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PROMOTION OF HYPERTENSION AND END-ORGAN INJURY BY INTERLEUKIN 17A MEDIATED BY ALDOSTERONE-MR AND ENaC

University of Southern Denmark

PHD THESIS

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PREFACE

The scientific work presented in this PhD thesis was performed at the Department of Cardiovascular and Renal Research, Institute of Molecular Medicine, University of Southern Denmark, Odense during the time June 2018 – June 2021. This thesis is structured into seven chapters including an introduction to the research topic, a chapter with materials and method used in this study, followed by four separate chapters presenting four manuscripts with original data (Study I-IV), and the final chapter with a discussion and conclusions of these studies.

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Included publications and manuscripts

The mineralocorticoid receptor blocker spironolactone lowers plasma Interferon-γ and Interleukin-6 in patients with type 2 diabetes and treatment-resistant hypertension.
Thangaraj SS, Oxlund CS, Fonseca MP, Ketelhuth DFJ, Svenningsen P, Stubbe J, Palarasah Y, Jacobsen IA, Jensen BL., J Hypertension, September 2021 (accepted)

Amiloride decreases Tumor Necrosis Factor and Interleukin-6 but not Interleukin-17A in patients with hypertension and type 2 diabetes
Thangaraj SS, Oxlund CS, Andersen H, Svenningsen P, Stubbe J, Palarasah Y, Fonseca MP, Ketelhuth DFJ, Henriksen JE, Jacobsen IA, Jensen BL. (Submission to AHA-Hypertension)

Mineralocorticoid receptor blockade with spironolactone has no direct effect on plasma IL-17A but lowers injury markers in urine from kidney transplant patients.
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Interleukin 17A lowers blood pressure at baseline and after angiotensin II-hypertension in conscious unrestrained male mice.
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Other scientific contributions

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ABBREVIATIONS

Aldosterone sensitive distal nephron	**(ASDN)**
Angiotensin II	**(ANGII)**
Angiotensin converting enzyme inhibitor	**(ACEi)**
Angiotensin receptor blocker	**(ARB)**
Antigen presenting cells	**(APCs)**
Calcineurin inhibitor	**(CNI)**
C-C Motif chemokine ligand	**(CCL)**
Cyclooxygenase	**(COX-2)**
C-C chemokine receptor-2	**(CCR2)**
C-X-C chemokine ligand	**(CXCL)**
Damage-associated molecular patterns	**(DAMP)**
Deoxycorticosterone acetate	**(DOCA)**
Experimental autoimmune encephalomyelitis	**(EAE)**
Epithelial sodium channel	**(ENaC)**
intracellular adhesion molecule	**(ICAM)**
inducible NO synthase	**(iNOS)**
Interleukin	**(IL)**
Knock out	**(KO)**
Macrophage colony-stimulating factor	**(MCS-F)**
Major histocompatibility complexes	**(MHC)**
Mineralocorticoid receptor	**(MR)**
Matrix metalloproteinases	**(MMP)**
Sodium channel co-transporter	**(NCC)**
Neutrophil gelatinase-associated lipocalin	**(NGAL)**
Na^+-K^+-Cl^{2-} co-transporter	**(NKCC)**
Pathogen associated molecular patterns	**(PAMP)**

Pathogen recognition receptor	**(PRR)**
Recombinase activating gene	**(RAG)**
Regulatory T-cells	**(Tregs)**
Renin-angiotensin-aldosterone system	**(RAAS)**
Severe combined immune deficient	**(SCID)**
Serum glucocorticoid kinase 1	**SGK-1**
Sodium-hydrogen exchanger 3	**(NHE3)**
Spontaneously hypertensive rats	**SHR**
T-cell receptor	**(TCR)**
Transforming growth factor β	**TGF-β**
T helper	**(Th)**
Toll-like receptor	**TLR**
Tumor necrosis factor	**TNF**
Type 2 diabetes mellitus	**(T2DM)**
Urine albumin/creatinine ratio	**(UACR)**
Mean arterial pressure	**(MAP)**
Systolic blood pressure	**(SBP)**
Diastolic blood pressure	**(DBP)**
Blood pressure	**(BP)**
Room temperature	**(RT)**

TABLE OF CONTENT

ABSTRACT

Hypertension is one of the most important risk factors for cardiovascular diseases and mortality worldwide. Despite available lifestyle modifications and pharmacological antihypertensive medication high blood pressure remains undiagnosed in more than 50% of hypertensive patients, and poorly controlled in 12-15% of patients. Furthermore, patients with type 2 diabetes have more prevalent resistant hypertension and rarely reach treatment goals.

Evidence from preclinical studies suggest that the adaptive and innate immune system, involving both T-cells, macrophages and dendritic cells play significant roles in the pathogenesis of hypertension. The major focus of this PhD project is to investigate the role of the pro-inflammatory interleukin 17A (IL-17A) produced by T-helper (Th)-17 lymphocytes in hypertension. Mineralocorticoid receptor (MR) is expressed on several adaptive and innate immune cells including T-lymphocytes, macrophages, and dendritic cells. Differentiation of naïve $CD4^+$ T-cells into pro-inflammatory Th17 cells is dependent on the MR in MR-dependent-hypertension in rats, and aldosterone-MR activation *in vitro*. Thus, a mechanistic link between aldosterone-MR and Th17-mediated inflammation contributing to hypertension can be suggested. This could also include the epithelial sodium channel ENaC that, besides being expressed in the distal nephron, also is expressed on lymphocytes and dendritic cells, and shown to be involved in promoting hypertension *in vivo*. We sought to translate these findings that IL-17A is a major culprit in immune-mediated hypertension in animals by two approaches: **1)** In retrospective explorative studies we examined whether well-known anti-hypertensive drugs (spironolactone and amiloride) target release of IL-17A and related upstream and downstream cytokines in patients *in vivo*. **2)** By direct intervention in mice, IL-17A was administered and effects on blood pressure and heart rate were measured. The following overall hypotheses were tested: **1)** IL-17A is directly pro-hypertensive by vascular and renal actions in a concentration-dependent manner; **2)** IL-17A contributes non-redundantly to immune mediated hypertension through aldosterone-MR interaction; and **3)** Dendritic or macrophage and/or Th17 lymphocyte-ENaC channels contribute to IL-17A production in hypertensive patients and accounts for salt-sensitivity. Hypotheses were addressed directly by IL-17 infusion into mice and indirectly, by testing the impact of MR inhibitors and ENaC blocker *in vitro* in inflammation-stimulated macrophages treated with MR and ENaC blockers, and *in vivo* in patient plasma samples from intervention studies, by measuring the cytokine IL-17A, and other cytokines that are either produced downstream of IL-17A production (IL-6),

involved in IL-17A production (IL-6, IL-1β), and produced by macrophage activation (TNF, IL-6, and IL-10) or Th-1 cell activation (IFN-γ). The obtained results and conclusions of this overall study resulted in 4 independent manuscripts as follows.

Study I: The mineralocorticoid receptor blocker spironolactone lowers plasma interferon-γ and interleukin-6 in patients with type 2 diabetes and treatment resistant hypertension

We hypothesized that MR antagonism by spironolactone selectively suppresses Th-17 derived IL-17A and other macrophage- and Th-1 cell derived cytokines, and that this relates to reduction in blood pressure, albuminuria, and a plasma potassium increase. Plasma samples from patients with type 2 diabetes mellitus (T2DM) and treatment resistant hypertension, included in a double-blind, placebo-controlled interventional trial, were assessed for plasma cytokine levels before and after intervention with spironolactone (25-50 mg/daily) for 16 weeks. It was previously shown that spironolactone intervention mediated blood pressure and urine albumin/creatinine ratio (UACR) decreases, and plasma potassium increase. In the present study it was shown that plasma IL-17A was unchanged upon spironolactone intervention, but plasma levels of IFN-γ and IL-6 were decreased with no changes observed in placebo treated patients. At baseline serum aldosterone concentrations and night-time diastolic blood pressure (DBP) correlated positively. There were no relations between blood pressure and plasma cytokine levels at baseline; between serum aldosterone and plasma cytokine levels at baseline; and between plasma cytokine and blood pressure changes after spironolactone treatment, except for IFN-γ, which was inversely related to blood pressure changes. Plasma potassium increases related to blood pressure decrease, but not to plasma cytokine decrease after spironolactone intervention. Furthermore, baseline plasma IL-6 related positively to baseline UACR. Finally, *in vitro* macrophage cell studies revealed a reduction in macrophage derived cytokines upon spironolactone intervention in LPS stimulated cells. We conclude that blood pressure lowering by MR blockade with spironolactone in T2DM patients with treatment resistant hypertension was not mediated by Th-17-derived IL-17A reduction. Instead, spironolactone exerts anti-inflammatory actions *in vivo* through suppression of Th-1 and macrophage-derived cytokines (IFN-γ and IL-6)

Study II: Amiloride decreases TNF and IL-6 but not IL-17A in patients with hypertension and type 2 diabetes

We hypothesized that ENaC inhibition with amiloride reduces plasma IL-17A in patients with T2DM and treatment resistant hypertension, and that this reduction in plasma IL-17A relates to amiloride-mediated reduction in blood pressure, albuminuria, and plasma potassium increase. In a follow-up, open label study of the previously described patient cohort (study I), T2DM patients with treatment resistant hypertension received amiloride for 8 weeks (5-10 mg/day). Previously, it was shown that amiloride lowered blood pressure and UACR and increased plasma potassium. In the present study, we show that amiloride decreased plasma TNF and IL-6 but had no effect on plasma IL-17A. At baseline, plasma IL-1β correlated positively to night-time mean arterial pressure (MAP) and DBP. Baseline plasma IL-17A correlated positively to systolic blood pressure (SBP). No correlations were observed between TNF, IL-6, and blood pressure decreases after amiloride intervention. Baseline UACR correlated positively with baseline SBP, and with baseline plasma IL-17A, TNF, IL-6, and IL-1β concentrations. Plasma potassium increase did not relate to blood pressure decrease or plasma TNF and IL-6 changes after amiloride intervention. *In vitro* studies show that LPS-stimulated macrophages increase release of cytokines whilst amiloride co-incubation caused a significant reduction in IL-6, IL-1β, and IL-10 but had no effect on TNF. Co-incubation with benzamil, a more potent ENaC inhibitor, reduced all 4 cytokines at nmol/L concentrations. In conclusion, amiloride-mediated blood pressure reduction in the hypertensive patients was not mediated by IL-17A reduction. By contrast, amiloride lowered TNF and IL-6 both *in vitro* and *in vivo*. Thus, ENaC contributes to macrophage stimulation and is a relevant non-renal target to suppress inflammation and potentially blood pressure in patients with type 2 diabetes and hypertension.

Study III: Mineralocorticoid receptor blockade with spironolactone has no direct effect on plasma IL-17A but lowers injury markers in urine from kidney transplant patients

We hypothesized that MR antagonism by spironolactone reduces IL-17A production in plasma and relates to spironolactone-mediated blood pressure changes and renal epithelial protection in kidney transplant patients. Plasma and urine samples were obtained from kidney transplant patients from a multicenter, double-blind, placebo-controlled intervention study including kidney transplant patients, that received either spironolactone (25-50 mg/daily) or placebo for 3 years. Plasma and urine samples from study inclusion and after 1 year intervention were

analyzed for cytokine or kidney injury markers, respectively. Plasma IL-17A was unchanged in response to spironolactone treatment but increased in the placebo group. No changes were observed in other T-cell and macrophage-derived cytokines. Plasma aldosterone did not relate to 24hr ambulatory MAP or plasma IL-17A at baseline. Plasma IL-17A did also not relate to 24h ambulatory MAP measurements at baseline. The kidney injury markers calbindin and trefoil factor 3 (TFF3) were decreased after 1-year spironolactone treatment with no changes in the placebo group. Baseline urine calbindin/creatinine and TFF3/creatinine ratios did not relate to baseline plasma aldosterone, but TFF3/creatinine ratios related positively to blood pressure at baseline. When assessing differences in cytokine response of patients that received angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs), and patients that did not receive these drugs, no difference in plasma cytokine production was observed. We conclude that MR blockade may stabilize IL-17A production and suppress epithelial injury markers associated with the distal nephron.

Study IV: Interleukin 17A lowers blood pressure at baseline and after ANGII-hypertension in conscious unrestrained male WT mice

*We hypothesized that **1)** chronic i.v. infusion of IL-17A in conscious mice would increase blood pressure; that **2)** ANGII-induced blood pressure elevation would be accentuated by IL-17A by renal mechanisms; and that **3)** acute bolus i.v. infusion of IL-17A would cause acute blood pressure increase by vascular effects*. When mice received chronic i.v. IL-17A infusion for 8 days in a step-up model, hypertensive effects were not observed, rather significant hypotensive effects were detected. IL-17A also did not accentuate ANGII-mediated hypertension in mice, but rather lowered ANGII-mediated blood pressure elevation compared to blood pressure in ANGII-vehicle co-infused mice. Acute bolus infusion did not show any acute vascular effects either. Plasma IL-17A levels were measured to be up to 3500 times above baseline levels and activation of the downstream inflammatory IL-6 cytokine was observed by increased plasma IL-6 in mice and *in vitro* in IL-17A stimulated fibroblasts. We concluded that IL-17A by itself, does not have any direct hypertensive effects, but instead lowers blood pressure.

