Supplemental material – Table of contents

Supplemental table 1. Baseline characteristics per treatment sequence

Characteristic	Total, n=13	HCT-Met-Plc n=2	HCT-Plc-Met n=2	Met-HCT-Plc n=2	Met-Plc-HCT n=2	Plc-HCT-Met n=3	Plc-Met-HCT n=2
Age (years)	45 ± 8	37.5	37	51.5	43.5	46	51
Female sex (N (%))	7 (54%)	1 (50%)	1 (50%)	1 (50%)	2 (100%)	1 (33%)	1 (50%)
Weight (kg)	91 ± 18	102	81	107	74	95	89
Height (m)	1.79 ± 0.13	1.82	1.80	1.87	1.64	1.80	1.81
Systolic BP (mmHg)	125 ± 13	138	139	116	116	114	118
Diastolic BP (mmHg)	77 ± 7	89	85	75	74	75	73
Measured GFR (mL/min/1.73m^2)	55 ± 11	50	58	50	66	56	50
Mayo risk class[a]							
1A/1B (low risk disease) (N (%))	4 (31%)	0 (0%)	0 (0%)	1 (50%)	1 (50%)	1 (33%)	1 (50%)
1C/1D/1E (high risk disease) (N (%))	9 (69%)	2 (100%)	2 (100%)	1 (50%)	1 (50%)	2 (67%)	1 (50%)
2 (atypical) (N (%))	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
RAASi user (N (%))	13 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	3 (100%)	2 (100%)
Urine volume (L/24h)	6.87 ± 1.39	7.6	5.9	6.0	6.5	8.2	6.5
Osmolar excretion (mOsm/24h)	1044 ± 362	1099	859	1092	756	1222	1148
Sodium excretion (mEq/24h)	169 ± 58	156	150	154	149	191	203
Urea excretion (g/24h)	14.0 ± 0.2	15.0	10.1	15.6	10.2	17.1	14.5
Copeptin (pmol/L)	25.4 (19.1 – 28.7)	28.7	27.2	23.7	24.6	20.5	27.2

Variables are presented as mean ± SD, as median (interquartile range) in case of non-normal distribution or as percentage for categorical variables.

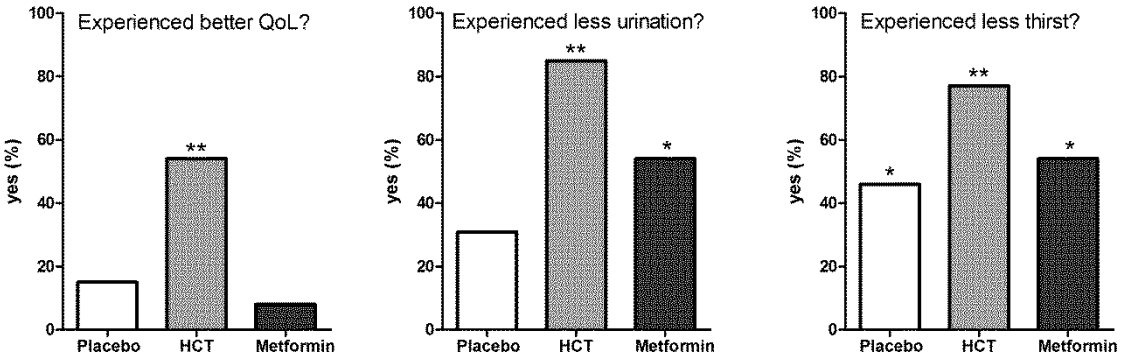
Abbreviations: n, number; BP, blood pressure; GFR, glomerular filtration rate; RAASi, renin-angiotensin II-aldosteron system inhibitor; HCT, hydrochlorothiazide; Plc, placebo; Met, metformin.

[a] Mayo ADPKD classification predicts prognosis and is based on total kidney volume indexed for height and age(1)

Supplemental Figure 1. Quality of life.

