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Proteomic and Mechanistic Analysis of Spironolactone in Patients at Risk for HF

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ABSTRACT

OBJECTIVES This study sought to further understand the mechanisms underlying effect of spironolactone and assessed its impact on multiple plasma protein biomarkers and their respective underlying biologic pathways.

BACKGROUND In addition to their beneficial effects in established heart failure (HF), mineralocorticoid receptor antagonists may act upstream on mechanisms, preventing incident HF. In people at risk for developing HF, the HOMAGE (Heart OMics in AGEing) trial showed that spironolactone treatment could provide antifibrotic and antiremodeling effects, potentially slowing the progression to HF.

METHODS Baseline, 1-month, and 9-month (or last visit) plasma samples of HOMAGE participants were measured for protein biomarkers (n = 276) by using Olink Proseek-Multiplex cardiovascular and inflammation panels (Olink, Uppsala, Sweden). The effect of spironolactone on biomarkers was assessed by analysis of covariance and explored by knowledge-based network analysis.

RESULTS A total of 527 participants were enrolled; 265 were randomized to spironolactone (25 to 50 mg/day) and 262 to standard care ("control"). The median (interquartile range) age was 73 years (69 to 79 years), and 26% were female. Spironolactone reduced biomarkers of collagen metabolism (e.g., COL1A1, MMP-2); brain natriuretic peptide; and biomarkers related to metabolic processes (e.g., PAPPA), inflammation, and thrombosis (e.g., IL17A, VEGF, and urokinase). Spironolactone increased biomarkers that reflect the blockade of the mineralocorticoid receptor (e.g., renin) and increased the levels of adipokines involved in the anti-inflammatory response (e.g., RARRES2) and biomarkers of hemostasis maintenance (e.g., tPA, UPAR), myelosuppressive activity (e.g., CCL16), insulin suppression (e.g., RETN), and inflammatory regulation (e.g., IL-12B).

CONCLUSIONS Proteomic analyses suggest that spironolactone exerts pleiotropic effects including reduction in fibrosis, inflammation, thrombosis, congestion, and vascular function improvement, all of which may mediate cardiovascular protective effects, potentially slowing progression toward heart failure. (HOMAGE [Bioprofiling Response to Mineralocorticoid Receptor Antagonists for the Prevention of Heart Failure]; NCT02556450)
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Preventing, rather than treating, heart failure (HF) might be a more effective way of increasing long-term survival and delaying health-related quality of life impairment. In addition to their beneficial effects in established HF, mineralocorticoid receptor (MR) antagonists (MRAs) might be useful for preventing incident HF (1).

Individuals at risk for developing HF often have higher levels of natriuretic peptides; increased activation of profibrotic, inflammatory and apoptotic pathways; elevation of markers of vascular and endothelial dysfunction; atherosclerosis; and increased activation of the renin-angiotensin-aldosterone system (RAAS) (2). Because of their pleiotropic effects beyond sodium and potassium regulation, MRAs (e.g., spironolactone, eplerenone, finerenone) may positively affect these aforementioned mechanisms (3). In the HOMAGE (Heart OMics in AGEing) trial, treatment with spironolactone decreased markers of collagen synthesis, decreased the circulating levels of N-terminal pro-brain-natriuretic peptide (NT-proBNP), reduced blood pressure, and improved cardiac remodeling in asymptomatic people at risk for developing HF (4).

In this pre-specified secondary analysis of the HOMAGE trial, we aimed to assess the effect of spironolactone on multiple circulating proteomic biomarkers to better characterize the biologic pathways that could be affected by spironolactone in individuals at risk of developing HF.

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METHODS

TRIAL DESIGN AND POPULATION. The HOMAGE trial had a prospective, randomized, open-label, blinded-endpoint, multicenter design in which people at increased risk of developing HF were randomly assigned to receive either spironolactone or the standard of care (control)—not receiving spironolactone or other MRAs (NCT02556450). The rationale, trial design, and main results have been published (1). The study was approved by all relevant ethics committees and regulatory bodies. All participants provided written informed consent before study-specific procedures. The main participation criteria included age of 65 years or older (amended to 60 years during the course of the trial) and cardiovascular risk, defined by the presence of coronary artery disease or at least 2 of the following: diabetes, treated hypertension, microalbuminuria, abnormal electrocardiogram, and an NT-proBNP level between 125 and 1,000 ng/l or BNP level between 35 and 280 ng/l. The main exclusion criteria were glomerular filtration rate of <30 ml/min/1.73 m^2, serum potassium level of >5.0 mmol/l, left ventricular ejection fraction of <45%, and diagnosis of HF or treatment with loop diuretic agents and atrial fibrillation/flutter.

HOMAGE TRIAL PATIENTS, FOLLOW-UP, AND ENDPOINTS. A total of 527 patients were randomized (265 to spironolactone and 262 to the standard of care). The median (interquartile range) follow-up time was 8.9 months (6.0 to 9.2 months). The primary endpoint was the interaction between the treatment and the baseline levels of galectin-3 for the change in serum concentrations of PIIINP (procollagen type III N-terminal propeptide) from baseline to the end of follow-up (the 9-month visit). Secondary aims were to investigate the effects of spironolactone on the change (from baseline to the end of follow-up) of other markers of collagen metabolism (procollagen type I C-terminal propeptide [PICP] and collagen type I-C terminal telopeptide [CITP]), NT-proBNP, echocardiographic measures of cardiac structure and function, and signs/symptoms. One-month changes were also assessed in exploratory analyses. At baseline, 1 month, and at the end of the study, participants performed clinical, biomarker, and echocardiographic measurements (4).

