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Published in: American Journal of Physiology: Renal Physiology

DOI: 10.1152/ajprenal.00025.2019

Publication date: 2019

Document version
Accepted manuscript

Citation for published version (APA):

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Download date: 03. Feb. 2021
EFFECT OF SPIRONOLACTONE FOR ONE YEAR ON ENDOTHELIAL FUNCTION AND VASCULAR INFLAMMATION BIOMARKERS IN RENAL TRANSPLANT RECIPIENTS

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Study type: Randomized clinical trial

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Running title: Effect of spironolactone on endothelial dysfunction

Key words: Mineralocorticoid receptor; nitric oxide; hypertension; aldosterone; nitrate
Authors Contributions

LM, HT and CB are the local investigators/sponsor. BLJ and JS supervised analyses of inflammation and aldosterone performed by LM. MC performed analyses of nitrite, nitrate and cGMP. AC and CW performed analyses of amino acids. YP and EB developed the assay for and analyzed tPA:Ag, PAI-1:Ag and vWF.

Study design and hypotheses were developed by LM, HT, CB and BLJ. LM drafted the manuscript. All co-authors contributed by critically revising and approving the final version of the manuscript.

Abbreviations

Abstract

Background: Kidney transplantation is associated with increased cardiovascular risk. Endothelial dysfunction and vascular inflammation contribute to negative outcome. In experimental models, mineralocorticoid receptor antagonists (MRA) improved endothelial function and reduced inflammation. The current study tested the hypothesis that the MRA spironolactone improves endothelial function and reduces vascular inflammation in renal transplant patients.

Methods: 80 prevalent renal transplant patients from an ongoing, double-blind randomized placebo-controlled trial were included. Paired plasma samples before and after one year of treatment (n=39 in spironolactone group, n=41 in placebo group) were used to determine markers of endothelial dysfunction (nitrite, nitrate, cyclic guanosine monophosphate, arginine, citrulline, ornithine, asymmetric dimethylarginine, symmetric dimethylarginine, NG-monomethyl-L-arginine, von Willebrand Factor, tissue-type plasminogen activator antigen, plasminogen activator inhibitor 1 antigen), and markers of inflammation (intercellular adhesion molecule, vascular adhesion molecule, high sensitivity C-reactive protein and serum amyloid protein A).

Results: The median time since the transplantation was 4.6 (0.12-22.3) years in the spironolactone group and 2.1 (0.17-13.9) years in the placebo group (p>0.05). Spironolactone increased plasma aldosterone (p<0.001) and potassium (p<0.001). Blood pressure did not change significantly. No significant differences were detected between groups in any of the measured markers of endothelial dysfunction or inflammation except in the subgroup analysis of diabetes patients, where spironolactone decreased nitrite compared to placebo.

Conclusions: In this study, MR antagonism did not improve biomarkers of endothelial dysfunction or vascular inflammation in prevalent renal transplant patients. Further studies
are needed to evaluate the potential beneficial effect of early or late MR antagonism on vascular outcomes in renal transplant patients.
Introduction

End stage renal disease is associated with a high risk of cardiovascular disease (CVD) (56). Transplantation improves outcome, but renal transplant patients remain at an elevated risk of CVD (50). Endothelial dysfunction (ED), defined as impaired endothelium-dependent vasodilatation (54), is an important predictor of CVD (33). ED can be evaluated non-invasively by flow-mediated dilatation (FMD) (10). In renal failure progressing towards end-stage renal disease, FMD gradually deteriorates (55). Renal transplantation improves FMD within the first month (32) and biomarkers of ED within 3 months (28). The beneficial effect of renal transplantation on ED is maintained up to 24 months (30). Nevertheless, FMD remains significantly impaired in renal transplant patients compared with healthy controls (42) serving as an indicator of persistent cardiovascular risk.

ED in renal transplant patients is aggravated by immunosuppressive regimens including the calcineurin inhibitors (CNI) cyclosporine or tacrolimus. Both cyclosporine (41) and tacrolimus (53) reduce endothelial nitric oxide synthase (eNOS) activity and hence the production of the potent vasodilator nitric oxide (NO) \textit{in vitro}. Oxidative stress with increased superoxide anion production related to cyclosporine treatment also decreases NO bioavailability (14). \textit{In vivo}, ED is aggravated by CNI side effects including hypertension (24) and diabetes mellitus (11). In accordance, renal transplant patients treated with cyclosporine had reduced basal and stimulated endothelial NO production compared with renal transplant patients on azathioprine and healthy controls (37).

\textit{In vitro} and animal studies showed that aldosterone contributes directly to cyclosporine-induced vascular changes (18), vascular inflammation (7) and ED (17). Aldosterone acting on the mineralocorticoid receptor (MR) stimulates the formation of reactive oxygen species, which leads to increased production of pro-inflammatory transcription factors. \textit{In vitro},
aldosterone reduces NO synthesis by uncoupling eNOS (39). MR-antagonism with
spironolactone increases NO-release in vitro (15) and alleviates ED in vivo in hemodialysis
patients (19, 35).

