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The mineralocorticoid receptor antagonist spironolactone significantly reduces albuminuria in subjects with diabetic kidney disease, albeit with a large variability between individuals. Identifying novel biomarkers that predict response to therapy may help to tailor spironolactone therapy. We aimed to identify a set of metabolites for prediction of albuminuria response to spironolactone in subjects with type 2 diabetes. Systems biology molecular process analysis was performed *a priori* to identify metabolites linked to molecular disease processes and drug mechanism of action. Individual subject data and urine samples were used from 2 randomized placebo controlled double blind clinical trials (NCT01062763, NCT00381134). A urinary metabolite score was developed to predict albuminuria response to spironolactone therapy using penalized ridge regression with leave-one-out cross validation. Bioinformatic analysis identified a set of 18 metabolites linked to a diabetic kidney disease molecular model and potentially affected by spironolactone mechanism of action. Spironolactone reduced UACR relative to placebo by median −42% (25th to 75% percentile −65 to 6) and −29% (25th to 75% percentile −37 to −1) in the test and replication cohorts, respectively. In the test cohort, UACR reduction was higher in the lowest tertile of the baseline urinary metabolite score compared with middle and upper tertiles −58% (25th to 75% percentile −78 to 33), −28% (25th to 75% percentile −46 to 8), −40% (25th to 75% percentile −52% to 31), respectively, $P = 0.001$ for trend). In the replication cohort, UACR reduction was −54% (25th to 75% percentile −65 to −50), −41 (25th to 75% percentile −46% to 30), and −17 (25th to 75% percentile −36 to 5), respectively, $P = 0.010$ for trend). We identified a set of 18 urinary metabolites through systems biology to predict albuminuria response to spironolactone in type 2 diabetes. These data suggest that urinary metabolites may be used as a tool to tailor optimal therapy and move in the direction of personalized medicine. (Translational Research 2020; 222:17−27)

Brief Commentary

Background

Patients with diabetic kidney disease suffer an enormous disease burden, in part due to heterogeneity in disease progression and drug response. The mineralocorticoid receptor antagonist spironolactone decreases albuminuria and can slow the progression of kidney function decline in patients with type 2 diabetes, although individual patients show a large variation in albuminuria response to this drug.

Translational significance

This study demonstrates that a metabolite score of 18 metabolites, identified through systems biology reflecting inflammation and fibrosis processes, predicts response to spironolactone relative to placebo. This metabolite score may help personalize treatment for people with diabetic kidney disease in the future.

INTRODUCTION

Diabetes is a major and rapidly growing health problem worldwide and is associated with considerable morbidity and health care expenditures.[1] Despite advances in the treatment of diabetes and its related renal and cardiovascular complications, risk of morbidity and mortality persists in many people.[2,3] The explanation may be, at least in part, that drug therapy is not being optimized to the individual patient.[4]

Inhibition of the renin-angiotensin-aldosterone-system with mineralocorticoid receptor antagonists (MRA, eg, spironolactone) decreases albuminuria and has the potential to slow progression of kidney function decline in people with diabetic kidney disease (DKD).[5] However, some individuals show a large variation in therapy response to MRA treatment. This variation is thought to be attributed at least in part to the heterogeneity in the pathophysiology of type 2 diabetes, and there is only limited knowledge which specific factors determine the individual renal response to MRAs.[6]

The measurement of metabolites in biological fluids such as serum and urine has emerged as a potential tool to unravel perturbations in biological systems in chronic diseases such as type 2 diabetes. Characterizing these biological features may lead to the characterization of biological pathways related to disease activity. Advancements in omics technologies coupled with high dimensional data integration via systems medicine approaches can provide new insights in molecular mechanism of action of drugs and molecular mechanisms of disease progression.[4] In a recent study we used publicly available and experimental data to develop a network-based molecular model of DKD,[7] which was subsequently used to select and test prognostic DKD biomarkers.[8] In this present work we analyze the impact of spironolactone on molecular DKD mechanisms and select a set of metabolites for measurements in urine samples from 2 independents cohorts. We assess the ability of a urinary metabolites to predict albuminuria response to spironolactone therapy in subjects with type 2 diabetes.

METHODS

In-silico modeling of spironolactone mechanism of action and DKD pathophysiology. Network-based molecular models reflecting spironolactone mechanism of action as well as DKD pathophysiology were generated following previously described and successfully applied computational workflows.[8,9]

In brief, molecular features associated with spironolactone were consolidated from 3 data sources, namely scientific literature, DrugBank, and a transcriptomics data set from DrugMatrix. Molecular features were defined as genes, transcripts, or proteins. Scientific articles annotated with spironolactone as major MeSH term were retrieved and genes were extracted using NCBI's gene2pubmed file. This set of genes was complemented by drug targets listed in DrugBank for spironolactone.[10] We further extracted transcripts being differentially expressed between spironolactone-treated and untreated kidney samples of animal models as stored in DrugMatrix.[11] The unique set of spironolactone associated molecular features was mapped onto a hybrid interaction network including protein-protein interaction data from IntAct, BioGrid, and Reactome together with computationally inferred relations.[12] Interactions between members of the spironolactone feature set were extracted and the MCODE algorithm was used to identify clusters of highly interconnected proteins.[13]