PROTEOMIC BIOMARKERS. Baseline, month 1, and month 9 (or last visit) plasma samples were analyzed for 276 protein biomarkers by the TATAA Biocenter by using the Olink Proseek Multiplex Cardiovascular CVD II, CVD III, and inflammation panels. These panels were selected by the balanced inclusion of proteins with already established associations with cardiovascular disease and HF (e.g., BNP or GDF-15) and many others, more exploratory, with less

ABBREVIATIONS AND ACRONYMS

BNP = brain natriuretic peptide
ECM = extracellular matrix
FDRq = false discovery rate q value
HF = heart failure
IGF = insulin growth factor
IGFBP = insulin growth factor binding protein
MMP = matrix metalloproteinase
MR = mineralocorticoid receptor
MRA = mineralocorticoid receptor antagonist
NT-proBNP = N-terminal pro-brain-natriuretic peptide
RAAS = renin-angiotensin-aldosterone system
VEGF = vascular endothelial growth factor

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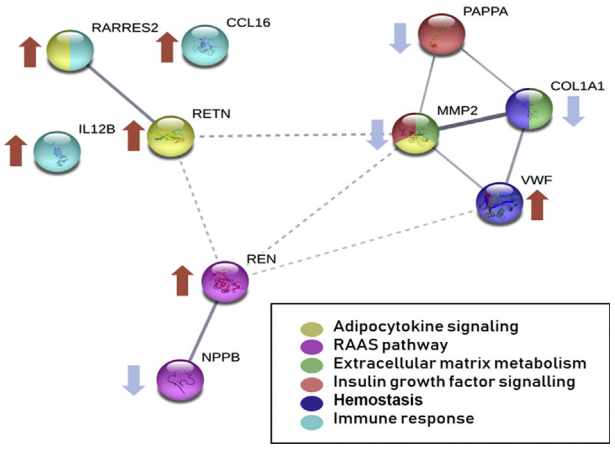
FIGURE 1 Focused Network of Biomarkers Influenced by Spironolactone Treatment From Baseline to the Last Visit With an FDRq of <0.05.



Knowledge-based network with the top plasma protein biomarkers that changed with spironolactone treatment. COL1A1 (collagen type I alpha 1 chain), MMP2 (matrix metalloproteinase 2), PAPPA (pappalysin 1), VWF (Von Willebrand factor), CCL16 (C-C motif chemokine ligand 16), RETN (resistin), IL12B (interleukin 12B), REN (renin), RARRES2 (retinoic acid receptor responder 2); NPPB (natriuretic peptide B). The **thickness of the line** indicates the strength of data support as defined by STRING, with the **dashed line** as weak confidence of evidence. The **colors of the nodes** indicate significant Kyoto Encyclopedia of Genes and Genomes and Gene Ontology pathways that were significantly overrepresented. FDRq = false discovery rate q value; RAAS = renin-angiotensin-aldosterone system; STRING = Search Tool for the Retrieval of Interacting Genes/Proteins.

TABLE 1 Changes in Biomarkers With Spironolactone Treatment (From Baseline to Last Visit)

Biomarker	β-Coefficient (95% CI)	p Value	FDRq
Decreased with spironolactone			
COL1A1	−0.14 (−0.20 to −0.08)	0.00001	0.0014
MMP2	−0.10 (−0.16 to −0.04)	0.0008	0.032
BNP	−0.28 (−0.44 to −0.11)	0.001	0.041
PAPPA	−0.13 (−0.20 to −0.05)	0.001	0.044
VEGFD	−0.08 (−0.13 to −0.03)	0.003	0.064
NOTCH3	−0.09 (−0.15 to −0.03)	0.003	0.068
EPCAM	−0.16 (−0.27 to −0.05)	0.005	0.090
BOC	−0.06 (−0.11 to −0.02)	0.007	0.10
IL4RA	−0.09 (−0.15 to −0.02)	0.007	0.10
IL17A	−0.15 (−0.27 to −0.04)	0.009	0.12
SELE	−0.09 (−0.16 to −0.02)	0.011	0.12
APN	−0.07 (−0.12 to −0.01)	0.013	0.13
THBS2	−0.04 (−0.08 to −0.01)	0.019	0.17
AXL	−0.07 (−0.13 to −0.01)	0.027	0.20
TIE2	−0.05 (−0.09 to −0.01)	0.027	0.20
ALCAM	−0.06 (−0.11 to −0.01)	0.027	0.20
CNTN1	−0.06 (−0.12 to −0.01)	0.033	0.23
IL17D	−0.06 (−0.11 to −0.01)	0.037	0.24

Continued on the next page

well-established roles (e.g., TRAIL or PAPPA [pappalysin 1]). The proteins were determined by using high-throughput Olink Proseek Multiplex 96×96 kits, which measures 92 manually selected proteins simultaneously in 1 μl of plasma per kit. Each kit uses proximity extension assay technology with a dual-recognition DNA-coupled readout, where 92 oligonucleotide-labeled antibody probe pairs are allowed to bind to their respective targets in the sample. The platform provides log2 normalized protein expression (NPX) values with relative quantification. A detailed description of the Olink technology is provided on the company's website (https://www.olink.com/). The abbreviations, full names, and respective Olink multiplex panels of the studied proteins are described in Supplemental Table 1. The assays were performed in a blinded fashion to the treatment allocation. The proteomic results were then merged into the database.