Altogether the 3 clinical studies including hypertensive patients treated with a MR inhibitor, spironolactone, or ENaC blocker, amiloride, revealed no effect on IL-17A production in plasma of these patients. Instead, our data indicated a minor, but non-redundant anti-inflammatory effect by detection of reduced levels of other T-cell and macrophage-derived cytokines including IFN-γ, IL-6, and TNF. Chronic and acute i.v. infusion of IL-17A in mice

showed no direct hypertensive effect, but rather a blood pressure lowering effect at non-physiologically high concentrations. Thus, it is concluded that IL-17A by itself is not a hypertensive protein but may in concert with other cytokines or unknown factors, contribute to inflammation-mediated hypertension.

Introduction

The following chapter provides an overview of studies implicating: The involvement of the pro-inflammatory cytokine interleukin 17A in hypertension in animals and humans; Promotion of hypertension via immune cells including T-lymphocytes, macrophages, and dendritic cells in humans and animals; Expression of the mineralocorticoid receptor (MR) and the epithelial sodium channel (ENaC) on immune cells may be related to IL-17A production and hypertension.

HYPERTENSION

Hypertension affects 1.2 billion individuals worldwide and is the most common chronic condition and leading risk factor for developing cardiovascular diseases including stroke, heart failure, and chronic kidney disease in the human population with increasing prevalence worldwide (1, 2). Hypertension is collectively referred to as the "silent killer" since symptoms are often less pronounced and therefore rarely discovered. Despite increasing awareness and availability of conventional treatment for blood pressure control, recommended blood pressure goals (<140/80 mmHg) are difficult to achieve in nearly half of the hypertensive population according to the international society of hypertension (3). Patients that do not reach blood pressure goals despite multidrug therapy, are diagnosed with resistant hypertension (4). Only 10% of hypertensive cases are cause-identified whereas 90% of the cases are identified as primary or essential hypertension with no etiology (3). Hypertension is a multifactorial condition, which is caused by both environmental and genetic factors, and the pathogenesis of hypertension involves many systems including the vasculature, central and sympathetic nervous systems, kidneys, hormonal regulation by the renin-angiotensin-aldosterone system (RAAS), and the immune system (5-8). However, the role of these systems in the pathogenesis of hypertension is less clearly defined. In this thesis the major focus will be on the inflammatory contribution to hypertension, with specific focus on the relation between the pro-inflammatory cytokine IL-17A and aldosterone-MR and ENaC mediated hypertension.

HYPERTENSION AND DIABETES

Hypertension occurs in nearly half of the diabetes mellitus (DM) population and consistent control of blood pressure in these patients is important for preventing and delaying cardiovascular complications (9). However, patients with T2DM and hypertension have more prevalent and resistant hypertension and rarely reach desired treatment goals set by international societies (130/80 mmHg) (3, 10, 11). A study showed that 50% of hypertensive patients experienced impaired glucose tolerance or T2DM, and 85% of patients with T2DM became hypertensive over time, suggesting that the combination of T2DM and hypertension is associated with elevated risk for cardiovascular diseases (12). Hypertension increases the risk factor for diabetes-associated vascular complications including macrovascular diseases involving atherosclerosis, and microvascular diseases involving nephropathy, retinopathy, and neuropathy (13-15). Hypertension and T2DM are associated with several similar pathophysiologic mechanisms including overactivation of the renin-angiotensin-aldosterone

system (RAAS) (16, 17), oxidative stress (15), increased activation of the sympathetic nervous system (18, 19), impaired insulin-mediated vasodilation, increased pro-inflammatory response by the innate and adaptive immune system and inappropriate renal handling of sodium (16, 20-22). Furthermore, studies have revealed that the adipose tissue contributes to chronic low-grade systemic inflammation with increased production of TNF, IL-6, and IL-1β in patients with T2DM, leading (23-26) to increased ANGII and aldosterone production which may also contribute to hypertension (27). Thus, the interconnection between the RAAS and low-grade inflammation in diabetic patients may further aggravate the pre-existing pro-inflammatory state resulting in hypertension in these patients (20). However, precise mechanisms need to be further elucidated.

HYPERTENSION IN KIDNEY TRANSPLANT PATIENTS

Kidney transplantation is considered the best treatment option for patients with end-stage renal disease (28). With successful kidney transplantation quality of life is improved and mortality is reduced (29). Despite of this, kidney allograft transplantation is associated with increased risk of graft failure which is associated with both immunological and non-immunological risk factors (30). Allogenic non-self-recognition induces activation of T-lymphocytes and other immune cells that contribute to cytotoxicity on graft cells, leading to renal fibrosis and eventually graft failure. Introduction of advanced immunosuppressive treatments like calcineurin inhibitors (CNIs) has reduced episodes of acute allograft rejections (31, 32), however the long-term graft survival is still a challenge since continuous treatment with CNIs is associated with renal fibrosis and ultimately leads to chronic allograft failure (33, 34).

About 90% of kidney transplanted patients develop arterial hypertension after transplantation which increases the risks of allograft failure (35). It is well-known that hypertension leads to progression of kidney failure in the general population (36, 37). In a large retrospective transplant study conducted over 7 years, it was shown that increased blood pressure post-transplantation was associated with a gradual increase of subsequent graft failure (38). Similarly, other studies have shown the same relation independently of baseline graft function and incidences of acute rejection (39, 40). The improvement of kidney function in primary kidney disease with antihypertensive agents is well documented (41, 42). Hypertension is a non-immunological risk factor for graft failure and therefore lowering blood pressure in these patients has been suggested to be beneficial (39). This was shown in a large retrospective study, where systolic blood pressure reduction was associated with improved graft function and patient survival studied up to 10 years (43). The 2021 KDIGO guidelines recommend to treat

adult kidney transplant recipients with blood pressure lowering drugs to reach target office blood pressure of <130/80 mmHg (44). The underlying mechanisms leading to development of hypertension in kidney transplant patients are less clearly defined, however renal- and inflammatory mechanisms have been suggested.

INFLAMMATION IN HYPERTENSION

For more than 50 years, evidence from several *in vitro,* pre-clinical and clinical studies have suggested that inflammation involving both the innate and adaptive immune systems contribute to the pathogenesis of hypertension. Early observations by Grollman, White, and Olsen in the 1960-1970s showed that immunosuppression of rats with partial renal infarction lowered blood pressure and that hypertension was recapitulated when transferring lymph node cells from the renal infarct rats into normo-tensive recipient rats (45-47). Studies by Olsen revealed mononuclear cell infiltration in blood vessels of ANGII-infused rats and occurrence of vascular immune cell accumulation in humans with hypertension (48, 49). Furthermore, thymus transplantation from healthy rats into spontaneously hypertensive rats (SHR) or anti-thymocyte serum administration showed to lower blood pressure in SHRs (50, 51). Adoptive transfer of splenocytes from deoxycorticosterone (DOCA)/salt hypertensive rats raised blood pressure in recipient rats (47). Nude and athymic rats were protected against DOCA/salt hypertension (52, 53). These early findings all indicate that immune cells, specifically T-lymphocytes, contribute significantly to the pathogenesis of hypertension, however underlying mechanisms are still poorly understood.

THE IMMUNE SYSTEM – AN OVERVIEW

The immune system consists of the innate and the adaptive branches. Innate immunity is the immediate, non-specific defense against a wide range of pathogens, involving myeloid cells like monocytes, macrophages, and dendritic cells. These cells express pathogen recognition receptors (PRR) like the Toll-like receptors (TLRs) and recognize pathogen associated molecular patterns (PAMPs) or cellular stress signals, danger-associated molecular patterns (DAMPs), and engage intracellular pathways that induce the assembly of the inflammasomes. This ultimately leads to production of a set of pro-inflammatory cytokines and chemokines that will induce clearance of the harmful element. The adaptive immune system is antigen-specific and involves T and B lymphocytes. T-cell activation is mediated by antigen presenting cells (APCs), including dendritic cells, macrophages, and B-cells. APCs process antigens and present antigen peptides via their major histocompatibility complexes (MHC) and bind to

specific T-cell receptors. MHCI will interact and activate $CD8^+$ T cells while MCHII activates $CD4^+$ T-cells. This is known as the first signal in T-cell activation. The second signal in T-cell activation is known as T-cell co-stimulation where an interaction between CD28 on T-cells binds B7 ligands (CD80 or CD86) on APCs. B7 ligands are upregulated during APC maturation and absence of this marker prevents T-cell activation and induce T-cell cell-death. Cytotoxic lymphocyte antigen 4 (CTLA4) prevent T-cell co-stimulation by B7 ligand interaction. T-cell activation leads to a series of inflammatory signaling with production of cytokines and chemokines leading to effector T-cell clonal expansion. Four distinct subpopulations of $CD4^+$ effector T-cells have been characterized – the Th-1, Th-2, Th17 and the regulatory T cells (Tregs) (54). Th-1 cells are involved in defense against intracellular infections and evoke cell-mediated immunity by producing the cytokines IL-2, IFN-γ, and TNF which further stimulates macrophage activation (55). Th-2 cells induce strong antibody responses by producing cytokines like IL-4, IL-5, IL-13, and IL-10 against extracellular pathogens (55). Th-17 cells are characterized by their production of IL-17 and are involved in defense against extracellular bacteria and fungi, and development of autoimmune diseases like psoriasis (56). The fourth T-cell effector subtype is the regulatory T-cells (Tregs). These cells exert immunosuppressive anti-inflammatory responses and are characterized by their production of transforming growth factor (TGF)-β and IL-10 (57). These cells are essential for balancing the immune response of the pro-inflammatory Th-17 cells since increased production of Tregs has shown to be involved in less pronounced differentiation of the pro-inflammatory Th17 cells and IL-17 (58, 59).

THE ROLE OF T-LYMPHOCYTES IN HYPERTENSION

More recent studies using different experimental hypertensive animal models have shown immense evidence to support the previous findings that inflammation plays a pivotal role in promotion of hypertension. A pioneering study by Guzik showed that mice lacking the recombinase activating gene1 ($RAG1^{-/-}$), deficient of T and B- lymphocytes, were protected against ANGII and DOCA/salt-induced hypertension and vascular dysfunction, since these mice developed blunted hypertension and their production of superoxides was reduced (60). Adoptive transfer of T-cells, but not B-cells, restored the hypertensive responsiveness of these $RAG^{-/-}$ mice (60). In another study, severe combined immune deficient (SCID) mice, that lack T and B- lymphocytes, have also been proven to be protected against experimentally induced hypertension by manifest of blunted ANGII-induced hypertension (61). Furthermore, T-lymphocyte deficient Dahl salt sensitive rats ($RAG^{-/-}$ and $CD247^{-/-}$) developed blunted

hypertension and had reduced salt-induced renal damage (62, 63). Treatment with the T-cell immunosuppressing reagent mycophenolate mofetil in hypertensive mice has been shown to lower blood pressure and reduce renal inflammation (64-66). This anti-hypertensive effect of mycophenolate mofetil has also been reported in a small population of hypertensive patients with psoriasis and rheumatoid arthritis (67). As mentioned earlier, for full T-cell activation two signals are needed, the TCR/MHC and the CD28-B7 ligand co-stimulation. Administration of chimeric molecules of CTLA4 linked to immunoglobulin (abatacept) to DOCA-salt or ANGII-induced hypertensive mice, mediated blood pressure lowering, and prevented T-cell activation and vascular infiltration (68). In this study, B7-deficient mice were protected against ANGII-mediated hypertension. Furthermore, several studies have confirmed the accumulation of T-cells in the kidneys of experimental hypertensive animals (69-72). Together these studies suggest that T-cells and T-cell activation play an important role in the development of hypertension in different experimental models in animals.

INTERLEUKIN 17

The above-mentioned studies all implicate the role of T-lymphocytes in general but do not specify the subset of effector T-cells involved. As mentioned, four subpopulations of $CD4^+$ T helper cells are characterized. Of these, Th17 cells are newly characterized and produce the pro-inflammatory cytokine IL-17, which has been associated with promotion of hypertension in preclinical and clinical studies (73). Other than Th17 cells, γ/δ T cells, T_C17 subset of $CD8^+$ T cells, B cells and natural killer T cells have been shown to produce this cytokine as well (74-76). IL-17 exist as 6 isoforms (IL-17A-F) of which IL-17A and IL-17F are the most abundant and thoroughly studied isoforms. These two cytokines are closely related and share 50% sequence homology and are mainly produced by the Th17 cells (77). IL-17A and IL-17F bind as homo- or heterodimers to the IL-17 receptor complex consisting of IL17RA and IL-17RC (77). Upon cytokine binding the receptor complexes heterodimerize and mediates downstream inflammatory signaling (77). In hypertension-related preclinical studies IL-17A has been shown to play a major role compared to IL-17F (78, 79). In the following section focus will be on the association between IL-17A and hypertension. IL-17A can induce expression of a wide range of proinflammatory cytokines, including TNF, IL-1β, IL-6, IL-21, TGF-β; chemokines (CXCL1, CXCL5, IL-8, CCL2, CCL7) (80, 81), antimicrobial peptides (defensins and S100 proteins) and matrix metalloproteinases (MMP1, MMP3, and MMP13) from fibroblasts, endothelial cells and epithelial cells. IL-17A also induce expression of the intracellular

adhesion molecule 1 (ICAM-1) in keratinocytes (82), as well as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in chondrocytes (83).

