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Proteomic and mechanistic analysis of spironolactone in patients at risk for HF

Short title: Spironolactone effect on multi-proteomics

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On behalf of HOMAGE “Heart Omics in AGEing” consortium

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Abstract

Background: Further 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 “Heart OMics in Aging” (HOMAGE) trial showed that spironolactone treatment could provide antifibrotic and anti-remodelling effects, potentially slowing the progression to HF.

Objectives: To further understand the mechanisms underlying spironolactone effect, we assessed its impact on multiple plasma protein biomarkers and respective underlying biological pathways.

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

Results: 527 participants were enrolled, 265 were randomized to spironolactone (25-50 mg/day) and 262 to standard care (“control”). The median (percentile₂₅₋₇₅) age was 73 (69-79) years and 26% were female. Spironolactone reduced biomarkers of collagen metabolism (e.g., COL1A1, MMP2), BNP, biomarkers related to metabolic processes (e.g., PAPP), inflammation and thrombosis (e.g., IL17A, VEGF and urokinase). Spironolactone increased biomarkers that reflect the blockade of the mineralocorticoid receptor (e.g., renin), increased the levels of adipokines involved in anti-inflammatory response (e.g., RARRES2), biomarkers of haemostasis maintenance (e.g., tPA, UPAR), myelosuppressive activity (e.g., CCL16), insulin suppression (e.g., RETN), and inflammatory regulation (e.g., IL12B).

Conclusion: 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.

Key-words: spironolactone; heart failure prevention; fibrosis; inflammation; renin-angiotensin-aldosterone system

Condensed abstract

We studied the impact of spironolactone on multiple plasma protein biomarkers and respective underlying biological pathways in the HOMAGE trial. Spironolactone reduced biomarkers of collagen metabolism, BNP, biomarkers related to metabolic processes, inflammation and thrombosis. Spironolactone increased biomarkers that reflect the blockade of the mineralocorticoid receptor, increased the levels of adipokines involved in anti-inflammatory response, biomarkers of haemostasis maintenance, myelosuppression, insulin suppression, and inflammatory regulation. Proteomic analyses suggest that spironolactone exerts pleiotropic effects including reduction in fibrosis, inflammation, thrombosis, congestion and vascular function, all of which may mediate cardiovascular protective effects potentially slowing progression toward heart failure.

Abbreviations list

HF, heart failure

MRA, mineralocorticoid receptor antagonist

RAAS, renin-angiotensin-aldosterone system

COL1A1, collagen type I alpha 1 chain

MMP2, matrix metalloproteinase 2

BNP, brain natriuretic peptide

PAPPA, pappalysin 1

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

IL17D, interleukin17D

REN, renin

RARRES2, retinoic acid receptor responder 2

VWF, Von Willebrand factor

CCL16, C-C motif chemokine ligand 16

RETN, resistin

IL12B, interleukin 12B

PGLYRP, peptidoglycan recognition protein 1

IL6RA, interleukin 6 receptor A

AMBP, alpha-1-microglobulin/bikunin precursor

CCL19, C-C motif chemokine ligand 19

MMP7, matrix metalloproteinase 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, TNF 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

PRTN3, proteinase 3

Introduction

Preventing, rather than treating HF, might be a more effective way of increasing long-term survival and delay health-related quality of life impairment. Further to their beneficial effects in established HF, mineralocorticoid receptor antagonists (MRAs) might be useful for preventing incident HF¹.

Individuals at risk for developing HF often have higher levels of natriuretic peptides, increased activation of pro-fibrotic, inflammatory and apoptotic pathways, elevation of markers of vascular and endothelial dysfunction, atherosclerosis, and increased activation of the renin-angiotensin-aldosterone system (RAAS)². Due to their pleiotropic effects beyond sodium and potassium regulation, MRAs (e.g., spironolactone, eplerenone, finerenone) may positively affect these aforementioned mechanisms³. 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-pro BNP), reduced blood pressure and improved cardiac remodelling in asymptomatic people at risk for developing HF (*Cleland et al. EHJ 2020 accepted*).