STATISTICAL ANALYSES. The primary analysis focused on the changes of the proteins from baseline to month 9 or the final visit (for consistency with the primary report [4]), using analysis of covariance comparing the difference of changes between the control and spironolactone groups in the regression model. A linear regression model was fitted, with protein change (last visit - baseline) as the outcome variable, a binary variable to indicate the treatment group (control/spironolactone), and the baseline protein value (normalized protein expression) as covariates. The treatment effect was the coefficient that resulted from the comparison of spironolactone vs. control in the regression model. Residual analysis was used to examine the fit of the model. No data transformation was required to meet the assumptions of linear regression. Similar analyses were performed for the protein change at 1 month. A correction for multiplicity of tests using a false discovery rate q value (FDRq) of <0.05, as described by Benjamini and Hochberg, was applied to the protein change from baseline to the last visit. All other analyses should be viewed as exploratory, including the interaction term between treatment and baseline protein level (below vs. above the median), to evaluate whether the effect of spironolactone on PIIINP and PICP could have varied by the baseline levels of the studied proteins. Statistical analyses were performed by using Stata, version 16 (StataCorp, College Station, Texas).

BIOINFORMATICAL AND NETWORK ANALYSES. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to analyze the functional enrichment (Gene Ontology biologic processes, Kyoto Encyclopedia of Genes and Genomes,

and Reactome pathways) and connections of proteins that were strongly changed by spironolactone. In addition, we used knowledge-based network analysis with the induced network approach by the consensuspathDB online server (accessed on February 17, 2020) from the Max Planck Institute for Molecular Genetics (Berlin, Germany) to identify the links among all significantly changed protein biomarkers, based on known knowledge of interactions (protein interactions, genetic interactions, biochemical interactions, and gene regulatory interactions) (5). The network analysis also identifies additional proteins limited to the first-degree interactors (intermediate nodes) linking our input proteins (seed nodes), with exclusion of low-confidence interactions and quantified by a z-score of ≤20 calculated for each intermediate node. The created network was reorganized in Cytoscape, version 3.7, to cluster proteins based on biologic functions and linked this network to spironolactone via MR-aldosterone-angiotensin receptor.

RESULTS

CHARACTERISTICS OF THE POPULATION. The median (interquartile range) age was 73 (69 to 79) years; 26% of participants were female, 71% had coronary artery disease, and 41% had diabetes (Supplemental Table 2).

EFFECT OF SPIRONOLACTONE ON PLASMA PROTEINS FROM BASELINE TO THE LAST VISIT. Compared with the control, 18 proteins decreased with spironolactone treatment at the $p < 0.05$ level, among which 4 proteins strongly decreased at the FDRq <0.05 level. These (FDRq <0.05) were COL1A1 (collagen type I alpha 1 chain), MMP2 (matrix metalloproteinase 2), BNP (brain natriuretic peptide), and PAPPA (pappalysin 1) (**Figure 1**), plus ($p < 0.05$) VEGFD (vascular endothelial growth factor D), NOTCH3 (notch receptor 3), EPCAM (epithelial cell adhesion molecule), BOC (BOC cell adhesion associated, oncogene regulated), IL4RA (interleukin 4 receptor subunit alpha), IL17A (interleukin 17A), SELE (selectin E), APN (aminopeptidase N), THBS2 (thrombospondin 2), AXL (AXL receptor tyrosine kinase), TIE2 (angiopoietin-1 receptor), ALCAM (activated leukocyte cell adhesion molecule), CNTN1 (contactin 1), and IL17D (interleukin17D) (**Table 1, Figure 2**).

Compared with control, 33 proteins increased with spironolactone treatment at the $p < 0.05$ level, among which 6 proteins strongly increased at the FDRq <0.05 level. These (FDRq <0.05) were REN (renin), RARRES2 (retinoic acid receptor responder 2), VWF (Von Willebrand factor), CCL16 (C-C motif chemokine ligand 16), RETN (resistin), and IL12B (interleukin 12B) (**Figure 1**), plus ($p < 0.05$) PGLYRP1 (peptidoglycan recognition protein 1), IL6RA

TABLE 1 Continued

Biomarker	β-Coefficient (95% CI)	p Value	FDRq
Increased with spironolactone			
REN	0.53 (0.42 to 0.63)	0.00001	0.0014
RARRES2	0.09 (0.04 to 0.14)	0.0002	0.018
VWF	0.39 (0.18 to 0.59)	0.0003	0.021
CCL16	0.12 (0.05 to 0.19)	0.0008	0.032
RETN	0.15 (0.06 to 0.24)	0.0008	0.032
IL12B	0.13 (0.05 to 0.22)	0.002	0.044
PGLYRP1	0.13 (0.05 to 0.22)	0.003	0.064
IL6RA	0.08 (0.03 to 0.14)	0.004	0.085
AMBP	0.05 (0.01 to 0.08)	0.006	0.10
CCL19	0.15 (0.04 to 0.26)	0.007	0.10
MMP7	0.10 (0.03 to 0.18)	0.009	0.12
PLC	0.06 (0.02 to 0.11)	0.010	0.12
CCL25	0.07 (0.02 to 0.13)	0.010	0.12
TRAIL	0.06 (0.01 to 0.11)	0.011	0.13
TPA	0.19 (0.04 to 0.34)	0.012	0.13
GAL9	0.06 (0.01 to 0.11)	0.013	0.13
NT3	0.11 (0.02 to 0.20)	0.018	0.17
SRC	0.22 (0.04 to 0.40)	0.019	0.17
CSTB	0.10 (0.02 to 0.19)	0.021	0.18
FABP4	0.12 (0.02 to 0.23)	0.024	0.20
GDF15	0.09 (0.01 to 0.16)	0.024	0.20
TNFRSF9	0.08 (0.01 to 0.15)	0.0269	0.20
CST5	0.07 (0.01 to 0.13)	0.0334	0.23
CCL3	0.09 (0.01 to 0.18)	0.034	0.23
CPA1	0.12 (0.01 to 0.22)	0.0352	0.23
MPO	0.04 (0.00 to 0.07)	0.0387	0.24
TFPI	0.06 (0.00 to 0.12)	0.0392	0.24
UPAR	0.07 (0.00 to 0.14)	0.0402	0.24
TFF3	0.07 (0.00 to 0.13)	0.042	0.25
CXCL9	0.11 (0.01 to 0.22)	0.0433	0.25
ADM	0.06 (0.00 to 0.11)	0.0459	0.26
KLK6	0.07 (0.00 to 0.13)	0.0488	0.27
PRTN3	0.05 (0.00 to 0.11)	0.0497	0.27

