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Mineralocorticoid receptor blockade with spironolactone has no direct effect on plasma IL-17A and injury markers in urine from kidney transplant patients

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Abstract

Kidney transplantation is associated with increased risk of cardiovascular morbidity. Interleukin-17A (IL-17A) mediates kidney injury. Aldosterone promotes T-helper-17 (Th-17) lymphocyte differentiation and IL-17A production through the mineralocorticoid receptor (MR). In this exploratory, post-hoc substudy, it was hypothesized that 1-year intervention with the MR antagonist spironolactone lowers IL-17A and related cytokines and reduces epithelial injury in kidney transplant recipients. Plasma and urine samples were obtained from kidney transplant recipients from a double-blind randomized clinical trial testing spironolactone (n=39) versus placebo (n=41). Plasma concentrations of cytokines IFN-γ, IL-17A, TNF-α, IL-6, IL-1β, and IL-10 were determined before and after 1-year treatment. Urine calbindin, clusterin, KIM-1, osteoactivin, TFF3, and VEGF/creatinine ratios were analyzed. Blood pressure and plasma aldosterone concentration at inclusion did not relate to plasma cytokines and injury markers. None of the cytokines changed in plasma after spironolactone intervention. Plasma IL-17A increased in the placebo group. Spironolactone induced an increase in plasma K^+ (0.4 ± 0.4 mmol/L). This increase did not correlate with plasma IL-17A or urine calbindin and TFF3 changes. Ongoing treatment at inclusion with angiotensin-converting-enzyme inhibitor and/or angiotensin II receptor blockers was not associated with changed levels of IL-17A and injury markers and had no effect on the response to spironolactone. Urinary calbindin and TFF3 decreased in the spironolactone group with no difference in between-group analyses. In conclusion, irrespective of ongoing ANGII inhibition, spironolactone has no effect on plasma IL-17A and related cytokines or urinary injury markers in kidney transplant recipients.

New & Noteworthy

The mineralocorticoid receptor antagonist spironolactone had no direct anti-inflammatory effects on pro-hypertensive IL-17A or distal nephron epithelial injury markers in kidney transplant recipients.

INTRODUCTION

Kidney allograft injury with decline in glomerular filtration rate (GFR) and proteinuria is progressive but also increases the risk of co-morbidities. A key mediator in inflammation and kidney injury is the T-helper (Th)-17 lymphocyte lineage. Th-17 lymphocytes are effector cells distinct from the Th-1 and Th-2 effector cells. Th-17 cells produce a family of pro-inflammatory IL-17 cytokines. Of these, IL-17A promotes allograft injury in human and preclinical mouse studies. Intragraft IL-17A mRNA and plasma IL-17A levels increased in kidney transplant recipients with acute rejection and plasma IL-6, IL-17, and TNF-α levels were associated with acute kidney injury after living donor liver transplantation (1, 2). In chronic kidney allograft dysfunction, there was an increase of Th-17 cells and IL-17A in plasma (3). Moreover, IL-17 levels increased in patients with ongoing acute rejection compared to patients with stable allograft function (4). In preclinical mouse kidney transplantation models, IL-17A deficiency attenuated kidney allograft injury and prolonged survival (5). Several preclinical studies have implicated the involvement of IL-17A in the pathology of kidney injury and hypertension. In mice, IL-17A overexpression was associated with hypertension and IL-17A infusion by osmotic minipumps was associated with inflammatory cell infiltration in the kidneys and increased level of the injury marker neutrophil gelatinase-associated lipocalin (NGAL) (6, 7). Mice with targeted deletion of IL-17A were protected against diabetic nephropathy-induced kidney fibrosis and pro-inflammatory cytokine expression, and anti-IL-17A treatment of these mice ameliorated

kidney dysfunction and disease progression (8, 9). Experimental hypertension in mice and rats is associated with elevated plasma IL-17A levels and deletion or inhibition of IL-17A production in mice protected against blood pressure elevation (10, 11). *In vivo* transfer of Th-17 cells into mice mediated rapid development of albuminuria, glomerular neutrophil infiltration, and increased levels of injury markers in the kidneys when compared to mice receiving Th-1 cells (12). However, only few clinical studies have examined involvement of IL-17A in kidney pathology in humans. IL-17A was expressed in kidney biopsies of hypertensive nephropathy patients (7); serum levels of IL-17A were elevated in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis (13), and urine IL-17A was elevated in glomerulonephritis (14, 15). The mechanistic role of IL-17A was not identified. IL-17A is implicated in autoimmune disorders like psoriasis, asthma, and multiple sclerosis and IL-17A neutralizing antibodies have beneficial clinical effects in patients with psoriasis and psoriatic arthritis (16-19). Taken together, IL-17A could be a non-redundant contributor to allograft dysfunction by direct effects and indirectly through hypertension.

Mineralocorticoid receptor (MR) is expressed in T-lymphocytes, B-lymphocytes, dendritic cells, and macrophages, and aldosterone promotes the Th-17 lineage differentiation (20-23). MR blockade with spironolactone mediated blood pressure and IL-17 reduction in DOCA/salt hypertensive rats (11). The acute effect of MR blockade with spironolactone over 5 days (25-100 mg/day) in kidney transplant recipients, reduced oxidative stress, urinary kidney injury molecule 1 (KIM-1), and NGAL in kidney transplant patients in double-blind randomized, placebo-controlled studies (24, 25). In a double-blind placebo-controlled study of treatment with eplerenone for 24 months in children with chronic allograft nephropathy, there was a tendency towards declining renal function and increased proteinuria in the placebo-treated group whereas the eplerenone-treated group remained stable, however these differences were non-significant. Furthermore, there was no change in urinary KIM-1

between the groups (26). The present post-hoc defined, exploratory sub-study with patients from the multicenter randomized double-blind placebo-controlled intervention trial with spironolactone, the SPIREN trial (ClinicalTrials.gov: NCT01602861), was conducted to address the specific hypotheses that spironolactone decreases plasma concentrations of IL-17A and other related T-lymphocyte and macrophage-derived cytokines and that this suppression relates to renal epithelial protection (27, 28). *In vitro* studies have shown that increased extracellular [K^+] causes depolarization of membrane potential in T-lymphocytes and suppression of Ca^{2+} influx which is needed for T-cell activation and T-cell cytokine production including IL-17A (29-31). Thus, elevated K^+ could suppress renal inflammation. It was therefore further hypothesized that the spironolactone-mediated plasma potassium increase (28) would relate inversely to plasma cytokine decrease. These hypotheses were addressed by determining plasma concentrations of cytokines in paired plasma samples from placebo- and spironolactone-treated adult, stable, kidney allograft patients and the excretion of established epithelial injury markers in spot urine samples expressed as creatinine ratios. The main outcome parameters of the SPIREN trial are glomerular filtration rate and kidney fibrosis, however these data have not been published yet (27).