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 biological pathways that could be affected by spironolactone in individuals at risk of developing HF.

Methods

Trial design and population

The HOMAGE trial was a prospective, randomized, open-label, blinded-endpoint (PROBE), multicentre design, in which people at increased risk of developing HF were randomly assigned to receive either spironolactone or standard of care (“control”) - not receiving spironolactone or other MRA (ClinicalTrials.gov Identifier: NCT02556450). The rationale, trial design and main results have been published¹. The study was approved by all relevant ethics committees and regulatory bodies. All participants provided written informed consent prior to study-specific procedures. The main participating criteria included age of 65 or older (amended to 60 years during the course of the trial), cardiovascular risk defined by the presence of coronary artery disease or at least 2 of the following: diabetes, treated hypertension, microalbuminuria, abnormal ECG, and a NT-pro BNP between 125 and 1,000 ng/L or BNP between 35 and 280 ng/L. The main exclusion criteria were glomerular filtration rate (eGFR) <30 mL/minute/1.73m², serum potassium >5.0 mmol/L, left ventricular ejection fraction <45%, a diagnosis of HF or treatment with loop diuretics and atrial fibrillation/flutter.

HOMAGE trial patients, follow-up and endpoints

A total of 527 patients were randomized (265 to spironolactone and 262 to standard of care). The median (percentile₂₅₋₇₅) follow-up time was 8.9 (6.0-9.2) 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 (“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-pro BNP, echocardiographic measures of cardiac structure and function, and signs/symptoms. One-month changes were

also assessed in exploratory analyses. At baseline, one month and at the end of the study, participants performed clinical, biomarker and echocardiographic measurements (*Cleland et al. EHJ 2020 accepted*).

Proteomic biomarkers

Baseline, month 1 and month 9 (or last visit) plasma samples were analysed for 276 protein biomarkers by the TATAA-biocenter 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 well-established role (e.g., TRAIL or PAPPa). The proteins were determined using high-throughput Olink Proseek® Multiplex 96x96 kits, which measures 92 manually-selected proteins simultaneously in 1 µl of plasma per kit. Each kit uses a proximity extension assay (PEA) technology with dual-recognition DNA-coupled readout, where 92 oligonucleotide-labeled antibody probe pairs are allowed to bind to their respective target in the sample. The platform provides Log₂ normalized protein expression (NPX) values with relative quantification. A detailed description of the Olink® technology is depicted on the website: <https://www.olink.com/>. The abbreviations, full names and respective Olink® multiplex panels of the studied proteins are described in the **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 (*Cleland et al. EHJ 2020 accepted*)) using analysis of covariance (ANCOVA) comparing the difference of changes between the control and spironolactone groups in the regression model. A linear regression model was

fitted, with the protein change (last visit – baseline) as outcome variable, a binary variable to indicate the treatment group (control/spironolactone), and the baseline protein value (NPX) 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 <0.05 (FDR5%) as described by Benjamini and Hochberg was applied to the protein change from baseline to the last visit. All the other analyses should be viewed as exploratory, including the interaction term between the treatment and the baseline protein level (below vs. above median) to evaluate if the effect of spironolactone on PIIINP and PICP could have varied by the baseline levels of the studied proteins. Statistical analyses were performed using Stata® (version 16, StataCorp LP).

Bioinformatical and network analyses

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to analyse functional enrichment (GO biological processes, KEGG and Reactome pathways) and connections of proteins that were strongly changed by spironolactone. In addition, we used knowledge-based network analysis with induced network approach by consensuspathDB (CPDB) online server (accessed on 17 February 2020) from Max Planck Institute for Molecular Genetics 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)⁷. 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 ≤ 20 calculated for each intermediate node. The created network was reorganized in Cytoscape (version 3.7) to cluster proteins based on biological functions and linked this network to spironolactone via mineralocorticoid receptor –aldosterone - angiotensin receptor.

