OPEN ACCESS

Original research

Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial

Yu-Ling Yu ,[1,2] Justyna Siwy,[3] De-Wei An,[1,2,4] Arantxa González ,[5] Tine Hansen,[2,6] Agnieszka Latosinska,[3] Pierpaolo Pellicori ,[7] Susana Ravassa,[5] Beatrice Mariottoni,[8] Job AJ Verdonschot ,[9] Fozia Ahmed,[10] Johannes Petutschnigg,[11] Patrick Rossignol,[7] Stephane Heymans,[9] Joe J Cuthbert,[12] Nicolas Girerd,[7] Andrew L Clark ,[12] Peter Verhamme,[13] Tim S Nawrot,[1,14] Stefan Janssens,[15] John G Cleland ,[16] Faiez Zannad,[7] Javier Diez,[5] Harald Mischak,[3] João Pedro Ferreira ,[7,17,18] Jan A Staessen ,[2,4,19] on behalf of the HOMAGE investigators

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/heartjnl-2023-323796).

For numbered affiliations see end of article.

Correspondence to
Dr Jan A Staessen, appremed, Research Institute Alliance for the Promotion of Preventive Medicine, Mechelen, BE-2800, Belgium;
jan.staessen@appremed.org

JPF and JAS contributed equally.

Received 14 December 2023
Accepted 28 April 2024

To cite: Yu Y-L, Siwy J, An D-W, *et al*. *Heart* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/heartjnl-2023-323796

ABSTRACT

Objective Heart failure (HF) is characterised by collagen deposition. Urinary proteomic profiling (UPP) followed by peptide sequencing identifies parental proteins, for over 70% derived from collagens. This study aimed to refine understanding of the antifibrotic action of spironolactone.

Methods In this substudy (n=290) to the Heart 'Omics' in Ageing Study trial, patients were randomised to usual therapy combined or not with spironolactone 25–50 mg/day and followed for 9 months. The analysis included 1498 sequenced urinary peptides detectable in ≥30% of patients and carboxyterminal propeptide of procollagen I (PICP) and PICP/carboxyterminal telopeptide of collagen I (CITP) as serum biomarkers of COL1A1 synthesis. After rank normalisation of biomarker distributions, between-group differences in their changes were assessed by multivariable-adjusted mixed model analysis of variance. Correlations between the changes in urinary peptides and in serum PICP and PICP/CITP were compared between groups using Fisher's Z transform.

Results Multivariable-adjusted between-group differences in the urinary peptides with error 1 rate correction were limited to 27 collagen fragments, of which 16 were upregulated (7 COL1A1 fragments) on spironolactone and 11 downregulated (4 COL1A1 fragments). Over 9 months of follow-up, spironolactone decreased serum PICP from 81 (IQR 66–95) to 75 (61–90) µg/L and PICP/CITP from 22 (17–28) to 18 (13–26), whereas no changes occurred in the control group, resulting in a difference (spironolactone minus control) expressed in standardised units of −0.321 (95% CI 0.0007). Spironolactone did not affect the correlations between changes in urinary COL1A1 fragments and in PICP or the PICP/CITP ratio.

Conclusions Spironolactone decreased serum markers of collagen synthesis and predominantly downregulated urinary collagen-derived peptides, but upregulated others. The interpretation of these opposite UPP trends might be due to shrinking the body-wide pool of collagens, explaining downregulation, while some degree of collagen synthesis must be maintained to sustain vital organ functions, explaining upregulation. Combining urinary and serum fibrosis markers opens new avenues for the understanding of the action of antifibrotic drugs.

Trial registration number NCT02556450.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Spironolactone reduces plasma markers reflecting collagen-1 synthesis and decreases myocardial fibrosis by inhibiting activation of the mineralocorticoid receptors.
⇒ Over 70% of urinary peptides are derived from collagens.

WHAT THIS STUDY ADDS

⇒ In patients prone to heart failure because of coronary heart disease, spironolactone compared with control reduced 16 urinary collagen fragments and increased 11 with no other differential changes in the urinary proteome.
⇒ Spironolactone did not affect the relation between urinary and serum fibrosis markers.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Combining urinary and serum fibrosis markers opens new avenues for discovery of antifibrotic drugs and refines insight in the action of antifibrotic drugs.

INTRODUCTION

Fibrosis is the common pathological response to inflammation and chronic tissue injury, such as those that occur with ageing, hypertension, diabetes mellitus or ischaemia. Activation of the mineralocorticoid receptor (MR) initiates a cascade of molecular events leading to cell growth and inappropriate

expansion and disorganisation of the extracellular matrix (ECM) in the myocardium,[1] which is a hallmark of heart failure (HF), irrespective of its cause.[2] The Heart 'Omics' in Ageing Study (HOMAGE) was an open-label randomised clinical trial in 527 patients at high risk of developing HF.[3] Patients were randomised to spironolactone 25–50 mg/day on top of usual therapy or usual therapy alone and were followed up for 9 months.[3] Spironolactone caused a fall in serum carboxyterminal propeptide of procollagen I (PICP), a rise in serum carboxyterminal telopeptide of collagen I (CITP) and a fall in the PICP/CITP ratio. PICP and CITP are circulating biomarkers of COL1A1 synthesis and degradation, respectively (online supplemental figure S1).[3 4]

Urine contains >20 000 endogenous peptides, of which many have been sequenced, thereby identifying the parental proteins.[5] The urinary proteomic profile (UPP) consists for over 70% of collagen fragments.[6] In a subgroup of patients randomised in the HOMAGE trial, we analysed the between-group differences in the UPP and the associations of urinary with serum fibrosis markers.

METHODS

Study participants

HOMAGE was a multicentre open-label trial with blinded end point evaluation (registration number: NCT02556450).[3] Each centre had its own recruitment strategies . Patients of either sex, aged ≥60 years, were eligible provided that they were at increased risk of developing HF, because they already had or were likely to develop coronary heart disease. Additionally, eligible patients had to have a serum N-terminal pro-B-type natriuretic peptide of 125–1000 ng/L or a serum brain natriuretic peptide of 35–280 ng/L. These ranges excluded patients at low HF risk as well as those with advanced disease requiring further investigation and intensive treatment. Of 877 screened patients, 527 were randomised to spironolactone 25–50 mg/day (n=265) on top of usual treatment or usual treatment alone (n=262). The UPP was analysed in urine samples at baseline and at months 1 and 9, if sequenced peptides had a detectable signal in ≥30% of patients. A flow chart (online supplemental figure S2) shows the derivation of the HOMAGE trial subgroup dataset currently analysed.

Urinary and circulating biomarkers

Mosaiques-Diagnostics, Hannover, Germany did the UPP profiling for all patients. The methods for sample preparation, capillary electrophoresis coupled with mass spectrometry (CE-MS), peptide sequencing and for the evaluation, calibration and quality control of the mass spectrometric data have been published[7] and are described in detail in the online supplemental (pp 3–5). In the CE-MS step, 29 abundant endogenous urinary peptides were run along with the samples as internal standards for calibration of the signal intensity. This procedure is highly reproducible and addresses in a single calibration step both analytical and dilution variances, such as the variability in renal function.[8] A total of 1498 sequenced urinary peptides with a detectable signal in ≥30% of participants were analysed. Undetectable peptides were set at the distribution minimum.[9] Glomerular filtration rate was estimated (eGFR) from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration formula.[10] Using methods described in the online supplemental (p 6), serum was analysed for PICP, a marker of COL1A1 synthesis and CITP a marker of COL1A1 degradation (online supplemental figure S1).[11] All intra-assay coefficients of variations were <10%.[3]

Statistical analysis

For database management and statistical analysis, SAS software, V.9.4, maintenance level 5, was used (SAS Institute, Cary, North Carolina, USA). Deviation from the normal distribution was assessed by the Shapiro-Wilk statistic. The distributions of the serum biomarkers (online supplemental figure S3) and urinary peptides (online supplemental figure S4) were rank normalised, by sorting measurements from the smallest to the highest value and then applying the inverse cumulative normal function.[12] Rank normalised variables have mean 0 and SD 1. For non-transformed data, the central tendency (spread) of the data is given as the arithmetic mean (SD) and for rank normalised variables as median (IQR). In unadjusted analyses, means were compared using the large-sample Z-test and proportions by the χ^2 statistic or the Fisher's exact test, as appropriate based on cell frequencies. Statistical tests were two-sided.

The within-group changes (time point in the trial) and between-group differences (randomisation group) in the urinary peptides and serum biomarkers were tested, using mixed model analysis of variance with the patient modelled as random effect as implement in the PROC GLM procedure in the SAS package. The general linear models were adjusted for the baseline levels of the urinary or serum biomarkers if follow-up data were tested and additionally for sex, age, body mass index (BMI), eGFR,

Table 1 Baseline characteristics of patients

Characteristic	Control	Spironolactone
Number in group (n (%))	144	146
Women	41 (28.5)	28 (19.2)
Past smoking	77 (53.5)	94 (64.4)
Current smoking	11 (7.6)	8 (5.5)
Drinking alcohol	35 (24.3)	34 (23.3)
Hypertension	106 (73.6)	111 (76.0)
Diabetes	33 (22.9)	31 (21.2)
Antihypertensive treatment	140 (97.2)	140 (95.9)
Lipid-lowering treatment	125 (86.8)	132 (90.4)
Antidiabetic agents	54 (37.5)	51 (34.9)
Antiplatelet drugs	106 (73.6)	112 (76.7)
History of ischaemic heart disease	116 (80.6)	119 (81.5)
Clinical characteristics		
Age (years)	73.7±5.9	73.8±6.1
Body mass index (kg/m^2)	28.2±4.7	29.3±5.5
Systolic blood pressure (mm Hg)	139.4±19.0	137.2±17.5
Diastolic blood pressure (mm Hg)	78.1±10.7	77.2±10.0
Heart rate (bpm)	60.9±8.6	62.0±8.8
Biochemistry		
Serum sodium (mmol/L)	139.6±2.7	139.2±3.0
Serum potassium (mmol/L)	4.35±0.37	4.37±0.37
eGFR (mL/min/1.73 m^2)	67.4±13.8	66.7±16.7
Total cholesterol (mg/dL)	151.9±44.4	147.7±40.3
HbA1c (%)	6.20±1.40	6.03±1.21
Markers of collagen turnover		
Serum PICP (µg/L)	81.8 (69.5–94.8)	77.9 (64.2–93.6)
Serum CITP (µg/L)	3.73 (3.15–4.89)	3.78 (3.05–4.89)
PICP/CITP ratio	20.8 (15.5–29.3)	21.9 (16.6–28.1)

Data are presented as arithmetic mean±SD, n (%) or median (IQR) for variables deviating from the normal distribution.
CITP, collagen type I C-terminal telopeptide; eGFR, glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation; HbA1c, glycated haemoglobin; PICP, procollagen type I C-terminal propeptide.