METHODS

Patient cohort

Kidney transplant patients were included in the SPIREN trial, a randomized, double-blind, placebo-controlled clinical trial designed to test the hypothesis that MR antagonism by spironolactone could improve long term kidney function (GFR) and reduce allograft fibrosis (27, 28, 32). This trial was approved by the Ethics Committee of Southern Denmark [project ID: s-20110095, protocol version 2 (07/28/2011)], amendment 2 (24/10/2017) and amendment 4 (13/11/2019)] and registered at ClinicalTrials.gov (5/17/2012; NCT01602861)

and EudraCT (5/31/2011; 2011-002243-98). The full study protocol has previously been published (27). In short, prevalent kidney transplant recipients were randomized to spironolactone (25-50 mg/day) or placebo. Spironolactone was given at a dose of 25 mg/day for the first 3 months. Next, the dose was doubled if tolerated by the patient. This dose was then continued for 3 years. Inclusion and exclusion criteria have previously been described in detail (27, 28). Compliance to the study drug was evaluated by tablet counts at each visit. Doses of calcineurin inhibitor (CNI) were titrated independently of the study to aim for a tacrolimus trough level of 5 μg/l or a cyclosporine 2-hour level of 600 μg/l according to local immunosuppressive protocol. The number of antihypertensive medications used did not change significantly within the groups or between groups. Patients in the present sub-study were selected only on the basis of completion of 1 year treatment. The present cohort is identical to the one published previously (28). Baseline data, urine, and blood samples collected at study inclusion and after 1 year intervention for the first 80 patients who completed the first year of the SPIREN trial, were used. At the time of the present analyses, the blinding of the original trial was not compromised since all patients had not completed the full treatment protocol before unblinding. The present analyses were performed blinded to the allocation. Plasma and urine samples were analyzed for cytokine and kidney injury marker concentrations. A spot urine and non-fasting EDTA-anticoagulated blood plasma sample was collected at their first visit at study inclusion after transplantation before starting the treatment (baseline), and after 1 year of treatment (28). Plasma samples used were thawed once before the present determinations.

Cytokine quantification

Using the multiplex electrochemiluminescence immunoassay**,** U-PLEX human group 1 multi-spot kit (*Mesoscale Discovery, Denmark*), 6 cytokines including IFN-γ, IL-17A, TNF-

α, IL-6, IL-1β, and IL-10 were quantified in kidney transplant patient plasma samples as per manufactures protocol. In short, 10-spot 96-well mesoscale plates were coated with 6 different biotinylated antibodies conjugated to 6 different plate linkers for 1 hour at room temperature (RT). Each antibody-linker complex would bind to one of the 10 spots in each well. All wells were washed 3 times with PBS with 0.05% Tween-20 (PBS/Tw). Then plasma samples and standard calibrators were added to the wells undiluted. Plasma from LPS-stimulated (*Sigma Aldrich Denmark*, 10 ng/ml) whole blood (24h, 37°C) was included on all plates as a positive control and to determine in-house interassay variation and applied to the wells as duplicates in a 1:4 dilution. All samples were incubated O/N at 4°C. After analyte incubation, wells were washed 3 times with PBS/Tw and incubated for 1 hour at RT with detection antibody. Finally, the plates were developed using Mesoscale 2X read buffer and read by the MESO QuickPlex SQ 120 reader (*Mesoscale, Discovery, Denmark).* Cytokine quantification was based on a 4-parameter analysis of fitted calibrator curve obtained by serial dilutions of mesoscale calibrator 1, assessed by the mesoscale Discovery Workbench software version 4.0 (*Mesoscale, Discovery, Denmark)*. Concentrations are given as pg/ml and data are presented as median with interquartile range. Intra-assay variation was determined in-house by assessing cytokine levels in normal human plasma samples obtained from healthy individuals. The same individual sample was applied to 8 wells in 1:10 dilution. Intra-assay variations were obtained for IL-10 (12,1%), IL-1β (7,3 %), and IL-6 (6,8%) and were within acceptable range. Repeated determinations of a positive control plasma sample (24h incubation of whole blood with 10 μg/ml LPS) on all plates yielded in-house coefficient of inter-assay variation at 6.5 %, 6.6%, 5.1%, 18.9% and 0.6% for IFN-γ, TNF-α, IL-6, IL-1β and IL-10 respectively. Paired samples from each patient were analyzed on the same plates so that any possible inter-assay variation would not interfere with the differences within each patient. Interassay variations of TNF-α, IL-6, IL-1β were based on values above detection

range with CV<20%. It was pre-hoc determined that values obtained below detection range and with CV>20% were excluded from the dataset. If one sample was excluded, its paired measurement in the same assay was excluded too. Therefore, the different numbers of "n" for each analyte is a result of this exclusion criterion (table 1). The samples (n) excluded based on this cutoff for each analyte has been stated (table S1).

To clarify the effect of data exclusion based on the above-mentioned criteria, and exclude bias, data was reassessed by two setups; 1) including datapoints obtained below and within detection range, except the ones with CV>20%, and 2) including all datapoints below and within detection range, expect data points that were too low to be calculated by the mesoscale Discovery Workbench software version 4.0 (table S1-S3). Using the last-mentioned exclusion criteria, only 0-3 data points were excluded in each group of each assay (table S1), and the reassessed analyses (table S3) was not different from the reassessed setup 1 (table S2). When including datapoints below detection range, the mesoscale software was able to calculate extrapolated values from the fitted 4-parametre curve, and these values were used for analyses. A few samples were below this fitted-curve range and concentrations could not be calculated for these, and therefore not included in analyses.

Urinary kidney Injury marker quantification

Spot urine samples complementary to the plasma samples were analyzed for six kidney injury markers including calbindin, clusterin, kidney injury molecule 1 (KIM-1), osteoactivin, trefoil factor 3 (TFF3) and vascular endothelial growth factor (VEGF) using the multiplex electrochemiluminescence immunoassay, urinary kidney injury marker panel 3 kit (*mesoscale discovery, Denmark*) as per manufactures protocol. The assay was designed to contain markers representative for all nephron segments. In short, pre-coated 96-well mesoscale plates were blocked for 30 min. at RT and washed with PBS/Tw 3 times. Then, 50

µl of diluted (1:10) patient urine samples, urine pool (positive control) and standard calibrator dilutions were added to the wells in duplicates. All samples were incubated for two hours at vigorous shaking at RT. Hereafter, wells were washed three times with PBS/Tw and detection antibodies were added and incubated for 2 hours. Finally, wells were washed 3 times with PBS/Tw and the plates were developed using mesoscale 2X read buffer and read using the MESO QuickPlex SQ 120 reader. Concentrations of kidney injury markers were calculated by the mesoscale Workbench software version 4.0 and given as pg/ml based on a 4-parameter analysis of fitted calibrator curve obtained by serial dilutions of Calibrator 1. Concentrations were normalized to urinary creatinine concentration. Data are presented as (ng/g) median with interquartile range. Inter-assay CV% was determined in house by applying a urine pooled from 2 healthy controls on all plates: Calbindin (3%), clusterin (1%), KIM-1 (15%), osteoactivin (10%), TFF3 (19%) and VEGF (13%). Baseline and after-intervention samples from each patient were analyzed on the same plate to avoid inter-assay differences to influence the differences within each patient. Values obtained below detection range or with CV>20% were excluded from the dataset. Since only few data points were excluded from this dataset, caused by CV>20% (less than 6 datapoints in each assay), the data was not reevaluated as for the cytokine data.

Urinary creatinine

Urinary creatinine measurements were analyzed by the Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark. Briefly, creatinase, sarcosine oxidase and peroxidase were added to the urine samples. The enzymes catalyze conversion of creatinine into a quinoniminchromogen. Absorbance of the color intensity was measured photometrically at 546 nm.

Plasma aldosterone and K^+ concentrations

Aldosterone and K^+ concentrations have previously been determined and reported in plasma samples before and after intervention in both the spironolactone and placebo groups (28).