Results

Characteristics of the population

The median (percentile₂₅₋₇₅) age was 73 (69-79) years, 26% were female, 71% had coronary artery disease, and 41% diabetes. **Supplemental Table 2.**

Spironolactone effect 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 PAPP A (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 (interleukin 6 receptor A), AMBP (alpha-1-microglobulin/bikunin precursor), CCL19 (C-C motif chemokine ligand 19), MMP7 (matrix metalloproteinase 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 (TNF 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 the studied proteins are presented in the supplemental material (**Supplemental Tables 3 & 4**). None of the studied proteins had a strong correlation between each other (Spearman $Rho < 0.7$ for all comparisons; **Supplemental Table 5**), nor protein-clinical parameter correlation (Spearman $Rho < 0.5$ for all comparisons; **Supplemental Table 6**). Information regarding network edges, nodes and interactions can be found in **Supplemental Table 7**.

Proteins that changed both at month 1 and last visit

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

Network analysis

The 10 proteins which strongly changed after spironolactone treatment (FDR q <0.05) were used as input to create a focused network. Overall, three clusters of proteins can be identified, collectively fitting six biological 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) signalling, haemostasis and adipocytokine signalling.

To get a broader overall picture of the biological network, we used all the 51 proteins that significantly changed with spironolactone treatment (p <0.05) to generate **Figure 2**. Spironolactone directly affected the RAAS-pathway and then activate 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 biological functions. Furthermore, ECM metabolism and haemostasis are two major biological 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 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 spironolactone effect 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 spironolactone effect with CCL28 levels above median (p for interaction =0.003). **Supplemental Table 8**. However, all

the studied interactions were statistically non-significant when corrected for multiple testing at a FDRq level <0.05.

Discussion

To the best of our knowledge this is the first report showing a 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 anti-fibrotic 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 adipokines involved in white adipose tissue formation and anti-inflammatory response (e.g., RARRES2), and markers of haemostasis 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 mineralocorticoid receptor (e.g., renin)

Spironolactone has been used for over 50 years. However, until around 20 years ago, spironolactone was thought to be mainly a potassium-sparing diuretic. Increasing evidence shows that the mineralocorticoid receptor (MR) is expressed in the vascular smooth muscle, endothelial cells, and macrophages, myocardium, kidney, brain, bone and several other tissues (e.g., intestines and eyes), has led to an intense investigation on the role of the MR and its blockade, especially after the publication of RALES (the effect of spironolactone on morbidity and mortality in patients with severe heart failure)^{4,5}. Particularly interesting was the observation that spironolactone could decrease markers of collagen synthesis, that were associated with adverse cardiac remodelling and poor prognosis in severe HF⁶. Later these

findings were also replicated with eplerenone in other patient-populations⁷⁻⁹. 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 the increase in reactive oxygen species, decrease in nitric oxide availability, increase in inflammatory cytokines, insulin resistance, activation and infiltration of macrophages, sodium retention and potassium loss, as well as an increase in systemic fibrosis leading to organ dysfunction and death^{10, 11}.

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 C1P, suggesting a favourable effect in collagen turnover (with decreased synthesis and increased degradation) and improvement in cardiac remodelling (also supported by the improvement of several echocardiographic parameters (*Cleland et al. EHJ 2020 accepted*)). Spironolactone did increase the levels of galectin-3 (a finding also reported in the Aldo-DHF trial)¹². 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 in consequence of the MR blockade (i.e., a feedback mechanism as discussed above)¹³. As the MR is present in many tissues it is likely that its blockade influences multiple biological pathways and pathophysiological mechanisms. In this regard, the present study provides important mechanistic insights.