Endothelial activation refers to the recruitment of inflammatory cells to the vessel wall
through expression of adhesion molecules (34) and is tightly associated with ED. Aldosterone
can induce inflammation through a direct effect on inflammatory cells (7) and by promoting
the vascular expression of adhesion molecules and the release of endothelium-derived
substances associated with ED such as von Willebrand Factor (vWF) and plasminogen
activator inhibitor (PAI-1) (7). vWF (21), PAI-1 (45) and markers of inflammation (45, 46)
are associated with adverse cardiovascular outcome in the general population. Markers of
both systemic and vascular inflammation are increased in renal transplant patients when
compared to healthy controls (12). Furthermore, systemic inflammation is associated with
adverse outcome in this population (1).

Thus, MR-antagonism may have a therapeutic vascular effect. This has never been tested in
renal transplant patients. The present sub-study from a randomized, double-blind clinical trial
tested the hypotheses that MR-antagonism with spironolactone for one year attenuates
general ED and vascular inflammation in renal transplant patients receiving CNI as
maintenance immunosuppression.

Materials and Methods

Participants

The study included a subgroup of participants in the ongoing double-blind, randomized
clinical trial the SPIREN trial. The full study protocol has previously been published (38). In
brief, 170 kidney transplant patients at any point after the transplantation were randomized to
spironolactone 25-50 mg daily or placebo for three years. Inclusion criteria were: Age >18
years, treatment with a CNI, proteinuria<3 g/day, creatinine clearance ≥ 30 mL/min, plasma-
potassium < 5.5 mmol/L and for women of childbearing potential, a negative pregnancy test
at inclusion and adequate contraception throughout the study. Exclusion criteria were
intolerance to spironolactone, current treatment with potassium-binding resins or digoxin,
pregnancy, expectation of non-compliance and clinically relevant organic or psychological
disorders. Compliance to the study drug was evaluated by tablet counts at each study visit.
Doses of 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 the local immunosuppressive
protocol. The morning dose of CNI was ingested prior to the study visit. At yearly visits,
patients were evaluated by chrome-EDTA clearance, ambulatory blood pressure
measurements, electrocardiography, plasma and urine samples. The current sub-study
analyzed data from baseline and after 1 year of treatment with spironolactone or placebo in
the first 80 patients to complete one year of participation. At the time of the current analysis,
all 80 patients had completed the full study participation of 3 years or had withdrawn from
the study, thus the blinding of the original trial was not compromised. All analyses were
performed blinded to the allocation.

The SPIREN trial was approved by the Research Ethics Committee of Southern Denmark on
the 24th of August 2011 (project ID: s-20110095, protocol version 2 (07/28/2011)). Oral and
written informed consent to participation was obtained from all study participants by study
personnel prior to any study related procedures.


Ambulatory blood pressure
Ambulatory blood pressure measurements were performed at yearly visits using the equipment available at the local center (Diasys Integra II, Novacor, United Kingdom/TM2430, A&D, Japan). Measurements containing at least 20 daytime values and seven nighttime values were considered valid. Blood pressure results are reported as mean blood pressure during the 24 hour period of measurement. Doses of concurrent antihypertensive medication were adjusted according to clinical indication and independently of the study protocol to maintain blood pressure within the recommended range. Due to technical error and reluctance among some subjects to participate in the measurements, only 57 patients completed valid blood pressure measurements at baseline and follow-up.

Plasma samples

Non-fasting EDTA-blood was obtained after 30 minutes of seated rest between 10 and 12 AM. Samples were kept on ice and centrifuged within 30 minutes at 4°C, 1,500 g for 15 minutes. Plasma aliquots were frozen at -80°C pending analyses.

Analyses

Plasma samples were analyzed for electrolytes, hemoglobin A1C (HbA1C), cholesterols and triglycerides and 24-hour urine samples were analyzed for electrolytes at the Department of Clinical Biochemistry of Odense University Hospital using standard equipment.

cGMP: Plasma analyses for cyclic guanosine mono phosphate (cGMP) were performed using the cGMP enzyme immunoassay Biotrak System (GE Healthcare, Fairfield, CT, USA). Analyses were performed according to the manufacturers’ instructions with the addition of 2 µL of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (750 mM) to prevent degradation of cGMP. The assay was read on a spectrophotometer (SpectraMax Plus) at 450 nm.
Nitrite and nitrate: Plasma was analyzed using high performance liquid chromatography (HPLC) in a system dedicated to measuring nitrite and nitrate (ENO-20, EiCom, Kyoto, Japan) using an auto-sampler (840, EiCom, Kyoto, Japan). The system separates nitrate by reverse-phase/ion exchange chromatography followed by the reduction of nitrate to nitrite using cadmium and reduced copper. Reduced nitrate was subsequently diazotized by the Griess reagent, and the level of diazo compounds was measured at 540 nm. 100 µL plasma samples were deproteinized using 100 µL ice-cold HPLC grade methanol (Chromasolv Solvent, Sigma-Aldrich), vortexed and centrifuged for 10 minutes (4°C, 10,000 g). 100 µL supernatant were transferred to a 96-well plate with conical wells (Costar, nitrate- and nitrite-free), sealed with an adhesive film and kept at 4°C in the autosampler. 10 µl of background control, standard or samples were injected. The needle was automatically flushed between each injection by the autosampler. A standard curve was prepared from sodium nitrite and sodium nitrate diluted with carrier solution. The concentration in the samples was measured as the area under the curve relative to the slope of the standard curve.