Blood pressure measurements

Twenty-four-hour blood pressure measurements were performed at baseline before and after 1-year of intervention in all patients in both groups using the equipment available at the local center (*Diasys Integra II, Novacor, UK; and TM2430, A&D, Japan*). Valid measurements consisted of 20 daytime and 7 night-time values. Of the 80 patients included in this study, valid blood pressure measurements were obtained from 57 patients in both the spironolactone (n=27) and placebo (n=30) groups before and after 1 year of intervention. Anti-hypertensive medication was adjusted according to clinical indication and independently of the study protocol, to maintain blood pressure within recommended range (28).

Statistics

Data were tested for normality using the D'Agostino & Pearson test. Paired t-tests compared changes in parameters before and after intervention in each group including only paired datasets with both baseline and 1 year sample measurements within detection range with CV<20%. Normally distributed data were analyzed by parametric paired, two-sample t-tests. Data that were not normally distributed even after log-transformation, were analyzed by a non-parametric Wilcoxon test. Comparison between groups of delta values was carried out by unpaired Mann Whitney tests. Data that were normally distributed or normally distributed after log-transformation, were also tested for significance on effect of treatment, time, and interaction by a two-way ANOVA analysis followed by Bonferroni's comparison post hoc test. Pearson correlations were carried out on normally distributed data and spearman

correlations were carried out on data that were not normally distributed. A p-value of 0.05 or less was considered significant. All statistical analyses were made in GraphPad Prism version 9.

RESULTS

Clinical Characteristics

Baseline patient characteristics have been reported previously (28). In brief, of the 80 patients included 39 patients received spironolactone and 41 patients received placebo. The two groups were comparable at baseline in terms of demographics, comorbidity, and renal function. At inclusion 94% of the patients received anti-hypertensive medication and 26% had diabetes. All patients received a CNI (tacrolimus or cyclosporine) and an antimetabolite (mycophenolate or azathioprine). More patients in the placebo group received prednisolone (22% vs. 5% ($p<0.05$)) (28). There were no episodes of acute rejection during the study period. Plasma samples from 3 patients in the spironolactone group and 1 patient from the placebo group were not available since sample volume was too small, due to usage in previous analysis. Thus, for plasma analyses spironolactone-treated patients included n=36 and placebo treated patients included n=40.

Effect of spironolactone on plasma concentration of T-cell derived cytokines

When comparing cytokine levels at inclusion between the spironolactone and placebo groups, no significant differences were apparent (table 1). While 1-year spironolactone intervention did not significantly change plasma concentrations of the T-cell derived cytokines IFN-γ and IL-17A (figure 1), IL-17A concentrations increased slightly but significantly from 6.7 (5.1-9.4) pg/ml to 7.5 (5.4-12.6) pg/ml in the placebo group (figure 1D). No differences were observed between the interventional groups after 1 year (table 1). Data analyses was based on

the data inclusion criteria CV<20% and within detection range. It was verified that the excluded datapoints did not affect the results by reassessing data in the two mentioned setups: 1) including datapoints below detection range except datapoints with CV>20%, and 2) including all datapoints. Number of datapoints excluded in each assay is shown (table S1). The reassessed data analyses did not change the result and no significant effect of spironolactone was uncovered (table S2-3).

Effect of spironolactone on plasma concentration of macrophage-derived cytokines

Baseline levels of the four macrophage-derived cytokines (IL-6, TNF-α, IL-1β and IL-10) were not different between groups (table 1). At 1-year follow-up, no changes were observed in the spironolactone-treated group and in the placebo-treated group (figure 2). Differences in cytokine levels between groups after 1 year of intervention were not observed (table 1). Reassessed analyses of data points 1) within and below detection range and 2) all data points, revealed significantly increased levels of plasma IL-β concentrations in placebo treated patients compared to spironolactone treated patients (table S2-3). This between-group difference was not observed in the analysis that only included datapoints within detection range of assay.

Blood pressure and plasma aldosterone did not correlate to plasma cytokine levels at baseline

Systolic blood pressure (SBP) and diastolic blood pressures (DBPs) were measured at entry of study and after 1 year of treatment. Mean arterial pressure (MAP) was not different after 1 year of treatment, and no MAP differences were observed between groups. Placebo-treated patients exhibited increased SBP by 7 mmHg after 1 year (28). When correlating SBP, DBP, or MAP to plasma cytokine levels at study entry, no significant correlations were observed

for any of the analyzed cytokines (table 3). Correlation of plasma IL-17A increase and SBP increase in the placebo group revealed no significant relation (table S3). Plasma aldosterone concentrations at baseline did not relate significantly to MAP, SBP or DBP at baseline in the patients (table 3). There was no significant correlation between plasma aldosterone and plasma cytokine levels at study entry (table 3). Blood pressure and plasma aldosterone changes upon spironolactone intervention did not relate to cytokine changes (table S3).

Subgroup analyses of ACEi- and ARB-treated patients (cytokines)

Angiotensin converting enzyme inhibitor (ACEi) or angiotensin receptor blockers (ARBs) were given to 52% of the patients included (28). Since treatment with ACEi and ARBs may impact levels of the measured cytokines, we performed a subgroup analysis of cytokine levels in patients treated with ACEi/ARB vs no treatment with ACEi/ARB (no drug) at baseline (figure S1) and after intervention (delta-values) in both the spironolactone and placebo groups (figure S2). At baseline, plasma cytokine levels were not different between patients receiving ACEi/ARB and no drug (figure S1A-E) except for plasma IL-10, which was significantly decreased in patients receiving ACEi/ARB compared to patients treated with no drug (figure S1F). Plasma cytokine differences (delta-values) after 1 year intervention were not different between patients that received ACEi/ARB or no drug in both spironolactone and placebo groups (figure S2).

Paired analyses of data from patients in placebo and spironolactone interventional groups were assessed in patients that received ACEi/ARB and no drug, comparing plasma IL-17A and IL-10 concentrations at baseline and after 1 year intervention (figure S4). Plasma IL-17A and IL-10 were unchanged after 1 year intervention with spironolactone and placebo in patients that did not receive ACEi/ARB treatment (figure S4). ACEi/ARB treatment was not associated with a change in IL-17A and IL-10 in spironolactone treated patients but in

placebo treated patients' plasma IL-17A and IL-10 was significantly increased (P=0.004** and P=0.05* respectively, figure S4).

Effect of spironolactone treatment in kidney transplant patients on epithelial injury markers

Individual urine injury marker concentration was normalized to urine creatinine concentration. Urine injury marker/creatinine ratios at baseline did not differ between spironolactone and placebo groups (table 2). In spironolactone-treated patients, calbindin and TFF3/creatinine ratios decreased significantly from 340 (253-448) pg/ml to 273 (214-347) pg/ml and 19 (7-48) pg/ml to 10 (4-31) pg/ml respectively ($p<0.05$, paired t-test) whereas no changes were observed in the placebo group (figure 3). This change was not significant in the between-group analysis. No significant differences after treatment were observed in clusterin, KIM-1, osteoactivin, and VEGF levels by paired t-tests. No correlation was observed between plasma aldosterone or plasma IL-17A and urinary kidney injury markers at baseline (table 3), however blood pressure (MAP, SBP and DBP) correlated positively with urinary TFF3 at baseline (table 3), but no relation was observed between blood pressure changes and TFF3 changes upon spironolactone intervention (table S3). DBP changes correlated positively with calbindin changes and plasma aldosterone changes correlated positively with KIM-1 changes (table S3).