IL-17A CONTRIBUTION TO HYPERTENSION

IL-17A is protective against bacterial and fungal infections but has also shown to play a major role in the development of several autoimmune disorders including psoriasis, arthritis, multiple sclerosis, and asthma (84-89). Treatment of these patients with neutralizing antibodies against IL-17A has shown promising clinical outcomes with improvement of condition, however, the effect on arterial blood pressure has not been stated (85, 89). In clinical studies plasma IL-17A has been shown to be significantly increased in hypertensive patients compared to normotensive individuals (72, 90-92). In women with polycystic ovary syndrome, associated with hypertension, serum IL-17A levels were positively correlated to blood pressure (93). In preclinical studies it was shown that experimental hypertensive animals, induced by either ANGII or DOCA/salt, have increased production of IL-17A systemically and Th17 differentiation (79, 90, 94). Deficiency of IL-17A or IL-17RA, by gene deletion or with neutralizing antibodies, in hypertensive animals protects against ANGII-induced hypertension, aortic T-cell infiltration, oxidative stress, endothelial dysfunction and end organ damage (79, 90). IL-17A knockout mice rescued salt excretion upon saline challenge and attenuated ANGII mediated increased activity of the proximal tubule transporter sodium hydrogen exchanger 3 (NHE3) in the kidneys (78, 95). IL-17A has also been shown to exert NO-dependent endothelial dysfunction in mice (96). This NO-dependent relaxation is mediated by increased phosphorylation of the inhibitory endothelial NO synthase (eNOS) Thr495 and Rho-kinase activity, which was prevented by anti-IL-17A or Rho-kinase inhibitor treatment (96). IL-17A also contributes to vascular dysfunction (97), aortic stiffening and vascular fibrosis through activation of p38 mitogen-activated protein kinase (MAPK) (98). These studies altogether suggest that IL-17A is playing an important role in the inflammatory contribution to promote hypertension. Mechanistic links between IL-17A and NO-dependent endothelial and vascular dysfunction; regulation of renal sodium handling; tissue fibrosis including kidney, vasculature, and heart have all been implicated in animal models with either experimental hypertension, knockout of IL-17A, or treatment with neutralizing antibodies against IL-17A. However, the direct inflammatory and hypertensive effects of IL-17A have been scarcely investigated and with inconsistent results. Ngyen et al. (2013) have shown that i.p. injections of 1 mg recombinant IL-17A per day for 7 days in mice caused a significant increase from approximately 95 to 130 mmHg systolic blood pressure measured by the tail-cuff method (96).

Dermal overexpression of IL-17A in mice mediated tail-cuff measured increased systolic blood pressure in these mice compared to controls (Karbach et al. 2014) (84). Orejudo et al. (2020) showed similar experiments with chronic subcutaneous infusion of IL-17A (1.5 ng/g) using osmotic minipumps and measured a significant increase from approximately 95 to 115 mmHg systolic blood pressure by the tail-cuff method (99). In 2019 however, Orejudo et al. showed that in transgenic mice overexpressing IL-17 in T-cells, by up to 15 times higher than IL-17A circulating levels in control mice, blood pressure was not affected as measured by carotid artery telemetry catheter assessment (100). Finally, Travis et al. (2019) also showed that chronic subcutaneous infusion of IL-17A by osmotic minipumps in pregnant rats caused an increase of 10 mmHg in mean arterial blood pressure compared to a vehicle group measured by carotid artery telemetry. The discrepancies of these studies could be caused by the different administration routes, concentrations of IL-17A, duration of administration, or the different ways of measuring blood pressure. The direct effect of IL-17A on blood pressure will be further investigated in this study, and the discrepancies will be further discussed.

IL-17A Contribution to Kidney Allograft Failure

Renal transplantation is a pro-inflammatory state associated with development of hypertension, graft injury and increased cardiovascular risk profile (101). In a murine transplantation model, IL-17A deficiency or neutralization was protective against kidney allograft injury and led to prolonged survival (102). IL-17A deletion in mice has also shown to protect against diabetic nephropathy-induced kidney fibrosis and pro-inflammatory cytokine expression including TNF, IL-6, CCL2, and TGF-β; and treatment with neutralizing antibodies against IL-17A ameliorated kidney dysfunction and disease progression (103, 104). Other studies have shown that systemic IL-17A administration to mice mediates increased levels of inflammatory cell infiltration in the kidneys and increased level of the injury marker neutrophil gelatinase-associated lipocalin (NGAL) (84, 100). In humans, it has been shown that plasma IL-17A concentrations and intragraft IL-17A mRNA levels were increased in kidney transplant patients with acute rejection (105). In patients with either ongoing acute rejection or chronic allograft dysfunction plasma IL-17A was elevated when compared to patients with a stable allograft function (106). These observations in both animals and patients could be interconnected with the IL-17A hypertensive effect and suggest a potential role of IL-17A in renal allograft failure.

Macrophages in Hypertension

Macrophages and dendritic cells are part of the innate immune system and function as APCs. Besides phagocytic functions to eliminate foreign components in the body, these cells produce

different cytokines and chemokines for T-cell recruitment and stimulation (54). In ANGII-hypertensive animals, augmented monocyte, macrophage, and dendritic cell-infiltration in the kidneys has been shown (64, 66, 107, 108). Macrophage colony stimulating factor (MCS-F) deficient Op/Op mice, have shown to be protected against ANGII and DOCA/salt-induced hypertension (109, 110). Inhibition of the monocyte chemoattractant protein (MCP)-1, the MCP-1 receptor, or the C-C chemokine receptor-2 (CCR2) reduced macrophage infiltration of kidneys and suppressed blood pressure elevation in both ANGII and DOCA-salt hypertensive animal models (111-113). Macrophage depletion in mice attenuated ANGII-mediated blood pressure elevation; induction of vascular adhesion molecules; vascular, endothelial, and smooth muscle dysfunction; and reduced vascular superoxide production (114). Adoptive transfer of monocytes into these macrophage depleted mice restored their hypertensive responsiveness, vascular dysfunction, and increased their production of superoxides (114). Clinical studies have shown that hypertensive patients have enhanced expression of systemic inflammatory markers of monocyte-endothelial cell adhesive interaction and macrophage-related cytokines including TNF, IL-1, IL-6, cell adhesion molecules (CAM), and MCP-1 (115-118). These studies together suggest that macrophages play an essential role in hypertension and could serve as a novel therapeutic target to treat hypertension. The exact mechanisms of these cells in promotion of hypertension and their potential targets for pharmacological intervention remain unclear and needs further investigation.

IMMUNE CELLS IN SALT-SENSITIVE HYPERTENSION

Excess salt consumption provokes and increase blood pressure and causes targeted organ damage (119). This has been proposed to be mediated through adaptive immune cells including macrophages and dendritic cells (120-122). High sodium chloride concentrations promote Th17 cell differentiation from naïve $CD4^+$ T-cells *in vitro*, and high salt diet enhanced the severity of experimental multiple sclerosis in mice with increased Th17 cell differentiation (123-125). ANGII-induced hypertension in WT mice decreases salt excretion upon saline challenge, but mice deficient in IL-17A maintains baseline salt excretion and show with decreased expression of the proximal tubule transporter sodium hydrogen exchanger 3 (NHE3) (95). IL-17A deficiency abolished activation of the Na^+-K^+-Cl^{2-} co-transporter (NKCC2) of the thick ascending limb, the sodium channel co-transporter (NCC), and the epithelial sodium channel (ENaC) of the distal tubules and protected against glomerular and tubular injury (78, 95). IL-17A administration to proximal tubule cells (HK-2) and mouse distal convoluted tubule cells (mDCT15) *in vitro* caused an increased expression of NHE3 and NCC, respectively (78).

These studies suggest a mechanistic link between IL-17A and renal sodium transporters, and that targeting IL-17A may improve renal function in hypertension. A few cell-culturing- and animal studies show the link between IL-17A, salt, and immune cells as well. Both *in vitro* and *in vivo* studies have shown that sodium chloride by itself can induce macrophage proliferation and increase pro-inflammatory macrophage-derived cytokines and promote kidney and vascular injury (111, 126, 127). Besides macrophages, dendritic cell activation and T-cell co-stimulation is increased in ANGII and DOCA-salt-induced hypertensive mice (68, 70, 128). Dendritic cells are essential for adaptive immunity because they prime naïve T-cells for differentiation into effector cells. Recently it has been demonstrated, both *in vitro* and *in vivo* (mice) that excess dietary salt can prime dendritic cell activation via ENaC and NHE1, which leads to NADPH-oxidase dependent superoxide production and the formation of highly reactive isoketals (fatty acid oxidation product) (129). Isoketals rapidly adducts and oxidatively modify proteins and are immunogenic (130). Salt-induced dendritic isoketal protein-adducts promote *ex vivo* Th-17 conversion from naïve $CD4^+$ T-cells; DOCA-salt- and ANGII-hypertension in mice; and elevate cytokine production by dendritic cells (IL-6, IL-1 b, and IL-23) and T-cells (IFN-γ and IL-17) (70, 129). Adoptive transfer of salt-stimulated dendritic cells into mice that received low dose ANGII, primed hypertension (129). Dendritic cells express the a and g subunits of ENaC and facilitate sodium entering and promote hypertension in a serum and glucocorticoid-induced kinase 1 (SGK-1)-dependent manner (128, 131). ENaC blockade with amiloride suppressed isoketal protein-adduct accumulation in salt-stimulated dendritic cells and decreased dendritic IL-1β production (70, 129). ENaC blockade with amiloride reduces blood pressure in hypertensive patients (132, 133). These studies implicate mechanistic links between salt-sensitive hypertension and dendritic cell ENaC-dependent activation with subsequent stimulation of Th17 and IL-17A in mice and *in vitro*. Patient data investigating these mechanisms are very scarce, therefore this will be further investigated in this study.

THE ROLE OF THE MINERALOCORTICOID RECEPTOR ON IMMUNE CELLS IN HYPERTENSION

The mineralocorticoid receptor is known for its role as an important regulator of blood pressure and electrolyte and fluid homeostasis in response to its ligand aldosterone (134). Although MR is best known for its expression in the so-called aldosterone sensitive distal nephron (ASDN), it is also expressed by other renal cell types as well as a wide range of other tissues including colon (135), lungs (136), salivary glands (137), liver (138), and also in immune cells including

macrophages, dendritic cells and T-lymphocytes (139-142). *In vitro* studies have shown that aldosterone primes dendritic cells to differentiate naïve $CD4^+$ T-cells into Th-17 cells and cause MAPK pathway activation with secretion of IL-6 and TGF-β (139). In experimental autoimmune encephalomyelitis (EAE), aldosterone administration resulted in enhanced progression of disease with increased Th17 cell differentiation. MR blockade with spironolactone prevented aldosterone effects in both *in vitro* and *in vivo* studies (139). A preclinical study showed that MR mediated Th17 proliferation and IL-17 production in peripheral mononuclear cells and in cardiac and renal tissue in DOCA-salt induced hypertensive rats (94). In these DOCA-salt-hypertensive rats MR blockade with either spironolactone, triple antihypertensive drug treatment (reserpine + hydralazine + hydrochlorothiazide), or IL-17 neutralizing antibody treatment, lowered blood pressure and prevented cardiac hypertrophy and glomerular damage (94). However, IL-17 levels remained elevated after triple-antihypertensive drug treatment only, implicating a specific role of IL-17A in MR-mediated hypertension and tissue damage, and that MR blockade not only lowers blood pressure but modulates IL-17 production (94). Taken together these studies provide a possible mechanistic link between aldosterone-MR mediated hypertension and IL-17A. This will be further investigated in the following chapters.

AIMS AND HYPOTHESES

The overall aim of the research projects described in this dissertation was to primarily investigate the association between MR-aldosterone and ENaC in mediating IL-17A production in relation to hypertension and kidney injury and secondarily to elucidate up- and downstream cytokines in relation to IL-17A. This was investigated in four studies and presented in four separate manuscripts. The hypotheses were addressed in existing, biobanked, samples from 3 clinical interventional studies with administration of spironolactone and amiloride, designed originally with other main outcomes (blood pressure, natriuresis, and GFR) and in a preclinical animal study testing the direct impact of IL-17A administration on blood pressure.

THE HYPOTHESES OF STUDY I

MR antagonism by spironolactone selectively suppresses Th-17 derived IL-17A along with other macrophage- (TNF, IL-6, IL-1β, and IL-10) and Th-1 (IFN-γ) cell-derived cytokines, and this relates to blood pressure, albuminuria, and plasma potassium increase in patients with T2DM and resistant hypertension. MR-blockade in *in vitro*-differentiated macrophages suppresses macrophage-derived pro-inflammatory cytokines during LPS stimulated inflammation.

THE HYPOTHESES OF STUDY II

ENaC inhibition with amiloride reduces plasma IL-17A along with other dendritic cell dependent cytokines including IFN-γ (T-cells), IL-1β, IL-6, and TNF (dendritic cells and macrophages) in patients with treatment resistant hypertension and T2DM. This reduction in plasma IL-17A relates to amiloride-mediated reduction in blood pressure, albuminuria, and plasma potassium increase. ENaC-blockade (amiloride and benzamil) in *in vitro*-differentiated macrophages suppresses macrophage-derived pro-inflammatory cytokines during LPS-stimulated inflammation.

THE HYPOTHESES OF STUDY III

MR antagonism by spironolactone reduces IL-17A along with other T-cell (IFN-γ), dendrite (IL-1β) and macrophage (IL-6, TNF, IL-1β)-derived cytokines in plasma and relates to spironolactone mediated blood pressure reduction, epithelial protection, and plasma potassium increase in kidney transplant patients.

THE HYPOTHESES OF STUDY IV

Chronic and acute i.v. infusion of IL-17A with increasing doses in conscious FVB/n mice increases blood pressure by renal and vascular effects respectively. Because of its suggested pro-hypertensive function from previous preclinical studies, IL-17A accentuate ANGII-mediated hypertension in FVB/n mice by renal mechanisms.

