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RESEARCH ARTICLE

Effect of spironolactone for 1 yr on endothelial function and vascular inflammation biomarkers in renal transplant recipients

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Mortensen LA, Bistrup C, Stubbe J, Carlström M, Checa A, Wheelock CE, Palarasah Y, Bladbjerg EM, Thiesson HC, Jensen BL. Effect of spironolactone for 1 yr on endothelial function and vascular inflammation biomarkers in renal transplant recipients. *Am J Physiol Renal Physiol* 317: F529–F539, 2019. First published June 5, 2019; doi:10.1152/ajprenal.00025.2019.—Kidney transplantation is associated with increased cardiovascular risk. Endothelial dysfunction and vascular inflammation contribute to negative outcome. In experimental models, mineralocorticoid receptor antagonists improved endothelial function and reduced inflammation. The present study tested the hypothesis that the mineralocorticoid receptor antagonist spironolactone improves endothelial function and reduces vascular inflammation in renal transplant patients. Eighty prevalent renal transplant patients from an ongoing, double-blind randomized placebo-controlled trial were included. Paired plasma samples before and after 1 yr of treatment ($n = 39$ in the spironolactone-treated group and 41 in the placebo-treated group) were used to determine markers of endothelial dysfunction (nitrite, nitrate, cGMP, arginine, citrulline, ornithine, asymmetric dimethylarginine, symmetric dimethylarginine, N^G-monomethyl-L-arginine, von Willebrand factor, tissue-type plasminogen activator antigen, and 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). The median time since the transplantation was 4.6 (0.12–22.3) yr in the spironolactone-treated group and 2.1 (0.17–13.9) yr in the placebo-treated group ($P > 0.05$). Spironolactone increased plasma aldosterone ($P < 0.001$) and K^+ ($P < 0.001$). 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 patients with diabetes, where spironolactone decreased nitrite compared with placebo. In this study, mineralocorticoid receptor 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 mineralocorticoid receptor antagonism on vascular outcomes in renal transplant patients.

aldosterone; hypertension; mineralocorticoid receptor; nitrate; nitric oxide

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 noninvasively by flow-mediated dilatation (FMD) (10). In renal failure progressing toward end-stage renal disease, FMD gradually deteriorates (55). Renal transplantation improves FMD within the first month (32) and biomarkers of ED within 3 mo (28). The beneficial effect of renal transplantation on ED is maintained up to 24 mo (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 (NO) synthase (eNOS) activity and hence the production of the potent vasodilator NO in vitro. Oxidative stress with increased superoxide anion production related to cyclosporine treatment also decreases NO bioavailability (14). 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).

In vitro and animal studies have shown 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 proinflammatory transcription factors. In vitro, aldosterone reduces NO synthesis by uncoupling eNOS (39). MR antagonism with spironolactone increases NO release in

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vitro (15) and alleviates ED in vivo in patients on hemodialysis (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 release of endothelium-derived substances associated with ED such as von Willebrand factor (vWF) and plasminogen activator inhibitor (PAI-1) (7). wWF (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 compared with 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 substudy from a randomized, double-blind clinical trial tested the hypotheses that MR antagonism with spironolactone for 1 yr attenuates general ED and vascular inflammation in renal transplant patients receiving CNI as maintenance immunosuppression.

MATERIALS AND METHODS

Participants

The present study included a subgroup of participants in an ongoing double-blind, randomized clinical trial (SPIREN trial). The full study protocol has previously been published (38). In brief, 170 kidney transplant patients at any point after transplantation were randomized to spironolactone (25–50 mg daily) or placebo for 3 yr. Inclusion criteria were as follows: age $>$ 18 yr, treatment with a CNI, proteinuria $<$ 3 g/day, creatinine clearance $\geq$ 30 ml/min, plasma $K^+ < 5.5$ 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 K^+-binding resins or digoxin, pregnancy, expectation of noncompliance, 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-h level of 600 μg/l according to the local immunosuppressive protocol. The morning dose of CNI was ingested before the study visit. At yearly visits, patients were evaluated by chrome-EDTA clearance, ambulatory blood pressure measurements, electrocardiography, and plasma and urine samples. The present substudy analyzed data from baseline and after 1 yr of treatment with spironolactone or placebo in the first 80 patients to complete 1 yr of participation. At the time of the present analysis, all 80 patients had completed the full study participation of 3 yr 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 August 24, 2011 [project ID: s-20110095, protocol version 2 (07/28/2011)]. Oral and written informed consent to participation were obtained from all study participants by study personnel before any study-related procedures.

The trial is registered at ClinicalTrials.gov (5/17/2012; NCT01602861) and EudraCT (5/31/2011; 2011-002243-98).

Ambulatory Blood Pressure

Ambulatory blood pressure measurements were performed at yearly visits using the equipment available at the local center (Diasys Integra II, Novacor, UK; and TM2430, A&D, Japan). Measurements containing at least 20 daytime values and 7 nighttime values were considered valid. Blood pressure results are reported as mean blood pressure during the 24-h 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. Because of technical error and reluctance among some patients to participate in the measurements, only 57 patients completed valid blood pressure measurements at baseline and followup.