Increases in plasma potassium concentration did not relate to IL-17A, calbindin or TFF3 changes upon 1 year of spironolactone treatment

Plasma potassium concentration increased significantly from 4.2±0.4 to 4.5±0.4 mmol/L in response to spironolactone intervention in the kidney transplant patients with no changes in the placebo group (table 1) (28). When correlating individual K^+ changes (delta) with plasma IL-17A changes (delta) after spironolactone intervention, no significant correlation was observed (figure 4A). Spironolactone induced plasma K^+ increase did not relate to the urinary calbindin and TFF3 decrease (figure 4B-C).

Subgroup analyses of ACEi- and ARB treated patients (kidney injury markers)

The subgroup analyses were performed as for the subgroup analyses of cytokines. Data revealed no association between ACEi/ARB treatment and kidney injury markers at baseline (figure S1G-L) or after spironolactone or placebo intervention (figure S3). Urine calbindin and TFF3/creatinine ratios were significantly decreased after spironolactone intervention in ACEi/ARB treated patients and not in spironolactone-treated patients that received no ACEi/ARBs. Calbindin and TFF3 were unchanged in placebo treated patients with and without ACEi/ARB (figure S4I-P).

DISCUSSION

In the present study, plasma IL-17A, IFN-γ, TNF-α, IL-6, IL-1β, and IL-10 concentrations were measured to analyze a hypothesized beneficial suppressive effect of spironolactone treatment selectively on the Th-17-derived IL-17A in kidney transplant patients. Cytokines related to Th-17 activation and IL-17 production (IL-1β and IL-6) (33), Th1-cell activation (IFN-γ), and macrophage activation (TNF-α, IL-6, and IL-10) were included in the analyses. Our results showed no effect of 1-year spironolactone treatment on plasma IL-17A or any of the other cytokines. A small but significant increase in 1-year placebo-treated patients was observed for IL-17A. When reassessing data including all datapoints and datapoints below detection range with CV<20%, plasma IL-1β levels increased in placebo-treated patients with a significant difference between groups (analyzed on log-transformed normally distributed data), however since the difference observed is based on very low concentrations of IL-1β and almost 50% of the data are below detection limit, the interpretation of this result should be done with caution.

The concentration of plasma IL-17A in the kidney transplant patients of the present study cohort at approximately 7 pg/ml corresponds to levels of IL-17A in serum of patients with

type 2 diabetes and hypertension or other pathological conditions and is higher than plasma IL-17A levels of normal healthy individuals (approximately 2.5 pg/ml), suggesting valid determination (10, 34-36). The increase in plasma IL-17A in the placebo group was not a result of baseline differences between groups. Blood pressure was independently controlled in the patients, but with a minor, significant, increase in blood pressure in the placebo group, the IL-17A increase could be causally related. However, there was no significant correlation. Since the difference was small and no difference between groups was observed, it cannot be excluded that the significant increase is a coincidental random finding. Cytokines circulate in comparatively low concentrations and are particularly sensitive to freeze-thaw cycles, but the present samples were only thawed once, and we found reasonable in-house inter- and intraassay coefficients of variation. Ninety-four percent of all patients received antihypertensive medication mostly including ACEi, ARBs, Ca^{2+} channel blockers, and β-blockers. Although there was no reduction in the number of antihypertensive drugs in both groups, it cannot be excluded that dosage adjustments occurred in some of the patients according to clinical indications and independently of the study protocol. This could have interfered with cytokine levels and also be the reason for undetectable changes, since antihypertensive medication like ACEi, ARBs, and statins have been shown to exert anti-inflammatory effects in hypertensive patients by reducing IL-17A as well (37-39). Preclinical studies have suggested that IL-17A production may depend on both ANGII-AT1 and aldosterone-MR actions in mice and rats (10, 11). If IL-17A is dependent on ANGII-AT1 actions, it is possible that IL-17A was already suppressed at baseline and the MR blockade-mediated suppression would be less pronounced. The data were re-analyzed according to antihypertensive treatment with ACEi and/or ARBs. When groups were compared, ACEi/ARB treatment was not associated with different cytokine concentration in kidney transplant recipient plasma compared with patients not treated with ACEi/ARBs in the

404 placebo group, and no cytokine-lowering effect was observed in spironolactone-treated
405 patients with and without ACEi/ARB. Of interest, in paired analyses, plasma IL-17A and IL-
406 10 increased in placebo and ACEi/ARB treated patients. These data indicate that ACEi/ARBs
407 are not potent inhibitors of IL-17A. Thus, in stable kidney transplant patients, ACEi/ARB
408 treatment does not exclude beneficial non-redundant effects of spironolactone.
409 One confounding factor could be the treatment with prednisolone. *In vivo* and *ex vivo* studies
410 in humans indicate that prednisolone reduce IL-17A levels both on transcript and protein
411 levels (40, 41). However, with more prednisolone-treated patients in the placebo group this
412 cannot account for increased IL-17A concentration. Rather, an underlying inflammatory
413 response in the placebo-treated patients could explain the IL-17A increase. We previously
414 reported that spironolactone increased plasma K^+ in this cohort (28). Increased extracellular
415 [K^+] is associated with suppression of T cell activation and T-cell cytokine production
416 including IL-17A *in vitro* (31, 42, 43). Moreover, an increased expression and activity of K^+-
417 channels correlate with pro-inflammatory cytokine response and blocking subtypes of
418 potassium channels inhibits T-cell proliferation and inflammation (29, 30). In the present
419 cohort, the increase in plasma potassium concentration in the spironolactone group did not
420 relate significantly to plasma IL-17A levels after 1 year. It cannot be excluded that the
421 increase in K^+ in the spironolactone-treated patients could have contributed to stabilize
422 plasma IL-17A, since K^+ did not change in the placebo group where plasma IL-17A
423 increased.
424 All patients in the included cohort received immunosuppressive calcineurin inhibitors (CNIs).
425 CNIs prevent graft rejection by predominantly inhibiting interleukin-2 (IL-2) production.
426 Interleukin-2 is a critical component in maintaining the regulatory T-cell (Treg)/Th-17 axis,
427 where IL-2 promotes polarization of Tregs and inhibits Th-17 cells. Deficiency of IL-2 in
428 mice caused an imbalance in the Treg/Th-17 axis leading to decreased Tregs and an increase

in Th-17 cells (44). Mice treated with the CNI tacrolimus showed enhanced Th-17 cells and reduced Tregs (45, 46). These studies implicate that CNI treatment of the kidney transplant patients in this study would rather increase IL-17A production and plasma levels. Also, in acute graft rejection episodes, calcineurin inhibition did not prevent IL-17A increases (1, 4). The majority of the patients included in this study received the CNI tacrolimus (79% and 83% in the spironolactone and placebo group, respectively). There were no systematic differences in the use of tacrolimus and cyclosporine between the spironolactone and placebo patient groups.