Amino acids: Aliquots of 25 µL per sample were thawed at 4°C. For protein precipitation, 250 µL of crash solution containing $^{15}$N$_4$-Arginine in 0.2% formic acid in isopropanol were added to each sample. Samples were then vortexed for 30 seconds and allowed to equilibrate with the internal standard for five minutes. Next, samples were centrifuged at 10,000 g for 15 minutes. Finally, 150 µL of supernatant were transferred to an LC-MS 700 µL insert and allocated in a 96-well autosampler plate. Arginine, ornithine, citrulline, NG-monomethyl-L-arginine (MNMA), symmetric dimethyl arginine (SDMA) and asymmetric dimethyl arginine (ADMA) were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS). Analyses were performed on an Acquity UPLC system coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, Massachusetts, USA) operating the positive mode as previously described(23).
Concentrations of vWF were determined by an in-house ELISA using rabbit anti-human vWF polyclonal IgG as capture and detecting antibodies (DAKO, Glostrup, Denmark, Ref. Nr. A0082).

Concentrations of tPA:Ag were determined by an in-house ELISA using mouse anti-human tPA monoclonal IgG as capture (clone 15-4-21) and detection (clone 15-4-6) antibodies. In brief, MaxiSorp ELISA plates (NUNC, Roskilde, Denmark) were coated with 2 μg/mL monoclonal anti–tPA antibody 15-4-21 in PBS overnight at 4°C. Plates were washed three times in PBS + 0.05% Tween-20 and the samples diluted in PBS + 20mM EDTA + 0.05% Tween-20, were applied to plates in duplicate and incubated for 2 hours at room temperature. The detection antibody (biotinylated anti–tPA monoclonal antibody 15-4-6) was applied at 2 μg/mL and incubated for 1 hour at room temperature. Secondary detection was performed with HRP-conjugated streptavidin (GE Healthcare; catalog no. RPN1051) diluted to 1/3000 and developed with o-phenylene-diamine (Kem-En-Tec, Taastrup, Denmark)/H₂O₂. The reaction was stopped by adding 1 M sulphuric acid. Plates were read at an optical density of 490 nm. Serial dilutions of plasma pool with known tPA were included for generation of the standard curve.

PAI:Ag levels were measured by an in-house ELISA as previously described (22). Samples were analyzed in duplicate, and the inter-assay coefficient of variation (CV) was 6% for vWF, 5% for tPA:Ag, and 5% for PAI:Ag.

Vascular inflammation: Markers of vascular (sVCAM-1 and sICAM-1) and general (high sensitivity C-reactive protein (hsCRP) and serum amyloid protein A (SAA)) inflammation were determined using a commercially available ELISA kit (Vascular Injury Panel 2, Meso Scale Discovery, Rockville, Maryland, USA) and read on Meso QuickPlex SQ120 reader (Meso Scale Discovery). All procedures were performed according to the manufacturers’
instructions. Paired samples were analyzed in duplicate on the same assay plate. Inter-assay CV was 6% for sICAM-1, 6% for sVCAM-1, 3% for hsCRP and 14% for SAA.

Aldosterone: Plasma aldosterone was determined using a commercially available ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturers’ instructions. Samples were analyzed in duplicate with paired plasma samples on the same plate. Inter-assay CV was 9%.

Statistics

The power calculation was based on plasma nitrite. Assuming a standard deviation of 15 nmol/L analogous to previous studies (31), the current sample size would be able to detect a difference of 35 nmol/L between the groups with a power>80% and a significance level of 5%. In comparison, plasma nitrite levels decreased by 10-90 nmol/L for each additional CV risk factor present in a previous study (31). All analyses were performed using STATA 15.1 software (STATACorp, College Station, TX, USA). For numerical data, normality was evaluated using histograms and QQ-plots. When appropriate, data were log-transformed to obtain normality. Unless specifically stated, all comparisons presented are between-group comparisons performed using a two-sample t-test for normally distributed data or a Mann-Whitney U test comparing differences from baseline to follow-up for all variables. For all variables the follow-up:baseline-ratio was also compared between the groups, but this did not change the results (not presented). Within-group comparisons were performed by paired t-test after evaluating the assumptions of having the same distribution by “y-against-x” plots and Bland-Altman plots or by Wilcoxon signed rank test. Categorical variables were evaluated using chi-squared tests or Fishers exact test for cell counts <5.