ADM = adrenomedullin; ALCAM = activated leukocyte cell adhesion molecule; AMBP = alpha-1-microglobulin/bikunin precursor; APN = aminopeptidase N; AXL = AXL receptor tyrosine kinase; BOC = BOC cell adhesion associated, oncogene regulated; BNP = brain natriuretic peptide; CCL16 = C-C motif chemokine ligand 16; CCL19 = C-C motif chemokine ligand 19; CCL25 = C-C motif chemokine ligand 25; CCL3 = C-C motif chemokine ligand 3; CI = confidence interval; CNTN1 = contactin 1; COL1A1 = collagen type I alpha 1 chain; CPA1 = carboxypeptidase A1; CST5 = cystatin D; CSTB = cystatin B; CXCL9 = C-X-C motif chemokine ligand 9; EPCAM = epithelial cell adhesion molecule; FABP4 = fatty acid binding protein 4; FDRq = false discovery rate q value; GAL9 = galectin-9; GDF15 = growth differentiation factor 15; IL12B = interleukin 12B; IL17A = interleukin 17A; IL17D = interleukin17D; IL4RA = interleukin 4 receptor subunit alpha; IL6RA = interleukin 6 receptor A; KLK6 (kallikrein related peptidase 6; MMP2 = matrix metalloproteinase 2; MMP7 = matrix metalloproteinase 7; MPO = myeloperoxidase; NOTCH3 = notch receptor 3; NT3 = neurotrophin 3; PAPPA = pappalysin 1; PGLYRP1 = peptidoglycan recognition protein 1; PLC = phospholipase C gamma 1; PRTN3 = proteinase 3; RARRES2 = retinoic acid receptor responder 2; REN = renin; RETN = resistin; SELE = selectin E; SRC = SRC proto-oncogene, non-receptor tyrosine kinase; TFF3 = trefoil factor 3; TFPI = tissue factor pathway inhibitor; THBS2 = thrombospondin 2; TIE2 = angiopoietin-1 receptor; TNFRSF9 = TNF receptor superfamily member 9; TPA = tissue-type plasminogen activator; TRAIL = TRAIL/TNF superfamily member 10; UPAR = plasminogen activator, urokinase receptor; VEGFD = vascular endothelial growth factor D; VWF = Von Willebrand factor.

(interleukin 6 receptor A), AMBP (alpha-1-microglobulin/bikunin precursor), CCL19 (C-C motif chemokine ligand 19), MMP7 (matrix metallopeptidase 7), PLC (phospholipase C gamma 1), CCL25 (C-C motif chemokine ligand 25), TRAIL (TRAIL/TNF superfamily member 10), TPA (tissue-type plasminogen activator), GAL9 (galectin-9), NT3 (neurotrophin 3), SRC (SRC proto-oncogene, non-receptor tyrosine kinase), CSTB (cystatin B), FABP4 (fatty acid binding protein 4), GDF15 (growth differentiation factor 15), TNFRSF9 (tumor necrosis factor receptor superfamily member 9), CST5 (cystatin D), CCL3 (C-C motif chemokine ligand 3), CPA1 (carboxypeptidase A1), MPO (myeloperoxidase), TFPI (tissue factor pathway inhibitor), UPAR (plasminogen activator, urokinase receptor), TFF3 (trefoil factor 3), CXCL9 (C-X-C motif chemokine ligand 9), ADM (adrenomedullin), KLK6 (kallikrein related peptidase 6), and PRTN3 (proteinase 3) (**Table 1, Figure 2**).

The results for all studied proteins are presented in the supplemental material (Supplemental Tables 3 and 4). None of the studied proteins had a strong correlation between each other (Spearman rho of <0.70 for all comparisons) (Supplemental Table 5) or protein-clinical parameter correlation (Spearman rho of <0.50 for all comparisons) (Supplemental Table 6). Information regarding network edges, nodes, and interactions can be found in Supplemental Table 7.

PROTEINS THAT CHANGED AT BOTH MONTH 1 AND THE LAST VISIT. Compared with control, 19 proteins significantly changed ($p < 0.05$) with spironolactone treatment at both month 1 and month 9. Five proteins decreased: COL1A1, MMP2, BNP, VEGFD, and NOTCH3; 14 proteins increased: renin, IL12B, AMBP, CCL19, CCL25, TRAIL, CSTB, FABP4, TNFRSF9, CST5, CCL3, CPA1, TFF3, CXCL9 (**Table 2**).

NETWORK ANALYSIS. The 10 proteins that strongly changed after spironolactone treatment (FDRq < 0.05) were used as input to create a focused network. Overall, 3 clusters of proteins can be identified, collectively fitting 6 biologic functions (**Figure 1**). The pathways with the strongest associations with the 10 proteins are the RAAS pathway, extracellular matrix (ECM) metabolism, insulin growth factor (IGF) signaling, hemostasis, and adipocytokine signaling.