Collagen type I alpha 1 chain (COL1A1), MMP2, BNP, and PAPPa were decreased by spironolactone. Collagen type I and BNP reflect the reduction in collagen synthesis and reverse cardiac remodelling, respectively; and their reduction is consistent with previously published findings using other procedures and biomarkers that reflect the same pathways (*Cleland et al. EHJ 2020 accepted*), 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 the ECM/collagen formation and cardiac remodelling occurs early after its administration and is sustained over time. By modulating collagen, MMP2 regulates the ECM, bone formation, tissue repair, angiogenesis and tumor invasion¹⁴. MMP-2 may contribute to perpetuating the pro-fibrotic response by generating matrikines in the process of collagen degradation, as well as by activating growth factors (e.g., transforming growth factor- β), which in turn induce collagen synthesis. Indeed, targeted deletion of MMP-2 reduced the development of myocardial fibrosis and improve hypertension-induced cardiac hypertrophy in mice with chronic pressure overload¹⁵. MMP2 also acts on several non-matrix proteins such as big endothelin-1 and beta-type calcitonin-gene related peptide, which promotes vasoconstriction¹⁶. The metalloproteinase further increases myocardial oxidative stress and regulate 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 death¹⁷. Furthermore, MMP2 plays a role in the IGF pathway by cleaving IGF binding proteins (IGFBP). Pappalysin-A or pregnancy-associated plasma protein-A (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 turnover¹⁸; it is thus possible that the reduction of PAPPA is related to the reduction of collagen markers.

Vascular endothelial growth factor D (VEGFD) and NOTCH3 were also reduced after 1-month 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 growth¹⁹. In patients with age-related macular degeneration refractory to anti-VEGF treatment, spironolactone could reduce neovascularization, suggesting that spironolactone might be able to modulate

angiogenesis²⁰. NOTCH3 interferes with cell proliferation and apoptotic programs. NOTCH3 has been found to be upregulated in some tumor tissues and may contribute to premature biological ageing²¹. 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 setting of MRA use, the measurements of renin and aldosterone have limited clinical utility²². In our study, and as part of normal physiological feedback mechanisms, renin was increased by spironolactone, which shows drug compliance.

Retinoic acid receptor responder 2 (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 oxide²³. Furthermore, RARRES2 results from the cleavage of pre-chemerin by proteases involved in fibrinolysis, and fibrinolysis is a major pathway in our extended network. Von Willebrand factor (VWF) is important for the maintenance of haemostasis as it promotes the adhesion of platelets to the sites of vascular injury by forming a molecular bridge between sub-endothelial 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 haemostasis by enhancing angiotensin II-induced plasminogen activator inhibitor (PAI-1), spironolactone may inhibit haemostasis and promote clot dissolution. The proteomic profile shows that spironolactone has a strong mediating role in pro-vasodilation, inhibiting platelet adhesion, suppression of coagulation and fibrinolysis; implying an anti-thrombotic 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 cells²⁴. Thus, the increase in the 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 haemoglobin concentration, while also having lower weight (*Cleland et al. EHJ 2020 accepted*). Resistin (RETN) has been found to be increased in the context of a low sodium diet where RAAS activity is physiologically high²⁵; a condition that is similar to that induced by MRAs. Interleukin-12 beta subunit (IL12B) is an inflammatory cytokine that has been associated with chronic kidney disease and renal function²⁶; it is possible that the rise in this cytokine may be associated with the slight rises in creatinine and galectin-3 with spironolactone treatment (*Cleland et al. EHJ 2020 accepted*). 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 non-specific effector SRC, which is a tyrosine kinase, involved in several biological 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 formation²⁷. Our extended network shows a central role for SRC in mediating all effects of spironolactone on the proteomic profile: the immune response, cell adhesion, haemostasis and fibrinolysis, and ECM metabolism. Thus, inhibiting the effects of aldosterone may initiate a cascade of downstream pathway mediated by SRC, which helps explaining the pleiotropic effects of spironolactone.

Limitations

We tested the effect of spironolactone on multiple proteins applying a correction for test multiplicity to limit the occurrence of false positive findings; however, as HOMAGE was a randomized controlled trial, other proteins, which levels were also significantly changed with spironolactone, might also be implicated in relevant pathways and biological processes and could be worth exploring in further studies. Additionally, many of the highlighted mechanisms should be furtherly replicated and confirmed at a cellular level. We measured three 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 to cardiovascular and inflammatory processes.