A previously published DKD molecular model was used which was constructed following the same logic using data from scientific literature as well as from Omics datasets in the context of DKD.[7] Proteomics data of the published CKD273 proteomics classifier were used in order to identify DKD processes linked with DKD progression by mapping the set of proteins in the CKD273 classifier onto the DKD molecular network thus defining progression-associated process units.[14]

Network interference analysis and identification of candidate metabolites. Network alignment method was used to identify DKD molecular processes linked to DKD prognosis affected by spironolactone treatment

on the molecular level. Metabolites linked to proteins in affected DKD molecular processes were identified via enzyme-metabolite associations as stored in the Human Metabolome Database and forwarded to measurements in clinical samples.[15] A schematic figure regarding the systems biology workflow is shown in Supplementary Fig 1.

Clinical study design and patient population. For the present study, individual subject data and biobanked urine samples were used from 2 randomized placebo controlled double blind clinical trials (RCT) performed in subjects with diabetes (NCT01062763, NCT00381134). Both RCTs assessed the albuminuria lowering effect of spironolactone in subjects with DKD in comparison or on top of other blood pressure lowering agents. Both RCTs were performed in accordance with the Declaration of Helsinki and approved by local ethical committees. All subjects gave informed consent for participation in the study before any study specific procedure commenced.

For the test cohort, we used data from an RCT conducted in 4 centers in Denmark.[16] Subjects included were diagnosed with resistant hypertension and type 2 diabetes, ranging in age from 18 to 75 years, and receiving 3 or more antihypertensive drugs including a diuretic and an angiotensin converting enzyme inhibitor or an angiotensin receptor blocker. At baseline, subjects were randomly assigned to double-blind treatment with spironolactone 25 mg or matching placebo. Spironolactone was titrated up to 50 mg/day if BP was >130/80 mmHg, but maintained if BP was >110/60 mmHg. After randomization subjects were followed for 16 weeks. For the present study, data and samples were available for 102 subjects (52 spironolactone and 50 placebo). We defined albuminuria change as the percentage change in urinary albumin: creatinine ratio from baseline to week 16.

For the replication cohort, we used data from an RCT conducted in Dallas, Texas, United States.[17] Subjects included were diagnosed with diabetes, hypertension, and albuminuria (urine albumin-to-creatinine ratio >200 mg/g) who all received lisinopril (80 mg once daily). Subjects were randomly assigned to placebo, losartan (100 mg daily), or spironolactone (started at 12.5 mg which was later up titrated to 25 mg/day) for 48 weeks. For the present study, we used data from subjects from the placebo and spironolactone arms. After randomization, follow-up visits were conducted at 4-week intervals for the duration of one year. Data and samples were available for 43 subjects (20 spironolactone and 23 placebo). We defined albuminuria change as percent change in urinary albumin:creatinine ratio (UACR) from baseline to week 12.

Sample processing and measurement of metabolites. For the test cohort, nonfasting, spot urine samples were collected between 2010 and 2012, and immediately stored as whole urine at $-80°C$ until metabolomic analysis. For the replication cohort, nonfasting, 24-hour urine samples were collected between 2003 to 2007. Samples were collected, then aliquoted separately for proteomic, iothalamate, clinical testing, and backups and stored at $-80°C$ upon receipt from the patient. Urines were stored unprocessed with no protease inhibitors added. Patients were instructed to keep urine receptacles in refrigerator at home. The urinary metabolites were measured in 2014 by Biomedical Metabolomics Facility Leiden (Leiden, the Netherlands). The workflow utilized in the wet lab utilized an ACQUITY UPLC system with autosampler (Waters, Etten-Leur, The Netherlands) which was coupled online with a Xevo Tandem quadrupole mass spectrometer (Waters). After processing these were samples were analyzed by UPLC-MS/MS using an Accq-Tag Ultra column (Waters). The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. The metabolite raw data were preprocessed using Agilent MassHunter Quantitative Analysis software (Agilent, Version B.05.01). The data were further processed using the MultiQuant software (AB SCIEX, Version 3.0.2) by employing the integration of assigned MRM (multiple reaction monitoring) peaks and normalization using proper internal standards. For analysis of amino acids, 13C15N-labeled analogs were used. For other amines, the closest-eluting internal standard was employed. Blank samples were used to determine blank effect. In-house developed algorithms were applied using the pooled quality control samples to compensate for shifts in the sensitivity of the mass spectrometer over the batches. Quality Control (QC) was performed in a similar way across the testing and replication cohort. Markers were tested with Relative Standard Deviation (RSD) which tested the reproducibility of metabolites. Metabolite values were reported if the RSD QC was >20%. Blanks were employed to characterize the background noise; if the noise was <5% the marker was reported.