Table 2 Urinary peptides with different levels on spironolactone versus control retained in the analyses

ID	Symbol	Parental protein	*r*	P value	pBH
e04960	COL1A1	Collagen alpha-1 (I) chain	0.93	0.00425	0.01392
e08916	COL1A1	Collagen alpha-1 (I) chain	1.26	0.00052	0.00010
e09408	COL1A1	Collagen alpha-1 (I) chain	1.34	0.00007	0.00252
e10266	COL1A1	Collagen alpha-1 (I) chain	1.28	0.00234	0.00649
e10395	COL1A1	Collagen alpha-1 (I) chain	1.08	0.00669	0.04818
e10437	COL1A1	Collagen alpha-1 (I) chain	1.42	0.00406	0.01217
e10657	COL1A1	Collagen alpha-1 (I) chain	1.52	0.00183	0.00470
e10863	COL1A1	Collagen alpha-1 (I) chain	1.13	0.00533	0.02132
e11972	COL1A1	Collagen alpha-1 (I) chain	0.81	0.00664	0.03985
e17425	COL1A1	Collagen alpha-1 (I) chain	0.81	0.00114	0.00273
e18740	COL1A1	Collagen alpha-1 (I) chain	0.92	0.00052	0.00111
e10563	COL2A1	Collagen alpha-1 (II) chain	1.49	0.01430	0.01716
e03506	COL3A1	Collagen alpha-1 (III) chain	0.77	0.00617	0.00723
e05700	COL3A1	Collagen alpha-1 (III) chain	0.90	0.00546	0.02457
e07668	COL3A1	Collagen alpha-1 (III) chain	2.79	0.00032	0.00033
e18839	COL3A1	Collagen alpha-1 (III) chain	0.55	0.00109	0.00245
e05473	COL4A1	Collagen alpha-1 (IV) chain	0.90	0.014689	0.01823
e17131	COL6A1	Collagen alpha-1 (VI) chain	2.03	0.00080	0.00085
e01100	COL7A1	Collagen alpha-1 (VII) chain	0.96	0.01862	0.02578
e02933	COL1A2	Collagen alpha-2 (I) chain	0.86	0.00346	0.00389
e04987	COL1A2	Collagen alpha-2 (I) chain	0.74	0.00444	0.01599
e16610	COL4A2	Collagen alpha-2 (IV) chain	0.93	0.01586	0.02039
e09267	COL5A2	Collagen alpha-2 (V) chain	0.95	0.02508	0.03311
e20509	COL11A2	Collagen alpha-2 (XI) chain	0.91	0.01593	0.02124
e15360	COL4A3	Collagen alpha-3 (IV) chain	1.43	0.00511	0.00593
e16874	COL5A3	Collagen alpha-3 (V) chain	0.90	0.03106	0.04659
e06373	COL4A6	Collagen alpha-6 (IV) chain	0.87	0.00323	0.00352

ID is the laboratory identification number of the peptide fragment. Using untransformed data, for each individual and for each peptide in the control and spironolactone group, the ratio of the 9-month-to-baseline ratio was computed. From the resulting distributions, the medians were computed; r is the ratio of the medians of these distributions (spironolactone/control). A ratio greater than unity indicates an increase in the level of the urinary peptide on spironolactone and vice versa. P value and pBH refer to the significance of the difference between control and spironolactone, respectively, without and with correction of the significance for multiple testing. Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. An ellipsis indicates that pBH was not computed, because p was not significant.
BMI, body mass index; eGFR, estimated glomerular filtration rate.

smoking and drinking, history of ischaemic heart disease and treatment at baseline and subsequent treatment changes during follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. The antihypertensive drugs applied for adjustment were diuretics (thiazides, thiazide-like agents and loop diuretics), β-blockers, vasodilators (calcium-channel blockers and α-blockers) and inhibitors of the renin-angiotensin system (ACE inhibitors and angiotensin-II receptor blockers). Lipid-lowering drugs included statins, fibrates and ezetimibe, and antiplatelet drugs aspirin and ADP receptor inhibitors. The antidiabetic drugs coded included insulin, metformin, sulfonylurea, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor antagonists, thiazolidinediones, and sodium-glucose co-transporter 2 inhibitors.

Urinary peptides with different levels at last follow-up between control and spironolactone-treated patients with a two-sided significance of 0.01 were selected, and only those keeping Benjamini-Hochberg-adjusted significance[13] of <0.05 were carried through to further analyses. Correlation coefficients of the changes from baseline to follow-up in the urinary peptides regressed on the serum biomarkers were compared between treatment groups (control vs spironolactone), using Fisher's Z transformation.[14] Between-group comparisons of the slopes of these associations were tested by linear regression models, including covariables, randomisation group, the changes in the serum biomarker and the interaction term between randomisation group and the changes in the serum biomarker.[15]

RESULTS

Patient characteristics

Descriptive data for the 290 patients in HOMAGE are shown in table 1. The HOMAGE patients were intensively treated with antihypertensive agents (n=280, 96.6%) and lipid-lowering drugs (n=257, 88.6%), mainly statins (n=251, 86.6%), antiplatelet drugs (n=218, 75.2%) and antidiabetic agents (n=105, 36.2%), insulin in 10 (3.4%) cases. None of the patients randomised in the HOMAGE trial and included in the current substudy was on treatment with sodium-glucose co-transporter-2 inhibitors. The analytical dataset of HOMAGE patients, randomised to control (n=144) or spironolactone (n=146), was well balanced with regard to risk factors, clinical characteristics, routine biochemistry and serum PICP, CITP and the PICP/CITP ratio (table 1). Compared with the 215 HOMAGE patients not included in the present analyses, the 290 patients analysed had a higher risk profile (online supplemental table S1).

Table 3 Between-group differences in the urinary peptide levels at last follow-up in HOMAGE trial

ID	Symbol	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)
e04960	COL1A1	3.9 (3.7–45.1)	3.9 (3.9–27.9)	−0.264 (−0.443 to 0.085)**
e08916	COL1A1	1946 (1432–2727)	2695 (1761–3011)	0.391 (0.173 to 0.609)***
e09408	COL1A1	685 (399–1168)	874 (627–1326)	0.461 (0.237 to 0.684)**
e10266	COL1A1	14 619 (9745–19 329)	17 047 (12 212–22 490)	0.352 (0.128 to 0.577)**
e10395	COL1A1	5997 (4727–7358)	6585 (5204–8017)	0.322 (0.091 to 0.553)**
e10437	COL1A1	359 (151–554)	459 (230–757)	0.341 (0.110 to 0.571)**
e10657	COL1A1	542 (207–1046)	784 (344–1369)	0.368 (0.139 to 0.597)**
e10863	COL1A1	2869 (2004–3984)	3301 (2606–4404)	0.299 (0.090 to 0.507)**
e11972	COL1A1	3216 (1617–5187)	2724 (1469–4216)	−0.275 (−0.473 to 0.078)**
e17425	COL1A1	58.4 (11.6–145)	19.7 (11.6–106)	−0.322 (−0.514 to 0.130)**
e18740	COL1A1	6.6 (2.1–60.9)	2.1 (2.1–31.5)	−0.350 (−0.546 to 0.155)***
e10563	COL2A1	55.3 (3.4–215)	132.5 (3.4–365)	0.292 (0.060 to 0.523)*
e03506	COL3A1	234 (55–415)	179.3 (16.4–332)	−0.316 (−0.540 to 0.092)***
e05700	COL3A1	25.8 (18.3–321)	18.8 (18.7–231)	−0.268 (−0.456 to 0.081)**
e07668	COL3A1	3.1 (3.0–158)	109 (3.1–292)	0.387 (0.179 to 0.595)**
e18839	COL3A1	185 (28.9–454)	113 (3.5–392)	−0.334 (−0.532 to 0.136)**
e05473	COL4A1	5.8 (5.7–435)	5.8 (5.7–21.4)	−0.245 (−0.441 to 0.050)*
e17131	COL6A1	150 (44–287)	211 (90.9–400)	0.381 (0.161 to 0.601)*
e01100	COL7A1	91.1 (2.5–308)	29.1 (2.5–168)	−0.234 (−0.428 to 0.040)*
e02933	COL1A2	481 (333–620)	398 (261–549)	−0.341 (−0.567 to 0.114)**
e04987	COL1A2	59.1 (6.1–281)	6.1 (6.1–161)	−0.279 (−0.470 to 0.088)**
e16610	COL4A2	5.8 (5.7–31.1)	5.8 (5.7–5.8)	−0.208 (−0.375 to 0.040)*
e09267	COL5A2	4.1 (4.0–14.0)	4.1 (4.1–6.8)	−0.211 (−0.394 to 0.027)*
e20509	COL11A2	953 (481–2149)	822 (248–1741)	−0.270 (−0.488 to 0.052)*
e15360	COL4A3	379 (186–589)	464 (193–779)	0.319 (0.098 to 0.541)*
e16874	COL5A3	4.9 (4.9–17.3)	4.9 (4.8–4.9)	−0.200 (−0.380 to 0.019)*
e06373	COL4A6	257 (94.7–433)	216 (75.2–371)	−0.278 (−0.462 to 0.945)*

ID is the laboratory identification number of the sequenced peptide. Values given for the sequenced peptides are medians of the non-normalised levels (IQR). Δ (95% CI) refers to the between-group difference (spironolactone minus control) with 95% CI. Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences in standardised units, as required by the general linear model. Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and change in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. Significance of the between-group differences was adjusted for multiple comparisons: *p≤0.05; **p≤0.01; ***p≤0.001 (see table 2 for p values).
BMI, body mass index; eGFR, estimated glomerular filtration rate; HOMAGE, Heart 'Omics' in Ageing Study.

Urinary peptides

The 1498 sequenced urinary peptides retained in the analysis were derived from 212 proteins and included 1109 (74.0%) collagen fragments and 389 (26.0%) peptides derived from other proteins. Peptide fragments were excluded from analysis if they were derived from albumin, β2-microglobulin and the fibrinogen α-chain (given their high concentration in blood); uromodulin because of its renal origin and osteopontin as prominent component of mineralised extracellular matrices.

At baseline, the levels of the selected urinary peptides were similar in both randomisation groups (online supplemental table S2). Given the balanced baseline characteristics of patients randomised to control or spironolactone, the between-group differences in the urinary and serum biomarkers and associated significance levels were not materially affected by adjustment. Table 2 shows the 27 peptides that differed at follow-up between spironolactone and control. All peptide fragments were derived from collagen. With correction of significance for multiple testing, 11 peptides were derived from COL1A1, 4 from COL3A1, 2 from COL1A2 and 1 peptide from each of 10 other collagens (table 2). Online supplemental table S3 lists the amino acid sequence of the peptides retained in analyses and the protein from which they were derived.