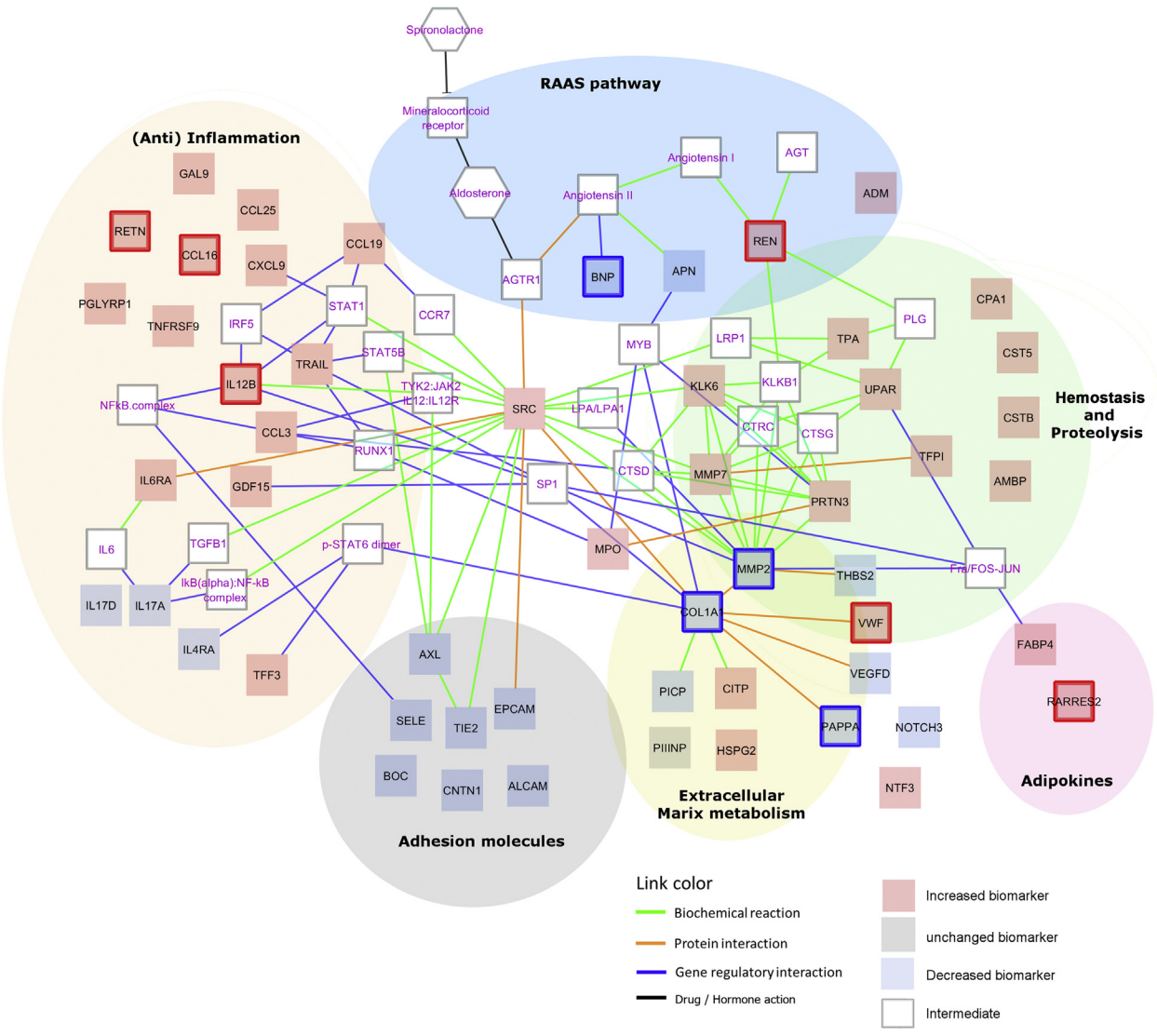
To get a broader overall picture of the biologic network, we used all of the 51 proteins that significantly changed with spironolactone treatment ($p < 0.05$) to generate **Figure 2**. Spironolactone directly affected the RAAS pathway and then activated downstream proto-oncogene tyrosine-protein kinase Src (SRC), which showed both the highest betweenness centrality (0.510) and the highest closeness centrality (0.519) of the network, making SRC a key hub through which spironolactone orchestrates its multiple biologic functions. Furthermore, ECM metabolism and hemostasis are 2 major biologic clusters affected by spironolactone, in line with the focused network (**Central Illustration**).

PROTEINS THAT COULD INFLUENCE THE EFFECT OF SPIRONOLACTONE ON COLLAGEN. We tested the interaction between all of the baseline proteins (below vs. above the median) and the effect of spironolactone on PICP and PIIINP. For PICP, the strongest interaction was with AXL (AXL receptor tyrosine kinase), whereby AXL levels above the median could predict a major PICP reduction by spironolactone, with no effect of spironolactone with AXL levels below the median (p for interaction = 0.005). For PIIINP, the strongest interaction was with CCL28 (C-C motif chemokine ligand 28), whereby CCL28 levels below the median could predict a major PIIINP reduction by spironolactone, with no effect of spironolactone with CCL28 levels above the median (p for interaction = 0.003) (Supplemental Table 8). However, all of the studied interactions were statistically nonsignificant when corrected for multiple testing at an FDRq level of <0.05.

DISCUSSION

To the best of our knowledge, this is the first report showing an MRA effect on a large panel of circulating proteins. This study confirms that spironolactone has a major effect in reducing markers of collagen metabolism (e.g., COL1A1, MMP2), likely reflecting its antifibrotic effects, and reduces BNP, the single most important prognostic marker of adverse cardiovascular events. Moreover, spironolactone reduces markers related to metabolic processes (e.g., PAPPA), inflammation, and thrombosis (e.g., IL17A, VEGF, and urokinase) and increases levels of the adipokines involved in white adipose tissue formation and anti-inflammatory response (e.g., RARRES2) and markers of hemostasis maintenance (e.g., VWF), myelosuppressive activity (e.g., CCL16), insulin suppression (e.g., RETN), and inflammatory regulation (e.g., IL12B). On the other hand, expectedly, spironolactone increases markers that reflect the blockade of the MR (e.g., renin).