As to epithelial injury, there was a spironolactone-mediated reduction in urine calbindin and TFF3 by paired testing, which was, however, not significant in the between-group analysis. The epithelial biomarkers calbindin, clusterin, KIM-1, osteoactivin, TFF3, and VEGF have all been recognized to reflect kidney injury and increase in urine in different kidney diseases (47, 48). Calbindin is an extracellular calcium-binding protein which is primarily expressed by distal tubular and collecting ducts cells (49, 50). Calbindin is associated with distal tubular cell injury and is upregulated *in vitro* after exposure to e.g., cisplatin (51, 52). Both preclinical animal experiments and patient data have shown increased urinary calbindin level in acute kidney injury (48, 53, 54). The TFF3 protein is secreted by tubular epithelial cells in the thick ascending limb of Henle's loop and the early portion of the distal tubule of the nephron (55, 56). Patients with chronic kidney disease (CKD) have increased serum and urinary levels of TFF3 compared to healthy controls, and the concentration of both serum and urinary TFF3 correlate with the stage of CKD severity (57-59). In CKD patients, TFF3 expression was in the tubular epithelial cells (57). The distal nephron localization of calbindin and TFF3 is in line with a direct action of the MR antagonist since MR is expressed from the distal convoluted tubules over connecting tubule and including the collecting ducts. In the present study no effect on TFF3 and calbindin were observed between groups, and

there was no relation between cytokines and injury markers in response to spironolactone. In conclusion spironolactone, irrespective of ACEi/ARB pretreatment and increases in plasma K^+ concentration, had no significant anti-inflammatory or reno-protective effect on a range of cytokines and urine markers in patients with a stable kidney transplant after 1 year treatment.

Perspectives and Significance

The present study finds that in stable kidney transplant patients with well-controlled blood pressure, administration of a mineralocorticoid receptor antagonist had no direct effect on T-cell derived cytokines, including IL-17A, or macrophage-derived cytokines in plasma. The progressive minor increases in plasma IL-17A seen in the placebo group and the biological importance of this needs further investigations but could indicate a stabilizing effect of spironolactone in the treatment group. Thus, blocking MR receptors in stable, immunosuppressed kidney transplant patients with an average age of the transplant of ~3 years have insignificant anti-inflammatory effects.

GRANTS

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AUTHOR CONTRIBUTION

H.C.T, C.B. and L.A.M. designed and conducted the SPIREN trial; S.S.T., L.A.M., and B.L.J. conceived the idea and designed the sub-study; S.S.T., B.L.J. and L.A.M. performed the experiments and analyzed the data; S.S.T., B.L.J. and L.A.M. interpreted results and experiments; S.S.T. prepared figures; All authors revised the manuscript and approved for final submission.

DISCLOSURE

The authors have nothing to disclose regarding conflict of interest with respect to this manuscript.

Figure Legends

Figure 1: Effect of spironolactone treatment for 12 months on T-cell cytokines in plasma samples from kidney transplant patients. A and B) Plasma IFN-γ concentrations were unchanged after 1 year intervention with spironolactone (*n*=31) and placebo (*n*=36). **C)** Spironolactone intervention for one year had no effect on plasma IL-17A concentrations (*n*=23). **D)** An increase of IL-17A was observed in the placebo group (*n*=26) ($p<0.05$*, paired t-test). Data are presented as pg/ml where intraindividual cytokine concentrations at baseline and after 1-year intervention are connected by lines. Normally distributed data were analyzed with paired t-test (IL-17A) and non-normally distributed data were analyzed by the Wilcoxon test (IFN-γ). P-values<0.05* were considered statistically significant.

Figure 2: Effect of spironolactone treatment for 12 months on macrophage-derived cytokines in plasma samples from kidney transplant patients.

Plasma concentrations of macrophage-derived cytokines TNF-α (spironolactone: *n*=32, placebo: *n*=37), IL-6 (spironolactone: *n*=27, placebo: *n*=36), IL-1β (spironolactone: *n*=14, placebo: *n*=15) and IL-10 (spironolactone: *n*=34, placebo: *n*=35) in kidney transplant patients before and after 1 year intervention. **A-H)** After 1 year intervention with spironolactone or placebo, no effect was seen in any of the cytokines in plasma. Data are presented as pg/ml where individual cytokine concentrations at baseline and after 1-year intervention are connected by lines. Normally distributed data were analyzed with a paired t-test and non-normally distributed data were analyzed by the Wilcoxon test (IL-6 spironolactone/placebo and IL-10 placebo).

519 **Figure 3: Effect of spironolactone treatment for 12 months on kidney injury markers in**
520 **spot urine samples from kidney transplant patients. A + I)** After 1-year spironolactone
521 intervention Calbindin and TFF3 were significantly decreased in urine samples of kidney
522 transplant patients (P<0.05* by paired t-test). **B-H + J-L)** No significant changes were
523 observed in any of the other kidney injury markers upon spironolactone or placebo
524 intervention. The variation in number of samples was caused by some samples being below
525 measuring range of the assay: Calbindin (spironolactone: *n*=36, placebo: *n*=38), Clusterin
526 (spironolactone: *n*=34, placebo: *n*=36), KIM-1 (spironolactone: *n*=37, placebo: *n*=37),
527 Osteoactivin (spironolactone: *n*=37, placebo: *n*=38), TFF3 (spironolactone: *n*=27, placebo:
528 *n*=21) and VEGF (spironolactone: *n*=37, placebo: *n*=39). Normally distributed data were
529 analyzed with a paired t-test and non-normally distributed data were analyzed by the
530 Wilcoxon test (KIM-1 spironolactone, osteoactivin placebo and TFF3 spironolactone). Data
531 are presented as ng/g creatinine where urinary kidney injury marker ratios at baseline and
532 after 1-year intervention are connected by lines. P-values<0.05* were considered statistically
533 significant.
534
535 **Figure 4: Relation between change in plasma K^+ concentration after spironolactone and**
536 **ΔIL-17A, ΔCalbindin and ΔTFF3 after spironolactone intervention.** No significant
537 correlation between K^+ and plasma IL-17A, urinary calbindin and TFF3 changes after 1-year
538 spironolactone treatment was observed. Data were not normally distributed and therefore
539 Spearman's correlations were carried out.
540

Table 1: Plasma cytokine and plasma K^+ levels

Plasma cytokine concentrations are presented as median values in pg/ml with interquartile range. Plasma K^+ concentrations are presented as mean ± SD in mmol/L. Within-group comparisons were performed by a paired t-test or a Wilcoxon test comparing changes from baseline to 1-year follow up. Between-group comparisons of Δ-values were performed by unpaired t-tests or Mann-Whitney tests comparing the changes from baseline to 1-year follow up between the placebo and spironolactone groups. Between-group comparison of baseline levels were analyzed by an unpaired t-test or a Mann-Whitney test comparing differences between the two groups at baseline. Furthermore, data were analyzed using a two-way ANOVA analysis with mixed effects model, which revealed no significant effect on all analyzed factors. $P<0.05$ was considered significant.

Table 2: Urinary kidney injury marker levels

Urinary kidney injury markers are presented as median values ng/g with interquartile range. Within-group comparisons were performed by a paired t-test or a Wilcoxon test comparing changes from baseline to 1-year follow up. Between-group comparisons of Δ-values were performed by unpaired t-tests or Mann-Whitney tests comparing the changes from baseline to 1-year follow up between the placebo and spironolactone groups. Between-group comparison of baseline levels were analyzed by an unpaired t-test or a Mann-Whitney test comparing differences between the two groups at baseline. Furthermore, data were analyzed using a two-way ANOVA analysis with mixed effects model, which revealed no significant effect on all analyzed factors. $P<0.05$ was considered significant.

564 **Table 3: Results of correlation analyses**

565 Univariate correlations between baseline plasma cytokine levels and MAP, SBP, DBP and
566 plasma aldosterone (p-aldo). Urinary kidney injury markers at baseline were tested for
567 correlation with p-aldosterone at baseline. Pearson's correlation was used for normally
568 distributed data and Spearman's correlation was used for data not normally distributed. Blood
569 pressure (MAP, SBP and DBP) correlated positively to urine TFF3/creatinine ratios.