Treatments were given in random order. At the end of each treatment period three questions were asked. The bar graphs below represent the percentage of affirmative answers on those questions (* p<0.05, ** P<0.01 using McNeymar's test).

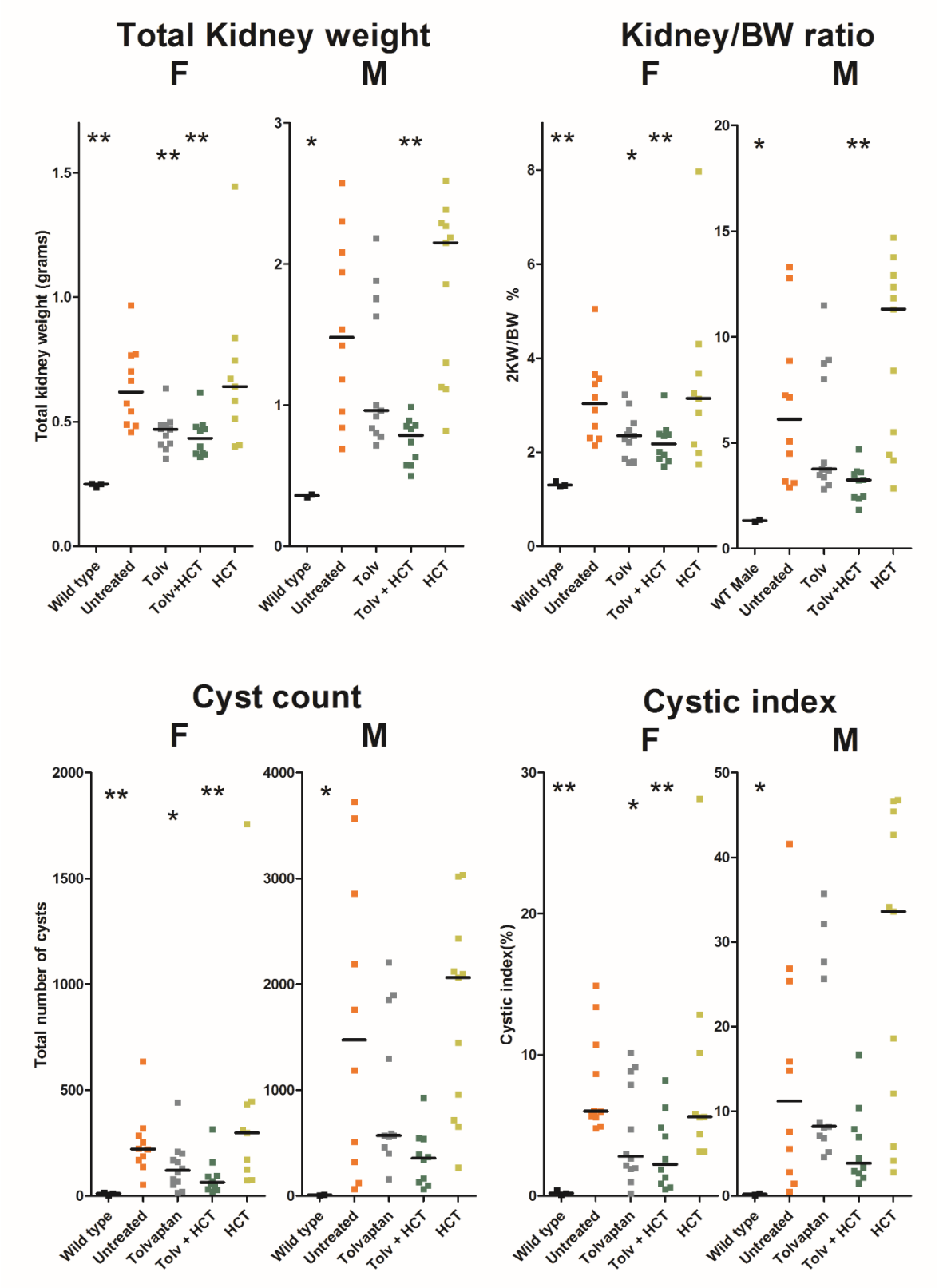
Abbreviations: QoL, quality of life; HCT, hydrochlorothiazide.