Plasma Samples

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

Analyses

Plasma samples were analyzed for electrolytes, hemoglobin A_1C (HbA_1C), cholesterol, and triglycerides, and 24-h urine samples were analyzed for electrolytes at the Department of Clinical Biochemistry of Odense University Hospital using standard equipment.

cGMP. Plasma analyses for cGMP were performed using the cGMP enzyme immunoassay Biotrak system (GE Healthcare, Fairfield, CT). Analyses were performed according to the manufacturers' instructions with the addition of 2 μl of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (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 HPLC in a system dedicated to measuring nitrite and nitrate (ENO-20, EiCom, Kyoto, Japan) using an autosampler (no. 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 Griess reagent, and the level of diazo compounds was measured at 540 nm. Plasma samples (100 μl) were deproteinized using 100 μl ice-cold HPLC-grade methanol (Chromasolv Solvent, Sigma-Aldrich), vortexed, and centrifuged for 10 min (4°C, 10,000 *g*). The supernatant (100 μl) was transferred to a 96-well plate with conical wells (Costar, nitrate and nitrite free), sealed with adhesive film, and kept at 4°C in the autosampler. Background control (10 μl), 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/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 was added to each sample. Samples were then vortexed for 30 s and allowed to equilibrate with the internal standard for 5 min. Next, samples were centrifuged at 10,000 *g* for 15 min. Finally, 150 μl supernatant was transferred to a LC-MS 700-μl insert and allocated in a 96-well autosampler plate. Arginine, ornithine, citrulline, N^G-monomethyl-L-arginine, symmetric dimethyl arginine (SDMA), and asymmetric dimethyl arginine (ADMA) were measured using LC-MS/MS. Analyses were performed on an Acquity UPLC system coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA) operating the positive mode as previously described (23).

vWF, tissue-type plasminogen activator antigen, and PAI-1 antigen. Concentrations of vWF were determined by an in-house ELISA using rabbit anti-human vWF polyclonal IgG as capture and detecting antibodies (catalog no. A-0082, DAKO, Glostrup, Denmark).

Concentrations of tissue-type plasminogen activator antigen (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 (clone 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 + 20 mM EDTA + 0.05% Tween 20 were applied to plates in duplicate and incubated for 2 h at room temperature. The detection antibody (biotinylated anti-tPA monoclonal antibody 15-4-6) was applied at 2 μg/ml and incubated for 1 h at room temperature. Secondary detection was performed with horseradish peroxidase-conjugated streptavidin (catalog no. RPN-1051, GE Healthcare), diluted to 1/3,000, and developed with *O*-phenylene-diamine (Kem-En-Tec, Taastrup, Denmark)/H_2O_2. 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 the generation of the standard curve.

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

Vascular inflammation. Markers of vascular [soluble VCAM-1 (sVCAM-1) and soluble ICAM-1 (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, MD) and read on a 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. The interassay coefficient of variation 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 manufacturer's instructions. Samples were analyzed in duplicate with paired plasma samples on the same plate. The interassay coeffient of variation was 9%.

Statistics

The power calculation was based on plasma nitrite. Assuming a SD of 15 nmol/l analogous to a previous study (31), the present sample size would be able to detect a difference of 35 nmol/l between 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 coefficient of variation risk factor present in a previous study (31). All analyses were performed using STATA 15.1 software (STATACorp, College Station, TX). For numeric 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 followup for all variables. For all variables, the followup-to-baseline ratio was also compared between the groups, but this did not change the results (not presented). Within-group comparisons were performed by a paired *t*-test after evaluating the assumptions of having the same distribution by "*y*-against-*x*" plots and Bland-Altman plots or by a Wilcoxon signed-rank test. Categorical variables were evaluated using χ^2-tests or Fishers exact test for cell counts of $<$5.

Two subgroup analyses were performed: *1*) diabetes/no diabetes and *2*) 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 means (SD) or medians (ranges) for numeric data and frequencies for categorical data. Figures were prepared in GraphPad Prism software (version 5 for Mac, GraphPad, San Diego, CA).

RESULTS

Baseline data are shown in Table 1. Of the 80 patients included, 39 patients received spironolactone treatment and 41 patients received placebo treatment. All patients 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 toward 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-treated group. Accordingly, more patients in the placebo-treated group received prednisolone (22% vs. 5%, $P < 0.05$).

There were no significant differences regarding plasma electrolyte concentrations, cholesterol, triglycerides, HbA_1C, 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. Twenty-nine 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 $<$ 1,000 copies/ml) during the first year, and, additionally, two patients in the placebo-treated group were treated with valganciclovir because of cytomegalovirus viremia. Two incidences of cardiovascular events occurred during the first year of treatment. One patient needed thrombendarterectomy of the left femoral artery (placebo-treated group), and one patient had a thrombosed aneurism in the right brachial artery, which was treated surgically (spironolactone-treated group).