Two subgroup analyses were performed: i) diabetes/no diabetes and ii) renin-angiotensin-system (RAS) antagonism/no RAS antagonism at baseline. Unless stated, the subgroup results were equal to those obtained in the main analyses. Data are described using mean(SD)
or median (range) for numerical data and frequencies for categorical data. Figures were prepared in GraphPad Prism software, version 5 for Mac (GraphPad, San Diego, CA, USA).

Results

Baseline data are listed in table 1. Of 80 patients included, 39 received spironolactone and 41 received placebo treatment. All subjects received immunosuppression with a CNI (tacrolimus or cyclosporine) and an antimetabolite (mycophenolate or azathioprine). Additionally, 14% of participants received prednisolone. The two groups were comparable at baseline regarding demographics, dialysis vintage, comorbidity and renal function. 94% of participants received antihypertensive therapy and 26% of participants had diabetes at inclusion. Although not significant, there was a tendency towards a higher number of previous rejections (24% vs. 10%) and more immunologically complex transplantations (defined as AB0 incompatibility or the presence of donor specific antibodies at the time of transplantation) (24% vs. 13%) in the placebo group. Accordingly, more subjects in the placebo group received prednisolone (22% vs. 5%, p<0.05).

There were no significant differences regarding plasma electrolyte concentrations, cholesterols, triglycerides, HbA1C, hsCRP or aldosterone at baseline. Likewise, baseline levels of arginine, nitrite, nitrate, vWF, PAI:Ag and tPA:Ag were comparable between groups (not shown). Donor specific antibodies were not measured. There were no incidences of acute rejection. 29 participants (36%) had renal biopsies performed at baseline and none of these had signs of chronic transplant glomerulopathy. Three patients in each group had one or more incidences of low-grade viremia (Epstein Barr virus, cytomegalovirus or BK-virus <1000 copies/mL) during the first year and additionally two patients in the placebo group were treated with valganciclovir due to cytomegalovirus viremia. Two incidences of cardiovascular events occurred during the first year of treatment. One patient needed
thrombendarterectomy of the left femoral artery (placebo group) and one patient had a thrombosed aneurism in the right brachial artery, which was treated surgically (spironolactone group).

Effect of spironolactone on aldosterone and electrolyte concentrations
Spironolactone treatment significantly increased plasma aldosterone (p<0.001) and plasma potassium (p<0.001) concentrations (figure 1). Four patients (one in the placebo group and three in the spironolactone group) experienced transient hyperkalemia above 5.5 mmol/L. In two of the patients in the spironolactone group, this resulted in a reduction of project medication dosage to 25 mg/day. In the third spironolactone patient, hyperkalemia coincided with hyperglycemia. He was kept on 50 mg/day and had stable plasma-potassium levels below 5.5 mmol/L with a few intermittent peaks above 5.8 mmol/L. The urinary sodium/potassium-ratio did not differ significantly between the groups (table 2).

Effect of spironolactone on markers of NO metabolism and endothelial dysfunction
Changes from baseline to follow-up in plasma levels of nitrite, nitrate, cGMP, arginine, citrulline, ornithine and the citrulline/arginine and ornithine/arginine ratios did not differ between the groups (figure 2). Spironolactone did not significantly impact plasma levels of the endogenous eNOS inhibitors ADMA, SDMA and MNMA (figure 3). Also, the markers of endothelial dysfunction PAI:Ag, tPA:Ag and vWF remained stable (figure 4).

Effect of spironolactone on markers of vascular inflammation
Soluble markers of vascular inflammation, sICAM-1 and sVCAM-1, remained stable from baseline to follow-up despite spironolactone treatment (figure 5). Similarly, the markers of general inflammation, hsCRP and SAA were unaltered (figure 5).
Effect of spironolactone on blood pressure and body weight

57 patients had valid ambulatory blood pressure measurements at both baseline and follow-up (30 in placebo group, 27 in spironolactone group). Systolic blood pressure was significantly lower in the placebo group at baseline (table 2). Systolic and diastolic blood pressures, mean arterial pressure (MAP) and body weight remained stable in the spironolactone group (table 2). Within the placebo group there was a significant 7 mmHg (SD 13) increase in systolic blood pressure from baseline to follow-up (p=0.01), which was paralleled by an increase in body weight of 0.9 kg (SD 2.7) (p=0.03). Both of these changes were not significant by between-group analysis. Diastolic blood pressure and MAP remained stable within and between groups (table 2). The observed increase in systolic blood pressure in the placebo group was due to an increase during daytime. Nighttime systolic and diastolic blood pressures were unaltered within and between groups (not shown). Office blood pressures were available in all subjects at baseline and although systolic blood pressure was lower in the placebo group (137 (SD 14) vs. 142 (SD 16) mmHg, p=0.10), this difference was not significant. Office diastolic blood pressure was comparable between the two groups at baseline (82 (SD 9) vs. 83 (SD 8) mmHg, p=0.47). The number of antihypertensive drugs used did not change significantly within the groups and did not differ between groups.