Conclusion

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 anti-apoptotic 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, many results that had been only described in experimental models. This highlights the usefulness of proteomics for pathophysiology and pharmacology clinical investigations.

Perspectives

Core Clinical Competencies

Spironolactone may delay the heart failure onset in people with high cardiovascular risk. The effects of spironolactone are mediated by a reduction in pro-fibrotic 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., PAPP A), inflammation and thrombosis (e.g., IL17A, VEGF and urokinase). Spironolactone increased biomarkers that reflect the blockade of the mineralocorticoid receptor (e.g., renin), increased the levels of adipokines involved in anti-inflammatory response (e.g., RARRES2), biomarkers of haemostasis 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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Disclosures

The authors have no relevant conflicts of interest to disclose with regards to the content of this manuscript.

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Table 1. Changes in biomarkers with spironolactone treatment (from baseline to the last visit)

Biomarker	β -coef. (95%CI)	P-value	FDRq
<i>Decreased with spironolactone</i>			
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
<i>Increased with spironolactone</i>			

REN	0.53 (0.42-0.63)	0.00001	0.0014
RARRES2	0.09 (0.04-0.14)	0.0002	0.018
VWF	0.39 (0.18-0.59)	0.0003	0.021
CCL16	0.12 (0.05-0.19)	0.0008	0.032
RETN	0.15 (0.06-0.24)	0.0008	0.032
IL12B	0.13 (0.05-0.22)	0.002	0.044
PGLYRP1	0.13 (0.05-0.22)	0.003	0.064
IL6RA	0.08 (0.03-0.14)	0.004	0.085
AMBP	0.05 (0.01-0.08)	0.006	0.10
CCL19	0.15 (0.04-0.26)	0.007	0.10
MMP7	0.10 (0.03-0.18)	0.009	0.12
PLC	0.06 (0.02-0.11)	0.010	0.12
CCL25	0.07 (0.02-0.13)	0.010	0.12
TRAIL	0.06 (0.01-0.11)	0.011	0.13
TPA	0.19 (0.04-0.34)	0.012	0.13
GAL9	0.06 (0.01-0.11)	0.013	0.13
NT3	0.11 (0.02-0.20)	0.018	0.17
SRC	0.22 (0.04-0.40)	0.019	0.17
CSTB	0.10 (0.02-0.19)	0.021	0.18
FABP4	0.12 (0.02-0.23)	0.024	0.20
GDF15	0.09 (0.01-0.16)	0.024	0.20
TNFRSF9	0.08 (0.01-0.15)	0.0269	0.20
CST5	0.07 (0.01-0.13)	0.0334	0.23

CCL3	0.09 (0.01-0.18)	0.034	0.23
CPA1	0.12 (0.01-0.22)	0.0352	0.23
MPO	0.04 (0-0.07)	0.0387	0.24
TFPI	0.06 (0-0.12)	0.0392	0.24
UPAR	0.07 (0-0.14)	0.0402	0.24
TFF3	0.07 (0-0.13)	0.042	0.25
CXCL9	0.11 (0.01-0.22)	0.0433	0.25
ADM	0.06 (0-0.11)	0.0459	0.26
KLK6	0.07 (0-0.13)	0.0488	0.27
PRTN3	0.05 (0-0.11)	0.0497	0.27

Legend: COL1A1 (collagen type I alpha 1 chain), MMP2 (matrix metalloproteinase 2), BNP (brain natriuretic peptide), PAPP A (pappalysin 1), 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), IL17D (interleukin17D), REN (renin), RARRES2 (retinoic acid receptor responder 2), VWF (Von Willebrand factor), CCL16 (C-C motif chemokine ligand 16), RETN (resistin), IL12B (interleukin 12B), PGLYRP1 (peptidoglycan recognition protein 1), IL6RA (interleukin 6 receptor A), AMBP (alpha-1-microglobulin/bikunin precursor), CCL19 (C-C motif chemokine ligand 19), MMP7 (matrix metalloproteinase 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 (TNF 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), PRTN3 (proteinase 3).