Supplemental Figure 2.

Differences in parameters of disease progression in male and female mice, separately. *p<0.05, ** p<0.01, *** p<0.001; compared to untreated cystic mice, Mann-Whitney U test.

Abbreviations: F, female; M, male, BW, body weight; tolv, tolvaptan; HCT, hydrochlorothiazide.



Supplemental Table 2. Changes in urinary excretion of kidney damage and metabolic markers.

	Baseline	Hydrochlorothiazide		Placebo		Metformin	
		Change	P-value	Change	P-value	Change	P-value
Kidney damage markers							
Albumin (mg/24h)	20 (0 – 34)	0 (-28 – 9)	0.3	0 (-9 – 0)	0.1	0 (-28 – 0)	0.2
β2MG (ng/24h)	261 (67 – 527)	-37 (-216 – 16)	0.1	2 (-92 – 98)	0.7	-15 (-208 – 93)	0.3
HFABP (ug/24h)	25.4 (15.3 – 38.3)	-7.6 (-17.5 – 13.7)	0.5	-0.1 (-17.4 – 13.3)	0.8	-2.0 (-16.9 – 11.5)	0.3
NGAL (ug/24h)	12.3 (9.3 – 27.2)	-0.9 (-2.5 – 2.1)	0.5	-0.2 (-2.9 – 1.8)	0.7	-0.6 (-6 – 0.01)	0.1
MCP-1 (ng/24h)	198 (114 – 549)	**-61 (-123 – 10)**	**0.02**	-79 (-176 – 17)	0.1	-56 (-226 – 2)	0.06
Metabolic markers							
Lactate (umol/24h)	46 (30 – 99)	**-20 (-44 – -1)**	**0.01**	-10 (-37 – 24)	0.7	-7 (-28 – 12)	0.3
Pyruvate (umol/24h)	46 (28 – 62)	**-10 (-21 – -8)**	**0.01**	-4 (-11 – 7)	0.7	-5 (-11 – 5)	0.2
Succinate (umol/24h)	63 (50 – 87)	-5 (-52 – 10)	0.2	17 (-14 – 67)	0.2	0 (-24 – 8)	0.7
PKM2 (pg/24h)	12.3 (10.2 – 16.6)	**-2.2 (-7.1 – -0.2)**	**0.04**	-1.6 (-2.9 – 1.8)	0.3	-1.8 (-4.2 – 0.9)	0.06

Data presented as median (IQR). P-value from Wilcoxon Signed Rank test.

Abbreviations: β2MG, β2 microglobulin; HFABP, heart-type fatty acid-binding protein; NGAL, neutrophil gelatinase-associated lipocalin; MCP-1, monocyte chemotactic protein-1; PKM2, pyruvate kinase isozyme M2.

Supplemental Table 3. Biochemistry analyses in mice.