Materials & Methods

MATERIALS

PATIENT SAMPLES FROM 2 STUDY COHORTS

SOUTHERN DANISH HYPERTENSION DIABETES STUDY (SDHDS)

Patients with T2DM and resistant hypertension were included in a multicenter, double-blind, randomized, placebo-controlled study (*n*=119) (143). Of these 119 patients 112 completed study protocol. Treatment-resistant hypertension in these patients was defined as daytime average blood pressure ≥ 130/80 mmHg despite treatment with 3 or more anti-hypertensive drugs of different classes, including a diuretic, ACEi and an ARB. Of the 112 patients 57 patients receive 25-50 mg/day spironolactone (MR-antagonist and diuretic) and 55 patients received placebo for 16 weeks (143). Spironolactone was dose titrated from 25mg/day to 50 mg/day after 4 weeks intervention if blood pressure was above 130/80 mmHg. Spironolactone study-dose was halved if office blood pressure was reduced to below 110/60 mmHg and plasma potassium increased to more than 5.5 mmol/L. After a two-week wash-out period, 80 patients were invited to an observational open-label extension study where they received 5-10 mg amiloride per day (132). Patients received 5 mg/day amiloride the first 4 weeks, whereafter study dose was titrated up to 10 mg/day if blood pressure had not reached <130/80 mmHg. Amiloride (ENaC blocker and diuretic) intervention was discontinued if plasma potassium levels increased more than 5.5 mmol/L. Detailed inclusion and exclusion criteria's have been documented previously (132, 143). Plasma samples were obtained at baseline before and after intervention from both studies. The primary outcome parameter of these studies was arterial blood pressure, and it was previously shown that blood pressure was significantly reduced by spironolactone and amiloride in these patients (132, 143). In the present study we measured cytokine concentrations in plasma samples at baseline and after spironolactone (study I) and amiloride intervention (study II). The study protocols were approved by the Ethical Committee of the Region of Southern Denmark (S-20090135, S-20180121) and the Danish Health and Medicines Agency, EudraCT 2009-017033-22. The study was performed in accordance with the Helsinki Declaration and Good Clinical Practice provided by the International Conference of Harmonization (ICH-GCP) rules and was registered at ClinicalTrials.gov as NCT01062763 and NCT02122731. GCP monitoring was done by the Department of Clinical Pharmacology, University of Southern Denmark.

Figure 1

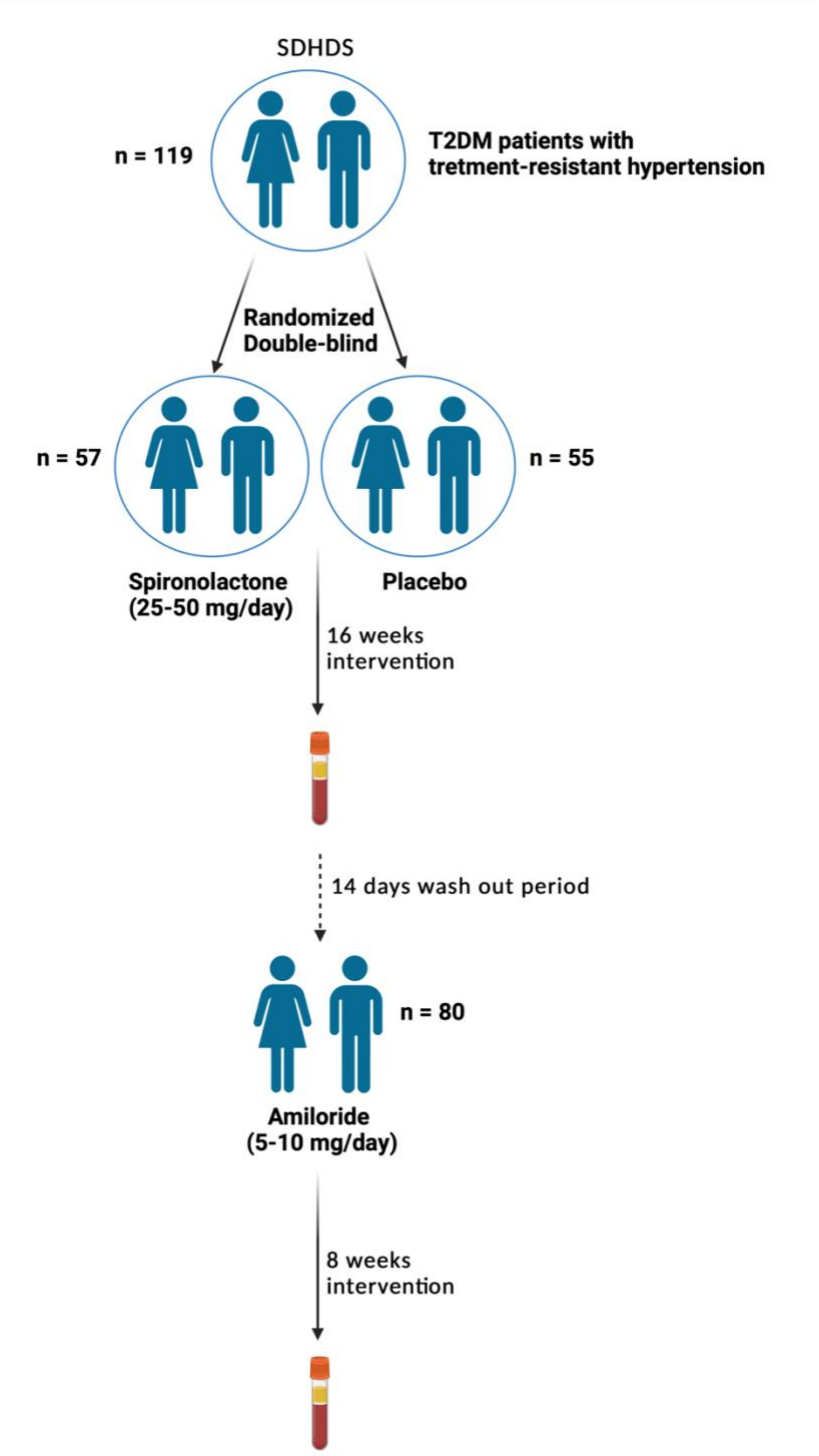

Figure 1: SDHDS study design. T2DM patients with treatment resistant hypertension (*n*=119) were included in a randomized, double-blind, placebo-controlled interventional trial with spironolactone for 16 weeks. After a two-week wash-out period 80 patients were invited to participate in an open-label interventional trial with amiloride (5-10 mg/day) for 8 weeks. In the present study, plasma samples from these patients at baseline and after spironolactone and amiloride intervention were used for cytokine analyses. Figure 1 is created in BioRender.

THE SPIREN TRIAL -KIDNEY TRANSPLANT PATIENTS

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 (144-146). The primary outcome parameters of this study are glomerular filtration rate and kidney fibrosis (data not published). Kidney transplant patients were randomized to spironolactone (25-50 mg/day, *n*=39) or placebo (*n*=41) for 3 years. Paraclinical data, spot urine- and EDTA plasma samples were collected at study inclusion after transplantation before starting the treatment, and after 1 year intervention for the first 80 patients who had completed the SPIREN trial and were used in a previous sub-study analysis (144) and in the present study (study III). In spot urine samples from kidney transplant patients included in the sub-study, urinary kidney injury markers were measured, including calbindin, clusterin, KIM-1, osteoactivin, TFF3, and VEGF at baseline and after 1 year spironolactone intervention, using a multiplex immunoassay (*Mesoscale*). In plasma samples, Th-17-derived (IL-17A), Th1-derived (IFN-γ), and macrophage-derived (TNF, IL-6, IL-1β, and IL-10) cytokines were measured at baseline and after 1 year intervention with spironolactone. 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 (144) (study III).

Figure 2

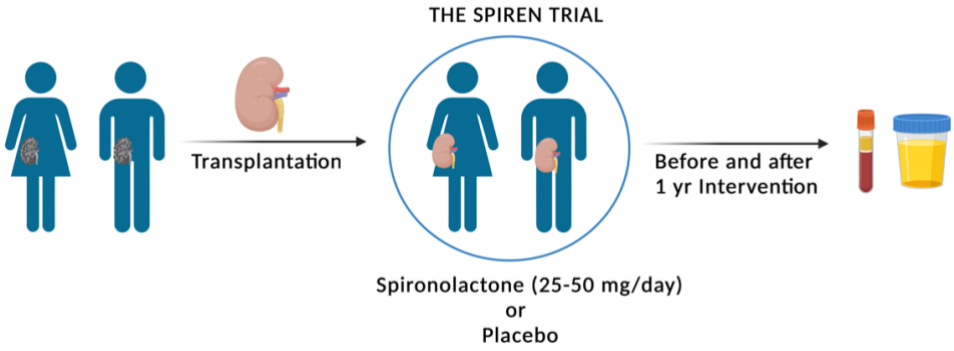

Figure 2: The SPIREN TRIAL. Kidney transplant patients (n = 80) were included in a randomized, double-blind, and placebo-controlled interventional study with spironolactone (25-50 mg/day) or placebo for 1 year. In the present study, plasma- and spot urine samples from these patients at baseline and after 1 year intervention were used for cytokine and urinary kidney injury marker analyses respectively. Figure is created in BioRender.

LABORATORY METHODS

CYTOKINE AND KIDNEY INJURY MARKERS (IMMUNOASSAY)

For measurements of cytokines in plasma and cell culture medium, and urinary kidney injury markers in spot urine samples, the multiplex mesoscale immunoassays were used (Mesoscale). The principle of these assays is similar to enzyme-linked immunosorbent assays (ELISAs) with capture and detection antibodies enabling detection of specific proteins in a well. The multiplex assay consists of a 96-well plate with 10 spot wells, enabling analyses of up to 10 proteins in each well. Biotinylated capture antibodies were coupled to specific linkers, that were able to self-assemble unto unique spots in each well. After analyte incubation, target proteins would have bound to the capture antibody, and recruitment of the detection antibodies (conjugated with an electro-chemiluminescent label - MSD SULFO-TAG) to the capture antibody-bound analytes completes the sandwich immunoassay. Different from a normal ELISA, these assays are developed for quantitative measurements by using an electro-chemiluminescent method, by applying a substrate buffer to the plate and voltage to the plate electrodes, which ultimately leads to light emission by the detection antibody label. The protocol for these multiplex immunoassays has been described in detail in manuscripts I-III.

Figure 3

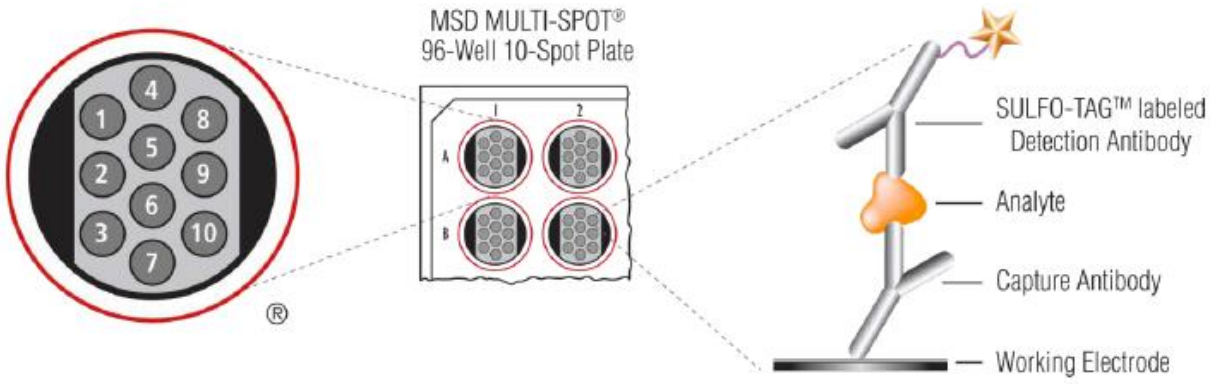

Figure 3: Multiplex plate spot-diagram with illustration of the assay principle. Each spot is connected to a working electrode. Capture antibodies are connected to each spot via specific linkers. Detection antibodies enables chemiluminescent development and quantitative measurements of analytes. Illustration is from Mesoscale protocols.

IN VITRO MACROPHAGE DIFFERENTIATION

The THP-1 cell line (ATCC) is widely used to study human monocytes and macrophages *in vitro* (147-149). In the present studies we used human monocytes from the THP-1 cell line and differentiated these into non-activated M0 macrophages using 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). The protocol has been described in detail in study I and II. The PMA concentration used in this experiment was based on prior titration experiments and determined based on differentiated macrophage cell morphology, heterogeneity, and baseline cytokine secretion levels. Based on prior titration studies, for inflammatory stimulation and conversion into M1 pro-inflammatory macrophages, 10 ng/ml LPS was used for stimulation. The incubation time with LPS was determine by measuring the cytokines TNF and IL-10 after 6 and 24hr where cytokine levels peaked, respectively. This is in accordance with the literature (150).

PARACLINICAL PARAMETERS

Paraclinical parameters from patient samples of each cohort were measured previously by the principal investigators. The parameters included for analyses in the present study are plasma aldosterone, plasma potassium, urine albumin, urine creatinine concentrations, and blood pressure (132, 143, 146, 151).

BLOOD PRESSURE MEASUREMENTS

Blood pressure was measured in all 3 cohorts analyzed for plasma cytokine abundance in this study. In the T2DM patients with resistant hypertension included in both the spironolactone and amiloride interventional trials, blood pressure was measured as 24hr ambulatory measurements and recorded every 15 min. during the day (0700 – 2200h), and every 30 min. in the night (2200 – 0700h) using a validated device (TM2430, A&D Company Ltd. Tokyo, Japan) (152). Appropriately sized cuffs were used covering at least 80% of the circumference of the upper arm. Ambulatory blood pressure measurements were considered valid if at least 2/3 of the planned measurements were available during day and night (132, 143). In kidney transplant patients, 24hr ambulatory blood pressure measurements were recorded at baseline and at yearly visits using the two monitors, Diasys Integra II (Novacor, UK) and TM2430 (A&D Company Ltd. Tokyo, Japan). Measurements were considered valid if at least 20 daytime and 7 nighttime values were obtained (146).