FIGURE 2 **Extended Network for the Biomarkers That Changed With Spironolactone Treatment From Baseline to the Last Visit With a p Value of <0.05**



Knowledge-based induced network with the plasma protein biomarkers that changed with spironolactone treatment and their intermediates. The full names of biomarkers are depicted in Supplemental Table 1. The full names of intermediates are depicted in Supplemental Table 8. Additionally, 3 collagen metabolism plasma biomarkers (PICP, CITP, and PIIINP) are also included in this network. RAAS = renin-angiotensin-aldosterone system.

Spironolactone has been used for more than 50 years. However, until approximately 20 years ago, spironolactone was thought to be mainly a potassium-sparing diuretic. Increasing evidence shows that the MR is expressed in the vascular smooth muscle, endothelial cells, macrophages, myocardium, kidney, brain, bone, and several other tissues (e.g., intestines and eyes). This has led to an intense investigation into the role of the MR and its blockade, especially after the publication of RALES (Randomized Aldactone Evaluation Study), which investigated the effect of spironolactone on morbidity and mortality in patients with severe HF (6,7). Particularly interesting was the observation that spironolactone could decrease markers of collagen synthesis, which were associated with adverse cardiac remodeling and poor prognosis in severe HF (8). Later, these findings were also replicated with eplerenone in other patient populations (4,9,10). The MR can be activated by aldosterone and cortisol in the setting of neurohormonal activation. Once activated, the MRs are associated with a number of cascade effects, including an

TABLE 2 Biomarkers That Changed at Both Month 1 and Last Visit

	Month 1		Last Visit	
Biomarker	β-Coefficient (95% CI)	p Value	β-Coefficient (95% CI)	p Value
Decreased with spironolactone				
COL1A1	−0.08 (−0.13 to −0.02)	0.0043	−0.14 (−0.20 to −0.08)	0.00001
MMP2	−0.10 (−0.16 to −0.04)	0.0008	−0.10 (−0.16 to −0.04)	0.0008
BNP	−0.46 (−0.61 to −0.32)	0.00001	−0.28 (−0.44 to −0.11)	0.0012
VEGFD	−0.09 (−0.14 to −0.04)	0.0003	−0.08 (−0.13 to −0.03)	0.0028
NOTCH3	−0.08 (−0.13 to −0.02)	0.0094	−0.09 (−0.15 to −0.03)	0.0032
Increased with spironolactone				
REN	0.48 (0.38 to 0.58)	0.00001	0.53 (0.42 to 0.63)	0.00001
IL12B	0.10 (0.03 to 0.17)	0.0058	0.13 (0.05 to 0.22)	0.0016
AMBP	0.05 (0.02 to 0.08)	0.0029	0.05 (0.01 to 0.08)	0.0058
CCL19	0.12 (0.03 to 0.21)	0.0063	0.15 (0.04 to 0.26)	0.0071
CCL25	0.10 (0.04 to 0.15)	0.0007	0.07 (0.02 to 0.13)	0.010
TRAIL	0.05 (0.01 to 0.10)	0.042	0.06 (0.01 to 0.11)	0.011
CSTB	0.13 (0.04 to 0.21)	0.0033	0.10 (0.02 to 0.19)	0.021
FABP4	0.12 (0.03 to 0.22)	0.010	0.12 (0.02 to 0.23)	0.024
TNFRSF9	0.11 (0.05 to 0.17)	0.0007	0.08 (0.01 to 0.15)	0.027
CST5	0.09 (0.03 to 0.15)	0.0019	0.07 (0.01 to 0.13)	0.033
CCL3	0.09 (0.01 to 0.17)	0.027	0.09 (0.01 to 0.18)	0.034
CPA1	0.13 (0.02 to 0.23)	0.016	0.12 (0.01 to 0.22)	0.035
TFF3	0.07 (0.01 to 0.13)	0.022	0.07 (0.00 to 0.13)	0.042
CXCL9	0.16 (0.05 to 0.27)	0.004	0.11 (0.00 to 0.22)	0.043

Abbreviations as in Table 1.

increase in reactive oxygen species; a decrease in nitric oxide availability; an increase in inflammatory cytokines, insulin resistance, activation and infiltration of macrophages, sodium retention, and potassium loss; and an increase in systemic fibrosis, leading to organ dysfunction and death (11,12).