With control patients as reference, the effect of spironolactone on urinary peptides is shown in table 3. Of the 11 peptides derived from COL1A1, 7 had higher and 4 had lower levels on spironolactone. Of four peptides derived from COL3A1, three had lower levels on spironolactone and one had a higher level. Of the two peptides derived from COL1A2, spironolactone decreased both. Of the 27 peptide fragments, spironolactone reduced 16, while the remaining 11 had higher urinary levels on spironolactone compared with spironolactone.

Serum biomarkers

In unadjusted analyses (figure 1), spironolactone shifted the whole distribution of serum PICP and the serum PICP/CITP ratio downwards. Fully adjusted analyses (online supplemental figure S5), in which the 9-month data were plotted against baseline values confirmed this downward shift. Analyses adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease, the baseline value of the biomarker and treatment at baseline and subsequent treatment changes during follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs (table 4) revealed no between-group differences in CITP at months 1 and 9 (table 4). However, at months 1 and 9, serum PICP and the serum PICP/CITP ratio were lower on spironolactone than control.

In fully adjusted analyses (figure 2 and online supplemental table S4), the correlations between the changes from baseline

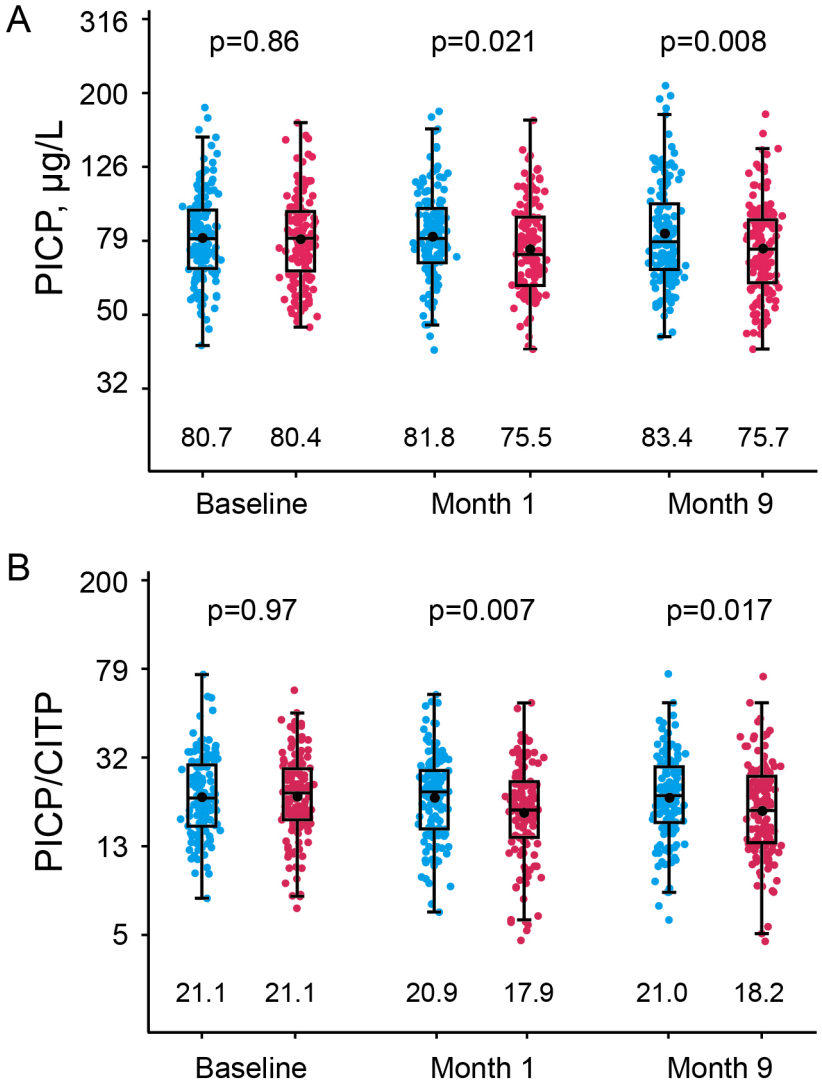

Figure 1 Distribution of serum PICP (A) and the serum PICP/CITP ratio (B) at baseline and during follow-up by treatment group. PICP is a marker of COL1A1 synthesis and CITP of COL1A1 (online supplemental figure S1). For each box plot, the central line, the upper and lower lines and the upper and lower caps represent the median, IQR and the 5th to 95th percentile interval. Dots represent individual levels with extremes falling outside the box and whiskers. The geometric means are given along the horizontal axes. P values are for the between-group differences at each time point. The shift in the distributions on spironolactone indicate decreased COL1A1 synthesis. CITP, carboxyterminal telopeptide of collagen I; PICP, carboxyterminal propeptide of procollagen I.

to follow-up in the urinary peptides and the corresponding changes in CITP, the biomarker reflecting degradation of mature COL1A1, were similar in patients randomised to control and spironolactone. None of the correlations with CITP was significant if the COL1A1 fragments decreased from baseline to follow-up (n=4), whereas the correlations with CITP were significant if the COL1A1 fragments increased during follow-up (n=7). Compared with the patients in control group (online supplemental table S5), serum sodium decreased by 0.90 mmol/L (95% CI 0.44 to 1.36 mmol/L), whereas serum potassium increased by 0.14 mmol/L (0.06 to 0.22 mmol/L) in the spironolactone group at the last follow-up visit. Moreover, compared with the control, eGFR (online supplemental table S5) decreased by 2.49 mL/min/1.73 m^2 (−4.94 to −0.47 mL/min/1.73 m^2) on spironolactone.

DISCUSSION

This preplanned substudy to the randomised controlled HOMAGE-RCT trial, to our knowledge for the first time assessed UPP changes in response to spironolactone in patients at high HF risk because of coronary heart disease. The following key observations summarise the results. First, the UPP differences between control and spironolactone were exclusively confined to 27 collagen fragments in line with the observation that collagen fragments constitute >70% of the UPP.[6] Of these 27 fragments, 16 were downregulated on spironolactone and 11 upregulated (table 3). Second, compared with control, serum PICP and the serum PICP/CITP ratio decreased on spironolactone (table 4). Finally, the correlations and regression slopes between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP were similar in patients randomised to control and spironolactone, but these correlations only reached significance, if the COL1A1 fragments were upregulated during follow-up (online supplemental table S4).

The current study shows associations, of which the interpretation remains speculative although based on literature data. The downward shift of serum PICP and the serum PICP/CITP ratio

Table 4 Serum fibrosis biomarkers at baseline and during follow-up in HOMAGE trial

Circulating biomarker	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)	P value
PICP, µg/L				
Baseline	80 (67–96)	81 (66–95)	−0.043 (−0.274 to 0.188)	0.72
Month 1	81 (69–97)	73 (60.1–92)	−0.253 (−0.413 to 0.093)	0.0025
Month 9	79 (66–100)	75 (61–90)	−0.321 (−0.501 to 0.142)	0.0007
CITP, µg/L				
Baseline	3.72 (2.89–5.09)	3.74 (2.90–4.67)	−0.058 (−0.283 to 0.168)	0.62
Month 1	3.80 (3.10–5.08)	3.93 (3.15–5.17)	0.105 (−0.048 to 0.257)	0.18
Month 9	3.82 (3.04–4.99)	4.00 (3.06–5.42)	0.079 (−0.101 to 0.259)	0.40
PICP/CITP				
Baseline	21 (16–29)	22 (17–28)	0.001 (−0.225 to 0.228)	0.99
Month 1	22 (15–28)	18 (14–25)	−0.240 (−0.406 to 0.074)	0.0056
Month 9	21 (16–29)	18 (13–26)	−0.256 (−0.451 to 0.061)	0.013

Values given for the circulating biomarkers are medians of the non-normalised non-adjusted levels (IQR). Δ (95% CI) refers to the between-group difference (spironolactone minus control) with 95% CI. Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences in standardised units, as required by the general linear model. P value denotes the significance of the between-group differences. All models were adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. The 1-month and 9-month models were additionally adjusted for the baseline value of the biomarker and the changes in the treatment status with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs.
BMI, body mass index; CITP, carboxyterminal telopeptide of collagen I; eGFR, estimated glomerular filtration rate; HOMAGE, Heart 'Omics' in Ageing Study; PICP, carboxyterminal propeptide of procollagen I.

on spironolactone replicates previous HOMAGE-RCT publications.[33] Given the stochiometric ratios relating PICP to collagen synthesis and CITP to the degradation of mature collagen-1 (online supplemental figure S1), the downregulation of urinary collagen fragments probably represent reduced synthesis of COL1A1 and by extension a lower body-wide pool of collagens available for degradation. Moreover, the decrease in serum PICP and the PICP/CITP ratio, respectively biomarkers of collagen synthesis and the ratio of collagen synthesis-to-degradation, might reflect the smaller collagen pool available for degradation.[16 17] Two mechanisms might explain why 11 collagen fragments (7 COL1A1 fragments) were upregulated. First, the correlations between the changes from baseline to follow-up in the urinary peptides and the corresponding changes in CITP, the serum marker reflecting degradation of mature COL1A1, were similar in patients randomised to control and spironolactone (online supplemental table S4), because in all conditions a certain degree of collagen turnover remains necessary to maintain physiological collagen scaffoldings. Another mechanism that might contribute to the discordant trends in the levels of the urinary peptides is a build-up of shorter collagen fragments due to the degradation of longer fragments by proteases along the nephron and the lower urinary tract.