Effect of Spironolactone on Aldosterone and Electrolyte Concentrations

Spironolactone treatment significantly increased plasma aldosterone ($P < 0.001$) and plasma K^+ ($P < 0.001$) concentrations (Fig. 1). Four patients (one patient in the placebo-treated group and three patients in the spironolactone-treated group) experienced transient hyperkalemia above 5.5 mmol/l. In two of the patients in the spironolactone-treated group, this resulted in a reduction of project medication dosage to 25 mg/day. In the third spironolactone-treated patient, hyperkalemia coincided with hyperglycemia. This patient was kept on 50 mg/day and had stable plasma K^+ levels below 5.5 mmol/l with a few intermittent peaks above 5.8 mmol/l. The urinary Na^+-to-K^+ ratio did not differ significantly between the groups (Table 2).

Effect of Spironolactone on Markers of NO Metabolism and ED

Changes from baseline to followup in plasma levels of nitrite, nitrate, cGMP, arginine, citrulline, ornithine, and citrulline-to-arginine and ornithine-to-arginine ratios did not differ between the groups (Fig. 2). Spironolactone did not significantly impact plasma levels of the endogenous eNOS inhibi-

Table 1. *Baseline characteristics of the included patients*

	Spironolactone-Treated Group	Placebo-Treated Group	*P* Value
n	39	41	
Men/women, *n* (%)	30/9 (77/23)	25/16 (61/39)	NS
Age, yr	56 (33–74)	56 (23–72)	NS
Body mass index	27.2 (SD 4.1)	27.0 (SD 3.9)	NS
Smoking (current/previous/never), *n*	5/16/18	5/20/16	NS
Previous dialysis, *n* (%)	34 (87)	34 (83)	NS
Time since transplantation, yr	4.6 (0.12–22.3)	2.1 (0.17–13.9)	NS
Previous rejections, *n* (%)	4 (10)	10 (24)	NS
Immunological high risk*, *n* (%)	5 (13)	10 (24)	NS
	Comorbidity		
Hypertension†, *n* (%)	37 (95)	38 (93)	NS
Diabetes, *n* (%)	10 (26)	11 (27)	NS
Previous cerebral ischemia, *n* (%)	7 (18)	3 (7)	NS
Previous myocardial infarction, *n* (%)	4 (10)	5 (12)	NS
Heart failure (ejection fraction < 45%), *n* (%)	1 (3)	0 (0)	NS
Ischemic heart disease‡, *n* (%)	4 (10)	6 (15)	NS
	Medication		
Antihypertensive			
Angiotensin-converting enzyme inhibitor, *n* (%)	17 (44)	12 (29)	NS
Angiotensin receptor blocker, *n* (%)	6 (15)	7 (17)	NS
Ca^{2+} channel blocker, *n* (%)	20 (51)	25 (61)	NS
β-Blocker, *n* (%)	19 (49)	23 (56)	NS
α-Blocker, *n* (%)	5 (13)	5 (12)	NS
Loop diuretic, *n* (%)	7 (18)	7 (17)	NS
Thiazide diuretic, *n* (%)	1 (3)	1 (2)	NS
Statins, *n* (%)	5 (15)	5 (12)	NS
Immunosuppressive			
Tacrolimus, *n* (%)	31 (79)	34 (83)	NS
Cyclosporine, *n* (%)	8 (21)	7 (17)	NS
Mycophenolate, *n* (%)	36 (92)	40 (98)	NS
Azathioprine, *n* (%)	3 (8)	1 (2)	NS
Prednisolone, *n* (%)	2 (5)	9 (22)	<0.05
Spironolactone (25 mg/day), *n* (%)	15 (38)		
Spironolactone (50 mg/day), *n* (%)	24 (62)		
	Paraclinical values		
Tacrolimus trough levels, μg/l	4.9 (2.0–11.7)	5.3 (2.3–15.2)	NS
Cyclosporine (2 h) levels, μg/l	502 (382–738)	462 (328–756)	NS
Chrome-EDTA clearance, $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$	47 (SD 17)	48 (SD 13)	NS
Plasma K^+, mmol/l	4.2 (SD 0.4)	4.2 (SD 0.5)	NS
Plasma cholesterol, mmol/l	5.0 (SD 1.0)	5.2 (SD 0.9)	NS
Plasma HDL, mmol/l	1.2 (0.8–2.4)	1.2 (0.6–1.9)	NS
Plasma LDL, mmol/l	3.0 (SD 0.8)	3.2 (SD 0.7)	NS
Plasma triglycerides, mmol/l	1.4 (SD 1.7)	1.7 (SD 1.6)	NS
Plasma hemoglobin A_1C, mmol/mol	36 (23–77)	39 (28–73)	NS
Plasma high-sensitivity C-reactive protein, mg/l	0.8 (SD 1.0)	1.2 (SD 1.3)	NS
Plasma aldosterone, pg/ml	70 (SD 23)	66 (SD 18)	NS

Data are presented as number of patients (*n*), means (SD), or medians (ranges). NS, not significant. *Immunological high risk was defined as AB0 incompatible or the presence of donor-specific antibodies at the time of transplantation. †Hypertension was defined as treatment with antihypertensive medication at inclusion. ‡Ischemic heart disease was defined as previous revascularization or coronary arteriography/heart computer tomography/myocardial scintigraphy indicative of coronary atherosclerosis.

tors ADMA, SDMA, and N^G-monomethyl-L-arginine (Fig. 3). Also, markers of ED (PAI:Ag, tPA:Ag, and vWF) remained stable (Fig. 4).