Five and 50 μL of urine was aliquoted for amine and organic acids profiling, respectively. In the test cohort, 102 baseline urine samples were available for metabolomic measurement; 48 amines and 24 organic acids were available for further analysis after quality control. In the replication cohort, 43 baseline urine samples were available for metabolomic measurement; 54 amines and 18 organic acids remained after quality control. Metabolite concentrations were corrected for urinary creatinine for all statistical analysis.

Machine learning & Statistical analysis. Analyses were performed using R version 3.4x employing the libraries: glmnet for regression analysis, ggplot2 and ggthemes for

plotting. Missing data were imputed using the R package Multivariate Imputation via Chained Equations by predictive mean matching (pmm) methodology.[18] Baseline characteristics with normal distribution are reported as mean (standard deviation), characteristics with skewed distribution are reported as median and 5th to 95th percentiles, and categorical variables are reported a number and percentage. A P value <0.05 (2-sided) was considered to indicate a statistically significant difference.

We defined Y as the log change of UACR between baseline and week 16 or week 12 for the testing and replication cohort, respectively. For X, a matrix consisting of the log-transformed metabolites and a metabolite * treatment interaction term was constructed. To predict the endpoint Y, matrix X was used to model Y as ridge penalized regression machine learning problem.[19] To assess the variation in estimates for subjects and markers, a random bootstrap combined with leave-one-out resampling was performed to select data for model construction.[20] The average predictions of all models using lambda.min,[21] excluding $i(x)$ the models used for predicting that $i(x)$, were saved for further analysis and plotting. Associations between clinical covariates: age, sex, systolic blood pressure, cholesterol, and HbA1c as X predicting Y were also assessed. R-squared was calculated by the Pearson's correlation to the power of 2 and was optimism corrected by subtracting correlation of the random permutations of Y (predicted) with Y (observed).[22]

Leave one out cross validation was employed and optimism correction was performed to counteract the positive predictions built in small datasets where $n \sim p$. Finally, an individual metabolite score is predicted by using all bootstrapped estimates of the penalized regression. This individual metabolite score is the predicted Y (Δ UACR) from the model of baseline metabolites and metabolite treatment interactions. This score was then used to stratify subjects into tertiles. Analysis of covariance was used to assess if this score is score metabolites could discriminate UACR response to spironolactone therapy. In both the test and replication cohorts, no clinical variables were significantly associated with the UACR change, and therefore not included in the final model (Supplementary Table 1).

RESULTS

Systems biology model construction and metabolite identification. The constructed spironolactone molecular model held 80 molecular features in 11 different processes ranging in size from 3 to 16. The DKD molecular model consisted of 688 molecular features in 34 process units. Two of the 5 progression associated process units, units 7 and 8, showed overlap with the spironolactone

molecular mechanism of action model and were thus considered for selection of metabolite marker candidates. Overlapping proteins between the progression associated process units and the spironolactone molecular mechanism of action model included NR3C2 (nuclear receptor subfamily 3 group C member 2), SERPINE1 (serpin family E member 1), PPARD (peroxisome proliferator activated receptor delta), PPARG (peroxisome proliferator activated receptor gamma), and SLC4A4 (solute carrier family 4 member 4). Eighteen metabolites were linked to proteins in unit 7 or unit 8 of the DKD molecular model (Fig 1). These metabolites were: alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, lysine, norepinephrine, phenylalanine, proline, serotonin, tryptophan, hydroxyproline, citric acid, 2-ketoglutaric acid, succinic acid, and pyroglutamic acid.

Subject characteristics. Baseline characteristics for the test and replication cohorts are presented in Table 1. In the test cohort, subjects were approximately 63 years of age, and 63% were male. In the replication cohort, subjects were approximately 50 years of age, and 58% were male. Large variability in UACR change was observed in the test and the replication cohort (Fig 2). In the test cohort, observed UACR change after 12 weeks was -1% [25th to 75th percentile -38 to 105] in the placebo arm and -43% [25th to 75th percentile -66 to 5] in the spironolactone arm. In the replication cohort, observed UACR change after 16 weeks was -14% [25th to 75th percentile -31 to 20] in the placebo arm and -43% [25th to 75th percentile -51 to 13] in the spironolactone arm (Figure 2).

Urine metabolites for prediction of UACR response to spironolactone. Figs 3A and B show the individual predictions of the leave-one-out cross-validated optimism corrected ridge regression to predict albuminuria response to spironolactone. A clear separation between the spironolactone and placebo arm can be observed in their predicted effects based on the combined metabolites and metabolite times treatment interactions.

When stratified by tertiles of the baseline urinary metabolite score, UACR reduction was higher in the lowest tertile compared to middle and upper tertiles -58% (25th to 75% percentile -78 to 33), -28% (25th to 75% percentile -46 to 8), -40% (25th to 75% percentile -52 to 31), respectively, $P = 0.001$ for trend) in the test cohort and in the replication cohort -54% (25th to 75% percentile -65 to -50), -41 (25th to 75% percentile -46% to 30), -17% (25th to 75% percentile -36 to 5), respectively, $P = 0.010$ for trend) as displayed in Fig 4. Individual contributions of the metabolites for the tertiles of the score are presented in Supplementary Fig 2.