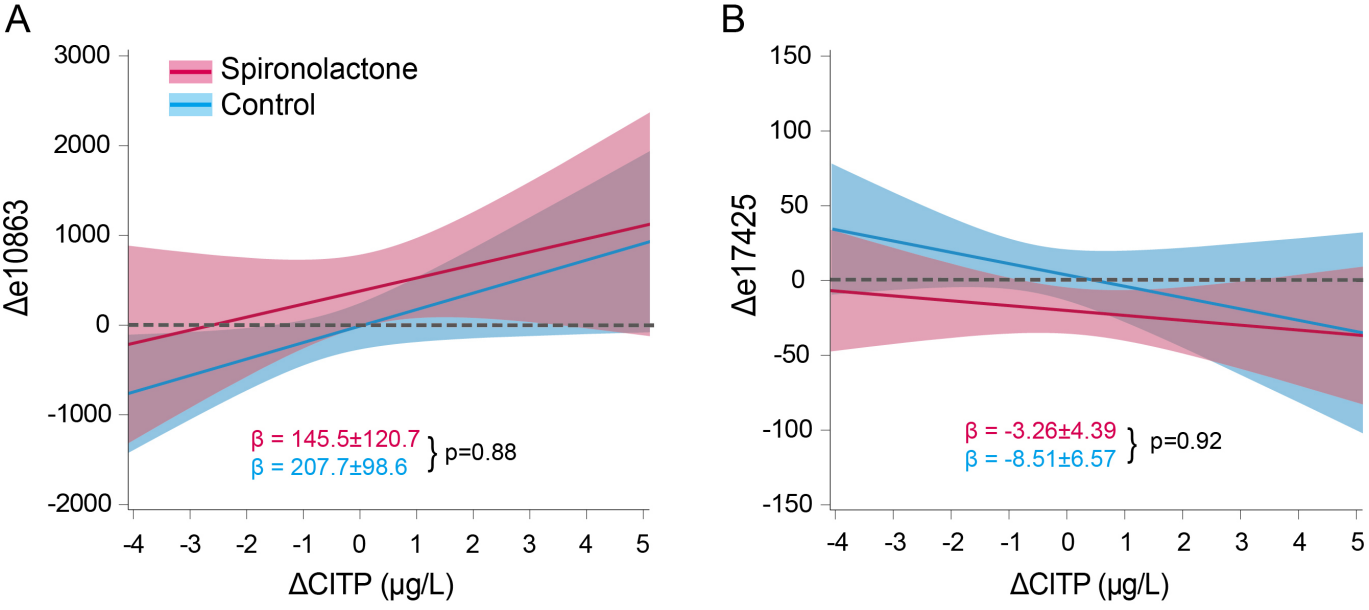

Figure 2 Linear associations between the changes over follow-up (Δ) in the COL1A1-derived peptides e10863 (A) and e17425 (B) regressed on the change (Δ) in carboxyterminal telopeptide of collagen I (CITP). e10863 increased during follow-up, whereas e17425 decreased. CITP is a serum biomarker of the degradation of mature COL1A1. The regression lines are presented with 95% CI. The regression slopes (β) are given with SE. The dotted lines indicate β=0. The p values refer to the between-group (control vs spironolactone) in the regression slopes. Models are adjusted for sex, age, body mass index, estimated glomerular filtration rate, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs.

The literature supports our interpretation of the current findings. In a random-effect meta-analysis[18] of 1038 patients randomised in HOMAGE (47.0%),[3] Aldosterone Receptor Blockade in Diastolic Heart Failure (ALDO-DHF, 37.2%)[19] and Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist (TOPCAT, 15.7%),[20] treatment with spironolactone for 9–12 months compared with placebo or usual care reduced PICP by 7.4 μg/L (95% CI 0.9 to 13.9 mg/L). This association between spironolactone and serum PICP was not mediated by blood pressure.[18] This meta-analysis was consistent with the concept that spironolactone reduces COL1A1 in patients with stages 3–4 of HF.[18] In a post hoc analysis of 1411 patients receiving spironolactone as add-on therapy in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA) trial, the serum concentrations of procollagen III amino-terminal propeptide (PIIINP) and PICP rose in controls but fell on spironolactone treatment. The adjusted mean changes were +0.52 (95% CI −0.05 to 1.09) vs −0.41 (−0.97 to 0.16) μg/L for PIIINP and +4.54 (−1.77 to 10.9) vs −6.36 (−12.5 to −0.21) μg/L for PICP.[21] An aptamer-based proteomic analysis used 5284 modified aptamers to 4928 unique proteins in 164 TOPCAT patients with paired plasma samples at baseline and 1 year.[22] The top four canonical pathways were enriched for multiple collagens that increased in the placebo group, but decreased on spironolactone.[22] In a previous HOMAGE-RCT report,[23] higher serum PICP was associated with left ventricular hypertrophy, left atrial enlargement and inversely with e' as index of left ventricular stiffness (all $p<0.05$). Moreover, the decrease in serum PICP in response to spironolactone was associated with a decline in E/e' ($p=0.022$).

Injury activates resident fibroblasts or mobilises bone marrow-derived circulating fibrocytes and epithelial or endothelial cells, and their transdifferentiate into α-smooth muscle actin-expressing myofibroblasts that secrete the ECM components. This process is required for wound repair in acute injury, but produce excessive ECM deposition in response to persistent injury.[24] Antifibrotic drugs remain a critically important unmet medical need, as nearly 45% of all natural deaths in the Western world are attributable to the complications of chronic fibroproliferative disorders.[25] Overall, the current findings might provide new perspectives in the search for refurbished or novel antifibrotic drugs and is therefore relevant for clinical practice.[26] Furthermore, non-steroidal MR antagonists and sodium-glucose co-transporter-2 inhibitors have potent anti-inflammatory and antifibrotic properties.[27] Given the present findings, UPP analysis combined with measurement of circulating fibrosis biomarkers offers novel perspectives in documenting the antifibrotic properties of novel drug classes. Of note, the serum PICP decrease produced by empagliflozin in the Empagliflozin Outcome Trial in Patients with Chronic Heart Failure (EMPEROR) was of the same order of magnitude as in the current study: 5% at 12 weeks and 8% at 52 weeks.[27]

Strengths and limitations

The randomised design of the current analysis, the first-time use of UPP data in the assessment of MR antagonism and the exploration of the changes in the urinary and serum fibrosis markers in response to spironolactone are among the strong points of the current study. However, the present study also has limitations. First, changes in CITP were not significant because of the smaller sample size compared with the full trial,[3] although the trends were similar. Second, one possible drawback of the CE-MS approach is the application of the ultrafiltration with the threshold set at 20 kDa, so that larger proteins escape analysis. Finally, proteases active along the nephron and distal urinary tract might affect the urinary peptide fragments detected by UPP analysis. However, in a placebo-controlled study of a dipeptidyl peptidase-4 inhibitor,[28] the UPP included pairs of peptide chains, that is, the substrate for the protease activity (eg, PPGP-PGKNGDDGEAGKPG) and the resulting breakdown product (eg, GPPGKNGDDGEAGKPG). In the current study, the UPP did not contain such peptide pairs, so that the assumption that spironolactone influenced the UPP by changing protease activity along the urinary tract could not be confirmed for the peptides retained in the analyses. However, this does not exclude degradation of peptides along the urinary tract, which were not retained in the analysis.

CONCLUSIONS

In patients prone to HF because of coronary heart disease, spironolactone compared with control reduced 16 urinary collagen fragments and increased 11 with no other differential changes in the urinary proteome. Spironolactone did not affect the relation between urinary and serum fibrosis markers. The interpretation of these factual observations is that MR antagonism predominantly downregulated urinary collagen-derived peptides, most likely by shrinking the body-wide pool of collagens. Why some urinary collagen fragments increased might be attributed to the maintenance of some degree of collagen synthesis and scaffolding to sustain vital organ functions or to the activity of proteases along the nephron and lower urinary tract. Combining urinary and serum fibrosis markers opens new avenues for discovery of antifibrotic drugs and refines insight in the action of antifibrotic drugs.

Author affiliations
[1]Research Unit Environment and Health, KU Leuven Department of Public Health and Primary Care, University of Leuven, Leuven, Belgium
[2]Non-Profit Research Association Alliance for the Promotion of Preventive Medicine (APPREMED), Mechelen, Belgium
[3]Mosaiques-Diagnostics GmbH, Hannover, Germany
[4]Department of Cardiovascular Medicine, Shanghai Key Laboratory of Hypertension, Shanghai Institute of Hypertension, State Key Laboratory of Medical Genomics, National Research Centre for Translational Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China
[5]Program of Cardiovascular Diseases, CIMA, Universidad de Navarra and IdiSNA, Pamplona, Spain CIBERCV, Carlos III Institute of Health, Madrid, Spain
[6]Steno Diabetes Center Copenhagen, the Capital Region of Denmark, Gentofte, Denmark
[7]Université de Lorraine, Inserm, Centre d'Investigation Clinique Plurithématique 1433, U1116, CHRU de Nancy, F-CRIN INI-CRCT, Nancy, France
[8]Department of Cardiology, Cortona Hospital, Arezzo, Italy
[9]Department of Cardiology, Maastricht University Medical Centre, Maastricht, The Netherlands
[10]Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
[11]Department of Internal Medicine and Cardiology, Campus Virchow Klinikum, Charité University Medicine Berlin, Berlin Institute of Health and German Center for Cardiovascular Research, Partner Site Berlin, Germany
[12]Department of Cardiology, University of Hull, Castle Hill Hospital, Cottingham, East Riding of Yorkshire, UK
[13]Centre for Molecular and Vascular Biology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium
[14]Centre for Environmental Sciences, Hasselt University, Hasselt, Belgium
[15]Research Unit Cardiology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium
[16]British Heart Foundation Centre of Research Excellence, School of Cardiovascular and Metabolic Health, University of Glasgow, Glasgow, UK
[17]Cardiovascular R&D Centre UniC@rRISE, Department of Physiology and Cardiothoracic Surgery, Faculty of Medicine, University of Porto Portugal, Porto, Portugal

[18]Portugal % Heart Failure Clinics, Department of Internal Medicine, Centro Hospitalar de Vila Nova de Gaia/Espinho, Vila Nova de Gaia/Espinho, Portugal
[19]Biomedical Science Group, University of Leuven, Leuven, Belgium

X Jan A Staessen @jasta49

Acknowledgements The authors are indebted to the many investigators, who were involved in HOMAGE. Their names are listed in the Data Supplement. This article was submitted for publication on their behalf.

Collaborators The HOMAGE investigators are listed in the online supplemental file (pp 2).

Contributors JGC, JD, JPF and JAS are the lead investigators who conceived the research idea and methodology. They are the guarantors for the overall content. Funding acquisition was done by FZ and JAS. NG and JPF supervised data acquisition. YY, D-WA and JAS performed the analyses and wrote the first draft of the manuscript. YY was supervised by PV and TSN. JS, AL and HM did the UPP analyses. AG, SR and JD supervised the measurements of the serum fibrosis markers. All coauthors critically revised the successive drafts of the manuscript and approved the final version.

Funding HOMAGE was funded by the European Union Seventh Framework Programme. OMRON Healthcare, Kyoto, Japan provided a non-binding grant to the Non-Profit Research Association Alliance for the Promotion of Preventive Medicine (APPREMED), Mechelen, Belgium.

Competing interests JS and AL are employees of Mosaiques-Diagnostics, Hanover, Germany. HM is the co-founder and co-owner of Mosaiques-Diagnostics. The other authors declare no conflict of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The HOMAGE trial was conducted in nine centres in the UK, France, Italy, Ireland, Germany and the Netherlands. The trial was approved by relevant ethics committees and regulatory bodies: Greater Manchester Central Research Ethics Committee (no. 16/NW/0012; EudraCT number: 2015-000413-48); Comité de Protection des Personnes Est-III, Hôpital de Brabois (no. 15.03.04); Comitato Etico Regione Toscana (no. 378/CEAVSE); Ethics and Medical Research Committee (no. 16/6/2015); Ethik-Kommission des Landes Berlin (no. 7.0.21/07/2016); De medisch-ethische toetsingscommissie (no. NL52729.068.15). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Anonymised participants data can be made available on request directed to the corresponding author. Proposals will be reviewed with scientific merit. After approval of a request, data can be shared via a secure online platform after signing a data access and confidentiality agreement. Data will be made available for a maximum of 2 years after a data sharing agreement has been signed.