Effect of Spironolactone on Markers of Vascular Inflammation

Soluble markers of vascular inflammation (sICAM-1 and sVCAM-1) remained stable from baseline to followup despite spironolactone treatment (Fig. 5). Similarly, markers of general inflammation (hsCRP and SAA) were unaltered (Fig. 5).

Effect of Spironolactone on Blood Pressure and Body Weight

Fifty-seven patients had valid ambulatory blood pressure measurements at both baseline and followup (30 patients in the placebo-treated group and 27 patients in the spironolactone-treated group). Systolic blood pressure was significantly lower in the placebo-treated group at baseline (Table 2). Systolic and diastolic blood pressures, mean arterial pressure, and body weight remained stable in the spironolactone-treated group (Table 2). Within the placebo-treated group, there was a significant 7 (SD 13) mmHg increase in systolic blood pressure

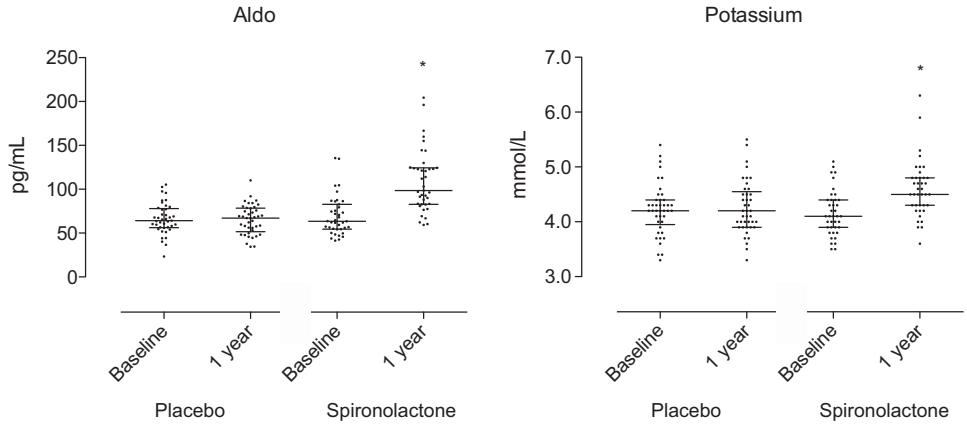

Fig. 1. Plasma levels of aldosterone (Aldo) and K^+. Medians and interquartile ranges are shown. Differences from baseline to followup were compared by two-sample *t*-tests with unequal (aldosterone) and equal (K^+) variances. *$P < 0.001$.

from baseline to followup ($P = 0.01$), which was paralleled by an increase in body weight of 0.9 (SD 2.7) kg ($P = 0.03$). Both of these changes were not significant by between-group analysis. Diastolic blood pressure and mean arterial pressure remained stable within and between groups (Table 2). The observed increase in systolic blood pressure in the placebo-treated 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 participants at baseline, and, although systolic blood pressure was lower in the placebo-treated 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-treated group at followup ($P = 0.04$) and cGMP was reduced at followup within the spironolactone-treated group (Table 3). All other components of the NO pathway, ED markers, and vascular inflammation markers were not affected by spironolactone. Likewise, HbA_1C levels and hsCRP levels were not significantly different between the groups (Table 3). Baseline levels of nitrite, hsCRP, and aldosterone did not differ between those with and without diabetes (not shown).

The results of the subgroup analyses in those without diabetes 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 ED (vWF, PAI:Ag, and tPA:Ag) and vascular inflammation (sICAM-1 and sVCAM-1) in renal transplant patients. Although spironolactone increased plasma aldosterone and K^+ concentrations, it did not affect measured NO products, upstream (arginine) or downstream (citrulline and 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 K^+, spironolactone reached therapeutic levels to block MR. It has been hypothesized that aldosterone can induce vasoconstriction independently of the MR (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 followup. This was corroborated by unaltered levels of the eNOS substrate arginine and the eNOS product citrulline as well as the citrulline-to-arginine ratio providing an estimation of eNOS activity. Also in line with these observations was the unchanged level of

Table 2. *Effect of spironolactone on the urinary Na^+-to-K^+ ratio, body weight, and ambulatory blood pressure*

	Spironolactone-Treated Group		Placebo-Treated Group		
	Baseline	1 yr	Baseline	1 yr	Between-Group Comparison of Δ Values
Urinary Na^+-to-K^+ ratio	3.1 (SD 1.6)	3.0 (SD 1.6)	3.0 (SD 1.5)	2.7 (SD 1.4)	NS
Body weight, kg	84.3 (SD 14.1)	84.3 (SD 16.0)	81.0 (SD 14.3)	81.9 (SD 14.5)†	NS
Systolic blood pressure, mmHg	138 (SD 11)	139 (SD 13)	131 (SD 13)*	138 (SD 11)†	NS
Diastolic blood pressure, mmHg	83 (SD 7)	82 (SD 8)	80 (SD 6)	80 (SD 6)	NS
Mean arterial pressure, mmHg	102 (SD 8)	101 (SD 9)	97 (SD 7)*	99 (SD 7)	NS

Data are reported as mean (SD). For urinary Na^+-to-K^+ ratio and body weight, $n = 39$ patients in the spironolactone-treated group and 41 patients in the placebo-treated group; for systolic, diastolic, and mean arterial pressures, $n = 27$ patients in the spironolactone-treated group and 30 patients in the placebo-treated group. Between-group comparisons were performed by an unpaired *t*-tests comparing the changes (Δ) from baseline to followup between groups. Within-group comparisons were performed by paired *t*-tests comparing baseline to followup values within groups. *$P < 0.05$ for between-group comparison of baseline values; †$P < 0.05$ for within-group comparison in the placebo-treated group.