	Wild-type N = 5	**Untreated** N = 20	**Tolvaptan** N=23	**Tolv + HCT** N = 20	**HCT** N =20
Blood (P117)					
Creatinine (umol/L)	8 (6 -12)	10 (8 – 15)	9 (8 – 13)	10 (9 – 12)	12 (8 – 32)
Urea (mmol/L)	10.3 (9.7 – 10.3)	12.3 (10.3 – 18.2)	11.5 (10.2 – 12.7)	13.9 (12.2 – 15.2)	**16.4 (13.0 – 58.8)***
Sodium (mmol/L)	153 (153 – 157)	156 (154 – 157)	157 (154 – 161)	156 (155 – 157)	158 (154 – 163)
Potassium (mmol/L)	**4.6 (4.4 – 4.8)***	5.0 (4.8 – 5.2)	4.8 (4.5 – 5.2)	**4.6 (3.8 – 4.8)***	4.6 (4.2 – 5.1)
24h urine (P90)					
Volume (g)	1.2 (1.1 – 2.1)	1.3 (0.9 – 1.6)	**6.4 (4.5 – 8.3)****	**3.9 (3.1 – 6.3)****	1.6 (1.1 – 2.3)
Creatinine (umol/24h)	2.2 (1.7 – 3.7)	2.6 (2.3 – 2.9)	2.2 (1.9 – 3.2)	2.8 (2.4 – 3.0)	2.4 (1.9 – 3.1)
Urea (mmol/24h)	1.5 (0.9 – 2.2)	1.4 (1.2 – 1.7)	**1.9 (1.4 – 2.2)***	1.7 (1.4 – 2.1)	1.7 (1.1 – 2.3)
Sodium (mEq/24h)	0.14 (0.11 – 0.22)	0.15 (0.12 – 0.19)	**0.20 (0.15 – 0.25)***	0.17 (0.15 – 0.24)	0.17 (0.14 – 0.22)
Potassium (mEq/24h)	0.34 (0.20 – 0.58)	0.32 (0.28 – 0.47)	**0.47 (0.34 – 0.57)***	0.39 (0.34 – 0.52)	0.38 (0.30 – 0.48)
Osmolality (mOsm/L)	1914 (1596 – 1922)	1789 (1559 – 2022)	**490 (442 – 580)****	**711 (597 – 846)****	1678 (1232 – 1964)
U/P Urea (mmol/mmol)	95 (64 – 119)	87 (54 – 110)	**26 (18 – 31)****	**31 (25 – 36)****	62 (12 – 95)

Data are displayed as median (IQR). *p<0.05; ** p<0.01 compared to untreated. *Abbreviations:* HCT, hydrochlorothiazide; U/P urea; urine to plasma urea ratio.

Supplemental methods

Clinical study

Specific examples of the exclusion criteria:
Potential safety risk
-Any other safety risk than those described below, in the opinion of the investigators.

Unlikeliness to adequately comply to the trials' procedures
- Medical conditions likely to require interruption or discontinuation
- History of substance abuse or non-compliance)

Concomitant use of medication likely to confound endpoint assessments
- NSAID
- Diuretics such as furosemide or spironolactone

Concomitant illnesses likely to confound endpoint assessments
- Diabetes mellitus for which medication is needed
- Diabetes insipidus

Pregnancy or breastfeeding

Known contra indications hydrochlorothiazide
- Gout
- Hepatic impairment
- Illnesses that cause potassium loss
- History of hypokalaemia
- Known allergy to hydrochlorothiazide,

Known contra indications metformin
- Illnesses that can cause tissue hypoxia (e.g. recent myocardial infarction, heart failure, respiratory failure)
- Known allergy to metformin

Randomization

An independent pharmacist used a computer program to randomize participants in blocks of six, for the six possible treatment orders of the three treatments. The pharmacist was not involved in the further conduct of the study. Patients were sequentially enrolled according to moment of recruitment. All patients, investigators and health-care providers were blinded to treatment allocation.

Procedures
In the two days before the baseline visit patients collected two 24-hour urine samples. During the baseline visit vital signs were assessed, and laboratory tests and physical examination were

performed. GFR was measured using a single shot iohexol technique(2-4). Blood samples for the measurement of iohexol plasma concentration were collected at 120, 150, 180, 210 and 240 minutes. After the baseline visit patients started with their first treatment period. A venous blood sample was drawn after the first week of treatment to assess safety and study medication was doubled. After the second week of treatment patients returned for a study visit (end of treatment visit). Subsequently, patients entered a one-week wash-out period before starting the following treatment period. This one week wash-out period was based on studies that show that after changes in sodium intake and/or excretion it takes around 2-4 days on average, up to 7 days maximum to reach a new steady state in case of dietary changes and after hydrochorothiazide use (5, 6). The second and third treatment periods were identical to the first one.