Ranking of individual metabolites for prediction of UACR response. The individual metabolites and their interaction with treatment resulted in 36 features in the
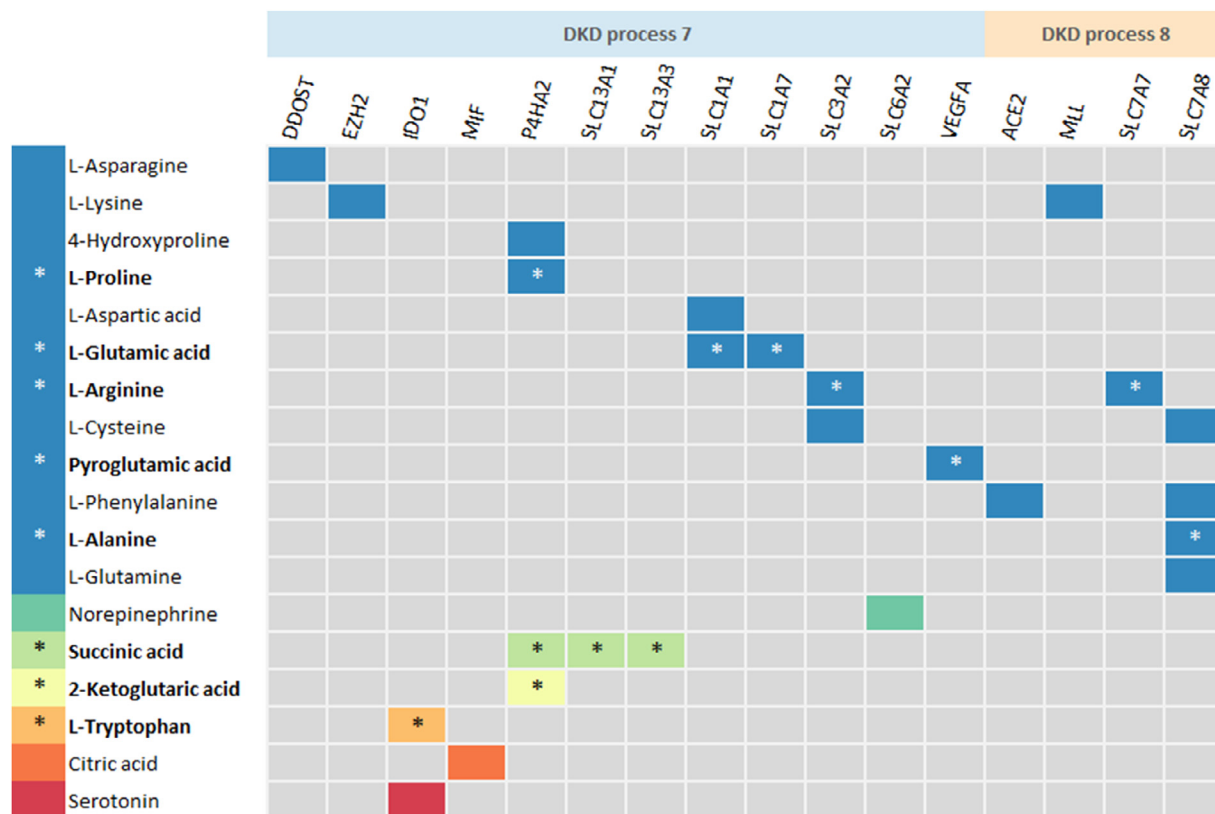
Fig 1. The 18 metabolites and their associations with proteins in process unit seven or 8 of the DKD molecular model are shown. Color-coding represents metabolite sub classes. Metabolites with the largest contributions to the urinary metabolite score are marked with an asterisk and written in bold. DDOST, dolichyl-diphosphooligo-saccharide−protein glycosyltransferase non-catalytic subunit; EZH2, enhancer of zeste 2 polycomb repressive complex 2 subunit; IDO1, indoleamine 2,3-dioxygenase 1; MIF, macrophage migration inhibitory factor; P4HA2, prolyl 4-hydroxylase subunit alpha 2; SLC13A1, solute carrier family 13 member 1; SLC13A3, solute carrier family 13 member 3; SLC1A1, solute carrier family 1 member 1; SLC1A7, solute carrier family 1 member 7; SLC3A2, solute carrier family 3 member 2; SLC6A2, solute carrier family 6 member 2; VEGFA, vascular endothelial growth factor A; ACE2, angiotensin I converting enzyme 2; MLL, lysine methyltransferase 2A (KMT2A); SLC7A7, solute carrier family 7 member 7; SLC7A8, solute carrier family 7 member 8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

ridge regression model. The beta estimates of each feature (per standard deviation increment) were ranked in terms of association with UACR change and in concordance between the test and replication cohort (eg, the larger the beta estimate, the stronger the association with UACR change and the largest contribution to the model). The metabolites with the largest contributions to the urinary metabolite score were proline, arginine, tryptophan, alanine, and pyroglutamic acid (Table 2 and Fig 1). Directionality of beta estimates and strengths of correlations are depicted in Supplementary Fig 3.