ANIMAL STUDIES

Experimental protocols were approved by the Danish Animal Experiment Inspectorate under the Ministry of Environment and Food of Denmark (approval number: 2020-15-0201-00470, and all procedures were in accordance with the Danish national Institutes of Health. Mice were housed at the Biomedical Laboratory at the University of Southern Denmark. Mice were kept on a 12:12 hours light/dark cycle and had free access to water and normal rodent chow. Three animal studies were conducted to assess the direct chronic and acute effects of IL-17A on blood pressure. For animal experiments male FVB/n mice, 10-12 weeks, (25-35 g) were included. The experimental protocols of these studies have been described in detail in study IV, and a protocol overview will be provided in the following as well.

CONTINUOUS BLOOD PRESSURE RECORDINGS IN CONSCIOUS LIVE NON-STRESSED MICE – IMPLANTATION OF INDWELLING CATHETERS IN THE FEMORAL ARTERY AND VEIN

CATHETERIZATION OF MICE

Mice were acclimatized for one week after arrival at the animal facility. Before surgery, the neck and left groin of the mice were shaved. Mice were anesthetized with ketamin (Ketalar®, 100 mg/kg) and xylazine (Rompun® 10 mg/kg) by i.p. injections. When fully anesthetized, mice were placed on a sterile operation cloth over a heating path (37 °C) that ensured stable body temperature during surgery, and inhalation of 100% oxygen was enabled through nasal tubes. To avoid eyes to dry out during surgery, viscotears gel (Novartis) was applied to the eyes, and to avoid dehydration during surgery, 0.5 ml isotonic NaCl was given s.c. prior to surgery. The surgical areas of the mice were disinfected with iodine, and small incisions were made in the neck and left groin of the mice so that the two catheters could be tunneled s.c. from the neck to the groin and exteriorized through the neck tunnel. At the groin, connective tissue was carefully removed, and the femoral artery and vein carefully separated and mobilized without disturbing the femoral nerves. To implant the catheters in the femoral artery and vein, sutures was placed in the top and bottom of the vein and artery. These sutures were used for arterial and venous stasis to avoid blood outflow when introducing an incision in each vessel, and to secure the inserted catheters. After introducing a small incision in the artery and vein, catheters were implanted and checked for proper i.v. and i.a. infusion and blood withdrawal without leakage (figure 5). Sutures and tissue adhesives ensured stabilization of catheters. At the end of surgery, the groin was sutured and prophylactic antibiotic ointment (Fucidin, 2%,

LEO Pharma) was applied on the sutures. Before suturing the neck, a steel disc covered with vliseline was placed below the skin. In order for the mice to move freely, the exteriorized catheters were protected via a lightweight tethering spring, which in one end was connected to the steel disc in the neck, and in the other end connected to a swivel. Hereby the mice were not disturbed by the catheters or able to grab them and had free mobility in the cages (figure 6). After implantation, catheters were always filled with glucose and heparin (100 U/ml isotonic glucose) at infusion rate 10 ml/hr. For analgesia, after surgery mice were given one bolus s.c. injection of 0.1 mg/kg Temgesic followed by 1 day of continuous infusion of 3.75 mg/kg/day Temgesic.

Figure 4

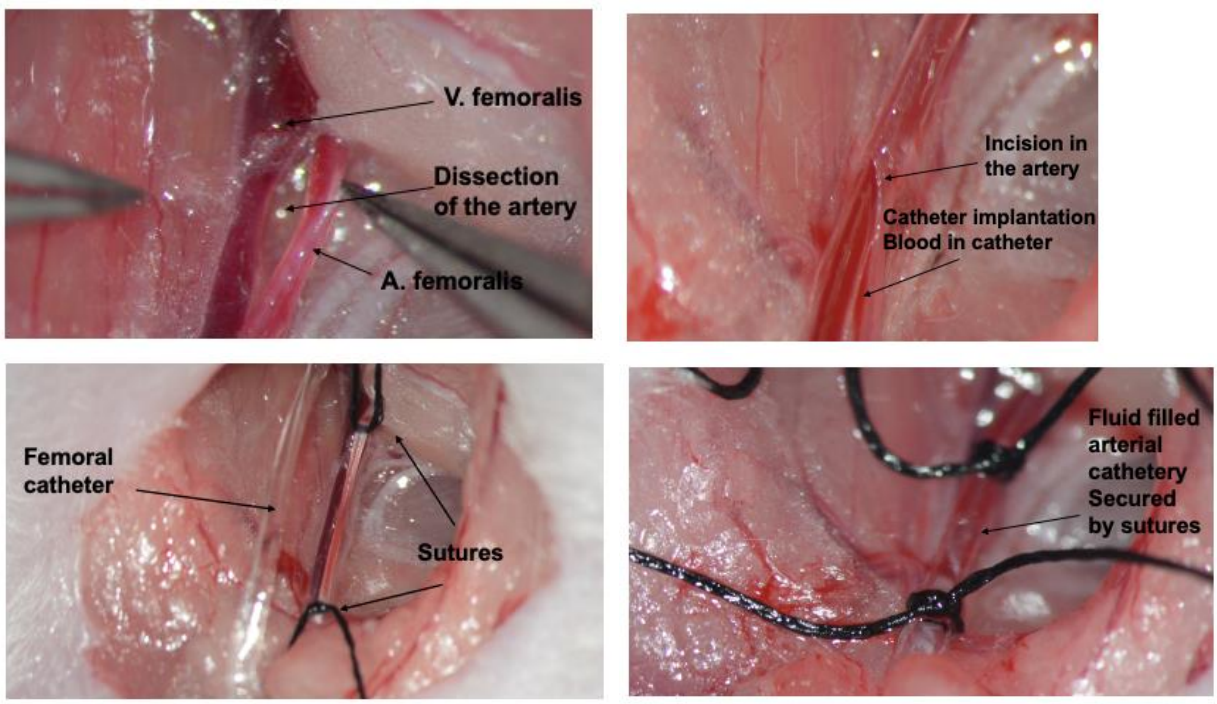

Figure 4: Catheterization of the femoral artery in a mouse. Upper left) Mobilization of the femoral vein and artery. Lower left) Placement of proximal and distal sutures around the artery with catheter in place ready to be inserted. Upper right) A small incision was made, grabbed with a surgical pincette, and the arterial catheter was placed in the artery. Blood is flowing back indicating proper insertion without clotting. Lower right) Catheters filled secured with sutures and filled with saline to test smooth infusion.

BLOOD PRESSURE RECORDINGS

After surgery, mice were housed individually and given a 4-day recovery period whereafter baseline continuous blood pressure recordings were measured, including SBP, DBP, MAP, and heart rate. This was enabled through the arterial catheter which was connected to a pressure transducer (Föhr Medical Instruments GmbH, Germany) that was connected to a computer with access to the LabView software (National Instruments, USA). The pressure transducer was calibrated every time before blood pressure recordings were initiated using a manometer. SBP, DBP, MAP, and heart rate were measured at 100 Hz every 5 min. In bolus infusion experiments, blood pressure was recorded every 10 seconds.

Figure 5

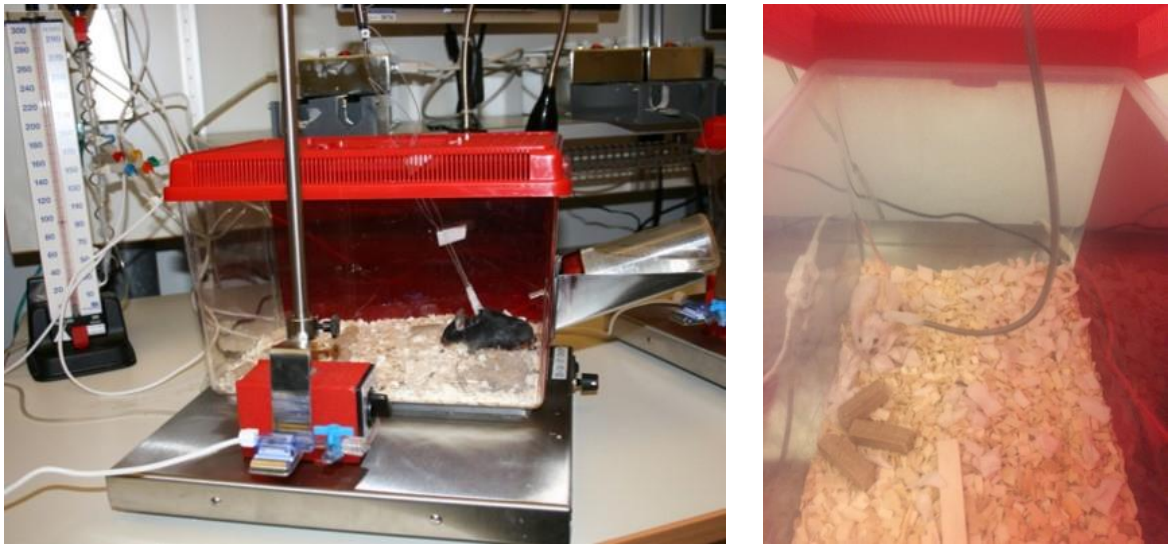

Figure 5: After surgery, each mouse was placed in cages with exteriorized catheter lines connected to a digital transduction system.

AN OVERVIEW OF THE 3 EXPERIMENTAL SERIES

SERIES 1: CHRONIC I.V. IL-17A INFUSION IN MICE – STEP-UP

In this series, the effect of chronic i.v. IL-17A infusion on blood pressure was tested. Three step-up doses of IL-17A were tested, of which the lowest dose was based on previously published experiments where IL-17A was given acutely by i.p. injections. Here 20-22 g male mice were given 1 ug recombinant murine IL-17A daily for 7 days (96, 153, 154). In our experiments, this corresponds to an infusion rate of IL-17A at 3.2 ng/kg/min, whereby mice weighing 22 g would receive 1 ug IL-17A per day. This experiment was conducted using two protocols (A and B)

PROTOCOL 1A)

After 2 days of baseline blood pressure measurements, FVB/n male mice were chronically infused with recombinant murine IL-17A (R&D Systems, E. Coli-expressed) at 3.2 ng/kg/min IL-17A for 2 days, followed by 16 ng/kg/min IL-17A for 2 days and finally 32 ng/kg/min IL-17A for 4 days (n=5). Since the effect of higher concentrations of IL-17 in mice had not been tested before, we only chose one 10-fold step-up, and an intermediate concentration of IL-17A. MAP, SBP, DBP, and heart rate were measured during the whole protocol and mean of every 5 min recordings were collected. Whole blood was collected from conscious undisturbed mice in EDTA vials (max 200 mL, unstressed blood, at baseline and after infusion) and Li-heparin vials (max 400 mL, stressed blood, only after infusion) from the arterial catheter at baseline before IL-17A infusion and after 8 days of IL-17A infusion. At the end of protocol, mice were euthanized by cervical dislocation, and organs were harvested.

PROTOCOL 1B)

After the first series of chronic i.v. infusion of IL-17A in mice, no blood pressure elevating effect of IL-17A was observed, but blood pressure was instead lowered during infusion with the highest dose of IL-17A (32 ng/kg/min). Therefore, the same protocol was repeated with recombinant murine IL-17A from another vendor (BioLegend, Nordic Biosite) using a different expression system (CHO-cells). In this protocol mice received 3.2 ng/kg/min, 32 ng/kg/min, and 320 ng/kg/min for 2, 2 and 4 days, respectively (n=4). Since IL-17A exerted blood pressure lowering effects during 32 ng/kg/min. infusion in the previous series, for this experiment one more step-up concentration was included (320 ng/kg/min.). In parallel with these infusion experiments, two mice received the same doses of the E. Coli expressed IL-17A (R&D systems).

Figure 6

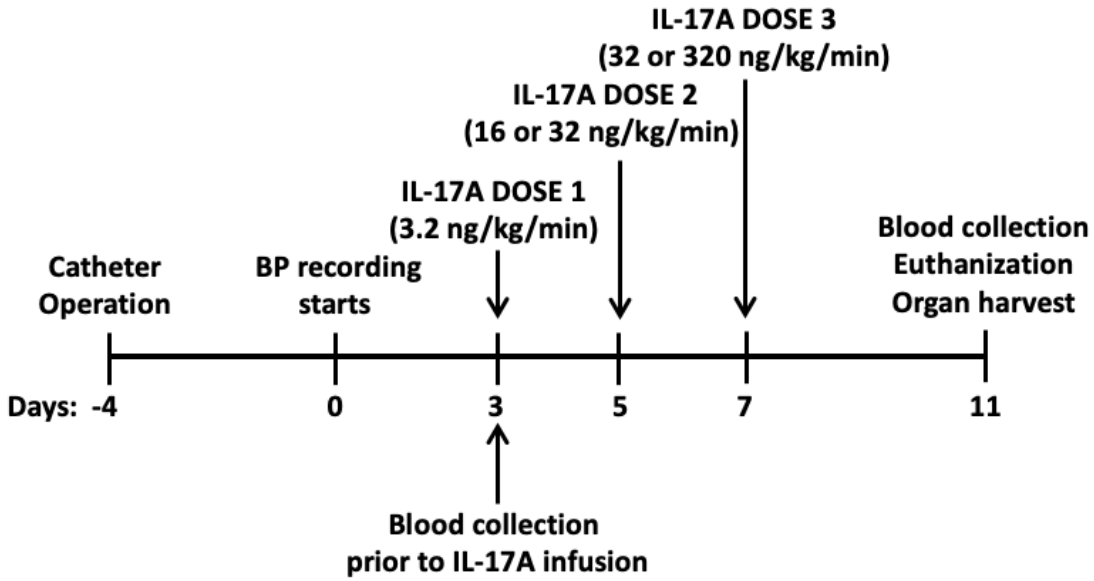

Figure 6: Study overview of protocol 1: Mice received IL-17A after 3 days baseline blood pressure recordings with step-up concentrations of 3.2-16-32 ng/kg/min (n=5) or 3.2-32-320 ng/kg/min. (*n*=4 (BioLegend), *n*=2 (R&D System)) for 2, 2 and 4 days, in two separate protocols.