ORCID iDs
Yu-Ling Yu http://orcid.org/0000-0002-8255-3770
Arantxa González http://orcid.org/0000-0001-5986-6528
Pierpaolo Pellicori http://orcid.org/0000-0001-7175-0464
Job AJ Verdonschot http://orcid.org/0000-0001-5549-1298
Andrew L Clark http://orcid.org/0000-0001-8362-668X
John G Cleland http://orcid.org/0000-0002-1471-7016
João Pedro Ferreira http://orcid.org/0000-0002-2304-6138
Jan A Staessen http://orcid.org/0000-0002-3026-1637

REFERENCES

1 Epstein M. A Podcast discussing aldosterone and mineralocorticoid antagonists in 2021: a paradigm shift. *Diabetes Ther* 2022;13:583–8.
2 Díez J. Mechanisms of cardiac fibrosis in hypertension. *J Clin Hypertens (Greenwich)* 2007;9:546–50.
3 Cleland JGF, Ferreira JP, Mariottoni B, *et al*. The effect of spironolactone on cardiovascular function and markers of fibrosis in people at increased risk of developing heart failure: the heart 'Omics' in ageing (HOMAGE) randomized clinical trial. *Eur Heart J* 2021;42:684–96.
4 Ravassa S, Kuznetsova T, Varo N, *et al*. Biomarkers of cardiomyocyte injury and stress identify left atria and left ventricular remodelling and dysfunction: a population-based study. *Int J Cardiol* 2015;185:177–85.
5 Latosinska A, Siwy J, Mischak H, *et al*. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis* 2019;40:2294–308.
6 Martens DS, Thijs L, Latosinska A, *et al*. Urinary peptidomics to address age-related disabilities: a prospective population study with replication in patients. *Lancet Healthy Longev* 2021;2:e690–703.
7 Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules* 2021;26:7260.
8 Jantos-Siwy J, Schiffer E, Brand K, *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res* 2009;8:268–81.
9 Lazar C, Gatto L, Ferro M, *et al*. Accounting for the multiple natures of missing values in label-free quantitative proteomics data SETS to compare imputation strategies. *J Proteome Res* 2016;15:1116–25.
10 Levey AS, Stevens LA, Schmid CH, *et al*. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150:604–12.
11 Ravassa S, López B, Ferreira JP, *et al*. Biomarker-based assessment of collagen cross-linking identifies patients at risk of heart failure more likely to benefit from spirolacttone effects on left atrial remodelling. *Eur J Heart Fail* 2022;24:321–31.
12 Blom G. Statistical estimates and transformed beta-variables. *Biom J* 1961;3:285.
13 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 1995;57:289–300.
14 Kleinbaum DG, Kupper LL, Nizam A, *et al*. *The Correlation Coefficient and Straight-Line Regression Analysis. Applied Regression Analysis and Other Multivariate Methods*. 4th edn. Andover, Hampshire, UK: BOOKS/COLE CENGAGE Learning, 2010:91–106.
15 Kleinbaum DG, Kupper LL, Nizam A, *et al*. *Straight-Line Regression Analysis. Applied Regression Analysis and Other Multivariate Methods*. 4th edn. Andover, Hampshire, UK: BOOKS/COLE CENGAGE Learning, 2010:41–90.
16 Magalhães P, Pejchinovski M, Markoska K, *et al*. Association of kidney fibrosis with urinary peptides: a path towards non-invasive liquid biopsies? *Sci Rep* 2017;7:16915.
17 Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Collagen-derived peptides in CKD: a link to fibrosis. *Toxins* 2022;14:10.
18 Ferreira JP, Cleland JG, Girerd N, *et al*. Spironolactone effect on circulating procollagen type I Carboxy-terminal propeptide: pooled analysis of three randomized trials. *Int J Cardiol* 2023;377:86–8.
19 Edelmann F, Wachter R, Schmidt AG, *et al*. Effect of spironolactone on diastolic function and exercise capacity in patients with heart failure with preserved ejection fraction. *JAMA* 2013;309:781.
20 Pitt B, Pfeffer MA, Assmann SF, *et al*. Spironolactone for heart failure with preserved ejection fraction. *N Engl J Med* 2014;370:1383–92.
21 Ferreira JP, Rossignol P, Pizard A, *et al*. Potential spironolactone effects on collagen metabolism biomarkers in patients with uncontrolled blood pressure. *Heart* 2019;105:307–14.
22 Javaheri A, Diab A, Zhao L, *et al*. Proteomic analysis of effects of spironolactone in heart failure with preserved ejection fraction. *Circ Heart Fail* 2022;15:e009693.
23 Kobayashi M, Girerd N, Ferreira JP, *et al*. The association between markers of type I collagen synthesis and echocardiographic response to spironolactone in patients at risk of heart failure: findings from the HOMAGE trial. *European J of Heart Fail* 2022;24:1559–68.
24 Fan D, Takawale A, Lee J, *et al*. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair* 2012;5:15.
25 Bollong MJ, Yang B, Vergani N, *et al*. Small molecule-mediated inhibition of myofibroblast transdifferentiation for the treatment of fibrosis. *Proc Natl Acad Sci U S A* 2017;114:4679–84.
26 Li X, Zhu L, Wang B, *et al*. Drugs and targets in fibrosis. *Front Pharmacol* 2017;8:855.
27 Ferreira JP, Butler J, Anker SD, *et al*. Effects of empagliflozin on collagen biomarkers in patients with heart failure: findings from the EMPEROR trials. *Eur J Heart Fail* 2024;26:274–84.
28 Siwy J, Klein T, Rosler M, *et al*. Urinary Proteomics as a tool to identify kidney responders to Dipeptidyl Peptidase-4 inhibition: a hypothesis-generating analysis of the MERLINA-T2D trial. *Proteomics Clin Appl* 2019;13:e1800144.

HEART

Supplementary information

Supplement to: *Urinary proteomic signature of mineralocorticoid receptor antagonism by spironolactone: evidence from the HOMAGE trial.*

YL Yu, J Siwy, DW An, A González, T Hansen, A Latosinska, P Pellicori, S Ravassa, B Mariottoni, JAJ Verdonschot, F Ahmed, J Petutschnigg, P Rossignol, S Heymans, JJ Cuthbert, N Girerd, AL Clark, P Verhamme, TS Nawrot, S Janssens, JG Cleland, F Zannad, J Díez, H Mischak, JP Ferreira, JA Staessen, on behalf of the HOMAGE investigators†

Table of contents

HOMAGE investigators

Robertson Centre for Biostatistics, Institute of Health and Wellbeing, University of Glasgow, Glasgow, Scotland, UK — John GF Cleland, MD; Pierpaolo Pellicori, MD; Javed Khan, MD.

Université de Lorraine, Inserm, Centre d'Investigation Clinique Plurithématique, CHRU de Nancy, Nancy, France — João P Ferreira, MD; Franco Cosmi, MD; Anne Pizard, PhD; Nicolas Girerd, MD; Patrick Rossignol, MD; Erwan Bozec, MD; María U Moreno, MD; Faiez Zannad, MD.

Department of Cardiology, Cortona Hospital, Arezzo, Italy — Beatrice Mariottoni, MD.

Department of Cardiology, University of Hull, Castle Hill Hospital, Cottingham, East Riding of Yorkshire, UK — Joe Cuthbert, MD; Andrew L Clark, MD.

Department of Cardiology, Maastricht University Medical Center, Maastricht, the Netherlands — Job AJ Verdonschot PhD; Hans P Brunner La Rocca, MD; Mark Hazebroek, PhD; Stephane Heymans, MD.

Department of Internal Medicine and Cardiology, Campus Virchow Klinikum, Charité, University Medicine Berlin, Berlin Institute of Health (BIH), and German Centre for Cardiovascular research (DZHK), Partner Site Berlin, Germany — Johannes Petutschnigg, MD; Frank Edelmann, MD; Burkert Pieske, MD.

Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK — Fozia Z Ahmed, MD; Mamas A Mamas, MD.

Centre for Prognosis Research, Institute for Primary Care and Health Sciences, Keele University, Newcastle, UK — Mamas A Mamas, MD.

German Heart Center Berlin, Berlin, Germany — Burkert Pieske, MD.

St. Vincent's University Healthcare Group, and School of Medicine, University College Dublin, Dublin, Ireland — Ken McDonald, MD.

Equipe obésité et insuffisance cardiaque, Université Paul Sabatier, Inserm I2MC, Toulouse, France — Philippe Rouet, MD.

Studies Coordinating Centre, Research Unit Hypertension and Cardiovascular Epidemiology, Department of Cardiovascular Sciences, University of Leuven, Leuven, Belgium — L Thijs, MSc.

Non-Profit Research Association Alliance for the Promotion of Preventive Medicine, Mechelen, Belgium — Jan A Staessen, MD; Kei Asayama, MD; Tine W Hansen, MD; Gladys E Maestre, MD.

Program of Cardiovascular Diseases, CIMA. Universidad de Navarra and IdiSNA, Pamplona, Spain CIBERCV, Carlos III Institute of Health, Madrid, Spain — Arantxa González, PhD; Suzanna Ravassa, PhD; Begoñia López, PhD; Javier Díez, MD.

Departments of Nephrology and Cardiology, Clínica Universidad de Navarra, Pamplona, Spain — Javier Díez, MD.

Department of Cardiovascular Medicine, Istituto di Ricerche Farmacologiche Mario Negri – IRCCS, Milan, Italy — Roberto Latini, MD.

Fondation Force, Research and Consulting Department, EDDH, Centre de Médecine Préventive, Vandoeuvre les Nancy, France — Stephanie Grojean, PhD.

Department of Medical Statistics, London School of Hygiene and Tropical Medicine, London, UK — Tim Collier, PhD.

Urinary proteomics

All steps of the CE-MS analysis and the performance of the analytical platform have recently been described in detail.[1]

Sample preparation and CE-MS analysis

Urine aliquots were thawed and 700 µL mixed with 700 µl of 2 M urea, 10 mM NH_4OH containing 0·02 % SDS. Subsequently, samples were ultrafiltered using a Centristat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) to eliminate high molecular weight proteins. The obtained filtrate was desalted using a PD 10 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden) to remove urea, electrolytes and salts as well as to enrich polypeptides. The samples were lyophilised and stored at 4°C before usage. Shortly before CE-MS analysis, the samples were re-suspended in 10 µL HPLC-grade H_2O. Samples were injected into CE-MS with 2 psi for 99 sec, resulting in injection volumes of ~280 nL.

A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker Daltronic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade water (Roth, Karlsruhe, Germany) supplemented with 0·94% formic acid (Sigma-Aldrich) was used as running buffer. For CE-MS analysis, the electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4·0 to -4·5 kV. Spectra were recorded over an *m*/*z* range of 350-3000 and accumulated every 3 s.