DISCUSSION

Using a systems biology and machine learning approach, we selected and tested urinary metabolites for predicting

Table 1. Subject characteristics

	Test cohort (*n* = 102)			Replication cohort (*n* = 43)		
	Placebo	Spironolactone	*P*	Placebo	Spironolactone	*P*
N	52	50		23	20	
Age, years	64.5 (7.1)	62.6 (6.9)	0.37	49.8 (8.4)	50.9 (9.69)	0.17
Male sex, *n*(%)	33 (63)	31 (62)	0.56	13 (56)	12 (60)	0.72
Systolic blood pressure, mm Hg	139 (15)	141 (15)	0.22	128 (16)	133 (15)	0.34
Diastolic blood pressure, mm Hg	75 (8)	77 (11)	0.29	72 (8)	71 (11)	0.71
Hba1C, %	7.2 (1.0)	7.5 (1.0)	0.57	8.0 (1.3)	7.2 (1.4)	0.18
Total cholesterol, mmol/L	3.9 (0.7)	4.1 (0.8)	0.59	4.7 (1.2)	4.5 (1.1)	0.59
Plasma potassium, mmol/L	3.8 (0.3)	3.9 (0.4)	0.12	4.5 (0.7)	4.6 (0.7)	0.92
UACR, mg/g	27 (14−64)	38 (14−284)	0.03	709 (399−1356)	1462 (481−2162)	0.34
eGFR	68 (19)	68 (21)	0.94	71 (33)	47 (25)	0.01

Data are presented as mean (standard deviation), number (proportion), and median (1st, 3rd quartile) for UACR. *P*-values are for the for comparison between placebo and spironolactone treatment arms.

the albuminuria lowering effect of the MRA spironolactone. These *a priori* selected urinary metabolites were tested in samples of 2 independent clinical trials. Our predictions suggest the use of urinary metabolites as a tool to tailor albuminuria lowering with spironolactone treatment.

We used a previously developed and validated network-based molecular interaction model of DKD to identify biomarkers associated with kidney disease progression.[7] Eighteen metabolites were selected based on network interference analysis between the DKD molecular model and the spironolactone mechanism of action molecular model. These 18 metabolites could be assigned to general molecular mechanisms of oxidative stress, inflammation, and fibrosis pathways. These urinary metabolites were able to predict the short-term albuminuria response to spironolactone. As albuminuria reduction is important for renal and cardiovascular protection, these urinary metabolites can help identify which people are more likely to respond to spironolactone therapy and receive renal and cardiovascular risk protection.

How do the identified metabolites mechanistically link to UACR response to spironolactone? Spironolactone has been proposed to influence expression of the 3 proteins SERPINE1, PPARG, and NR3C2 which were also part of the DKD molecular model and are linked to collagen synthesis and turnover. SERPINE1, a major inhibitor of ECM degradation, is increased by aldosterone,[23] and spironolactone administration leads to a significant decrease in SERPINE1 levels.[24,25] PPARG in turn is known to mediate anti-inflammatory and antifibrotic effects. NR3C2 is a direct target of spironolactone and causes collagen gene expression by proxy of aldosterone signaling.[26] Combination therapy with telmisartan and spironolactone has been reported to increase PPARG levels and thereby prevent renal tissue injury in spontaneously hypertensive rats.[27] Additionally, both proteins seem to be associated with collagen type 1 synthesis. SERPINE1 deficiency in animal models,[28] and activation of PPARg in glucose stimulated mesangial cells reduce collagen I expression,[29] thereby alleviating pro-fibrotic processes. Some of the metabolites
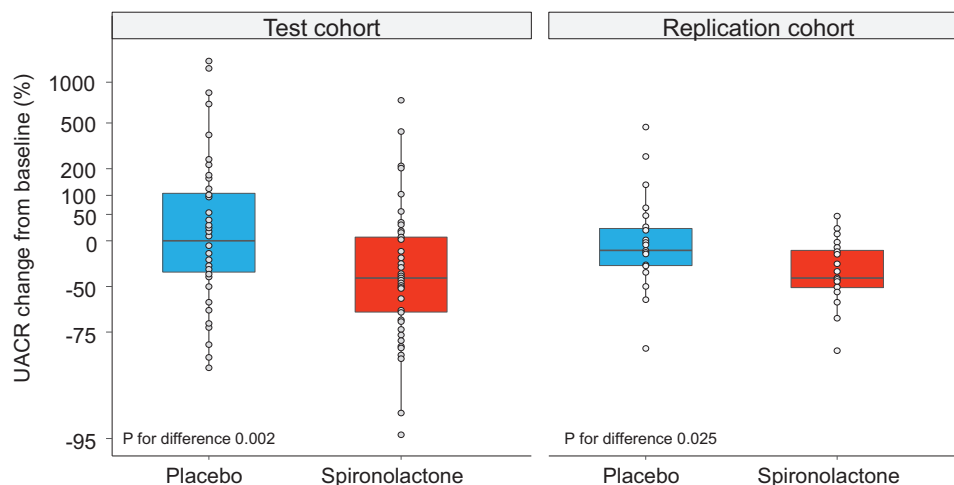

Fig 2. Variability in UACR response spironolactone treated subjects.