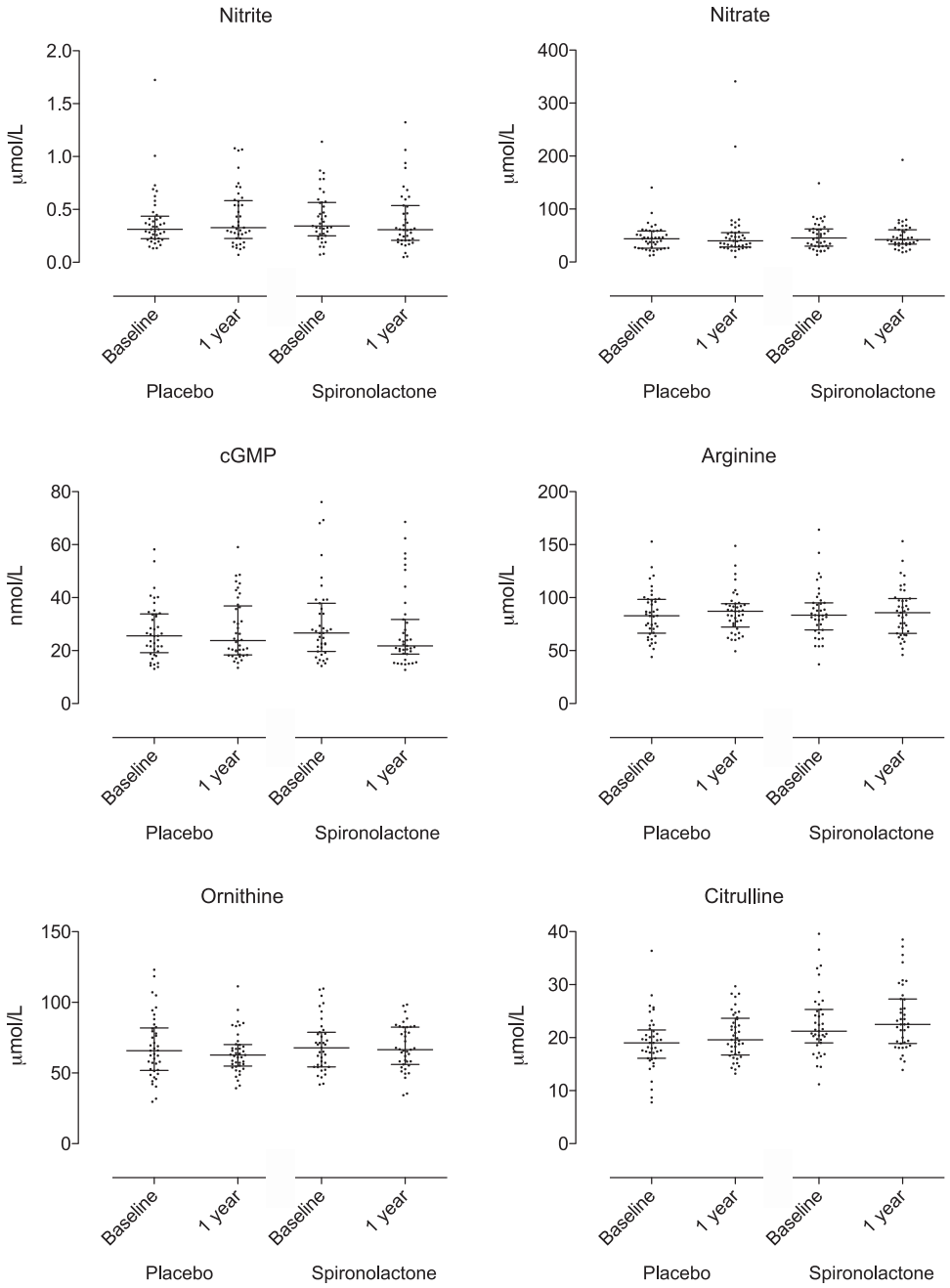

Fig. 2. Plasma levels of components of nitric oxide metabolism. Medians and interquartile ranges are shown. Differences from baseline to followup were compared by two-sample *t*-tests with equal variances (arginine and citrulline) or by a Mann-Whitney *U*-test (nitrite, nitrate, cGMP, and ornithine).

cGMP. In contrast, Syngle et al. (51) described a decrease in plasma nitrite without changes in blood pressure after spironolactone in patients with active rheumatoid arthritis and ankylosing spondylitis (52) and normal renal function. These patients were characterized by active inflammation at inclusion and exhibited markedly higher baseline and followup 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 NO synthase stimulated during inflammation (51).