Measurements

Heart-type fatty acid-binding protein (HFABP, a distal tubular damage marker) and neutrophil gelatinase-associated lipocalin (NGAL, damage marker of the collecting duct) were measured because these damage markers are known to decrease after initiation of tolvaptan treatment(7). Monocyte chemotactic protein-1 (MCP-1, a marker of inflammation) and β2 microglobulin (β2MG, a marker of the proximal tubule) were measured because these are strongly associated with eGFR decline in ADPKD(8, 9). All four damage markers (MCP-1, β2MG , HFABP and NGAL) were measured by ELISA as described previously(8, 9). Albuminuria was measured during the trial as general kidney damage marker with a colorimetric assay using bromocresol green (BCG; Sigma Aldrich Co. LLC., St. Louis, MO, USA). Lithium analyses were performed using inductively coupled plasma mass spectrometry (ICP-MS). AQP2 was measured by a direct ELISA (Santa Cruz Biotechnology, Dallas, TX, USA). NT-proBNP was measured using an electro-chemiluminescence immunoassay.

Following the manufacturer's instructions, lactate, pyruvate, and succinate were measured using a colorimetric assay kit (BioVision, Milpitas, CA, USA), and pyruvate kinase isozyme M2 (PKM2) was measured using an ELISA kit (MyBiosource Inc, San Diego, CA, USA) in thawed 24-hour urine samples along with appropriate standards. Optical density or fluorescence of samples on a 96-well plate were measured using a Synergy HTX multi-well plate reader with corrections for sample background control readings. All reported concentrations were normalized by the 24-hour urine volumes for each sample.

Serum- and 24-h urine lithium were measured to calculate the fractional lithium excretion, as a proxy for proximal tubular sodium reabsorption(10). AQP2 excretion as a measure of presence of this water channel in the apical cell membranes of the distal collecting duct. An estimate of extracellular volume was made using the single shot iohexol clearance technique, using the formula by Brochner-Mortensen(2-4, 11).

Experimental study

83 mice were treated with tamoxifen dissolved in ethanol in sun flower oil (150 mg/kg, by oral gavage) at postnatal days 18 and 19 (P18 and P19) to inactivate *Pkd1*, while five mice (littermates of cystic mice with similar genetic background) received sunflower oil only to serve as wild-type controls. This model is known to induce a severe cystic phenotype in approximately four months(12).

Interventions

We used spray-dried tolvaptan (Tolvaptan-SD), a formulation of tolvaptan designed to improve the drug's oral bioavailability in experimental studies (provided by Otsuka Pharmaceuticals, Tokyo, Japan). Treatment groups received food pellets (SSNIFF Spezialdiäten, Soest, Germany), supplemented with either 0.15% tolvaptan-SD and/or 0.035% hydrochlorothiazide (H2910, Sigma-Aldrich). For tolvaptan-SD, 0.15% is the equivalent dose of 0.1% 'regular' (non-SD) tolvaptan that is commonly used in preclinical research. For hydrochlorothiazide, the 0.035% dose has been shown efficacious as treatment for nephrogenic diabetes insipidus in a murine model(13).

Procedures

Mice were housed in groups of two or three animals per cage, with *ad libitum* access to food and water. Animals were sacrificed at P117 or in case of 20% weight loss (humane end-point), whichever occurred first. The animals were weighed and anesthetized with isoflurane (0.5% isoflurane with a flow of 0.6 L/min). Blood was obtained by cardiac puncture for determination of plasma electrolytes, creatinine and urea. Both kidneys were removed, weighed on a precision scale and placed into formaldehyde. The tissues were embedded in paraffin for histomorphometry and immunohistochemistry.

Measurements

To analyze the total cystic area within the transversal sections of the kidneys, transverse tissue sections (4 μm), including cortex, medulla and papilla, were stained for periodic acid-Schiff (PAS). The total area and number of the cysts were quantified on a transversal mid-slice of the right kidney using QuPath 0.1.2 and ImageJ 1.52 software. Total area of cysts was divided by the total area of tissue and multiplied by 100% to obtain a cystic index, expressed as percentage. The investigator performing these measurements was blinded to disease and treatment status.