Table 2. Ranking of beta estimates of the ridge regression

Feature	Test cohort	Replication cohort	Rank*
Proline	−0.21 (−0.53 to −0.03)	−0.09 (−0.29 to 0)	1
Arginine	0.1 (−0.06 to 0.42)	0.07 (−0.01 to 0.36)	2
Tryptophan	0.08 (−0.04 to 0.28)	0.05 (0 to 0.31)	3
Alanine* treatment	−0.01 (−0.02 to 0.01)	−0.12 (−0.5 to 0.01)	4
Pyroglutamic acid* treatment	0.11 (−0.24 to 0.54)	0.01 (−0.26 to 0.29)	5
Alanine	0.12 (0−0.39)	0 (−0.07 to 0.14)	6
2 ketoglutaric acid treatment	0.11 (−0.08 to 0.39)		7
Succinic acid* treatment	0.06 (0−0.18)	0.05 (0−0.2)	8
Glutamic acid	0.07 (−0.07 to 0.3)	0.01 (−0.07 to 0.2)	9
Asparagine	−0.04 (−0.35 to 0.07)	−0.03 (−0.27 to 0.03)	10
Glutamine	0 (−0.21 to 0.11)	−0.06 (−0.26 to 0)	11
Arginine* treatment	0 (−0.02 to 0.03)	−0.03 (−0.21 to 0.01)	12
Lysine* treatment	0 (−0.01 to 0.03)	0.01 (−0.12 to 0.2)	13
Succinic* treatment	−0.01 (−0.14 to 0.14)	0 (−0.14 to 0.1)	14
Glutamine* treatment	−0.01 (−0.01 to 0)	−0.01 (−0.21 to 0.13)	15
Pyroglutamic acid	−0.2 (−0.56 to 0.01)	0 (−0.25 to 0.23)	16
Serotonin treatment	−0.13 (−0.47 to 0.17)		17
Phenylalanine	0.1 (−0.02 to 0.39)	0 (−0.14 to 0.13)	18
Citric acid	0.06 (−0.07 to 0.32)	−0.03 (−0.17 to 0.04)	19
Cysteine	0 (−0.34 to 0.36)	0.06 (−0.01 to 0.35)	20
Cysteine* treatment	−0.03 (−0.13 to 0.01)	0.03 (−0.05 to 0.2)	21
Serotonin	−0.05 (−0.3 to 0.08)		22
Norepinephrine treatment	−0.05 (−0.39 to 0.25)		23
Phenylalanine* treatment	−0.03 (−0.13 to −0.01)	0.02 (−0.07 to 0.41)	24
Aspartic acid	−0.05 (−0.19 to 0.09)		25
Proline* treatment	−0.03 (−0.11 to 0.02)	0.01 (−0.05 to 0.1)	26
Citric acid* treatment	0.03 (−0.02 to 0.17)	−0.01 (−0.28 to 0.16)	27
Asparagine* treatment	−0.01 (−0.03 to 0)	0.02 (−0.05 to 0.17)	28
Hydroxyproline	−0.03 (−0.13 to 0.03)		29
Tryptophan* treatment	−0.01 (−0.02 to 0)	0.02 (−0.09 to 0.37)	30
Hydroxyproline treatment	−0.02 (−0.1 to 0.06)		31
Glutamic acid* treatment	−0.01 (−0.05 to 0.05)	0.01 (−0.06 to 0.07)	32
Lysine	0.01 (−0.12 to 0.14)	0 (−0.21 to 0.09)	33
Norepinephrine	−0.01 (−0.21 to 0.17)		34
2 ketoglutaric acid	−0.01 (−0.2 to 0.16)		35
Aspartic acid treatment	0 (−0.05 to 0.1)		36

Rank was calculated by the absolute sum of the medians of the penalized beta estimates of test and replication only if both were available and concordant in directionality. The 5 highest ranks are in bold. The formula used to calculate this rank number is $Rank = \sqrt[2]{\left(\beta i(replication) + \beta i(discovery)\right)^2}$. In this table metabolites are sorted by the sum of their estimates (Rank). Estimates for metabolites in italic were not available in the replication cohort and thus not ranked but the height of estimate of the test was used as an indication of the importance for predicting response to spironolactone thereby giving more weight to the test cohort.
*Treatment indicated the metabolite*treatment interaction.

included in the developed metabolite score also associate with collagen synthesis and fibrosis. Proline, 2-ketoglutaric acid and succinic acid were among the highest ranked metabolites associated with UACR change. The associated enzyme of these metabolites is prolyl 4-hydroxylase subunit alpha 2 (P4HA2), a key enzyme in collagen synthesis and previously associated with DKD.[30] Thus, the included metabolites, as marker of P4HA2 activity, may represent a pro-fibrotic environment which appears to be associated with response to spironolactone (Fig 5).