Surprisingly, the subgroup analysis of patients with diabetes also found a decrease in nitrite in the spironolactone-treated group compared with the placebo-treated group. This was supported by decreased cGMP within the spironolactone-treated group. These patients did not display signs of inflammation; thus, there was no evidence to support increased inducible NO synthase activity. HbA_1C was unaltered by spironolactone. The purpose of the subgroup analysis in patients with diabetes was to investigate whether the higher cardiovascular 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 patients with diabetes needs further investigation because the present study was not powered to examine patients with diabetes specifically.

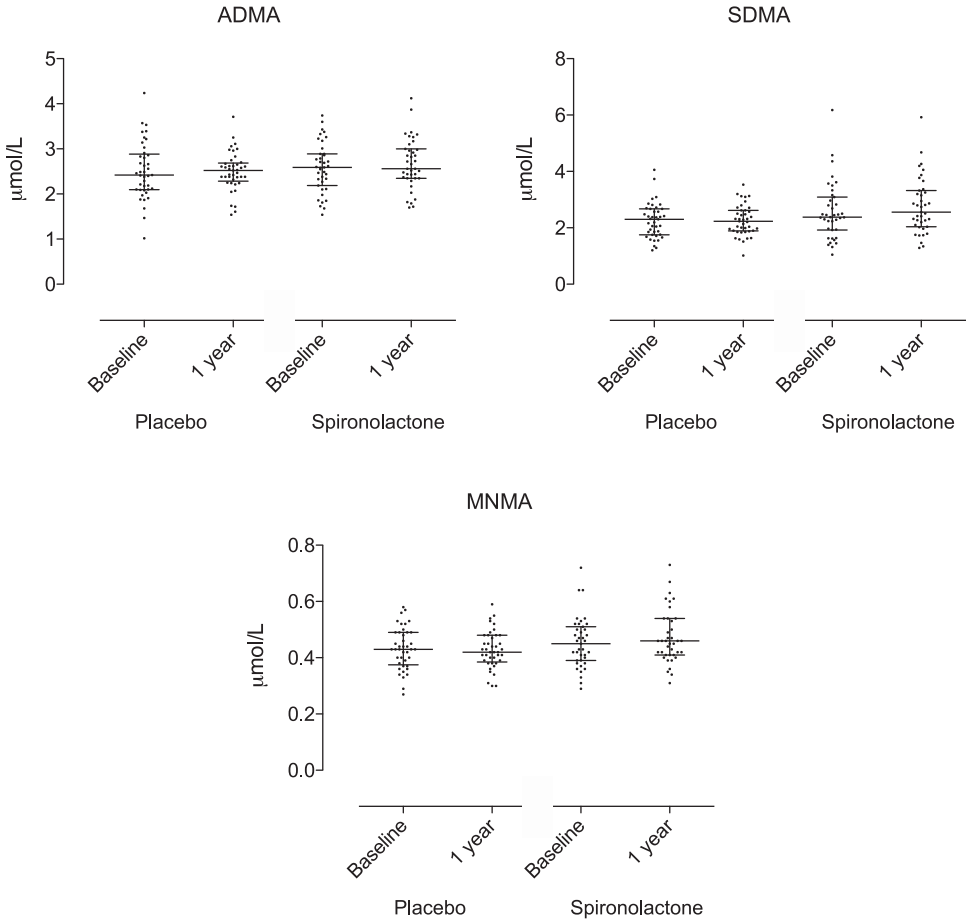

Fig. 3. Plasma levels of the endogenous endothelial nitric oxide synthase inhibitors. Medians and interquartile ranges are shown. Differences from baseline to followup were compared by two-sample *t*-tests with equal variances. ADMA, asymmetric dimethylarginine; MNMA, N^G-monomethyl-L-arginine; SDMA, symmetric dimethylarginine.

Functional measures of NO bioavailability in vivo include forearm blood flow and the noninvasive equivalent FMD (10). Spironolactone increases forearm blood flow in congestive heart failure (16) and FMD in patients on hemodialysis (19, 35) and in patients with active rheumatoid arthritis (51) and ankylosing spondylitis (52) but not in individuals with metabolic syndrome (27, 29) or obesity (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 and, hence, a suspected high degree of ED (43, 44, 58). Of note, in the present study, blood pressure in the spironolactone-treated 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 present study is the absence of functional measures of ED at baseline or followup. Several previous studies have found increased ED in renal transplant patients compared with healthy controls in the early (14 days) (32) and late (41 ± 9 mo) (42) phase after transplantation. Furthermore, Kensinger et al. (30) concluded that ED in renal transplant patients did not change from 1 to 24 mo after the transplantation. Based on these studies, it seems reasonable to assume a degree of ED at baseline in the present study.

In the present study, there was no effect of spironolactone on plasma levels of the endogenous eNOS inhibitors ADMA, SDMA, or N^G-monomethyl-L-arginine. 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 with mortality, cardiovascular morbidity, and impaired graft function (2). The present data suggest that there is no direct action of aldosterone via the MR on this pathway.

