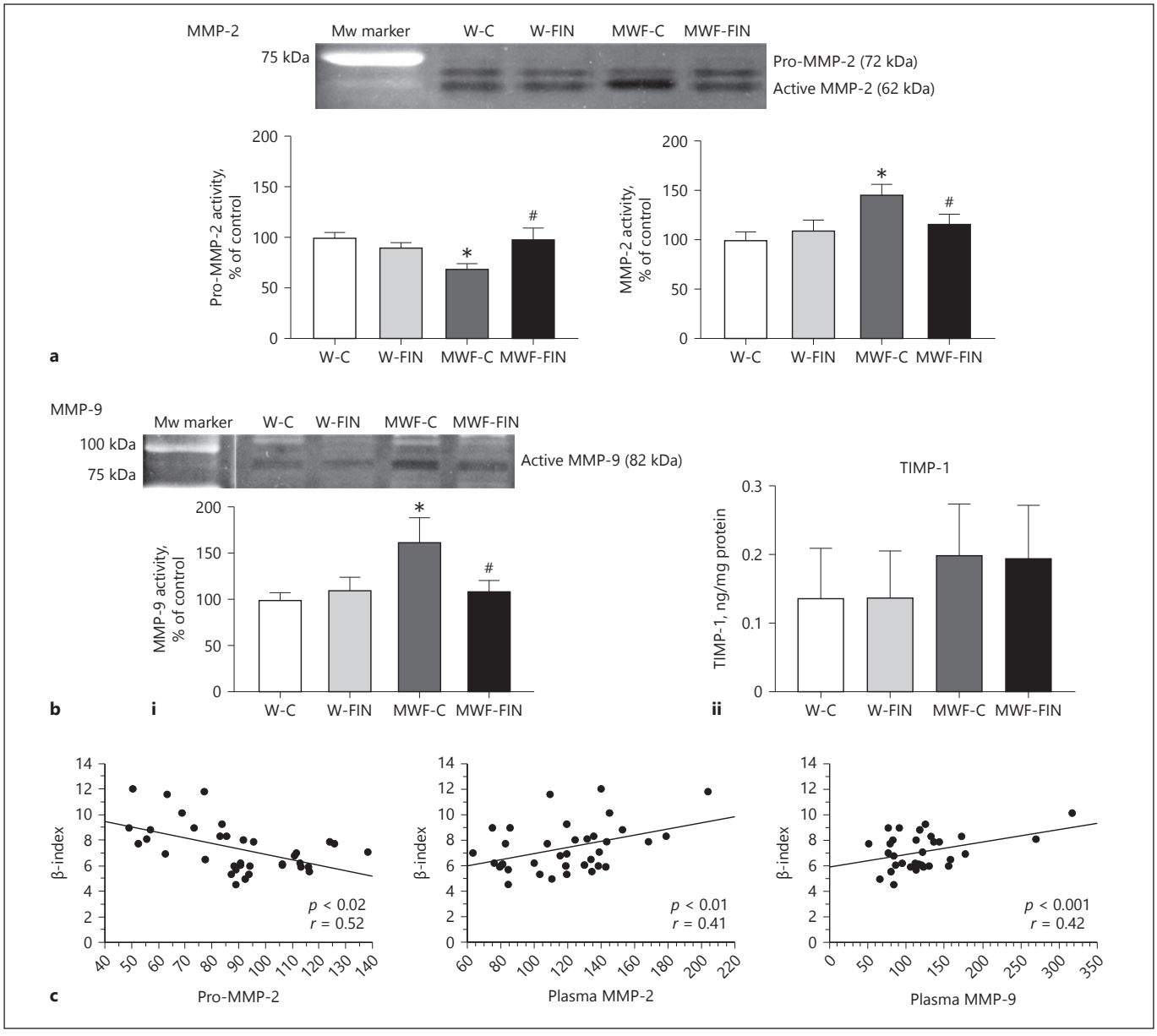

Fig. 3. MMPs (2 and 9) activities and TIMP-1 levels in plasma. **a** Representative gelatinase zymography and quantification of pro- and active MMP-2 activity in plasma of C and FIN-treated W and MWF rats. **bi** Representative gelatinase zymography and quantification of MMP-9 activity (82-kDa band) in plasma of C and FIN-treated W and MWF rats. **bii** Quantification of TIMP-1 levels in plasma of C and FIN-treated W and MWF rats. **c** Correlation between pro-MMP-2, MMP-2, or MMP-9 and β-values in MWF rats. Data are expressed in % of controls and as mean ± SEM of $n = 10$. * $p < 0.05$ compared with W. # $p < 0.05$ compared with MWF. MWF, Munich Wistar Frömter; W, Wistar; FIN, finerenone; C, control; MMP, matrix metalloproteinase.