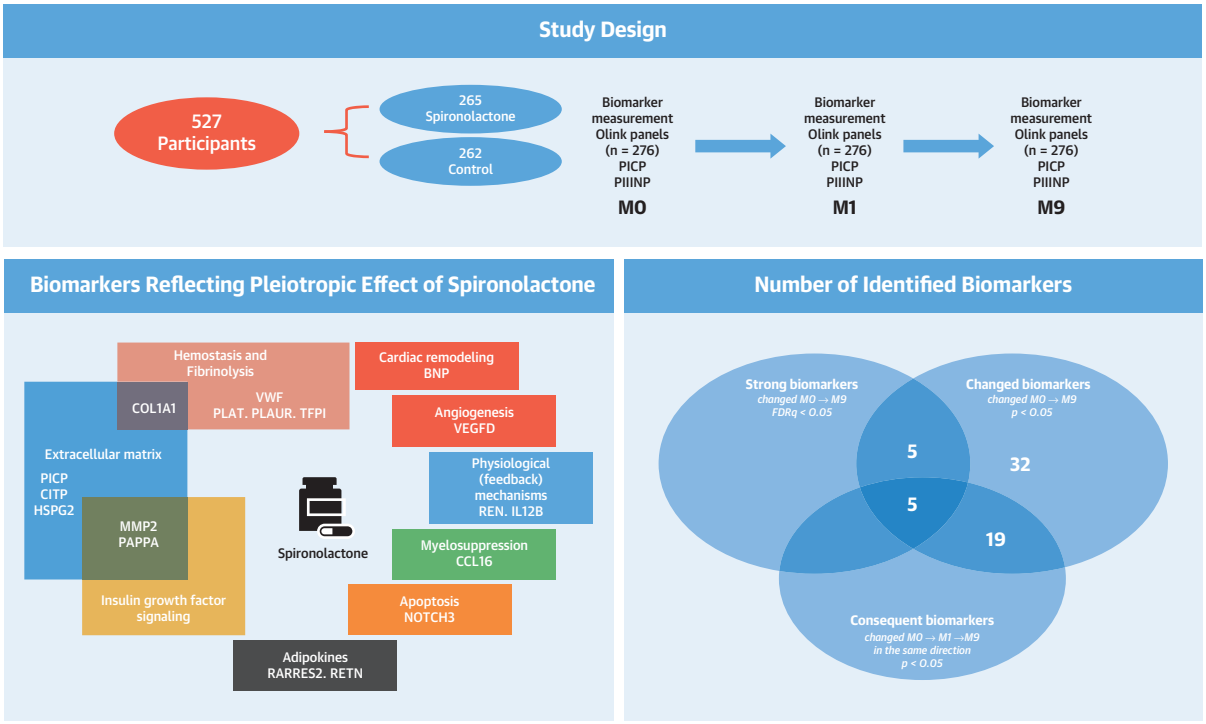
In the HOMAGE trial, treatment with spironolactone did not reduce the circulating levels of PIIINP but reduced PICP and NT-pro BNP while also increasing CITP, suggesting a favorable effect on collagen turnover (with decreased synthesis and increased degradation) and improvement in cardiac remodeling (also supported by the improvement of several echocardiographic parameters (4). Spironolactone did increase the levels of galectin-3 (a finding also reported in the Aldo-DHF trial) (13). Galectin-3 is one mediator of the fibrotic effect of aldosterone, and an increase in galectin-3 production might have been mediated by a rise in aldosterone as a consequence of the MR blockade (i.e., a feedback mechanism, as discussed earlier) (14). Because the MR is present in many tissues, it is likely that its blockade influences multiple biologic pathways and pathophysiologic mechanisms. In this regard, the present study provides important mechanistic insights.

COL1A1, MMP2, BNP, and PAPPA were decreased by spironolactone. Collagen type I and BNP reflect the reduction in collagen synthesis and reverse cardiac remodeling, respectively; their reduction is consistent with previously published findings using other procedures and biomarkers that reflect the same pathways (4), thus reinforcing the robustness of our results. Both COL1A1 and BNP, as well as MMP2, were reduced after 1 month of treatment, suggesting that the effect of spironolactone on ECM/collagen formation and cardiac remodeling occurs early after its administration and is sustained over time. By modulating collagen, MMP2 regulates the ECM, bone formation, tissue repair, angiogenesis, and tumor invasion (15). MMP2 may contribute to perpetuating the profibrotic response by generating matrikines in the process of collagen degradation, as wells as by activating growth factors (e.g., transforming growth factor β), which in turn induce collagen synthesis. Indeed, targeted deletion of MMP2 reduced the development of myocardial fibrosis and improved hypertension-induced cardiac hypertrophy in mice with chronic pressure overload (16). MMP2 also acts on several nonmatrix proteins, such as big endothelin-1 and beta-type calcitonin-gene related peptide, that promote vasoconstriction (17). The metalloproteinase further increases myocardial oxidative stress and regulates myocardial cell death pathways. The reduction of MMP2 by spironolactone supports the potential beneficial effects of the drug in reducing vasoconstriction, oxidative stress, and cardiac cell death (18). Furthermore, MMP2 plays a role in the IGF pathway by cleaving IGF binding proteins (IGFBPs). PAPPA is a metalloproteinase that also cleaves IGFBP. In animal models, the deletion of PAPPA increased circulating IGFBP-5 levels and was associated with a reduction in the collagen markers of bone turnover (19); it is thus possible that the reduction of PAPPA is related to the reduction of collagen markers.

VEGFD and NOTCH3 were also reduced after 1 month of spironolactone treatment and remained reduced until the end of the study (despite not passing the multiple test correction threshold). VEGFD plays an active role in angiogenesis, lymphatic angiogenesis, and endothelial cell growth (20). In patients with age-related macular degeneration refractory to anti-VEGF treatment, spironolactone could reduce neovascularization, suggesting that spironolactone might be able to modulate angiogenesis (21). NOTCH3 interferes with cell proliferation and apoptotic programs. NOTCH3 has been found to be up-regulated in some tumor tissues and may contribute to premature biologic ageing (22). Whether spironolactone can delay these processes is worth investigating.

MRAs increase renin and aldosterone by a feedback mechanism after blockade of the MR. Thus, in the

CENTRAL ILLUSTRATION **Overall Integration of the Effects of Spironolactone on the Proteomic Profile of People at Risk for Developing Heart Failure**



Ferreira, J.P. et al. J Am Coll Cardiol HF. 2021;9(4):268–77.