No difference in PAI:Ag was detected between the two groups (Fig. 5). In a previous study, endogenous aldosterone correlated positively with PAI:Ag in humans (8), whereas spironolactone prevented the increase in PAI:Ag despite increased aldosterone levels (47). In the present 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-treated group. We found no effect of spironolactone on markers of vascular and general inflammation between groups or within the spironolactone-treated group. A previous post hoc analysis from 4 pooled randomized controlled trials including 69 patients with diabetes with varying degrees of albuminuria likewise found no effect of spironolactone on circulating markers of vascular and systemic inflammation (40). Although a previous study has indicated a correlation between plasma aldosterone and levels of ICAM-1 (36), the present data sug-

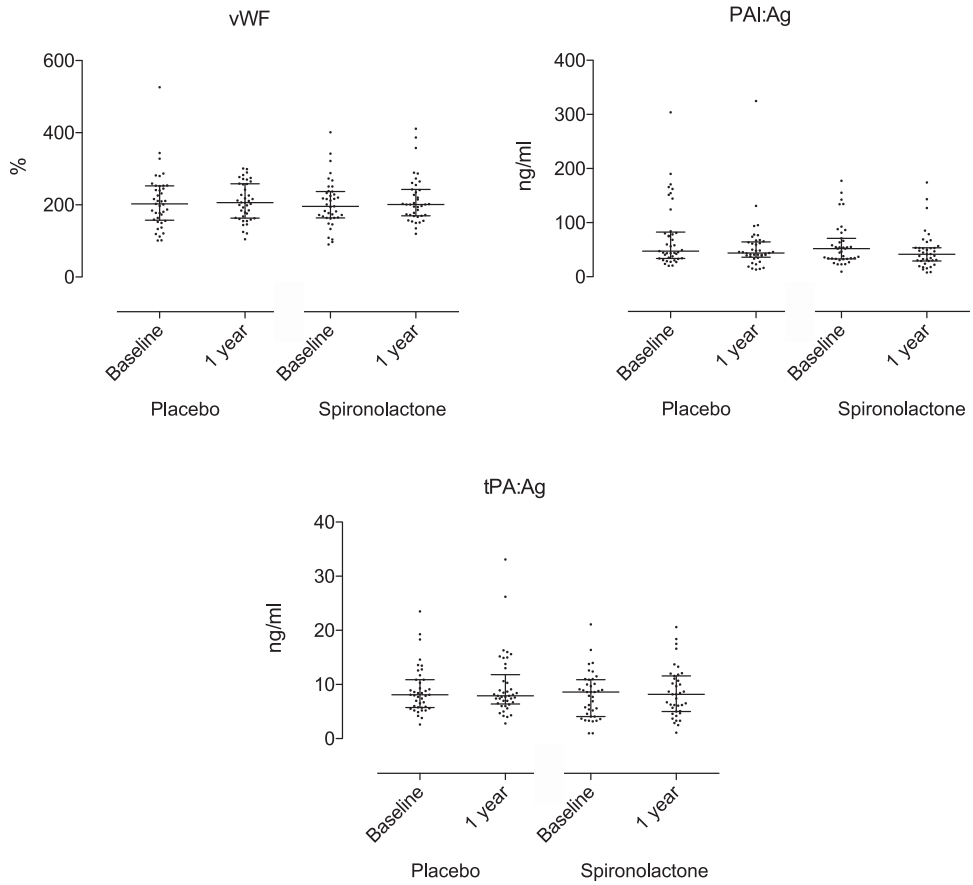

Fig. 4. Plasma levels of activated endothelial cell markers. Medians and interquartile ranges are shown. Differences from baseline to followup were compared by a Mann-Whitney *U*-test [plasminogen activator inhibitor type 1 antigen (PAI:Ag) and von Willebrand factor (vWF)] or a two-sample *t*-test with equal variances [tissue-type plasminogen activator antigen (tPA:Ag)].

gest 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 present 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 present study.

Limitations and Methodological Considerations

The double-blind, randomized design, and number of participants minimize selection bias and theoretically distribute potential unknown confounders evenly between groups. However, the outcomes of this substudy 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 followup. 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 participants 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.

Participants were included at any time after transplantation; thus, there was a large variation in the time since transplantation and a tendency toward older grafts in the spironolactone-treated 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-treated group at baseline. It is feasible that later vascular changes are less reversible, which may have limited a potentially beneficial effect in the spironolactone-treated group.