CE-MS data processing

After the CE-MS analysis, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software.[2] Only signals with z>1 observed in a minimum of 3 consecutive spectra with a signal-to-noise

ratio of at least 4 were considered. The resulting peak list characterises each polypeptide by its mass and migration time. Data were calibrated utilizing 3151 internal standards as reference data points for mass and migration time by applying global and local linear regression, respectively. Reference signals of 29 abundant peptides were used as internal standards for calibration of signal intensity using linear regression. This procedure is highly reproducible and addresses both analytical and dilution variances in a single calibration step.[3] The obtained peak list characterises each polypeptide by its calibrated molecular mass [Da], calibrated CE migration time [min] and normalised signal intensity. All detected peptides are deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Sequencing of peptides

Candidate biomarkers were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail.[4] MS/MS experiments were using an Ultimate 3000 nano-flow system (Dionex/LC Packings, USA) or a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), both connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific, Germany) equipped with a nano-electrospray ion source. The mass spectrometer is operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 300–2,000) were acquired in the Orbitrap. Ions were sequentially isolated for fragmentation. Data files were searched against the UniProt human nonredundant database using Proteome Discoverer 2·4 and the SEQUEST search engine. Relevant settings were: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high-confidence peptides were defined by cross-correlation (Xcorr) >1.9 and rank = 1. Precursor

mass tolerance and fragment mass tolerance were 5 ppm and 0.05 Da, respectively. For further validation of obtained peptide sequences, the correlation between peptide charge at the working pH of 2 and CE-migration time was utilised to minimise incorrect sequence assignment:[5] calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time.

References

1 Mavrogeorgis E, Mischak H, Latosinska A, *et al*. Reproducibility evaluation of urinary peptide detection using CE-MS. *Molecules* 2021;26:7260.

2 Latosinska A, Siwy J, Mischak H, *et al*. Peptidomics and proteomics based on CE-MS as a robust tool in clinical application: the past, the present, and the future. *Electrophoresis* 2019;40:2294-308.

3 Jantos-Siwy J, Schiffer E, Brand K, *et al*. Quantitative urinary proteome analysis for biomarker evaluation in chronic kidney disease. *J Proteome Res* 2009;8:268-81.

4 Klein J, Papadopoulos T, Mischak H, *et al*. Comparison of CE-MS/MS and LC-MS/MS sequencing demonstrates significant complementarity in natural peptide identification in human urine. *Electrophoresis* 2014;35:1060-4.

5 Zürbig P, Renfrow MB, Schiffer E, *et al*. Biomarker discovery by CE-MS enables sequence analysis via MS/MS with platform-independent separation. *Electrophoresis* 2006;27:2111-25.

Serum biomarkers

PICP was quantified by an enzyme linked immunosorbent assay (Quidel Corporation, San Diego, CA) and CITP by a quantitative radio-immunoassay (Orion Diagnostica, Espoo, Finland). The detection limits were 0.2 μg/L for PICP and 0.6 μg/L for CITP. All inter- and intra-assay coefficients of variation were <10%.

References

López B, Ravassa S, González A, et *al*. Myocardial collagen cross-linking is associated with heart failure hospitalization in patients with hypertensive heart failure. *J Am Coll Cardiol* 2016;67:251-60.

Ravassa S, López B, Ferreira JP, et *al*. Biomarker-based assessment of collagen cross-linking identifies patients at risk of heart failure more likely to benefit from spironolactone effects on left atrial remodelling. Insights from the HOMAGE clinical trial. *Eur J Heart Fail* 2022;24:321-31.

Ethics approval

The HOMAGE trial was conducted in nine centres in the United Kingdom, France, Italy, Ireland, Germany and the Netherlands. The trial was approved by relevant ethics committees and regulatory bodies. The name of the Ethics Committee for each country and the reference numbers of the ethical approvals are listed below.

- Greater Manchester Central Research Ethics Committee (No. 16/NW/0012).
- Comité de Protection des Personnes Est-III, Hôpital de Brabois (No. 15.03.04).
- Comitato Etico Regione Toscana (No. 378/CEAVSE).
- Ethics and Medical Research Committee (No. 16/6/2015).
- Ethik-Kommission des Landes Berlin (No. 7.0.21/07/2016).
- De medisch-ethische toetsingscommissie (No. NL52729.068.15).

Table S1 Comparison of patients analysed and not analysed (starts)

Characteristic	Analysed	Not analysed	p
Number in group	290	215	…
Number (%)			
Women	69 (23.8)	57 (26.5)	0.4851
Past smoking	171 (59.0)	117 (54.4)	0.3074
Current smoking	19 (6.5)	23 (10.7)	0.0693
Drinking alcohol	69 (23.8)	20 (9.3)	<0.0001
Hypertension	217 (74.8)	177 (82.3)	0.0442
Diabetes	64 (22.1)	20 (9.3)	0.0001
Antihypertensive treatment	280 (96.6)	92 (42.8)	0.1096
Lipid-lowering treatment	257 (88.6)	159 (74.0)	<0.0001
Antiplatelet drugs	218 (75.5)	142 (66.0)	0.0250
Antidiabetic agents	257 (88.6)	201 (93.5)	0.1337
History of IHD	235 (81.1)	130 (60.5)	<0.0001
Clinical characteristics			
Age (y)	73.8±6.0	73.5±7.3	0.5879
Body mass index (kg/m^2)	28.8±5.1	28.9±5.0	0.7618
Systolic blood pressure (mm Hg)	138.3±18.2	139.5±17.7	0.4758

Table S1 Comparison of patients analysed and not analysed (ends)

Characteristic	Analysed	Not analysed	p
Diastolic blood pressure (mm Hg)	77.6±10.3	77.8±9.6	0.8491
Heart rate (bpm)	61.4±8.7	62.1±8.5	0.4201
Routine biochemistry			
Serum sodium (mmol/L)	139.2±2.6	139.1±2.6	0.5734
Serum potassium (mmol/L)	4.36±0.37	4.33±0.33	0.3787
eGFR (mL/min/1.73 m^2)	67.1±15.3	70.8±17.4	0.0122
Total cholesterol (mg/dL)	149.8±42.4	141.4±53.1	0.0479
HbA1C (%)	6.11±1.31	6.13±1.02	0.8979

Data are presented as arithmetic mean ± standard deviation, *n (%)* or median (interquartile range) for variables deviating from the normal distribution. p-values were computed by the large sample Z test (after rank normalization, if appropriate) for continuously distributed variables or by the χ^2 statistic or Fisher's exact test, as appropriate according to the cell frequencies, for categorical variables. Abbreviations: eGFR, glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation; HBA1C, glycated haemoglobin.

Table S2 Urinary peptide levels at baseline by randomised groups

ID	Symbol	Control (n=144)	Spironolactone (n=146)
e04960	COL1A1	4.81 (4.31-33.9)	4.81 (4.71-37.8)
e08916	COL1A1	2133 (1644-2984)	1981 (1495-2649)
e09408	COL1A1	725 (415-1297)	679 (455-1034)
e10266	COL1A1	14,824 (10,672-20,817)	15,567 (9956-20,058)
e10395	COL1A1	6150 (4872-7577)	5887 (4909-7096)
e10437	COL1A1	415 (197-649)	374 (170-637)
e10657	COL1A1	691 (309-1245)	488 (211-1084)
e10863	COL1A1	2901 (2127-3916)	2803 (2071-3953)
e11972	COL1A1	3179 (1469-4696)	3267 (1818-4775)
e17425	COL1A1	45.4 (11.0-126)	55.9 (11.1-151)
e18740	COL1A1	6.50 (6.49-47.9)	6.62 (6.50-43.2)
e10563	COL2A1	129 (8.15-312)	109 (8.15-312)
e03506	COL3A1	190 (63.5-351)	196 (66.5-355)
e05700	COL3A1	5.17 (4.36-327)	5.52 (5.36-305)
e07668	COL3A1	60.5 (13.3-274)	17.7 (13.3-259)
e18839	COL3A1	209 (23.8-519)	193 (63.9-486)
e05473	COL4A1	2.62 (2.44-26.7)	2.62 (2.44-50.5)
e17131	COL6A1	156 (61.1-347)	157.1 (3.10-339.1)
e01100	COL7A1	94.7 (4.85-253)	87.7 (4.85-206.3)
e02933	COL1A2	468 (273-619)	441 (295-656)
e04987	COL1A2	66.6 (9.97-279)	43.9 (9.97-290.4)
e16610	COL4A2	5.26 (4.89-23.5)	5.26 (4.88-31.7)
e09267	COL5A2	3.34 (2.96-10.8)	3.34 (3.08-13.3)
e20509	COL11A2	879 (362-1776)	1025 (444-1858)
e15360	COL4A3	358 (175-678)	339 (176-643)
e16874	COL5A3	4.56 (4.07-17.0)	4.56 (3.75-20.8)
e06373	COL4A6	190 (72.8-367)	230 (88.0-409)

ID is the laboratory identification number of the peptide fragment. Symbol refers to the parental protein (see table S3). Tabulated values are median (interquartile range) of the urinary peptides retained in the statistical analysis. There were no between-group differences in the urinary peptide levels ($p \geq 0.29$).

Table S3 Urinary peptide sequences retained in the analyses (starts)

ID	Symbol	Amino-acid sequence	Protein (*Uniprot ID*)
e04960	COL1A1	GppGpPGSpGEQGPSG	collagen alpha-1 (I) chain (*P02452)*
e08916	COL1A1	DDGEAGKPGRpGERGpPGP	collagen alpha-1 (I) chain (*P02452*)
e09408	COL1A1	GDDGEAGKpGRPGERGPPGp	collagen alpha-1 (I) chain (*P02452*)
e10266	COL1A1	NGDDGEAGKpGRpGERGPPGP	collagen alpha-1 (I) chain (*P02452*)
e10395	COL1A1	NGDDGEAGKpGRPGERGpPGp	collagen alpha-1 (I) chain (*P02452*)
e10437	COL1A1	AEGSPGRDGSpGAKGDRGETGP	collagen alpha-1 (I) chain (*P02452*)
e10657	COL1A1	AEGSpGRDGSpGAKGDRGETGp	collagen alpha-1 (I) chain (*P02452*)
e10863	COL1A1	DGQPGAKGEpGDAGAKGDAGPPGp	collagen alpha-1 (I) chain (*P02452*)
e11972	COL1A1	ADGQpGAKGEPGDAGAKGDAGppGPA	collagen alpha-1 (I) chain (*P02452*)
e17425	COL1A1	AGPTGARGAPGDRGEPGPpGpAGFAGpPGADGQPGAK	collagen alpha-1 (I) chain (*P02452*)
e18740	COL1A1	DKGETGEQGDRGIKGHRGFSGLQGppGPPGSPGEQGP	collagen alpha-1 (I) chain (*P02452*)
e10563	COL2A1	NPGEPGEpGVSGPMGpRGPpGP	collagen alpha-1 (II) chain (*P02458*)
e03506	COL3A1	SpGERGETGppGP	collagen alpha-1 (III) chain (*P02461*)
e05700	COL3A1	TGpGGDKGDTGPpGPQG	collagen alpha-1 (III) chain (*P02461*)
e07668	COL3A1	GTGGPpGENGKpGEpGPKG	collagen alpha-1 (III) chain (*P02461*)
e18839	COL3A1	GPPGMPGPRGSPGpQGVKGESGKpGANGLSGERGpPGPQG	collagen alpha-1 (III) chain (*P02461*)
e05473	COL4A1	GPpGFTGPPGPPGPPGP	collagen alpha-1 (IV) chain (*P02462*)