Table 2. Biomarkers that changed both at month 1 and last visit

Biomarker	Month 1		Last visit	
	β -coef. (95%CI)	P-value	β -coef. (95%CI)	P-value
<i>Decreased with spironolactone</i>				
COL1A1	-0.08 (-0.13 to -0.02)	0.0043	-0.14 (-0.2--0.08)	0.00001
MMP2	-0.10 (-0.16 to -0.04)	0.0008	-0.1 (-0.16--0.04)	0.0008
BNP	-0.46 (-0.61 to -0.32)	0.00001	-0.28 (-0.44--0.11)	0.0012
VEGFD	-0.09 (-0.14 to -0.04)	0.0003	-0.08 (-0.13--0.03)	0.0028
NOTCH3	-0.08 (-0.13 to -0.02)	0.0094	-0.09 (-0.15--0.03)	0.0032
<i>Increased with spironolactone</i>				
REN	0.48 (0.38-0.58)	0.00001	0.53 (0.42-0.63)	0.00001
IL12B	0.10 (0.03-0.17)	0.0058	0.13 (0.05-0.22)	0.0016
AMBP	0.05 (0.02-0.08)	0.0029	0.05 (0.01-0.08)	0.0058
CCL19	0.12 (0.03-0.21)	0.0063	0.15 (0.04-0.26)	0.0071
CCL25	0.10 (0.04-0.15)	0.0007	0.07 (0.02-0.13)	0.010
TRAIL	0.05 (0.01-0.10)	0.042	0.06 (0.01-0.11)	0.011
CSTB	0.13 (0.04-0.21)	0.0033	0.1 (0.02-0.19)	0.021
FABP4	0.12 (0.03-0.22)	0.010	0.12 (0.02-0.23)	0.024
TNFRSF9	0.11 (0.05-0.17)	0.0007	0.08 (0.01-0.15)	0.027
CST5	0.09 (0.03-0.15)	0.0019	0.07 (0.01-0.13)	0.033
CCL3	0.09 (0.01-0.17)	0.027	0.09 (0.01-0.18)	0.034
CPA1	0.13 (0.02-0.23)	0.016	0.12 (0.01-0.22)	0.035
TFF3	0.07 (0.01-0.13)	0.022	0.07 (0-0.13)	0.042

CXCL9	0.16 (0.05-0.27)	0.004		0.11 (0-0.22)	0.043
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Legend: COL1A1 (collagen type I alpha 1 chain), MMP2 (matrix metalloproteinase 2), BNP (brain natriuretic peptide), VEGFD (vascular endothelial growth factor D), NOTCH3 (notch receptor 3), REN (renin), IL12B (interleukin 12B), AMBP (alpha-1-microglobulin/bikunin precursor), CCL19 (C-C motif chemokine ligand 19), CCL25 (C-C motif chemokine ligand 25), TRAIL (TRAIL/TNF superfamily member 10), CSTB (cystatin B), FABP4 (fatty acid binding protein 4), TNFRSF9 (TNF receptor superfamily member 9), CST5 (cystatin D), CCL3 (C-C motif chemokine ligand 3), CPA1 (carboxypeptidase A1), TFF3 (trefoil factor 3), CXCL9 (C-X-C motif chemokine ligand 9).

Figure 1. Focused network of biomarkers influenced by spironolactone treatment from baseline to the last visit with a $FDRq < 0.05$

Caption: Knowledge-based network with the top plasma protein biomarkers that changed with spironolactone treatment.

Legend: COL1A1 (collagen type I alpha 1 chain), MMP2 (matrix metalloproteinase 2), PAPP (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, BNP or brain natriuretic peptide. 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 colours of the nodes indicate significant KEGG and GO pathways which were significantly overrepresented.

Figure 2. Extended network for the biomarkers that changed with spironolactone treatment from baseline to the last visit with a $p < 0.05$

Caption: 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 the 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.

Central Illustration. Overall integration of the spironolactone effects on the proteomic profile of people at risk for developing heart failure.

Caption: This figure summarizes the main effects of spironolactone in the circulating proteins measured the HOMAGE trial.