570

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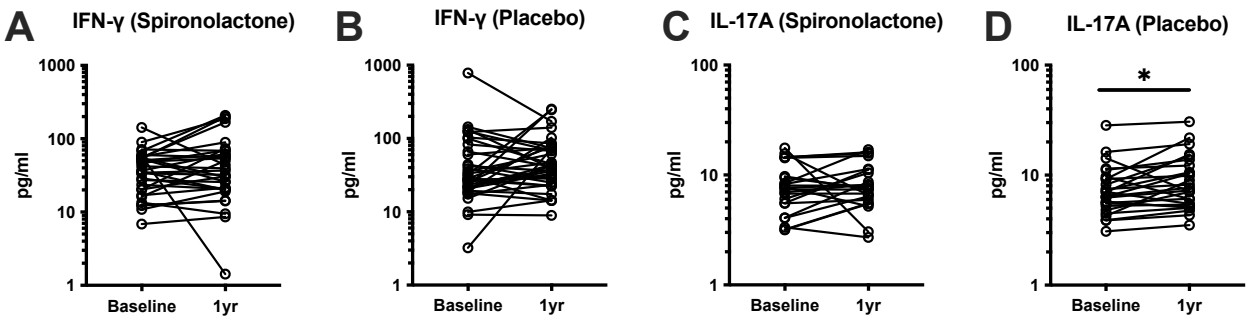
Figure 1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IFN-γ (Placebo)
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Baseline
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C
IL-17A (Spironolactone)
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IL-17A (Placebo)
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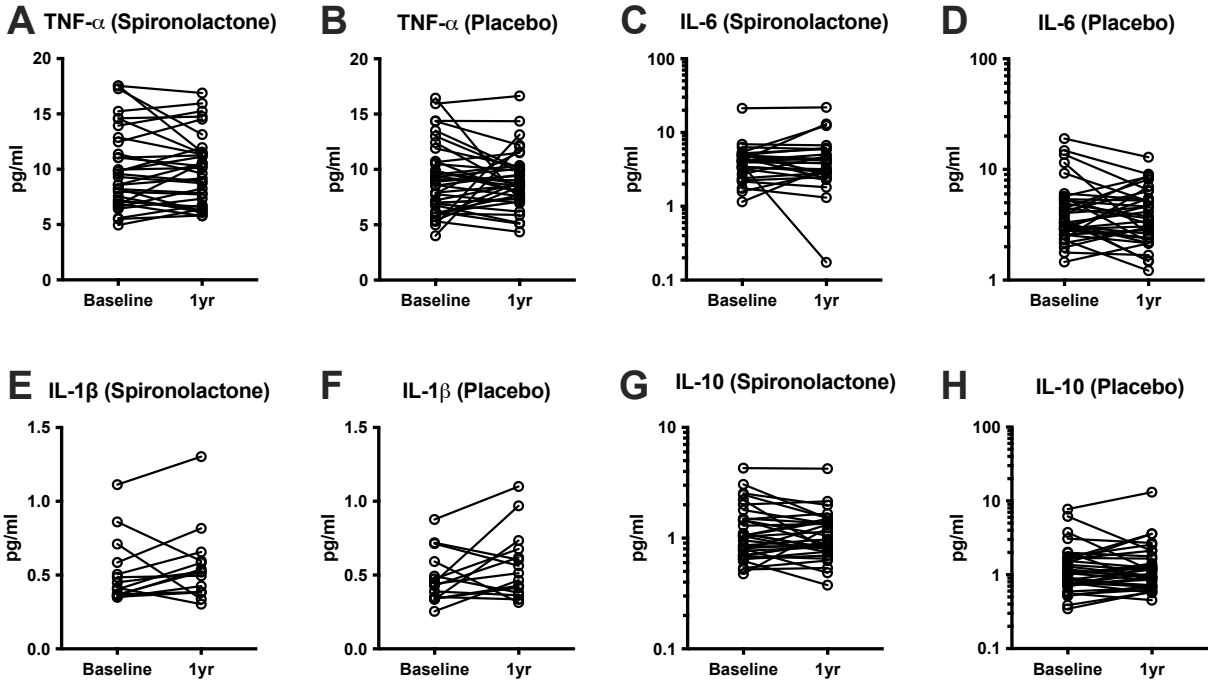
pg/ml
Baseline
1yr

Figure 2


A TNF-α (Spironolactone)
B TNF-α (Placebo)
C IL-6 (Spironolactone)
D IL-6 (Placebo)
E IL-1β (Spironolactone)
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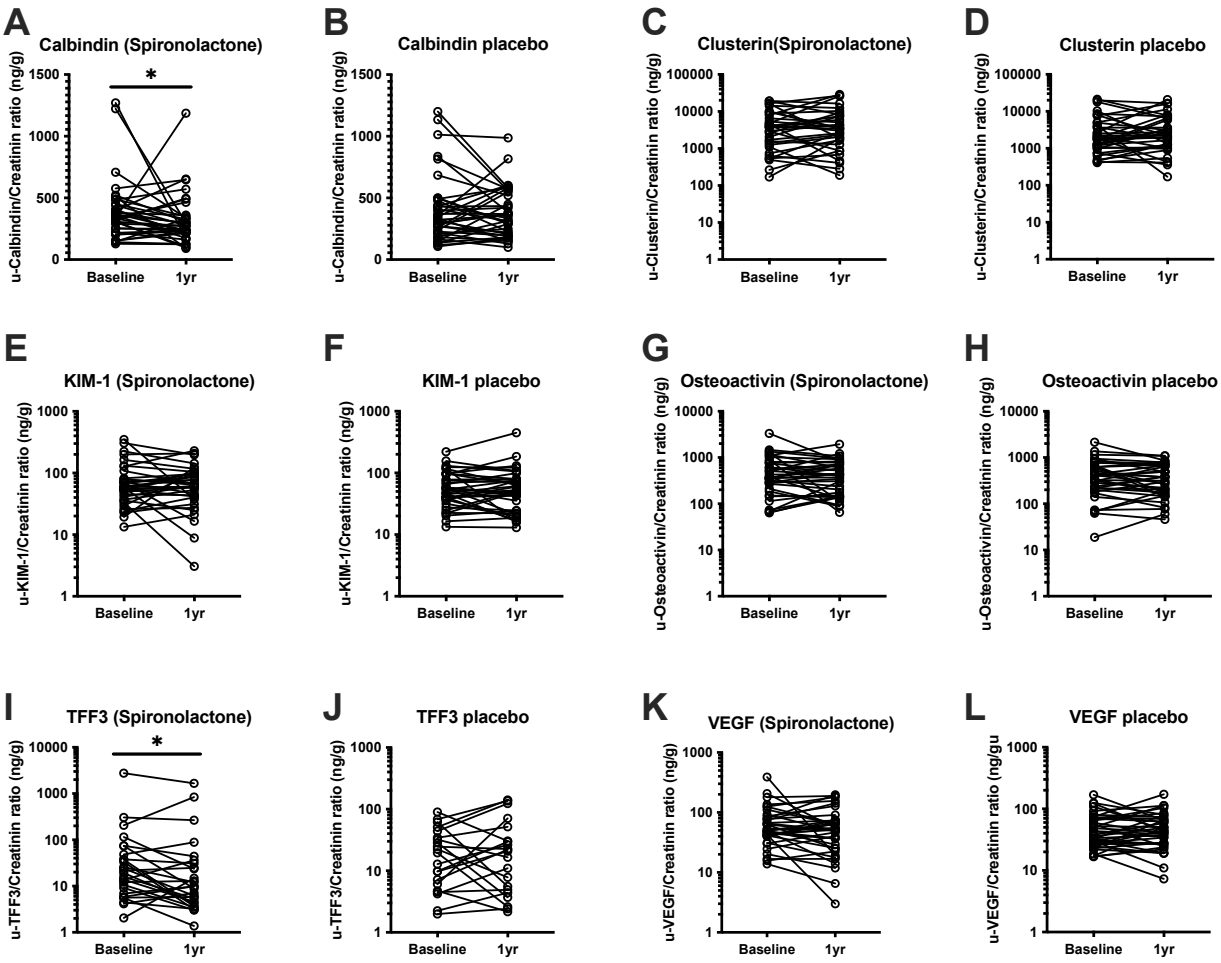
G IL-10 (Spironolactone)
H IL-10 (Placebo)
pg/ml
Baseline
1yr