A previous study conducted in our test cohort suggests that the response to MRA may be enhanced in individuals with a larger degree of fibrosis.[31] Lindhart et at. tested the urinary proteomic CKD273 score to predict responders to spironolactone.[31] This urinary proteomic score consists of 273 peptides and has been associated with progression of tubulointerstitial fibrosis.[14] Subjects with type 2 diabetes and a higher proteomic score, purportedly reflecting larger tubulointerstitial fibrosis, showed a larger reduction in albuminuria after 16 weeks spironolactone treatment.[31] As fibrosis is a driver of progression of DKD, preventing the development of fibrosis with MRA therapy may ameliorate albuminuria. Currently, a large prospective randomized controlled trial is being conducted, first identifying individuals at high risk for progression of DKD with the CKD273 score and then randomly
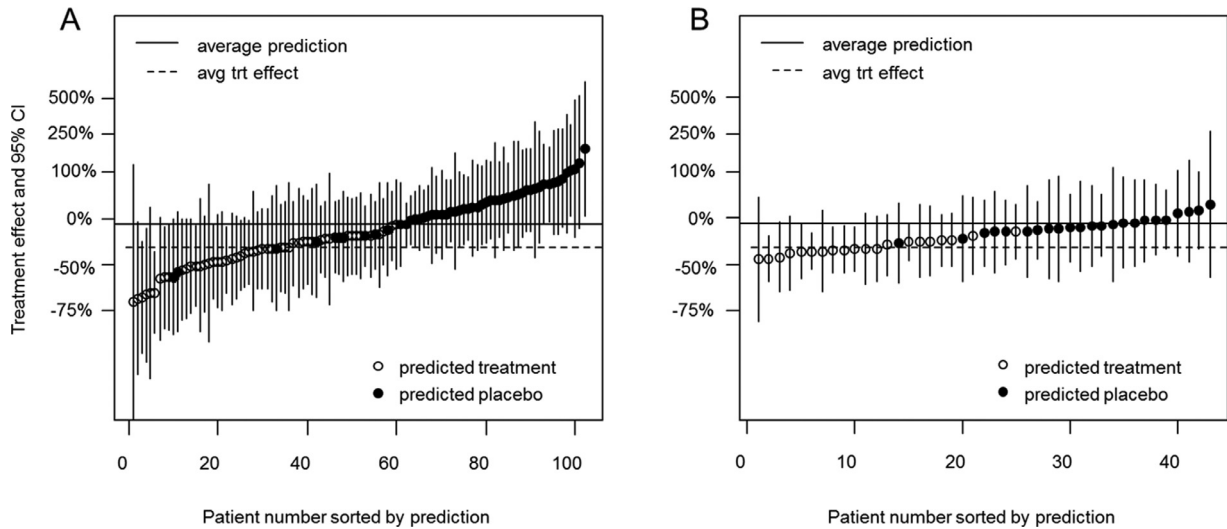
Fig 3. (Panel A) Personalized prediction plot in test cohort: (Panel B) Personalized prediction plot in replication cohort. The personalized predictions (*Y*) are the predicted change in UACR from the model of baseline metabolites and metabolite treatment interactions.

assigning these high risk subjects to spironolactone or placebo (PRIORITY, NCT02040441).[32] Results of this trial will give further insights into tailoring therapy to those at highest risk who might respond best to treatment.

Identified metabolites at the interference of the DKD and spironolactone model also represented sodium-dependent amino acid and dicarboxylate transporters which have been linked to mitochondrial function.[33] Emerging evidence support a role for mitochondrial dysfunction in kidney disease progression.[34,35] Whether potential beneficial effects of spironolactone therapy are

mediated by improvements in mitochondrial function requires confirmation in future studies.