Summary of the main effects of spironolactone in the circulating proteins measured the HOMAGE (Heart Omics in AGEing) trial. BNP = brain natriuretic peptide; CITP = collagen type I-C terminal telopeptide; CCL16 = C-C motif chemokine ligand 16; COL1A1 = collagen type I alpha 1 chain; HSPG2 = Basement membrane-specific heparan sulfate proteoglycan core protein; IL12B = interleukin 12B; MMP2 = matrix metalloproteinase 2; NOTCH3 = notch receptor 3; PAPPA = pappalysin 1; PICP = procollagen type I C-terminal propeptide' PLAT = tissue plasminogen activator; PLAUR = plasminogen activator; RARRES2 = retinoic acid receptor responder 2; REN = renin; RETN = resistin; TFPI = tissue factor pathway inhibitor; VEGFD = vascular endothelial growth factor D; VWF = Von Willebrand factor.

setting of MRA use, the measurements of renin and aldosterone have limited clinical utility (23). In our study, and as part of normal physiologic feedback mechanisms, renin was increased by spironolactone, which shows drug compliance.

RARRES2 or chemerin is an adipokine that regulates adipogenesis in a process associated with the expansion of white adipose tissue and may have anti-inflammatory properties via increased production of nitric oxide (24). Furthermore, RARRES2 results from the cleavage of pre-chemerin by proteases involved in fibrinolysis, and fibrinolysis is a major pathway in our extended network. VWF is important for the maintenance of hemostasis because it promotes the adhesion of platelets to sites of vascular injury by forming a molecular bridge between the subendothelial collagen matrix and platelets. Spironolactone enhances collagen degradation, which may contribute to increased plasma free form of VWF. In addition, several proteins involved in fibrinolysis were elevated after 9 months of spironolactone treatment: tissue plasminogen activator (PLAT), plasminogen activator (PLAUR), and tissue factor pathway inhibitor (TFPI). As aldosterone promotes hemostasis by enhancing angiotensin II-induced plasminogen activator inhibitor (PAI-1), spironolactone may inhibit hemostasis and promote clot dissolution. The proteomic profile shows that spironolactone has a strong mediating role in provasodilation, inhibiting platelet adhesion and suppressing coagulation and fibrinolysis, implying an antithrombotic potential. Overexpression of CCL16 reduced the collagen content in hepatic fibrotic cell lines. CCL16 also has potent myelosuppressive activity and suppresses the proliferation of myeloid progenitor cells (25). Thus, the increase in circulating levels of CCL16 may be one of the mechanisms by which spironolactone both reduces collagen content and exerts a mild myelosuppressive activity. Patients taking

spironolactone tended to have a lower hemoglobin concentration and lower weight (4). Resistin (RETN) has been found to be increased in the context of a low-sodium diet where RAAS activity is physiologically high (26), a condition that is similar to that induced by MRAs. IL12B is an inflammatory cytokine that has been associated with chronic kidney disease and renal function (27); it is possible that the rise in this cytokine may be associated with the slight rises in creatinine and galectin-3 with spironolactone treatment (4). This hypothesis is supported by the positive, albeit weak, correlation between IL12B and creatinine.

The central hub of our extended network was the angiotensin receptor nonspecific effector SRC, which is a tyrosine kinase, involved in several biologic process, including immune response, cell adhesion, cell cycle progression, apoptosis, migration, and transformation. The SCR pathway is an important effector of angiotensin receptor, which controls aldosterone formation (28). Our extended network shows a central role for SRC in mediating all effects of spironolactone on the proteomic profile: the immune response, cell adhesion, hemostasis and fibrinolysis, and ECM metabolism. Thus, inhibiting the effects of aldosterone may initiate a cascade of the downstream pathway mediated by SRC, which helps explain the pleiotropic effects of spironolactone.

STUDY LIMITATIONS. We tested the effect of spironolactone on multiple proteins by applying a correction for test multiplicity to limit the occurrence of false positive findings; however, because HOMAGE was a randomized controlled trial, other proteins, the levels of which were also significantly changed with spironolactone, might also be implicated in relevant pathways and biologic processes and could be worth exploring in further studies. Additionally, many of the highlighted mechanisms should be further replicated and confirmed at a cellular level. We measured 3 Olink panels (CVDII, CVDIII, and inflammation), and measuring more proteins could have provided further insight on the mechanisms of action and effects of spironolactone. However, the hypothesis is that spironolactone would mainly influence processes associated with cardiovascular and inflammatory processes.

CONCLUSIONS

Proteomic analyses suggest that spironolactone may have a pleiotropic mode of action that includes the reduction of biomarkers associated with fibrosis, congestion, inflammation, and vascular function. Additional metabolic and potentially antiapoptotic effects could also be found. Our proteomic approach for examining the underlying mechanisms of MRA action confirms, for the first time in a clinical setting, to our knowledge, many results that had been described only in experimental models. This highlights the usefulness of proteomics for pathophysiology and pharmacology clinical investigations.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Spironolactone may delay the onset of HF in people with high cardiovascular risk. The effects of spironolactone are mediated by a reduction in profibrotic factors but also by reducing inflammation, improving angiogenesis and metabolism.

TRANSLATIONAL OUTLOOK: Spironolactone reduced biomarkers of collagen metabolism (e.g., COL1A1, MMP2), BNP, biomarkers related to metabolic processes (e.g., PAPPA), inflammation, and thrombosis (e.g., IL17A, VEGF, and urokinase). Spironolactone increased biomarkers that reflect the blockade of the mineralocorticoid receptor (e.g., renin); levels of adipokines involved in anti-inflammatory response (e.g., RARRES2); and biomarkers of hemostasis maintenance (e.g., tPA, UPAR), myelosuppressive activity (e.g., CCL16), insulin suppression (e.g., RETN), and inflammatory regulation (e.g., IL12B). These biomarkers and pathways could be further tested as potential therapeutic targets.

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APPENDIX For supplemental tables, please see the online version of this paper.