Figure 3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Calbindin (Spironolactone)
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1yr
B
Calbindin placebo
u-Calbindin/Creatinin ratio (ng/g)
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1000
500
0
Baseline
1yr
C
Clusterin(Spironolactone)
u-Clusterin/Creatinin ratio (ng/g)
100000
10000
1000
100
10
1
Baseline
1yr
D
Clusterin placebo
u-Clusterin/Creatinin ratio (ng/g)
100000
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1000
100
10
1
Baseline
1yr
E
KIM-1 (Spironolactone)
u-KIM-1/Creatinin ratio (ng/g)
1000
100
10
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1yr
F
KIM-1 placebo
u-KIM-1/Creatinin ratio (ng/g)
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u-TFF3/Creatinin ratio (ng/g)
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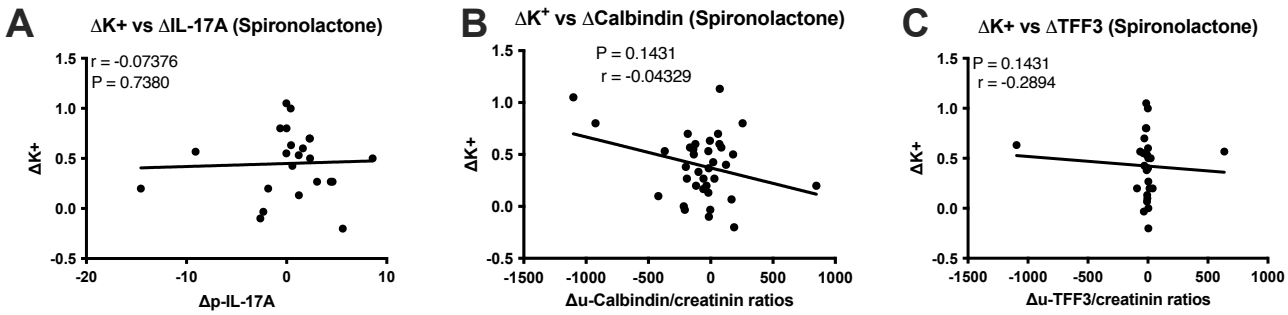
1yr

Figure 4


A
ΔK+ vs ΔIL-17A (Spironolactone)
r = -0.07376
P = 0.7380
ΔK+
Δp-IL-17A
B
ΔK+ vs ΔCalbindin (Spironolactone)
P = 0.1431
r = -0.04329
ΔK+
Δu-Calbindin/creatinin ratios
C
ΔK+ vs ΔTFF3 (Spironolactone)
P = 0.1431
r = -0.2894
ΔK+
Δu-TFF3/creatinin ratios

Tables

Table 1: Plasma cytokine, plasma K^+, and urinary kidney injury marker levels in kidney transplant recipients before (baseline) and after treatment with placebo or spironolactone for 12 months.

Assay	Spironolactone			Placebo			Between-group (baseline) p-value	Between-group (1yr) p-value	Between-group (Δ-values) p-value	Between-group Two-way ANOVA (1 yr) p-value
	Baseline cytokines (pg/ml)	1yr (pg/ml) (mmol/L)	Within-group p-value	Baseline (pg/ml)	1yr (pg/ml)	Within-group p-value				
IFN-γ	36.9 [20.2-55.9] n = 31	36.9 [20.6-68.6] n = 31	0.7	28.5 [21.0-65.0] n = 36	42.2 [25.8-74.7] n = 36	0.6	0.8	0.4	0.6	-
IL-17A	7.6 [5.5-9.7] n = 23	7.6 [5.9-11.2] n = 23	0.7	6.7 [5.1-9.4] n = 26	7.5 [5.4-12.6] n = 26	<0.05*	0.8	0.6	0.4	0.9
TNF-α	9.4 [7.1-12.8] n = 32	9.9 [5.5-9.7] n = 32	0.6	8.6 [6.5-10.7] n = 37	8.7 [7.3-10.2] n = 37	0.8	0.2	0.2	0.8	0.5
IL-6	4.1 [2.7-5.0] n = 27	3.8 [2.8-5.9] n = 27	0.7	3.5 [2.8-5.5] n = 36	3.5 [2.4-5.5] n = 36	0.3	1.0	0.8	0.3	-
IL-1β	0.4 [0.4-0.6] n = 14	0.5 [0.4-0.6] n = 14	0.5	0.5 ± 0.2 n = 15	0.6 ± 0.2 n = 15	0.4	0.2	0.9	0.5	0.9
IL-10	1.0 [0.7-1.5] n = 34	0.9 [0.8-1.4] n = 34	0.3	1.0 [0.8-1.6] n = 35	1.0 [0.7-1.7] n = 35	0.6	0.8	0.6	0.2	-
K+	4.2 ± 0.4	4.5 ± 0.4	<0.0001****	4.2 ± 0.5	4.3 ± 0.4	0.5	0.5	0.002**	<0.0001***	<0.0001***

Table 2: Plasma cytokine, plasma K^+, and urinary kidney injury marker levels in kidney transplant recipients before (baseline) and after treatment with placebo or spironolactone for 12 months.

	Spironolactone			Placebo						
Assays	**Injury markers (ng/g)**	**1yr (ng/g)**	**Within-group p-value**	**Baseline (ng/g)**	**1yr (ng/g)**	**Within-group p-value**	**Between-group (baseline) p-value**	**Between-group (1yr) p-value**	**Between-group (Δ-values) p-value**	**Between-group Two-way ANOVA 1 yr p-value**
Calbindin	340 [253-448] n = 36	273 [214-347] n = 36	<0.05*	329 [216-481] n = 38	347 [203-552] n = 38	0.9	0.9	0.1	0.4	0.3
Clusterin	3760 [1214-8236] n = 34	3391 [1585-7743] n = 34	0.7	1868 [1029-4732] n = 36	2094 [1108-6324] n = 36	0.8	0.3	0.4	1.0	0.5
KIM-1	58.7 [39.3-80.7] n = 37	64.3 [41.0-99.4] n = 37	0.9	53.6 [36.1-95.3] n = 37	51.5 [23.3-77.5] n = 37	0.4	0.3	0.2	0.7	-
Osteoactivin	368 [241-791] n = 37	424 [151-729] n = 37	0.5	402 [213-668] n = 38	344 [167-648] n = 38	0.2	0.6	0.5	0.6	-
TFF3	19.2 [6.9-48.4] n = 27	9.8 [4.3-31.1] n = 27	<0.05*	19.6 [5.4-39.2] n = 21	20.9 [4.7-41.1] n = 21	0.8	0.5	0.9	0.07	-
VEGF	49.7 [40.9-82.7] n = 37	51.2 [25.2-74.2] n = 37	0.1	37.8 [26.6-69.5] n = 39	43.1 [27.5-65.3] n = 39	0.9	0.08	0.8	0.8	0.9

Table 2: Correlations between blood pressure and cytokines; between aldosterone and cytokines; and aldosterone and epithelial injury markers in kidney transplant recipients before randomization to placebo or spironolactone.