SERIES 2: ACUTE I.V. BOLUS INFUSIONS

Since there was no difference between the effect exerted by IL-17A from the two different vendors, CHO-expressed recombinant murine IL-17A from the vendor BioLegend, was used in the next two experimental series. Acute bolus infusions were enabled by attaching an infusion pin port™ 25ga with injection (PNP3M-F25, Instech) to the exterior i.v. line from the mice. Mice were infused with 50-100 ul of solution using a manual constant and persistent pressure. At first, one bolus infusion was given with glucose to ensure that the acute infusion by itself did not promote blood pressure changes. Acute administration of IL-17A was done with different doses with a 10 min. interval. After IL-17A infusion, mice were given an i.v. bolus of ANGII (60 ng/kg) and Ach (0.005 mg/kg) by slow infusion over 10 min. to ensure catheter patency. EDTA whole blood was collected at baseline before glucose bolus, and after IL-17A infusion (before ANGII and ACh infusions).

PROTOCOL 2A)

In the first series, mice were first given a 50 ml glucose-heparin bolus followed by IL-17A bolus infusions with the concentrations 455 mg/kg and 1820 mg/kg (*n*=5). The lowest dose of IL-17 bolus (455 mg/kg) corresponds to the daily dose of IL-17A mice would have received in the step-up protocol, i.e., during the 32 ng/kg/min infusion with IL-17A.

PROTOCOL 2B)

In the second series of bolus experiments, mice were given one glucose-heparin bolus followed by four bolus infusions of 50-100 ml of IL-17A with the concentrations 455 mg/kg, 910 mg/kg, 1820 mg/kg, and 3640 mg/kg (*n*=5).

Figure 7

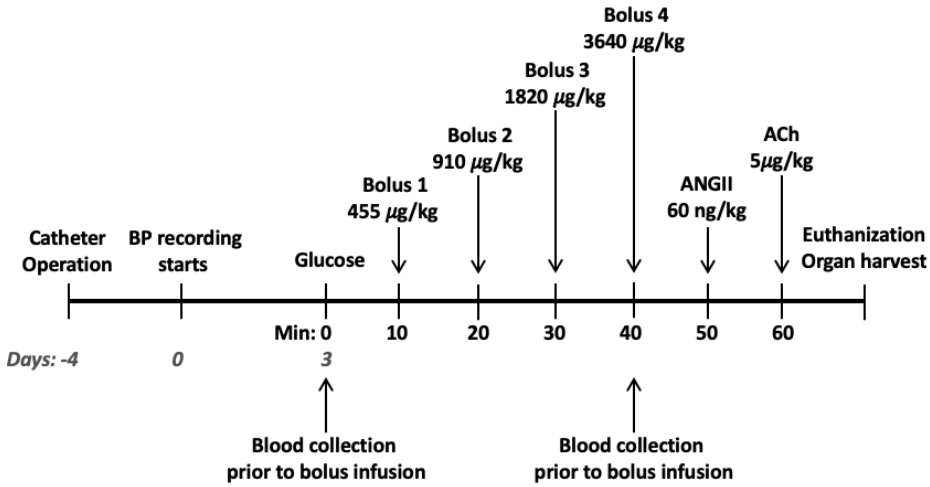

Figure 7: Study overview of protocol 2B: Mice received bolus infusion of IL-17A after 3 days baseline blood pressure recordings. First, a control infusion with glucose was given, then IL-17A was administered with 4 different concentrations of IL-17A (455-910-1820-3640 ug/kg) with a 10 min. interval. At the end of protocol, ANGII and Ach slow infusions were given to ensure catheter patency.

SERIES 3: CONTINUOUS I.V. ANGII CO-INFUSION WITH IL-17A OR VEHICLE

In this experiment, after 3 days baseline blood pressure measurements, mice received either vehicle (saline) or IL-17A (32 ng/kg/min.) for 2 consecutive days, and hereafter ANGII (60 ng/kg/min.) was co-infused with either saline or IL-17A for 7 days. Blood pressure recordings were obtained for the whole protocol every 5 min. Whole blood was collected at baseline and after ANGII infusion as described in protocol 1.

Figure 8

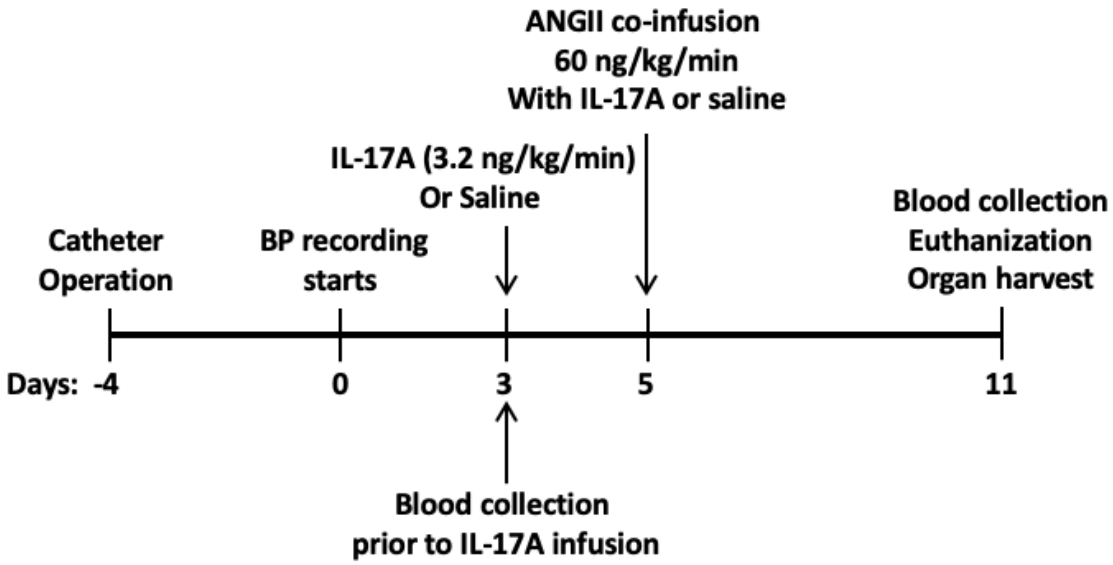

Figure 8: Study overview of protocol 3: Mice received continuous i.v. infusion if IL-17A or saline in two separate groups for 2 days, whereafter mice were ANGII co-infusion with IL-17A or saline for 7 days. Blood was collected at baseline before and after IL-17A infusion.

STATISTICAL ANALYSES

All data were tested for normal distribution using the D'Agostino omnibus or Shapiro-Wilk test. Data that were not normally distributed were subjected to log-transformation to achieve normal distribution. If data were not normally distributed non-parametric tests were used. For cytokine and kidney injury marker analyses, data were presented as mean ± SD (normally distributed data) or median with interquartile range (non-normally distributed data). If data were normally distributed after log-transformation, concentrations were presented as median with interquartile range in the graphical illustrations and in tables. For statistical comparisons between paired samples, paired student's t-test or the Mann Whitney test was used. For comparison between drug intervention at different concentrations in macrophages, and blood pressure at different IL-17A infusion doses, One-way ANOVA with multiple comparisons using Tukey's post-hoc test or the Friedman test with multiple comparisons followed by Dunn' post-hoc test was applied. Blood pressure differences between saline and IL-17A infused mice in ANGII co-infusion studies was compared using the two-way ANOVA with mixed analyses followed by Tukey's multiple comparison test. All statistical analyses were made in GraphPad Prism version 9.

STUDY I

Original Article

The mineralocorticoid receptor blocker spironolactone lowers plasma interferon-γ and interleukin-6 in patients with type 2 diabetes and treatment-resistant hypertension

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Background: The mineralocorticoid receptor antagonist spironolactone lowers blood pressure in patients with resistant hypertension despite antihypertensive treatment with angiotensin-converting inhibitors (ACEi) and angiotensin-II receptor blockers (ARB). In preclinical studies, spironolactone suppresses pro-hypertensive interleukin 17A (IL-17A).

Objectives: Plasma samples were analysed from a randomized, double-blind placebo-controlled trial with spironolactone given to patients with type 2 diabetes mellitus (T2DM) and resistant hypertension on three antihypertensive drugs. We tested the hypothesis that spironolactone-induced antihypertensive effects are associated with suppression of IL-17A and related cytokines.

Methods: Interferon-γ (IFN-γ), IL-17A, tumor necrosis factor-α (TNF-α), IL-6, IL-1β and IL-10 were assessed in plasma with immunoassay in samples before and after 16 weeks of treatment with placebo or spironolactone (12.5-25–50 mg/day).

Results: Spironolactone significantly reduced plasma IFN-γ and IL-6 IL-17A, while TNF-α, IL-1β and IL-10 were unchanged. IL-6 was more sensitive to higher doses of spironolactone. At baseline, serum aldosterone correlated positively with diastolic night blood pressure. Urine albumin/creatinine-ratios correlated positively with plasma IL-6 at baseline. There were no relations between aldosterone and cytokine concentrations at baseline; between cytokine concentration and blood pressure at baseline; and between cytokine concentration decrease and blood pressure decrease, except for IFN-γ, after treatment. The spironolactone-induced elevation in plasma potassium related inversely to blood pressure but not to changes in cytokines. In macrophages *in vitro*, spironolactone suppressed lipopolysaccharide (LPS)-induced TNF-α, IL-6, IL-1β and IL-10 levels.

Conclusion: The antihypertensive action of spironolactone in resistant hypertensive patients is associated with suppressed IFN-γ and IL-6 and not IL-17A. Spironolactone exerts anti-inflammatory actions *in vivo* on macrophages and T-cells.

Keywords: cytokines, macrophages, mineralocorticoid receptor-aldosterone, spironolactone, Th17 cells, treatment-resistant hypertension, type 2 diabetes

Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; ANGII, angiotensin II; ARB, angiotensin receptor blocker; AT1, angiotensin II type 1; BP, blood pressure; DOCA, deoxycorticosterone acetate; IFN, interferon γ; IL, interleukin; IL-17RA, interleukin 17A receptor; LPS, lipopolysaccharide; RAG, recombinase activating gene; RT, room temperature; T2DM, type 2 diabetes mellitus; TNF, tumour necrosis factor

INTRODUCTION

Despite the availability of pharmacologic antihypertensive therapy, 10% of hypertensive patients remain resistant to treatment [1,2]. Patients with diabetes have more prevalent resistant hypertension [2–4]. We showed previously in a double-blind, placebo-controlled, randomized trial that addition of the mineralocorticoid receptor antagonist spironolactone to a three-drug treatment regimen, including a diuretic, in patients with diabetes and poorly controlled hypertension, resulted in a significant decline in SBP and DBPs, decline in albuminuria and achievement of recommended blood pressure goal in 36% of patients [5]. Spironolactone had antihypertensive

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effects despite the fact that patients were treated with angiotensin-converting inhibitor (ACEi) or angiotensin II receptor blocker (ARB) and in 10% of patients, both drugs [5]. This effect was demonstrated by others including the PATHWAY studies [6–9]. As spironolactone is a weak natriuretic/diuretic drug, other targets than increased renal Na^+ excretion are relevant to account for the effect. Several preclinical and clinical studies show that the immune system contributes to the elevated blood pressure in hypertensive patients [10]. When mice lacking T and B lymphocytes (RAG-$1^{-/-}$ mice) were exposed to angiotensin II (ANGII) or deoxycorticosterone acetate (DOCA)-salt to induce experimental hypertension, blood pressure increase was not sustained implicating that T-lymphocytes could promote hypertension [11]. Studies have shown mineralocorticoid receptor expression in T-lymphocytes, dendritic cells and macrophages [12–15]. An ex-vivo study showed that spironolactone decreased lipopolysaccharide (LPS)-induced pro-inflammatory cytokines [16]. Aldosterone-mineralocorticoid receptor activation in dendritic cells promoted Th17 differentiation sensitive to spironolactone and eplerenone [12]. Th17 lymphocytes are source of pro-inflammatory interleukin 17A (IL-17A). DOCA-salt hypertensive rats had increased plasma IL-17A levels compared to rats treated with spironolactone, and anti-IL-17 treatment ameliorated hypertension and heart and kidneys fibrosis [17]. Targeting IL-17A and its receptor (IL-17RA) in ANGII-induced hypertensive mice reduced blood pressure [18]. Interleukin 17A knockout mice are protected against ANGII-induced hypertension [19]. Although mineralocorticoid receptor activation affects Th17 differentiation in mice and plasma IL-17A was increased in hypertensive patients, less is known about a possible effect of MR activation on IL-17A levels in patients [12,19–21]. Anti-IL-17A treatment is beneficial in patients with rheumatoid arthritis and psoriasis but has not been tested in trials on hypertension [22–24].