Results of the subgroup analyses

In the subgroup analysis in patients with diabetes (n=21), plasma nitrite concentrations were significantly lower in the spironolactone group at follow-up (p=0.04) and cGMP was reduced at follow-up within the spironolactone group (table 3). All other components of the NO pathway, endothelial dysfunction markers and vascular inflammation markers were not affected by spironolactone. Likewise, HbA1C levels and hsCRP levels were not significantly
different between the groups (table 3). Baseline levels of nitrite, hsCRP and aldosterone did not differ between diabetics and non-diabetics (not shown).

The results of the subgroup analyses in non-diabetics and in patients treated with or without RAS-inhibition at baseline were similar to those obtained in the main analyses.

Discussion

The present study tested the hypothesis that MR-antagonism by spironolactone increased markers of endothelial NO synthesis and reduced markers associated with endothelial dysfunction (vWF, PAI:Ag, tPA:Ag) and vascular inflammation (sICAM-1, sVCAM-1) in renal transplant patients. While spironolactone increased plasma aldosterone and potassium concentrations, it did not affect measured NO-products, upstream (arginine) or downstream (citrulline, cGMP) molecules in the NO pathway, markers of ED (vWF, PAI:Ag and tPA:Ag) or general- (hsCRP and SAA) and vascular inflammation markers (sVCAM-1 and sICAM-1).

Based on the observed increase in plasma aldosterone and potassium, spironolactone reached therapeutic levels to block MR. It has been hypothesized that aldosterone can induce vasoconstriction MR-independently (6), however, the excellent long term outcomes found with MR-antagonism in congestive heart failure (43, 44, 58) indicate that a potentially harmful effect of increased aldosterone levels was superseded by the benefits of preventing MR-mediated effects.

Baseline levels of nitrite and nitrate were comparable between groups and stable from baseline to follow-up. This was corroborated by unaltered levels of eNOS substrate arginine and eNOS product citrulline and citrulline/arginine-ratio providing an estimation of eNOS activity. Also in line with these observations was the unchanged level of cGMP. By contrast, Syngle et al described a decrease in plasma nitrite without changes in blood pressure after spironolactone in patients with active rheumatoid arthritis (51) and ankylosing spondylitis.
and normal renal function. These subjects were characterized by active inflammation at inclusion and exhibited markedly higher baseline and follow-up nitrite levels (up to 7.9 µmol/L) than the present patient group (range 0.1-1.7 µmol/L). It is likely that these high levels of nitrite were secondary to inducible nitric oxide synthase (iNOS) stimulated during inflammation (51).

Surprisingly, the subgroup analysis of diabetic patients also found a decrease in nitrite in the spironolactone group compared to the placebo group. This was supported by decreased cGMP within the spironolactone group. These subjects did not display signs of inflammation thus, there was no evidence to support increased iNOS activity. HbA1C was unaltered by spironolactone. The purpose of the subgroup analysis in diabetics was to investigate whether the higher CV risk in this group would result in a greater effect of spironolactone on the vascular markers, which was not the case. A previous intervention trial in type 2 diabetics found that spironolactone aggravated ED, partly through worsened glycemic control (13), which was also previously described (57). The impact of spironolactone on ED and glycemic control in diabetic patients needs further investigation since the present study was not powered to examine diabetic patients specifically.

Functional measures of NO bioavailability in vivo include forearm blood flow (FBF) and the non-invasive equivalent FMD (10). Spironolactone increases FBF in congestive heart failure (16) and FMD in hemodialysis patients (19, 35), in patients with active rheumatoid arthritis (51) and ankylosing spondylitis (52), but not in subjects with metabolic syndrome (27, 29) or in obese people (20, 26). These somewhat contradictory results can relate to the degree of ED at baseline. Indeed, the beneficial effects of MR-antagonists on mortality were demonstrated in groups of patients with an already established cardiovascular burden, hence a suspected high degree of ED (43, 44, 58). Of note, in the present study blood pressure in the spironolactone group was stable. Part of previously found beneficial effects of MR-
antagonists could be related to the antihypertensive action (19, 35), which is difficult to
discriminate from a potential direct action on vascular inflammation and endothelial function.

A limitation to the current study is the absence of functional measures of ED at baseline or
follow-up. Several previous studies have found increased ED in renal transplant patients
compared to healthy controls in the early (14 days) (32) and late (41±9 months) (42) phase
after the transplantation. Furthermore, Kensinger et al. concluded that ED in renal transplant
patients did not change from 1 to 24 months after the transplantation (30). Based on these
studies, it seems reasonable to assume a degree of ED at baseline in the current study.

In the present study there was no effect of spironolactone on plasma levels of the endogenous
eNOS inhibitors ADMA, SDMA or MNMA. ADMA and SDMA may have direct adverse
cardiovascular effects beyond eNOS inhibition and both compounds are correlated to
mortality and cardiovascular events in high- and medium-risk populations (49) through
increased vascular inflammation (48). ADMA and SDMA have also been associated with
worsening renal function (49). In renal transplant patients, ADMA was correlated to
mortality, cardiovascular morbidity and impaired graft function (2). The present data suggest
that there is no direct action of aldosterone-MR on this pathway.