FIN Reduces Oxidative Stress in MA from MWF Rats

NO bioavailability showed a significant increase in MWF-FIN compared to MWF-C rings (ΔAUC_{MWF-C} = 83.1 ± 19.2; $\Delta AUC_{MWF-FIN}$ = 197.5 ± 11.4; $p < 0.001$). No differences were observed between W groups (ΔAUC_{W-C} = 95.4 ± 13.4; ΔAUC_{W-FIN} = 64.3 ± 7.9; online suppl. Fig. 3c).

Superoxide anion bioavailability showed a significant decrease in MWF-FIN compared to MWF-C rings ($\Delta AUC_{MWF-C} = 102.4 \pm 9.1$; $\Delta AUC_{MWF-FIN} = 33.9 \pm 7.5$; $p < 0.001$). No differences were observed between W groups ($\Delta AUC_{W-C} = 41.5 \pm 11.3$; $\Delta AUC_{W-FIN} = 47.1 \pm 6.4$; online suppl. Fig. 4a, b). A similar reduction was observed for hy-

drogen peroxide availability (ΔAUC_{MWF-C} = 87.7 ± 12.2; $\Delta AUC_{MWF-FIN}$ = 47.8 ± 13.6; $p < 0.05$; ΔAUC_{W-C} = 50 ± 11.2; ΔAUC_{W-FIN} = 63.6 ± 8.1; online suppl. Fig. 4c, d).

Discussion

This study demonstrates the efficacy of FIN to reduce intrinsic arterial stiffness in MA from MWF, associated with a reduction in UAE. The reduction in arterial stiffness correlates with the increase in plasma pro-MMP-2 together with the reduction in plasma MMP-2 and MMP-9 activities, as well as with an increase in elastin amount and in the IEL fenestrae area. FIN also increases NO bioavailability and a reduction in superoxide and hydrogen peroxide levels in MA.

Albuminuria has been suggested as an early marker for vascular damage including arterial stiffness [42] since both phenotypes are linked in patients with hypertension [14, 15] or diabetes [16–18, 42]. We have previously shown that spontaneous albuminuria in MWF rats is associated with an increased intrinsic arterial stiffness as determined by the β-index of the stress-strain relationship in MA [32]. Here we show that FIN leads to a significant reduction of arterial stiffness in these vessels of MWF rats as evidenced by the rightward shift of the stress-strain relationship and the lower β-index, regardless of the small effect observed separately on stress and strain. Interestingly, there is a significant correlation between UAE and β-index in MWF rats.

The changes observed in elastin organization are essential for the change in elastic properties and reduction of arterial stiffness by FIN. It is well known that alterations in elastin content and organization determine mechanical properties of the vascular wall and compromise arterial elasticity contributing to arterial stiffness [44, 45]. In fact, we previously showed a negative correlation between IEL fenestrae size and β-values in MA from spontaneously hypertensive rats (SHR) [44, 45] and from MWF [32]. Changes in elastin structure associated to arterial stiffness reduction have been demonstrated for atorvastatin [46]. However, this is the first report showing that MRA treatment leads to enlarged elastin fenestrae area in the IEL associated to reduced intrinsic arterial stiffness in resistance arteries.

MMPs are important markers of the deleterious remodeling in the progression of CVD and CKD [47–49]. Although MMPs were classically viewed as antifibrotic tissue components, it is now accepted that a defective extracellular matrix (ECM) turnover managed by MMPs is associated with inflammation, deleterious remodeling, and oxidative stress [49, 50]. The increase in pro-MMP-2/MMP-2 ratio and the decrease in MMP-9 elicited by FIN treatment correlate with the reduction in arterial stiffness. Previous studies by the group showed that plasma MMP-9 activation is specifically linked to albuminuria and not to hypertension development and that increased plasma MMP-9 activity parallels increased renal MMP-9 activity [33]. This is also highlighted in this study by the positive correlation between albuminuria and both MMP-2 and MMP-9 plasma activities. Moreover, we previously demonstrated that arterial stiffness found in MWF was completely restored by albuminuria suppression in both consomic MWF-6^{SHR} and MWF-8^{SHR} rats correlating with elastin changes [32]. In this regard, a study performed in Asiatic patients with Type-2 diabetes has recently suggested arterial stiffness as a potential predictor for albuminuria progression [51]. Therefore, FIN could contribute to reducing arterial stiffness by preventing the development of albuminuria. In fact, since UAE at treatment start, that is, 12 week-old MWF, is around 40 mg/24 h [32], FIN seems to prevent a further increase in albuminuria but does not reverse already established UAE levels before treatment.