Table S3 Urinary peptide sequences retained in the analyses (ends)

ID	Symbol	Amino-acid sequence	Protein (*UniProt ID*)
e17131	COL6A1	PPGDPGLMGERGEDGpAGNGTEGFpGFPGYPGN	collagen alpha-1 (VI) chain (*P12109*)
e01100	COL7A1	DRGEpGPpGP	collagen alpha-1 (VII) chain (*Q02388*)
e02933	COL1A2	GppGPDGNKGEpG	collagen alpha-2 (I) chain (*P08123*)
e04987	COL1A2	pGpQGVQGGKGEQGp	collagen alpha-2 (I) chain (*P08123*)
e16610	COL4A2	DTGNPGAPGTpGTKGWAGDSGpQGRpGVFGLPG	collagen alpha-2 (IV) chain (*P08572*)
e09267	COL5A2	PGPVGApGDAGQRGDPGSRGP	collagen alpha-2 (V) chain (*P05997*)
e20509	COL11A2	GEHGpPGPPGPIGPVGQPGAAGADGEPGARGPQGHFGAKGDEGTRGFNGP	collagen alpha-2 (XI) chain (*P13942*)
e15360	COL4A3	GpKGDpGIpGLDRSGFpGETGSPGIPGHQ	collagen alpha-3 (IV) chain (*Q01955*)
e16874	COL5A3	DLGPpGDpGVSGIDGSpGEKGDPGDVGGPGPPGASG	collagen alpha-3 (V) chain (*P25940*)
e06373	COL4A6	SGpPGFPGLGTTGEKGE	Collagen alpha-6(IV) chain (*Q14031*)

ID is the laboratory identification number of the peptide fragment. Bolded IDs indicate urinary peptide fragments upregulated on spironolactone compared to control (see table 3). A lower case “p” in the amino-acid sequence indicates hydroxyproline. The protein identification number was obtained from the UniProt database (www.uniprot.org).

Table S2 Correlations between the changes over follow-up in COL1A1-derived urinary peptides and CITP

Biomarkers	Control (n=144)		Spironolactone (n=146)		p_{DIF}
	r (95% CI)	p	*r* (95% CI)	p	
Increasing peptide levels					
Δ e08916 (*COL1A1*)	0.079 (-0.085, 0.240)	0.34	0.025 (-0.138, 0.187)	0.77	0.12
Δ e09408 (*COL1A1*)	0.122 (-0.042, 0.280)	0.14	0.081 (-0.083, 0.240)	0.33	0.053
Δ e10266 (*COL1A1*)	0.145 (-0.019, 0.302)	0.080	0.051 (-0.112, 0.212)	0.54	0.33
Δ e10395 (*COL1A1*)	0.208 (-0.046, 0.359)	0.012	-0.072 (-0.232, 0.091)	0.39	0.47
Δ e10437 (*COL1A1*)	0.259 (0.100, 0.406)	0.0016	0.165 (-0.003, 0.319)	0.045	0.61
Δ e10657 (*COL1A1*)	0.188 (0.025, 0.341)	0.024	0.149 (-0.013, 0.304)	0.071	0.078
Δ e10863 (*COL1A1*)	0.198 (0.035, 0.350)	0.017	0.221 (0.060, 0.370)	0.0071	0.36
Decreasing peptide levels					
Δ e04960 (*COL1A1*)	-0.003 (-0.167, 0.160)	0.97	0.101 (-0.063, 0.259)	0.23	0.97
Δ e11972 (*COL1A1*)	-0.033 (-0.131, 0.195)	0.69	-0.073 (-0.232, 0.091)	0.38	0.81
Δ e17425 (*COL1A1*)	-0.076 (-0.237, 0.089)	0.36	-0.089 (-0.248, 0.075)	0.28	0.66
Δ e18740 (*COL1A1*)	-0.076 (-0.237, 0.089)	0.36	0.078 (-0.085, 0.238)	0.35	0.38

Δ, change from baseline to month 9; CITP, collagen type I C-terminal telopeptide (marker of degradation of mature collagen-1). Tabulated values are partial correlation coefficients (*r*) given with 95% confidence interval. Models are adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. Peptide levels increasing or decreasing from baseline to month 9 are listed in Table 3. *p* and p_{DIF} refer to the significance of the correlation coefficients and the significance of the between-group differences in the partial correlation coefficients.

Table S3 Between-group differences in serum electrolytes and glomerular filtration at last follow-up visit

Trial Biomarker	Control (n=144)	Spironolactone (n=146)	Δ (95% CI)	p
Serum Na^+ (mmol/L)	139.3±0.2	138.4±0.2	-0.90 (-1.36, -0.44)	0.0001
Serum K^+ (mmol/L)	4.39±0.03	4.53±0.03	+0.14 (+0.06, +0.22)	0.0008
eGFR (mL/min/1.73 m^2)	70.7±0.86	68.18±0.85	-2.49 (-4.94, -0.47)	0.047

Values are means ± SE or median (interquartile range). Δ (95% CI) provides multivariable-adjusted estimates of the between-group differences . Models were adjusted for sex, age, BMI, eGFR, the baseline value of the urinary marker, current smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs. eGFR is the glomerular filtration rate estimated from serum creatinine according to the Chronic Kidney Disease Epidemiology equation.

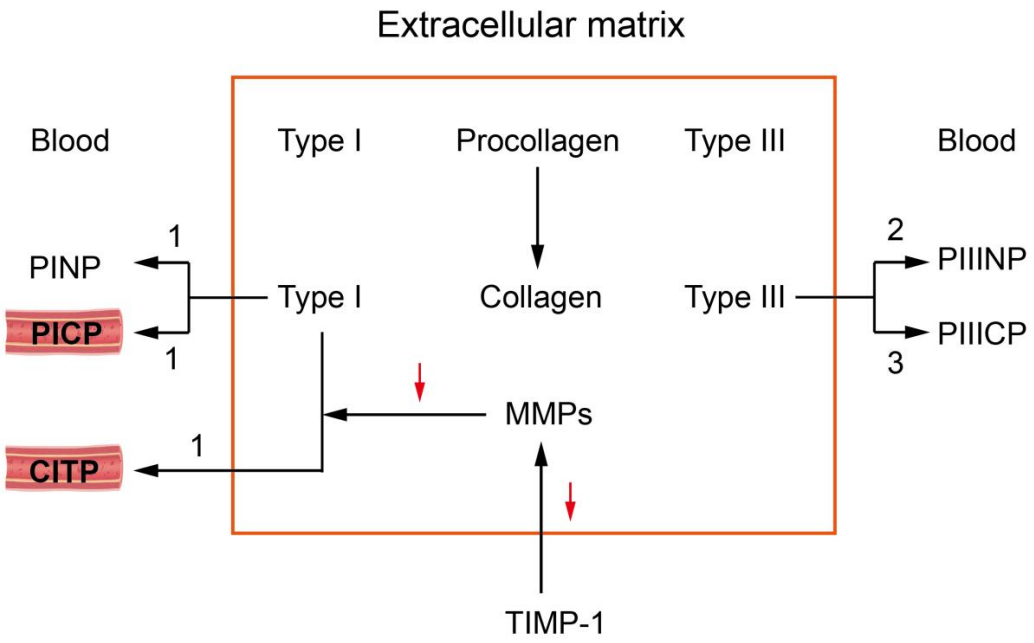

Figure S1

Circulating biomarkers of collagen turnover. Procollagen type-I amino-terminal propeptide (PINP) and procollagen type-I carboxy-terminal propeptide (PICP) are released during conversion of procollagen type-1 to collagen type-1 and carboxyterminal telopeptide of type-I collagen (CITP) during the degradation of collagen type-1 by matrix metalloproteinases (MMPs), which are inhibited by tissue inhibitor of the matrix metalloproteinase type-1 (TIMP1). Procollagen type-III amino-terminal propeptide (PIIINP) and procollagen type-III carboxy-terminal propeptide (PIIICP) are released during conversion of procollagen type-III to collagen type-III. Bracketed numbers indicate the stoichiometric ratio. PICP and CITP, which were analysed in the current HOMAGE Trial dataset, are serum markers of collagen type-1 synthesis and degradation, respectively. PIIINP is an indirect indicator of collagen-III synthesis, because cleavage at the amino-terminus proceeds at a relatively slow rate and, thus, partially processed procollagen molecules remain bound to the surface of collagen type-III fibres (*JACC* 2015;65:2449-2456).

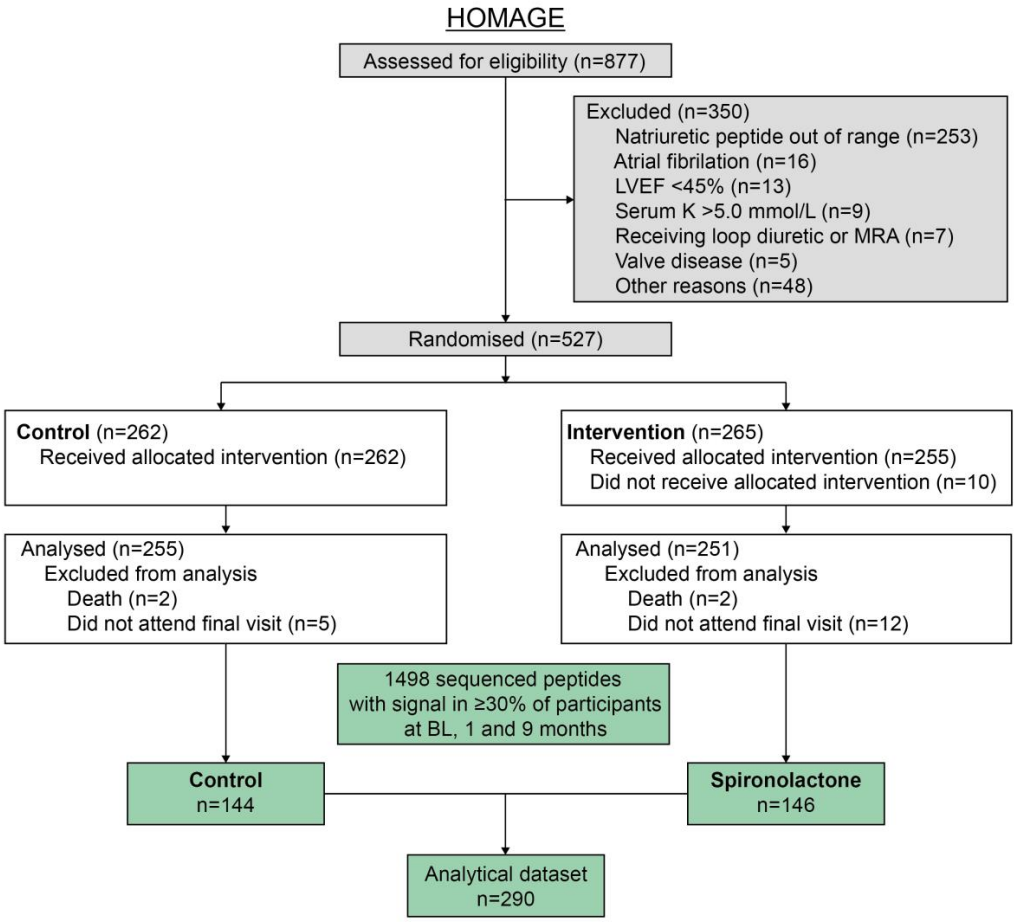

Figure S2

Flow chart of the HOMAGE analytical dataset. LVEF, left ventricular ejection fraction; BL, baseline.