No difference in PAI:Ag was detected between the two groups (figure 5). In a previous study,
derogenous aldosterone correlated positively with PAI:Ag in humans (8), whereas
spironolactone prevented the increase in PAI:Ag despite increased aldosterone levels (47). In
the current study, we found no such association.

The measured markers of inflammation need to be interpreted in light of concurrent treatment
with CNI and prednisolone, where the latter was more prevalent in the placebo group. We
found no effect of spironolactone on markers of vascular and general inflammation between
groups or within the spironolactone group. A previous post-hoc analysis from four pooled
randomized controlled trials including 69 diabetic patients with varying degrees of albuminuria likewise found no effect of spironolactone on circulating markers of vascular and systemic inflammation (40). Although previous studies indicated a correlation between plasma aldosterone and levels of ICAM-1 (36), the present data suggest that MR antagonism has little influence on vascular inflammation *in vivo*.

Hyperkalemia is a well-known side-effect of MR-antagonism aggravated by reduced renal function and concurrent treatment with CNI. In the current study, spironolactone treatment did not cause serious hyperkalemia. We conclude that spironolactone is safe for renal transplant patients with regard to hyperkalemia. Previously, a safety study similarly found the MR-antagonist eplerenone safe to use in renal transplant patients with a glomerular filtration rate above 30 mL/min (3).

The subgroup analyses of patients with and without RAS-inhibition at baseline was performed to investigate whether concurrent RAS-inhibition masked a potentially beneficial effect of MR-antagonism, however, this was not the case in the current study.

*Limitations and methodological considerations*

The double blind, randomized design and number of subjects minimize selection bias and theoretically distribute potential unknown confounders evenly between groups. However, the outcomes of this sub-study were defined *post-hoc*, hence there were no measures taken to ensure a consistent and comparable lifestyle (physical activity and dietary habits) from baseline to follow-up. Components of the NO pathway are sensitive to dietary intake of arginine (5), nitrite and especially nitrate (25), which is found in high levels in certain vegetables and is known to lower blood pressure (9).
The hypotheses of this study were based on the assumption that the included subjects had a degree of ED at baseline as previously found (32, 42), however as previously mentioned, no functional measures were performed to confirm this. Baseline nitrite values were within normal range (31). Absence of ED at baseline could account for the negative results.

Subjects were included at any time after the transplantation, thus there was a large variation in the time since transplantation and a tendency towards older grafts in the spironolactone group. Although this difference was not statistically significant, one could speculate if such a difference would impact the degree and reversibility of ED in the spironolactone group at baseline. It is feasible that later vascular changes are less reversible, which may have limited a potentially beneficial effect in the spironolactone group.

Evaluation of renal function was outside the scope of the current study. Plasma concentrations of some biomarkers may depend on renal function and an effect of spironolactone on renal function may thus have confounded the results. The absence of marked changes within the spironolactone group speaks against such an effect.

Concurrent medication was adjusted according to clinical indication, and such adjustments might introduce important confounders for the results. Although there was no reduction in the number of antihypertensive drugs in the spironolactone group, we cannot exclude the possibility that dosages have been changed. Particularly, adjustments of drugs targeting the renin-angiotensin-aldosterone system may have confounded the results.

We conclude that aldosterone-MR has little, if any, direct effect on components of NO metabolism, markers of ED or markers of vascular inflammation in vivo in prevalent renal transplant patients. The beneficial vascular effects on ED found in other studies are possibly enhanced through antihypertensive effects. The results from this study, however, do not rule out an implication of the MR in the vascular dysfunction seen in these patients nor a
beneficial effect of spironolactone on long-term cardiovascular and renal outcomes in renal transplant patients.
Acknowledgements

The authors wish to thank laboratory technicians Annika Olsson and Carina Nihlen for their help performing analyses of nitrite, nitrate and cGMP, laboratory technician Gitte Kitlen for help analyzing aldosterone, technicians Anette Larsen and Kathrine Overgaard for analyzing vWF, tPA:Ag and PAI:Ag, and clinical staff Liselotte Buus Sommer and Birgitte Broholm for their continuous and invaluable help during recruiting and follow-up of study subjects. Professor Paul M. Vanhoutte is thanked for his valuable contributions regarding points of discussion.

Funding

The SPIREN trial is an investigator initiated trial. Takeda Pharma supplies the trial medication including placebo tablets free of charge, but is otherwise not involved in collecting or analyzing data. Funding has been obtained by grants from the following independent public and private funds: Odense University Hospital Free Research Fund, the Region of Southern Denmark Research- and PhD-funds, the Danish Kidney Association Research Fund, Helen and Einar Bjørnows Fund, Danish Society of Nephrology Travel Fund, King Christian X Fund, the Danish Medical Association Research Fund, the Medicine Fund of the Danish Regions and Odense University Hospital Board of Consultants Research Fund. The analyses of amino acids, nitrate, nitrite and cGMP were funded by the Novo Nordisk foundation (grant number TrIC NNF15CC0018486), Swedish Heart and Lung Foundation (ID: 20170124) and the Swedish Research Council (ID: 2016-01381).