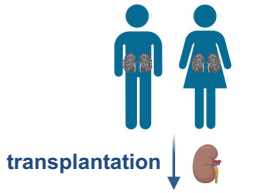
	Baseline levels (spironolactone and placebo)			
Parameters	**r-values**	**P-values**	**Test**	**Paired samples (n)**
MAP vs IFN-γ	-0.1	0.4	Pearson	59
MAP vs IL-17A	-0.1	0.4	Pearson	49
MAP vs TNF-α	-0.05	0.7	Pearson	62
MAP vs IL-6	0.03	0.8	Spearman	61
MAP vs IL-1β	0.1	0.4	Pearson	41
MAP vs IL-10	0.01	0.9	Spearman	62
MAP vs p-aldo	0.1	0.2	Spearman	67
SBP vs IFN-γ	-0.1	0.3	Pearson	59
SBP vs IL-17A	-0.2	0.2	Pearson	49
SBP vs TNF-α	-0.1	0.5	Pearson	62
SBP vs IL-6	0.1	0.5	Spearman	59
SBP vs IL-1β	0.1	0.5	Pearson	41
SBP vs IL-10	-0.07	0.6	Spearman	62
SBP vs p-aldo	67	0.8	Pearson	67
DBP vs IFN-γ	-0.08	0.5	Pearson	61
DBP vs IL-17A	-0.06	0.7	Pearson	50
DBP vs IL-1β	0.1	0.4	Pearson	41
DBP vs TNF-α	-0.01	0.9	Pearson	64
DBP vs IL-10	0.06	0.7	Spearman	64
DBP vs IL-6	-0.01	0.9	Spearman	61
DBP vs p-aldo	0.2	0.2	Pearson	69
p-aldo vs IFN-γ	-0.2	0.2	Pearson	71
p-aldo vs IL-17A	0.04	0.8	Pearson	58
p-aldo vs TNF-α	-0.09	0.5	Pearson	74
p-aldo vs IL-6	0.02	0.9	Spearman	71
p-aldo vs IL-1β	-0.1	0.5	Pearson	45
p-aldo vs IL-10	0.1	0.2	Spearman	73
MAP vs u-calbindin/crea ratio	0.06	0.7	Pearson	64
MAP vs u-clusterin/crea ratio	0.2	0.2	Pearson	60
MAP vs u-KIM-1/crea ratio	0.003	1.0	Pearson	63
MAP vs u-Osteoactivin/crea ratio	0.002	1.0	Pearson	63
MAP vs u-TFF3/crea ratio	0.4	0.005**	Pearson	47
MAP vs u-VEGF/crea ratio	0.1	0.4	Pearson	63
SBP vs u-calbindin/crea ratio	0.07	0.7	Pearson	64
SBP vs u-clusterin/crea ratio	0.2	0.2	Pearson	60
SBP vs u-KIM-1/crea ratio	0.02	0.9	Pearson	63
SBP vs u-Osteoactivin/crea ratio	0.09	0.5	Pearson	63
SBP vs u-TFF3/crea ratio	0.4	0.01**	Pearson	47
SBP vs u-VEGF/crea ratio	0.2	0.2	Pearson	63
DBP vs u-calbindin/crea ratio	0.002	1.0	Pearson	66
DBP vs u-clusterin/crea ratio	0.1	0.3	Pearson	62
DBP vs u-KIM-1/crea ratio	0.01	0.9	Pearson	65

DBP vs u-Osteoactivin/crea ratio	-0.05	0.7	Pearson	65
DBP vs u-TFF3/crea ratio	0.4	0.008**	Pearson	49
DBP vs u-VEGF/crea ratio	0.03	0.8	Pearson	65
P-aldo vs u-calbindin/crea ratio	-0.03	0.8	Spearman	77
P-aldo vs u-clusterin/crea ratio	0.2	0.1	Spearman	72
P-aldo vs u-KIM-1/crea ratio	0.07	0.5	Spearman	76
P-aldo vs u-Osteoactivin/crea ratio	0.03	0.8	Spearman	76
P-aldo vs u-TFF3/crea ratio	0.1	0.5	Spearman	55
P-aldo vs u-VEGF/crea ratio	-0.08	0.5	Spearman	76
P-IL-17A vs u-calbindin/crea ratio	0.08	0.6	Pearson	47
P-IL-17A vs u-TFF3/crea ratio	0.2	0.2	Pearson	33

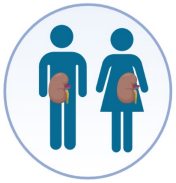
Spironolactone has no direct effect on plasma IL-17A, but lowers injury markers in urine from kidney transplant recipients

METHODS

Randomized double blind study, *THE SPIREN TRIAL*
Patiens with a kidney allograft



transplantation



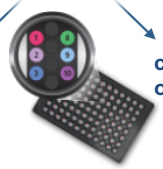
1 yr intervention with:
Spironolactone (n = 39)
Placebo (n = 41)

Before and after 1 year treatment

Plasma

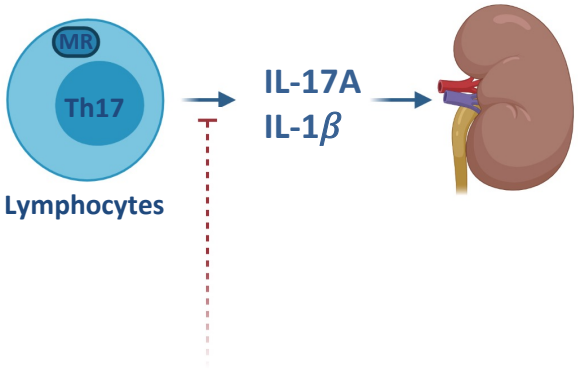
Urine

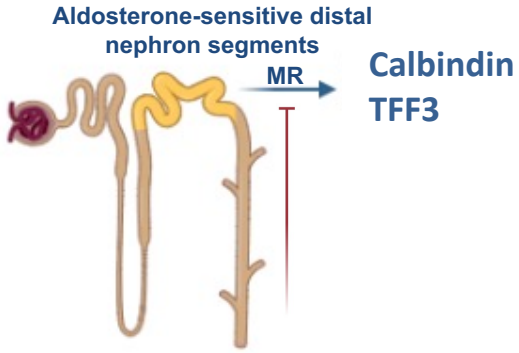
IFN-γ, IL-17A, TNF-α, IL-6, IL-1β and IL-10



calbindin, clusterin, osteoactivin, KIM-1, TFF3 and VEGF

OUTCOME





SUMMARY

Treatment of stable kidney transplant recipients with spironolactone had no direct effect on plasma cytokines. A small but significant increase of IL-17A and IL-1β in placebo-treated patients was observed. Spironolactone treatment suppressed distal nephron-epithelial injury markers calbindin and TFF3 in urine with no effect in placebo-treated patients.

CONCLUSION

In immunosuppressed, stable, kidney transplant patients, spironolactone has no direct anti-inflammatory effect on 6 selected cytokines. Spironolactone protects distal nephron segments.