Studies performed with other MRAs such as spironolactone have also shown a significant reduction of arterial stiffness in animal models of obesity [52] or hypertension as well as in patients with early CKD [35]. Eplerenone has shown to reduce arterial stiffness in hypertensive patients or in subjects with CKD as well [34, 53]. However, steroidal MRAs are currently often underused, not approved, or even contraindicated in patients with CKD because of their risk of hyperkalemia and worsening of renal function [54]. In contrast, administration of FIN in diabetic kidney disease patients with albuminuria already receiving RAS blockade resulted in dose-dependent, significant reductions in albuminuria at doses of 7.5, 10, 15, and 20 mg after 90 days of treatment [54]. Hyperkalemia leading to discontinuation was not observed in the placebo and FIN 10 mg groups and the incidences in the other groups were only between 1.7 and 3.2% [54].

In nonhypertensive and nondiabetic conditions, there is an association between albuminuria and pulse wave velocity (PWV) [25]. Despite the increase of intrinsic stiffness of MA from MWF rats, there are still no changes in PWV, probably because the age of 16 weeks is too early to detect the overall impact of functional and mechanic changes on PWV. Since arterial stiffness is influenced by arterial blood pressure, its reduction normally decreases the "pressure-dependent" component of PWV. In this

context, it must be noted that FIN is impacting the mechanical component of the blood vessel wall independently of blood pressure reduction.

We and others have shown an association between vascular alterations and increased vascular oxidative stress both in patients with albuminuria [55–57] and in MWF rats [32, 58–60], suggesting a pathophysiological link between cardiovascular and renal injury. This strain shows an increased vascular and renal oxidative stress [32, 61]. The current beneficial effect of FIN on the increase in NO availability and reductions in superoxide anion availability adds to recent findings from our group [38], demonstrating that FIN normalizes endothelial dysfunction in conductance vessels of MWF rats, such as the aorta. In the aortic wall, FIN decreased O_2^- and H_2O_2 levels due to an upregulation in peNOS, Mn-SOD and Cu, Zn-SOD expression at a blood pressure-lowering dosage [38]. In accordance, MA show an improvement of endothelial relaxations associated to lower O_2^- and H_2O_2 levels, higher NO availability, and a decrease in albuminuria (online suppl. Fig. 3, 4, Table 2). Similar results have been observed with other MRAs. Spironolactone [62] or eplerenone [62, 63] normalizes endothelial function by reducing O_2^- and oxidative stress in several models of hypertension [64]. Spironolactone reduces moreover urinary H_2O_2 levels in recipients of a kidney transplant [65].

In conclusion, the current study shows that FIN elicits a beneficial effect on both arterial distensibility and albuminuria in the MWF CKD model. These effects are thus contributing to an overall improvement of vascular function in this setting. Thus, our beneficial experimental findings support further clinical translational studies with FIN to explore its therapeutic potential on decreasing arterial stiffness and improving overall vascular function in patients with albuminuria and CKD. FIN at daily doses of 10 and 20 mg has currently been investigated in 2 large outcome trials in patients with CKD in T2DM (FIGARO-DKD, NCT02545049 [66] and FIDELIO-DKD, NCT02540993 [67]).

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Author Contributions

M.G.-O. contributed to structural and mechanical studies, to PWV measurements, study design, data analysis, and manuscript writing. E.V.-M. contributed to structural and mechanical studies and PWV measurements. M.M.-R. contributed to treating and handling animals and collaborated with vascular function studies. B.S. contributed to vascular function studies, study design, data analysis, and manuscript writing. R.G.-B., H.P.-O., and G.R.-H. contributed to zymography studies. A.S.: contributed to animals breeding. L.M.R. and P.K. designed the study, interpreted the critical results, and wrote the manuscript. R.K. and M.S.F.-A. designed the study, supervised the experiments, interpreted the critical results, and wrote the manuscript. All authors have given a final approval of the manuscript.

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