The funding bodies had no influence on the design of the study, the collection, analysis and interpretation of data or in writing the manuscript.

Disclosures
The authors declare no conflicts of interest.
References


Table 1. Baseline characteristics of included patients

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<th>Spironolactone (n=39)</th>
<th>Placebo (n=41)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex, n</td>
<td>30 (77%)</td>
<td>25 (61%)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56 (33-74)</td>
<td>56 (23-72)</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>27.2 (SD 4.1)</td>
<td>27.0 (SD 3.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>5/16/18</td>
<td>5/20/16</td>
<td>NS</td>
</tr>
<tr>
<td>(current/previous/never)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous dialysis, n</td>
<td>34 (87%)</td>
<td>34 (83%)</td>
<td>NS</td>
</tr>
<tr>
<td>Time since transplantation (years)</td>
<td>4.6 (0.12-22.3)</td>
<td>2.1 (0.17-13.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous rejections, n</td>
<td>4 (10%)</td>
<td>10 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>Immunological high risk a, n</td>
<td>5 (13%)</td>
<td>10 (24%)</td>
<td>NS</td>
</tr>
<tr>
<td>Comorbidity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension b, n</td>
<td>37 (95%)</td>
<td>38 (93%)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>10 (26%)</td>
<td>11 (27%)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous cerebral ischemia, n</td>
<td>7 (18%)</td>
<td>3 (7%)</td>
<td>NS</td>
</tr>
<tr>
<td>Previous MI, n</td>
<td>4 (10%)</td>
<td>5 (12%)</td>
<td>NS</td>
</tr>
<tr>
<td>Heart failure (EF&lt;45%), n</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Ischemic heart disease c, n</td>
<td>4 (10%)</td>
<td>6 (15%)</td>
<td>NS</td>
</tr>
<tr>
<td>Medication</td>
<td>n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>------------</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Antihypertensive:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ACE-inhibitor, n</em></td>
<td>17 (44%)</td>
<td>12 (29%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Angiotensin receptor</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blocker, n</em></td>
<td>6 (15%)</td>
<td>7 (17%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Calcium channel</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blocker, n</em></td>
<td>20 (51%)</td>
<td>25 (61%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>β-blocker, n</em></td>
<td>19 (49%)</td>
<td>23 (56%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>α-blocker, n</em></td>
<td>5 (13%)</td>
<td>5 (12%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Loop diuretic, n</em></td>
<td>7 (18%)</td>
<td>7 (17%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Thiazide diuretic, n</em></td>
<td>1 (3%)</td>
<td>1 (2%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Statins, n</em></td>
<td>5 (15%)</td>
<td>5 (12%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Immunosuppressive:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tacrolimus, n</em></td>
<td>31 (79%)</td>
<td>34 (83%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Cyclosporine, n</em></td>
<td>8 (21%)</td>
<td>7 (17%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Mycophenolate, n</em></td>
<td>36 (92%)</td>
<td>40 (98%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Azathioprine, n</em></td>
<td>3 (8%)</td>
<td>1 (2%)</td>
<td>NS</td>
</tr>
<tr>
<td><em>Prednisolone, n</em></td>
<td>2 (5%)</td>
<td>9 (22%)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td><strong>Spironolactone dosage,</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>n</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg/day</td>
<td>15 (38%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg/day</td>
<td>24 (62%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Paraclinical values</strong></td>
<td>Tacrolimus trough levels (µg/L)</td>
<td>Cyclosporine 2 hour levels (µg/L)</td>
<td>Chrome-EDTA clearance (mL/min/1.73 m²)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>---------------------------------</td>
<td>----------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>4.9 (2.0-11.7)</td>
<td>5.3 (2.3-15.2)</td>
<td>NS</td>
<td>47 (SD 17)</td>
</tr>
<tr>
<td>502 (382-738)</td>
<td>462 (328-756)</td>
<td>NS</td>
<td>48 (SD 13)</td>
</tr>
</tbody>
</table>

Data are presented as frequencies (n), mean (SD) or median (range)

ACE: Angiotensin converting enzyme, BMI: Body mass index, MI: Myocardial infarction, EF: Ejection Fraction, HDL: High density lipoprotein, LDL: Low density lipoprotein, HbA1C: hemoglobin A1C, hsCRP: High sensitivity C-reactive protein, NS: Not significant

*aImmunological high risk is defined as AB0 incompatible or the presence of donor specific antibodies at the time of transplantation

*bHypertension is defined as treatment with antihypertensive medication at inclusion
Ischemic heart disease is defined as previous revascularization or coronary arteriography/heart CT/myocardial scintigraphy indicative of coronary atherosclerosis
Table 2. Effect of spironolactone on urinary sodium/potassium ratio, bodyweight and ambulatory blood pressure