Evaluation of renal function was outside the scope of the present 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-treated 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-treated 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

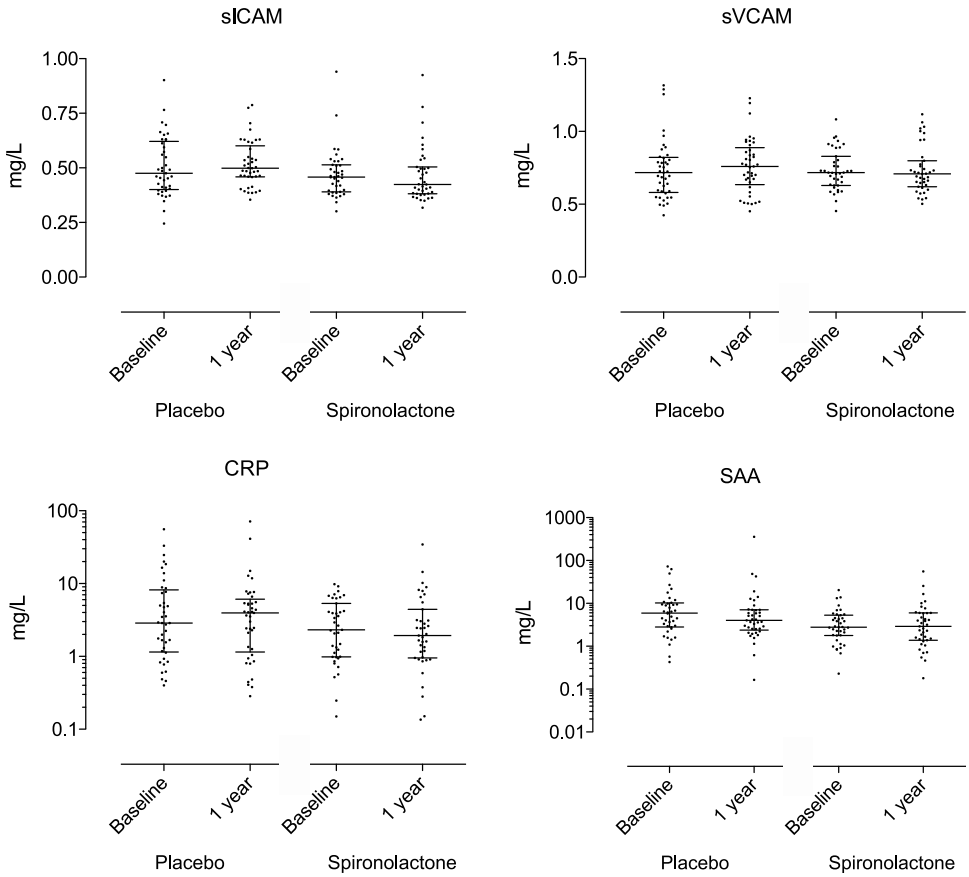

Fig. 5. Plasma levels of inflammatory markers. Medians and interquartile ranges are shown. Differences from baseline to followup were compared by Mann-Whitney *U*-tests. *y*-axes have been log transformed for C-reactive protein (CRP) and serum amyloid protein A (SAA) graphs to allow for outliers. sICAM, soluble ICAM-1; sVCAM, soluble VCAM-1.

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.

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Table 3. *Selected results from the diabetic subgroup analyses*

	Spironolactone-Treated Group			Placebo-Treated Group			
	Baseline	1 yr	Within-group comparison	Baseline	1 yr	Within-group comparison	Between-Group Comparison of Δ Values
Plasma nitrite, μmol/l	0.35 (0.08–0.69)	0.23 (0.09–0.59)	$P = 0.09$	0.29 (0.16–0.69)	0.43 (0.16–0.89)	$P = 0.21$	$P = 0.04$*
Plasma cGMP, nmol/l	32.9 (16.1–69.3)	28.6 (15.0–62.4)	$P = 0.03$*	25.5 (15.2–40.7)	22.0 (17.0–48.3)	$P = 0.72$	$P = 0.18$
Plasma hemoglobin A_1C, mmol/mol	49 (31–77)	50 (34–74)	$P = 0.37$	52 (28–73)	60 (32–77)	$P = 0.19$	$P = 0.56$
Plasma high-sensitivity C-reactive protein, mg/l	2.8 (0.5–6.5)	1.5 (0.1–10.1)	$P = 0.58$	2.7 (0.4–33.0)	3.6 (0.4–71.4)	$P = 0.93$	$P = 0.94$

Data are reported as medians (ranges); $n = 10$ patients with diabetes in the spironolactone-treated group and 11 patients with diabetes in the placebo-treated group. Between-group comparisons were performed by unpaired *t*-tests comparing the changes (Δ) from baseline to followup between groups (nitrite and hemoglobin A_1C) or Mann-Whitney *U*-test (cGMP and high-sensitivity C-reactive protein). Within-group comparisons were performed by a paired *t*-test (hemoglobin A_1C) or Wilcoxon signed-rank test (nitrite, cGMP, and high-sensitivity C-reactive protein). *Statistical significance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.A.M., C.B., H.C.T., and B.L.J. conceived and designed research; L.A.M., J.S., M.C., A.C., C.E.W., Y.P., E.M.B., and B.L.J. performed experiments; L.A.M. analyzed data; L.A.M., C.B., J.S., M.C., A.C., C.E.W., Y.P., E.M.B., H.C.T., and B.L.J. interpreted results of experiments; C.B. prepared figures; L.A.M. drafted manuscript; L.A.M., C.B., J.S., M.C., A.C., C.E.W., Y.P., E.M.B., H.C.T., and B.L.J. edited and revised manuscript; L.A.M., C.B., J.S., M.C., A.C., C.E.W., Y.P., E.M.B., H.C.T., and B.L.J. approved final version of manuscript.

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