We hypothesized that treatment with spironolactone suppresses IL-17A in patients, and that this suppression relates to decreases in blood pressure and albuminuria, and that elevated K^+ (+0.3 mmol/l increase) could be involved in cytokine suppression. Increased extracellular [K^+] suppressed T-cell activation and T-cell cytokine production including IL-17A *in vitro* [25–27]. The hypothesis was tested in paired plasma samples collected at entry and after 16 weeks of placebo or spironolactone treatment from 119 patients with type 2 diabetes mellitus (T2DM) and resistant hypertension in a multicentre randomized, double-blind placebo-controlled interventional trial with spironolactone [5]. Patients with T2DM and treatment resistant hypertension typically have low-grade systemic inflammation which made the samples particularly relevant [28,29]. Plasma IL-17A was the major outcome parameter in this study, but other cytokines related to IL-17A production (IL-1β and IL-6), T-cell activation (IFN-γ) and macrophage activation (IL-6, TNF-α and IL-10) were also assessed, as T-lymphocytes and macrophages promote hypertension [11,30]. Thus, the effect of treatment with spironolactone on plasma IL-17A was compared to other T-lymphocyte and macrophage-derived pro- and anti-inflammatory cytokines. Because of positive in-vivo findings, the effect of spironolactone on the production of pro-inflammatory cytokines by macrophages was further analysed *in vitro* with differentiated THP-1 macrophages.

MATERIALS AND METHODS

Patient cohort

Patients with T2DM and resistant hypertension were included in a multicentre, double-blind, randomized, placebo-controlled study ($n = 119$), which has been described previously [5,31]. Patients were recruited by four specialized diabetes clinics in Denmark, two university hospitals (Odense University Hospital, Gentofte Hospital – Steno Diabetes Center Copenhagen) and two general hospitals (Fredericia hospital, Esbjerg Hospital). In short, 119 T2DM patients aged between 30 and 75 years with treatment-resistant hypertension defined as daytime average blood pressure at least 130/80 mmHg despite treatment with three or more antihypertensive drugs of different classes, including a diuretic, ACEi or an ARB, were included [5]. Inclusion and exclusion criteria have been described previously [5]. Of the 119 patients included, 112 completed the study, 57 patients were allocated to receive 25–50 mg spironolactone per day and 55 patients received placebo for 16 weeks. For the first 4 weeks, patients received one tablet of 25 mg spironolactone or placebo, and hereafter, if tolerated, spironolactone was titrated to 50 mg spironolactone daily. The study dose was titrated by halving or doubling the dose from 12.5 up to 50 mg/day according to desired blood pressure (110/60 to 130/80 mmHg). The study dose was halved if office blood pressure was below 110/60 mmHg or if plasma K^+ increased to more than 5.0 mmol/l. Study compliance was evaluated at each visit by counting returned tablets and by interviews. The primary outcome parameter that determined power of the intervention study was arterial blood pressure. Blood was collected in EDTA vials from each patient at their first visit (baseline) and after 16 weeks of intervention. Plasma was separated by centrifugation at 2000 x *g* for 30 min at room temperature (RT) and stored at -80°C. Plasma potassium concentrations at baseline and after intervention were determined previously [5]. Baseline serum aldosterone levels were determined in a previous study [31].

Ethics statement

The clinical trial was conducted in accordance with the Helsinki Declaration and the International conference on Harmonization–Good Clinical Practice rules and was registered at ClinicalTrials.gov as NCT01062763. GCP monitoring was done by the Department of Clinical Pharmacology, University of Southern Denmark. Original study protocols were approved by the Regional Scientific Ethics Committee for Southern Denmark (S-20090135 and S-20180121) and the Danish Health and Medicines Authority (2009–017033-22). The present study was conducted on plasma samples stored in an approved biobank and was *de novo* applied and approved by the regional ethics committee of the Region of Southern Denmark (S-20180121).

Blood pressure measurements

Blood pressure measurements were conducted at baseline and after 16 weeks of intervention in all patients. SBP and DBP were measured by ambulatory monitoring for 24 h every 15 min at daytime and every 30 min at night-time using

a validated device (TM2430; A&D Company Ltd. Tokyo, Japan) [32]. Blood pressure measurements were considered valid if at least two-third of the planned measurements were available during both day and night-time [33].

Cytokine quantification

Plasma samples before and after intervention as well as macrophage culture supernatants were analysed for cytokine concentration by commercially available mesoscale (MSD) multiplex electrochemiluminescence immunoassay (*Mesoscale*, Copenhagen Denmark). Six different cytokines IFN-γ, IL-10, IL-6, IL-1β, IL-17A and TNF-α were assessed using the mesoscale U-PLEX kit. Assays were carried out according to manufactures protocol. In short, 10-spot 96-well plates were coated with six different biotinylated antibodies conjugated to six different plate linkers for 1 h at RT and shaking at 750 rpm on an ELISA plate shaker. Each antibody-linker complex would bind to one of the 10 spots in each well. After coating, all wells were washed three times with PBS with 0.05% Tween-20 (PBS/Tw). Then, analytes and standard calibrators were added to the wells in duplicates. Plasma from LPS stimulated blood (1 μg/ml, 24 h incubation) were included on all plates as a positive control and applied to the wells as duplicates in 1:4 dilution. All samples were incubated for 1 h at RT, shaking at 750 rpm on an ELISA plate shaker. After analyte incubation, wells were washed three times with PBS/Tw and incubated for 1 h with detection antibody. Finally, the 96-well plates were developed and read using Mesoscale 2X read buffer and the MSD instrument reader MESO QuickPlex SQ 120 (*Mesoscale*, Copenhagen, Denmark). Cytokine quantification was based on a four-parameter analysis of fitted calibrator curve obtained by serial dilutions of mesoscale calibrator 1, assessed by the mesoscale Workbench software. Concentrations are given as pg/ml and depending on distribution, data are presented as mean ± SD or median with interquartile range. Interassay variations were assessed for cytokines IFN-γ (12%), IL-10 (8%), IL-1β (11%) and TNF-α (13%). It was not possible to obtain interassay variations for IL-17A and IL-6 since the positive controls detected these two cytokines below and above detection range, respectively, thus no comparable measurements were obtained. Paired samples from the individual patient were analysed on the same plate so that any possible inter-assay variation would not interfere with the differences within each patient. It was pre-hoc determined that values below detection range and low-range values with CV more than 20% were excluded from the dataset. Plasma samples from 56 patients from the spironolactone group and 49 patients from the placebo group were analysed. One patient sample from the spironolactone group and six samples from the placebo group were missing because of usage of plasma for previous analyses.

Cell culture

Human monocytes from the THP-1 cell line were cultured in RPMI medium (Gibco, Thermofisher, Denmark) supplemented with 10% heat-inactivated FBS (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), L-glutamine 292 μg/ml (Gibco) and Sodium pyruvate 1 mmol/l (Gibco) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every third day during culturing. After 3 days of monocyte culturing in 96-well plates (25 000 cells/well in 200 μl), cells were differentiated to macrophages with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h. Then, to wash out PMA, medium was removed and new fresh medium was added to the wells. The cells were allowed to rest for 6 days with change of medium every third day. Differentiated THP-1 macrophages were stimulated with 10 ng/ml LPS (Sigma Aldrich, Denmark) with and without spironolactone at different concentrations (10^{-11}–10^{-7} mol/l) for 6 h. Culture supernatants were then harvested and stored at -20°C for cytokine quantification.

Statistics

Data were tested for normality using the D'Agostino & Pearson test. All results were presented as mean ± SD or if passing normality test otherwise median with interquartile range was applied. Statistically significant differences before and after intervention within each group of intervention in patients were determined using paired Student's *t*-test or Wilcoxon's comparison for normally and non-normally distributed data, respectively. Differences between groups (delta-values) were assessed using unpaired *t*-test (normally distributed) or Mann–Whitney test (non-normally distributed). Pearson correlations were carried out on normally distributed data and spearman correlations were carried out on non-normally distributed. A difference was considered significant if the *P* value was 0.05 or less. Drug interventional studies on THP-1 cells were analysed using one-way analysis of variance (ANOVA) with multiple comparisons using Dunnett posthoc test (normally distributed data) or the Friedman test with multiple comparisons followed by Dunn' posthoc test (non-normally distributed data after log-transformation). All statistical analyses were made in GraphPad Prism version 9 (GraphPad Software, San Diego, California, USA).

RESULTS

Effect of spironolactone on T-cell derived cytokine plasma concentrations in patients with Type 2 diabetes mellitus and hypertension

At baseline, there were no differences in the T-cell-derived cytokines IFN-γ and IL-17A between the spironolactone and placebo groups (Table 1). After 16 weeks of spironolactone intervention, no effect was observed on IL-17A concentration in patients, while IFN-γ plasma concentration was significantly reduced and no changes in the placebo-treated patients were observed (Fig. 1). The difference was robust significant both within and between groups (Table 1). No plasma IL-17A differences after intervention between groups (delta values) were observed (Table 1).

Effect of spironolactone on macrophage-derived cytokine production in patients with type 2 diabetes mellitus and hypertension

Macrophage-derived cytokines IL-6, IL-1β, TNF-α and IL-10 showed no significant difference between the placebo and

TABLE 1. Plasma cytokine levels

Assay	Spironolactone			Placebo			Between group comparison (baseline)	Between group comparison (16 weeks)	Between group comparison of Δ values
	Baseline (pg/ml)	16 weeks (pg/ml)	Within-group comparison	Baseline (pg/ml)	16 weeks (pg/ml)	Within group Comparison			
IFN-γ	11.2 [5.1–17.6] $n=47$	7.6 [4.5–13.7] $n=47$	0.02*	7.9 [5.4–15.0] $n=43$	8.0 [5.2–13.3] $n=43$	0.9	0.1	1.0	0.05*
IL-17A	5.7 [1.3–12.6] $n=37$	7.5 [1.3–14.0] $n=37$	0.1	8.0 [1.1–13.3] $n=25$	7.1 [1.4–13.5] $n=25$	0.9	1.0	0.7	0.4
TNF-α	1.5 ± 0.5 $n=43$	1.5 ± 0.6 $n=43$	0.9	1.4 ± 0.6 $n=41$	1.3 ± 0.6 $n=41$	0.2	0.4	0.4	0.2
IL-6	1.0 [0.6–1.5] $n=43$	0.9 [0.5–1.3] $n=43$	0.03*	1.0 [0.7–1.7] $n=39$	1.0 [0.7–1.4] $n=39$	0.5	0.9	0.4	0.3
IL-1β	0.1 [0.1–0.2] $n=12$	0.2 [0.1–0.2] $n=12$	0.7	0.1 [0.1–0.2] $n=16$	0.1 [0.1–0.3] $n=16$	0.9	0.9	0.7	0.4
IL-10	0.2 [0.2–0.4] $n=43$	0.2 [0.1–0.3] $n=43$	0.1	0.2 [0.2–0.4] $n=37$	0.2 [0.2–0.3] $n=37$	0.0001****	1.0	0.6	0.4

Cytokine analyses. Data are presented as mean ± SD for normally distributed data and median [interquartile range] for data that are not normally distributed. IFN-γ and IL-6 decreased significantly after spironolactone intervention, and IL-10 was likewise decreased after placebo intervention. IFN-γ changes were significantly different between groups, with bigger differences in spironolactone treated patients. No differences were observed between groups (Δ-values). Between and within-group comparisons indicate *P* values of unpaired *t*-test or Mann–Whitney tests. *Indicates $P < 0.05$. ****Indicates $P < 0.0001$. $P < 0.05$ were considered statistically significant.

spironolactone-group at inclusion before treatment (Table 1). Spironolactone reduced significantly the plasma concentration of IL-6 in patients without any changes observed in the placebo-treated group of patients (Fig. 2). This decrease was only seen within and not between groups (Table 1). IL-10 showed a decline in the placebo-treated patients after 16 weeks of intervention (Fig. 2D). No differences were observed between spironolactone and placebo groups when comparing delta-values (Table 1).

Interferon-γ and interleukin-6 production is more sensitive to higher spironolactone doses

Plasma Il-17A, TNF-α, IL-1β and IL-10 were unchanged after 16 weeks of spironolactone treatment with 12.5, 25 or 50 mg/day (figure S1, http://links.lww.com/HJH/B760). Patients treated with the lowest dose of spironolactone (12.5 mg/day) showed no change in plasma IFN-γ and IL-6 upon 16 weeks of treatment (figure S1, http://links.lww.com/HJH/B760). At a dose of 25 mg/day spironolactone, plasma IFN-γ was significantly reduced (figure S1, http://links.lww.com/HJH/B760). Patients treated with 50 mg/day spironolactone showed reduced IL-6, but IFN-γ was unchanged (figure S1, http://links.lww.com/HJH/B760).

Blood pressure relates to serum aldosterone but not to plasma cytokine levels at baseline

Serum aldosterone concentration did not relate significantly to any measured cytokines at baseline (Table 2). In the published trial, average daytime and night-time placebo-corrected blood pressures were reduced by 8.9/3.7 and 9.8/3.2 mmHg, respectively, by spironolactone [5]. No significant correlation was observed between night-time mean arterial pressure (MAP), SBP or DBP and plasma cytokine levels at baseline (Table 2). A statistically significant and positive correlation was found between baseline night-time diastolic blood pressure and serum aldosterone (Fig. 3a). When correlating night-time MAP or SBP with serum aldosterone at ($P = 0.06$ and $P = 0.5$, respectively), there were no significant correlations although at borderline significance for nighttime MAP (Fig. 3b,c). Blood pressure reductions (delta-values) related to plasma cytokine changes only for IFN-γ with a negative correlation to SBP decrease at the level of significance ($P = 0.05$) and a negative relation to night-time MAP at $P = 0.06$ (Table 3).