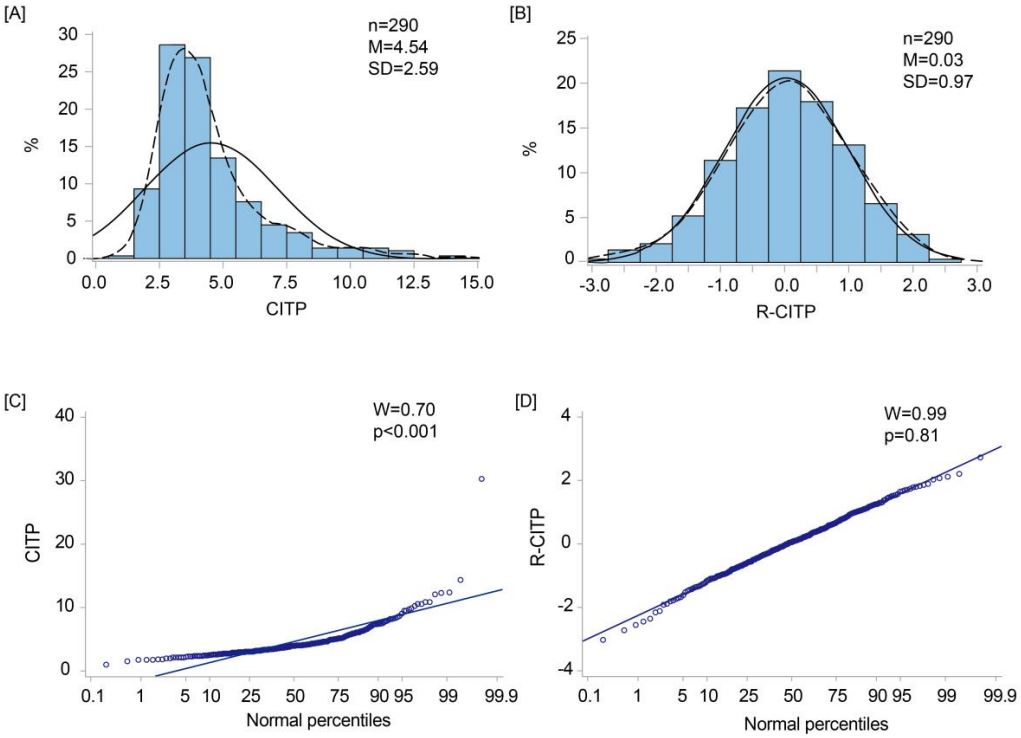

Figure S3

Rank normalisation of a carboxyterminal telopeptide of collagen I (CITP), a serum marker of collagen I degradation. Panels A and B show the distribution plots before (A) and after (B) rank-normalisation; panels C and D show the normal percentile plots before (C) and after (D) rank normalisation. The solid and dotted lines represent the normal and kernel density distributions. *n*, M and SD refer to the number of patients, the arithmetic means and standard deviation. W is the Shapiro-Wilk statistic and p is the associated significance. A significant Shapiro-Wilk test indicates deviation from the normal distribution.

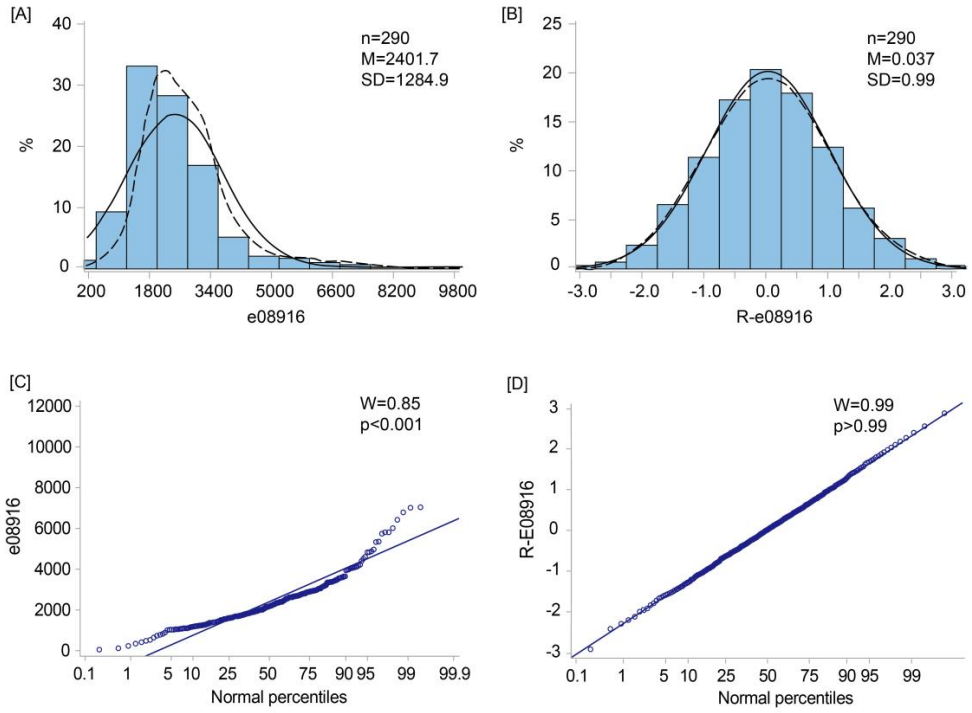

Figure S4

Rank normalisation of urinary peptide fragment e08916 derived from collagen I. Panels A and B show the distribution plots before (A) and after (B) rank-normalisation; panels C and D show the normal percentile plots before (C) and after (D) rank normalisation. The solid and dotted lines represent the normal and kernel density distributions. *n*, M and SD refer to the number of patients, the arithmetic means and standard deviation. W is the Shapiro-Wilk statistic and p is the associated significance. A significant Shapiro-Wilk test indicates deviation from the normal distribution.

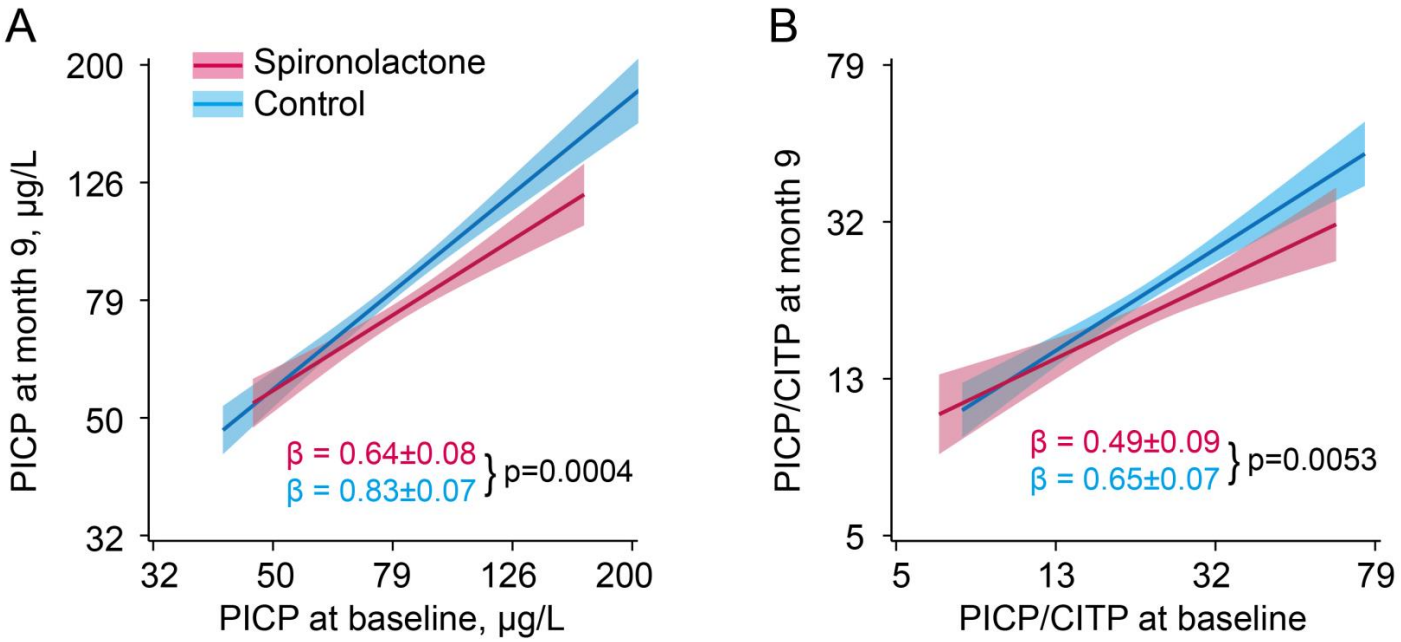

Figure S5

Linear associations between the 9-month serum PICP (A) and the serum PICP/CITP ratio (B) regressed on the baseline levels by treatment group. PICP (carboxyterminal propeptide of procollagen I) is a marker of COL1A1 synthesis and CITP (carboxyterminal telopeptide of collagen I) of COL1A1 degradation (see supplemental figure 1). The regression lines are presented with 95% confidence interval. The regression slopes (β) are given with standard error. p values refer to the between-group (control *vs* spironolactone) in the regression slopes. Models are adjusted for sex, age, BMI, eGFR, smoking and drinking, history of ischaemic heart disease and treatment at baseline and changes in treatment at last follow-up with antihypertensive, lipid-lowering, antiplatelet and antidiabetic drugs.