In the animal study, creatinine, urea, potassium and sodium were measured in plasma and urine, using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY). Urinary osmolality was calculated as 2 × (sodium concentration + potassium concentration) + urea concentration.

Statistics

Background power calculation clinical study

For the clinical study we calculated that enrollment of at least 10 patients was needed in the trial to be able to detect 1.8 L reduction in urine volume on a 6 L pre-treatment average (thirty percent). A decrease in urine volume of thirty percent (30% of 6 L, 1.8 L) was considered attainable and relevant based on literature of hydrochlorothiazide treatment in patients with nephrogenic diabetes insipidus(14-16). Standard deviation of urine volume was 1.8 L in a previous study of ADPKD patients treated with tolvaptan(17) (similarity to expected treatment effect is coincidental) and assumed to be equal across time periods, we assumed an internal correlation coefficient of 0.5 based on repeated 24-hour urine volume measurements in previous studies of ADPKD(18). We calculated that enrollment of 10

patients was needed to have 80% power to detect a difference in urine volume with a two-tailed α of 0.025 (0.05/2, applying a Bonferroni correction for the two treatments) using a paired sample T-test. To allow for drop-outs, we aimed to include 12 patients.

Background power calculation animal experiment

For the animal experiment we calculated that we needed to enroll 6 mice in each group to be able to detect a 6 mL/24hr (thirty percent) reduction in urine volume during tolvaptan of 20.0±3.5 mL/24hr average(19) with 80% power and a two-tailed α of 0.05. To be able to show non-inferiority with regard to disease progression during tolvaptan use (assessed as a kidney to body weight ratio of 3.9%±2.1(19)) by a margin of 40%, an α of 0.05 and with 80% power 22 animals per group would be needed.

Supplemental results

Testing for carry-over effect

The treatment effect of HCT was similar if all participants that used metformin in a period prior to HCT were excluded (n=7, urine volume compared to baseline -20±8%, p<0.001). The treatment effect of metformin was also similar if all participants that used HCT in a period prior to metformin were excluded (n=6, urine volume compared to baseline -25±13%, p=0.005).

Moreover, we compared the treatment effect of metformin on urine volume in participants who used metformin in the period directly before HCT (-1276±570) to the treatment effect of metformin in participants who used metformin in the period directly after HCT (-1308±126). The treatment effects were not significantly different (p=0.9), indicating no carry-over effect. Similarly, treatment effect of HCT if it was used in the treatment period directly before metformin (-1420±607) was not different from the treatment effect of HCT in the treatment period directly following metformin (-1778±1142), p=0.6, indicating no carry-over effect.

Osmolar excretion and free water clearance

Osmolar excretion (as measures of osmolar intake) was similar in all treatment periods (**Table 2**), suggesting a stable diet. Furthermore, osmolar excretion at baseline was strongly correlated with osmolar excretion during hydrochlorothiazide treatment (St. β 0.89, p<0.001), placebo treatment (St. β 0.95, p<0.001) and metformin treatment (St. β 0.95, p<0.001) . Free water clearance decreased significantly during both hydrochlorothiazide and metformin treatment (by 1.67±0.90 L and 1.14±0.49 L, respectively, p<0.001, (**Table 2**), indicating that the decrease in water clearance was also significant independent of any potential (non-significant) changes in total osmolar excretion.

Urinary excretions of metabolic markers and damage markers

During hydrochlorothiazide treatment albumin, β2 microglobulin (β2MG), heart-type fatty acid-binding protein (HFABP) and neutrophil gelatinase-associated lipocalin (NGAL) did not change, but the inflammation marker monocyte chemotactic protein-1 (MCP-1) decreased (p=0.02). During metformin and placebo treatment, the urinary excretion of all damage markers remained unchanged. Regarding the metabolic markers, 24-hour excretions of the metabolites lactate, pyruvate and the metabolic enzyme pyruvate kinase isozyme M2 (PKM2) showed a significant decrease during hydrochlorothiazide treatment, while succinate excretion did not change. There were no changes during metformin and placebo treatment.

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