<table>
<thead>
<tr>
<th></th>
<th>Spironolactone group (n=39)</th>
<th>Placebo group (n=41)</th>
<th>Between group comparison of Δ values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline 1 year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>u-Na^+/K^+ ratio</td>
<td>3.1 (SD 1.6)</td>
<td>3.0 (SD 1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>84.3 (SD 14.1)</td>
<td>81.0 (SD 14.3)</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>138 (SD 11)</td>
<td>131 (SD 13)</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>83 (SD 7)</td>
<td>80 (SD 6)</td>
<td>NS</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>102 (SD 8)</td>
<td>97 (SD 7)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline 1 year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>u-Na^+/K^+ ratio</td>
<td>1.6)</td>
<td>1.5)</td>
<td></td>
</tr>
<tr>
<td>Bodyweight (kg)</td>
<td>14.1 (SD 16.0)</td>
<td>14.3</td>
<td>14.5§</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>139 (SD 13)</td>
<td>131 (SD 13)</td>
<td>11)§</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>82 (SD 8)</td>
<td>80 (SD 6)</td>
<td>80 (SD 6)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>101 (SD 9)</td>
<td>97 (SD 7)</td>
<td>99 (SD 7)</td>
</tr>
</tbody>
</table>

Data are reported as mean (SD) or median (range). Between group comparisons were performed by unpaired t-tests comparing the changes from baseline to follow-up between groups (for u-protein by Mann-Whitney U test). Within group comparisons were performed by paired t-tests comparing baseline to follow-up within groups (for u-protein Wilcoxon signed rank test).

u-Na^+/K^+ ratio: Urinary sodium/potassium ratio, SBP: Ambulatory systolic blood pressure, DBP: Ambulatory diastolic blood pressure, MAP: Mean arterial pressure
*p<0.05 for between group comparison of baseline values

$\text{p}<0.05$ for within group comparison in placebo group
Table 3. Selected results from diabetic subgroup analyses

<table>
<thead>
<tr>
<th></th>
<th>Spironolactone (n=10)</th>
<th>Placebo (n=11)</th>
<th>Between group comparison of Δ values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>1 year</td>
<td>Within group comparison</td>
</tr>
<tr>
<td>p-nitrite (µmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 (0.08- 0.69)</td>
<td>0.23 (0.09- 0.59)</td>
<td>p=0.09</td>
<td>0.29 (0.16- 0.69)</td>
</tr>
<tr>
<td>p=0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-cGMP (nmol/L)</td>
<td>32.9 (16.1- 69.3)</td>
<td>28.6 (15.0- 62.4)</td>
<td>p=0.03</td>
</tr>
<tr>
<td>p=0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-HbA1C (mmol/mol)</td>
<td>49 (31-77)</td>
<td>50 (34-74)</td>
<td>p=0.37</td>
</tr>
<tr>
<td>p=0.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-hsCRP (mg/L)</td>
<td>2.8 (0.5-6.5)</td>
<td>1.5 (0.1- 10.1)</td>
<td>p=0.58</td>
</tr>
<tr>
<td>p=0.94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are reported as median (range). Between group comparisons were performed by unpaired t-tests comparing the changes from baseline to follow-up between groups (nitrite, HbA1C) or Mann-Whitney U test (cGMP, hsCRP). Within group comparisons were performed by paired t-test (HbA1C) or Wilcoxon signed rank test (nitrite, cGMP, hsCRP).

cGMP: Cyclic guanosine monophosphate, HbA1C: Hemoglobin A1C, hsCRP: High sensitivity C reactive protein
Figure legends:

**Figure 1.** Plasma levels of aldosterone and potassium. Median and interquartile range are indicated. Differences from baseline to follow-up were compared by two-sample t-tests with unequal (aldosterone) and equal (potassium) variances. *: p<0.001

**Figure 2.** Plasma levels of components of nitric oxide metabolism. Median and interquartile range are indicated. Differences from baseline to follow-up were compared by two-sample t-tests with equal variances (arginine, citrulline) or by Mann Whitney U test (nitrite, nitrate, cGMP, ornithine).

cGMP: Cyclic guanosine monophosphate

**Figure 3.** Plasma levels of the endogenous eNOS inhibitors. Median and interquartile range are indicated. Differences from baseline to follow-up were compared by two-sample t-tests with equal variances.

ADMA: Asymmetric dimethylarginine, SDMA: Symmetric dimethylarginine, MNMA: NG-monomethyl-L-arginine

**Figure 4.** Plasma levels of activated endothelial cell markers. Median and interquartile range are indicated. Differences from baseline to follow-up were compared by Mann Whitney U test (PAI:Ag, vWF) or two sample t-test with equal variances (tPA:Ag).

vWF: von Willebrand factor, PAI:Ag: Plasminogen activator inhibitor type 1 antigen, tPA:Ag: Tissuetype plasminogen activator antigen
Figure 5. Plasma levels of inflammatory markers. Median and interquartile range are indicated. Differences from baseline to follow-up were compared by Mann Whitney U tests.

Y-axis have been log-transformed in CRP and SAA figures to allow for outliers.

sICAM: Soluble intercellular adhesion molecule 1, sVCAM: Soluble vascular adhesion molecule 1, CRP: C-reactive protein, SAA: Serum amyloid protein A