This study has limitations, the first one is that marker measurements were restricted to urine samples. Unfortunately, we were unable to measure plasma metabolites in these subjects and can therefore not assess systemic processes reflected by these metabolites. Furthermore, we identified a large number of subjects in both placebo groups with a reduction in albuminuria from baseline to the end of study. For both cohorts, the study population included were defined as having hypertension and
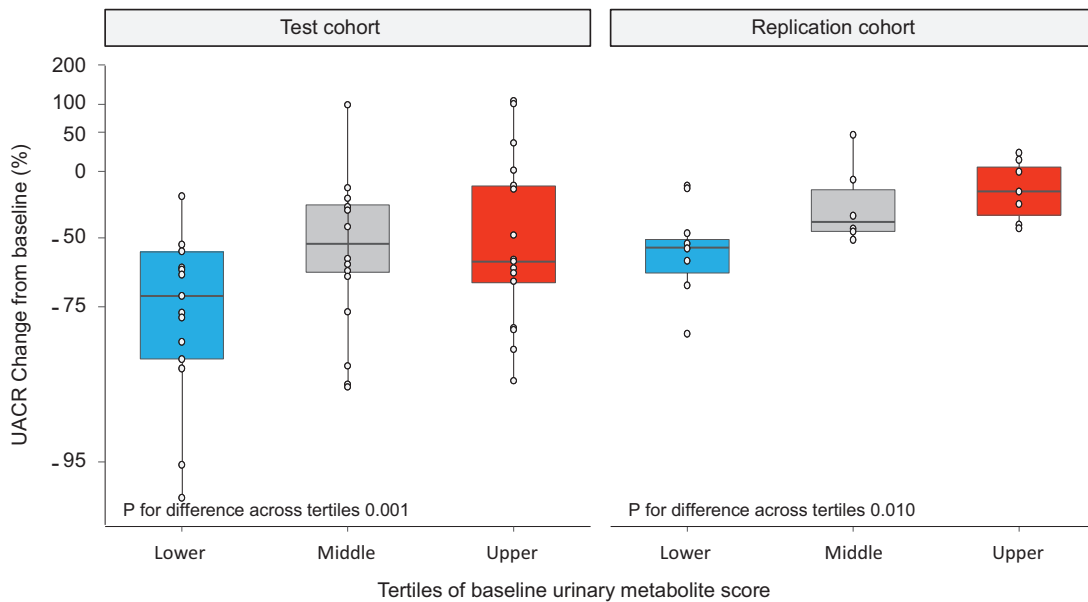
Fig 4. Albuminuria change from baseline % in response to spironolactone, stratified by tertiles of baseline urinary metabolite score.
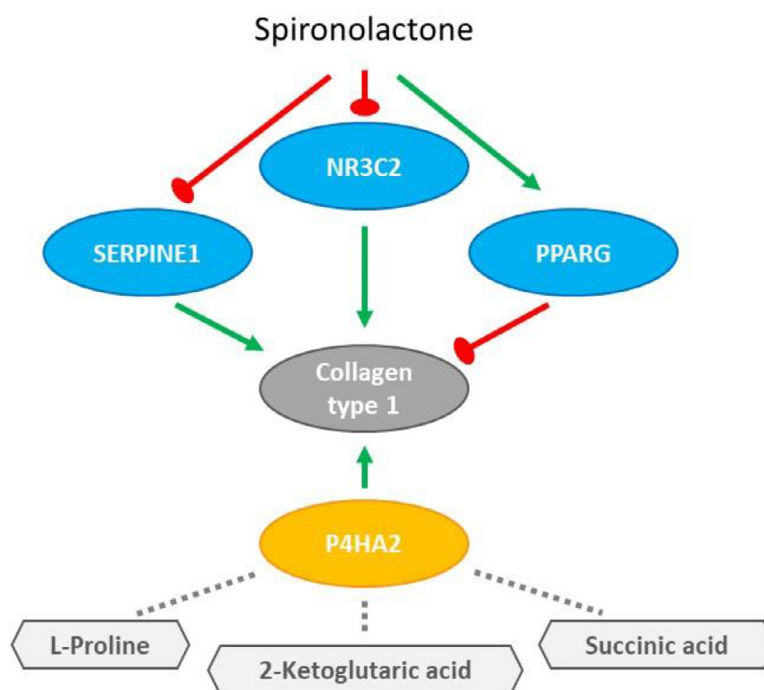
Fig 5. Proposed link of spironolactone treatment with collagen synthesis and turnover. Spironolactone affects the 3 proteins SERPINE1, NR3C2, and PPARG (in blue) which are also part of the DKD molecular model and linked to collagen synthesis and turnover. Measured metabolites in this study that are linked to the DKD-associated protein P4HA2 are shown in grey hexagons. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

received at least concomitant renin angiotensin aldosterone system inhibition. We were not able to determine the effect of other co-medication in individual subjects' expression of metabolites. The regression model predicting our set of metabolites is less accurate than a set of serum metabolites able to predict response to angiotensin receptor blockers in subject with diabetes.[36] This variation could be related to the preselection which may not be able to cover all processes related to drug response or intraindividual variability in albuminuria which is not directly related to active disease processes.[37]

In conclusion, we used a systems biology approach to *a priori* select urinary metabolites for predicting the albuminuria lowering effect of the MRA spironolactone in type 2 diabetes. We tested and replicated our findings in 2 independent clinical trials. These data suggest that systems biology can identify urinary metabolites to predict albuminuria response to spironolactone to tailor optimal therapy and move in the direction of personalized medicine in type 2 diabetes.

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SUPPLEMENTARY MATERIALS

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