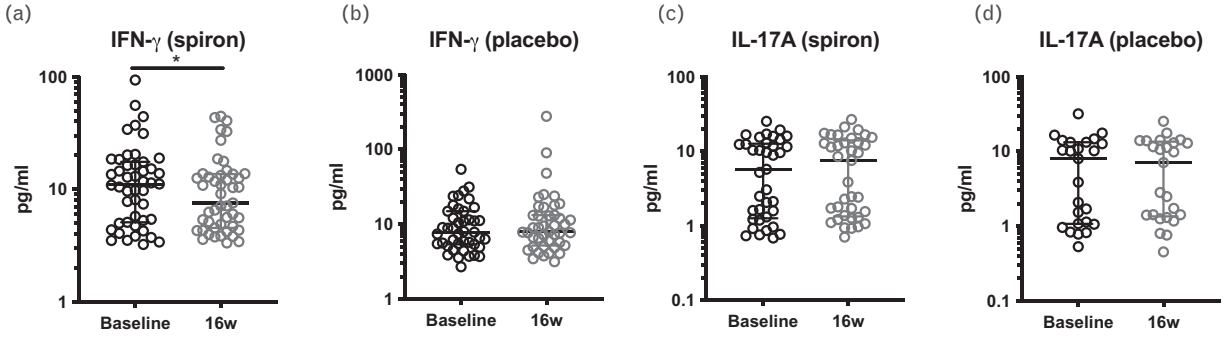

FIGURE 1 T-cell produced cytokine concentrations in plasma from patients with type-2 diabetes and treatment-resistant hypertension before and after 16 weeks treatment with spironolactone or placebo. (a,b), Plasma IFN-γ is significantly reduced ($P < 0.05^*$, $n = 47$) after 16 week spironolactone intervention with no effect in placebo-treated patients ($n = 43$). (c,d) IL-17A plasma concentration was not changed in patients receiving either spironolactone ($n = 37$) or placebo ($n = 25$). Data are presented as median [interquartile range]. $P < 0.05$ corresponds to significant change. Comparisons from baseline to 16 weeks treatement were carried out by paired *t*-test for normally distributed data and Wilcoxon test for data not normally distributed. *P* values less than 0.05 were considered significant.

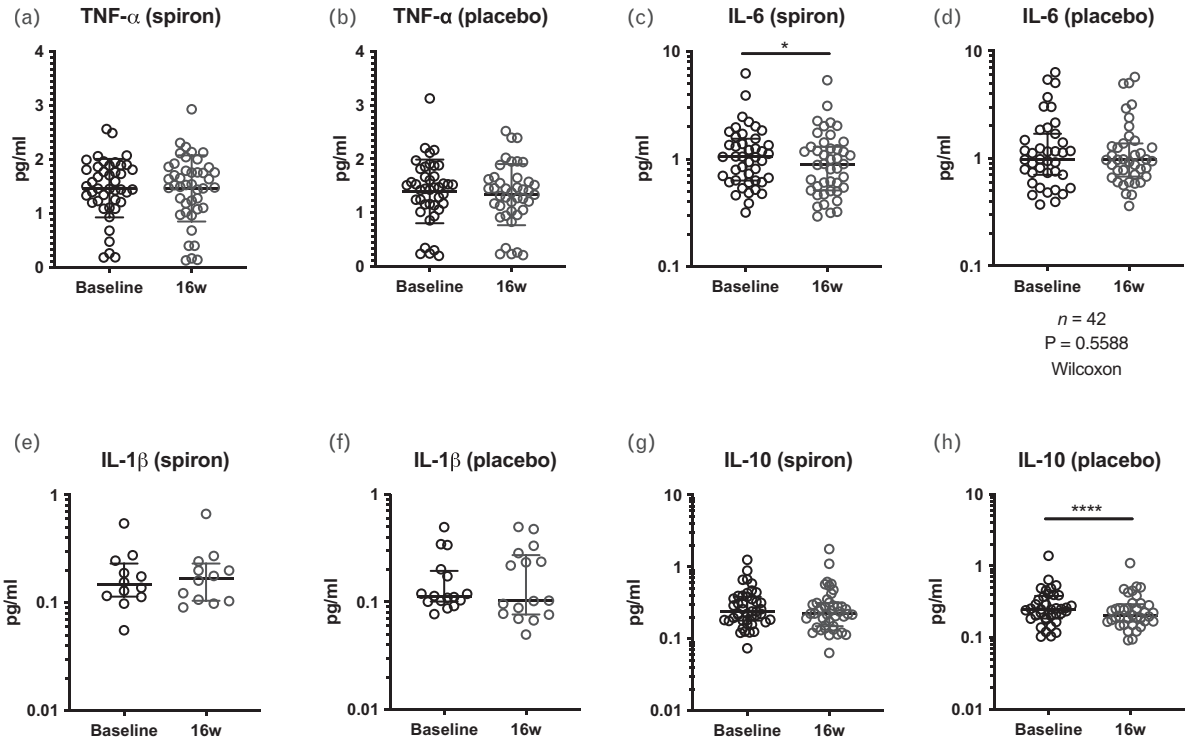

FIGURE 2 Macrophage-produced cytokine concentration in plasma from patients with type-2 diabetes and treatment-resistant hypertension before and after 16 weeks treatment with spironolactone or placebo. (a, b, e, f and g) Plasma TNF-α ($n = 43$), IL-1β ($n = 12$) and IL-10 ($n = 43$) were unchanged after spironolactone intervention. (c,d), Plasma IL-6 ($n = 42$) was significantly reduced ($P < 0.05$ *) after spironolactone intervention with no effect in placebo-treated patients ($n = 39$). (h) Plasma IL-10 decreased significantly after placebo treatment ($P < 0.0001$ ****, $n = 37$). Data are presented as median [interquartile range] for non-normally distributed data (IL-6, IL-1β), and IL-10) and as mean ± SD TNF-α for normally distributed data (TNF-α). $P < 0.05$ were considered statistically significant.

Spironolactone-mediated increase in potassium did not relate to plasma cytokine decrease

Plasma potassium concentration increased significantly from 3.9 (0.4) to 4.2 (0.4) mmol/l upon spironolactone intervention in patients with T2DM and treatment-resistant hypertension [5]. This potassium increase was negatively related to blood pressure changes at a level of significance, $P < 0.01$ (Table 3). The spironolactone-mediated plasma potassium increase (Δ[K+]) did not relate significantly to the spironolactone-induced decrease in plasma IFN-γ or IL-6 levels (Table 3).

Relation between cytokines and albuminuria in response to spironolactone treatment

At baseline, urine albumin/creatinine ratio (UACR) was similar in the two groups, but spironolactone intervention for 16 weeks caused a significant decrease in UACR [5]. Baseline UACR correlated positively with plasma IL-6 levels

TABLE 2. Correlations: p-aldo, MAP or SBP and plasma cytokine (baseline)

	IFN-γ	IL-17A	TNF-α	IL-6	IL-1β	IL-10
MAP	$P = 0.1$ $r = -0.2$ $n = 98$	$P = 1.0$ $r = -0.007$ $n = 79$	$P = 0.8$ $r = -0.03$ $n = 96$	$P = 1.0$ $r = -0.004$ $n = 88$	$P = 0.8$ $r = 0.04$ $n = 46$	$P = 0.8$ $r = 0.03$ $n = 90$
SBP	$P = 0.1$ $r = -0.2$ $n = 98$	$P = 1.0$ $r = 0.007$ $n = 79$	$P = 0.7$ $r = -0.04$ $n = 97$	$P = 0.9$ $r = -0.01$ $n = 89$	$P = 0.7$ $r = 0.06$ $n = 46$	$P = 0.8$ $r = 0.03$ $n = 90$
DBP	$P = 0.08$ $r = -0.2$ $n = 97$	$P = 0.5$ $r = -0.08$ $n = 79$	$P = 0.9$ $r = -0.02$ $n = 96$	$P = 0.9$ $r = 0.009$ $n = 88$	$P = 0.9$ $r = 0.02$ $n = 46$	$P = 0.9$ $r = -0.02$ $n = 96$
s-aldo	$P = 0.5$ $r = 0.07$ $n = 98$	$P = 0.2$ $r = -0.2$ $n = 78$	$P = 0.4$ $r = 0.1$ $n = 97$	$P = 0.3$ $r = 0.1$ $n = 89$	$P = 0.1$ $r = -0.2$ $n = 45$	$P = 0.4$ $r = -0.1$ $n = 92$
UACR	$P = 0.8$ $r = -0.03$ $n = 90$	$P = 1.0$ $r = -0.001$ $n = 74$	$P = 0.1$ $r = 0.2$ $n = 91$	$P = 0.02$* $r = 0.3$ $n = 84$	$P = 0.6$ $r = 0.08$ $n = 43$	$P = 0.07$ $r = 0.2$ $n = 84$
p-IL-6	$P = 0.08$ $r = -0.2$ $n = 87$					

Relation between serum aldosterone, blood pressure, urine albumin/creatinine ratios (UACR) and plasma cytokines at baseline. Serum aldosterone, mean arterial pressure (MAP) or SBP did not relate to plasma cytokine levels of IFN-γ, IL-17A, TNF-α, IL-6, IL-1β and IL-10 at baseline. UACR relates significantly to IL-6 at baseline but not to any other of the other cytokines.

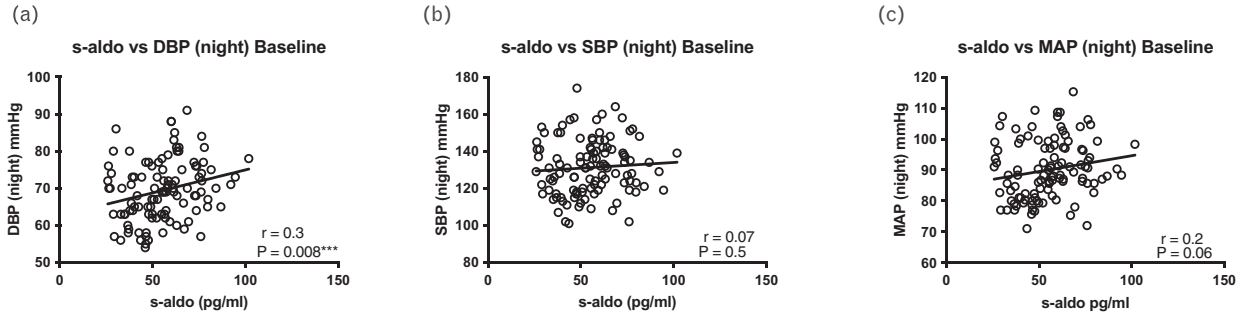

FIGURE 3 Serum aldosterone concentration and blood pressure correlations at baseline. (a) Night-time mean DBP correlates positively with serum aldosterone (s-aldo) concentration at baseline ($P = 0.008^{***}$, $n = 109$). (b,c) Night-time SBP and MAP did not relate to s-aldo ($P = 0.5$, $n = 110$; $P = 0.06$, n = 109, respectively). (c) ($P = 0.06$, $n = 109$). *P* values less than 0.05 were considered significant.

(Table 2). No correlation was observed between UACR and any of the other cytokines at baseline (Table 2). The spironolactone-induced reduction in UACR (delta-value) did not relate to the decreases (delta-value) in plasma IFN-γ or IL-6 concentrations (Table 3). Baseline plasma IL-6 and IFN-γ concentrations were not related (Table 2).

Effect of spironolactone on human macrophages *in vitro*

Addition of LPS to the medium of differentiated THP-1 macrophages resulted in a significant increase of the cytokines IL-10, IL-6, IL-1β and TNF-α upon 6 h of incubation compared with vehicle-treated macrophages (Fig. 4). The largest increase in response to LPS was seen in TNF-α and IL-6 (Fig. 4). As expected, T-cell cytokines IL-17A and IFN-γ were not detected in the conditioned media from THP-1 cells. Coincubation of macrophages for 6 h with LPS and spironolactone (10^{-11}–10^{-7} mol/l) caused a significant concentration-dependent reduction in cytokine release beginning at a concentration of spironolactone at 1×10^{-9} mol/l for TNF-α, IL-6, IL-1β and IL-10 (Fig. 4).

The association of cytokines with BMI, weight, age, sex, smoking and lipids in type 2 diabetes mellitus patients with resistant hypertension

At baseline, plasma IL-6 and TNF-α correlated positively to triglycerides and inversely to HDL and LDL. Baseline plasma IL-10 concentrations related positively to triglycerides, HDL and LDL. Baseline plasma IFN-γ, IL-17A and IL-1β levels did not relate to any of the lipid parametres, and none of the cytokines related to weight, BMI, age, sex, smoking or HbA1c at baseline (table S1, http://links.lww.com/HJH/B761).

DISCUSSION

The present study sought to address mechanisms by which the mineralocorticoid receptor antagonist spironolactone lowers blood pressure in patients with T2DM and treatment-resistant hypertension despite therapy with ACEi and/or ARBs and diuretics [5]. The focus was on inflammation and the pro-hypertensive cytokine IL-17A. We show that baseline aldosterone related to DBP and that spironolactone reduced circulating levels of INF-γ and IL-6 while IL-17A was unchanged. As the anti-inflammatory IL-10 concentration decreased in the placebo-treated group but not in the spironolactone group, IL-10 could be supported or maintained by spironolactone and suppressed by aldosterone. The decrease in IFN-γ related inversely to blood pressure decline and there was a significant relation between IL-6 and albuminuria. The increase in plasma potassium levels related to blood pressure decline but not cytokines. In-vitro experiments confirmed a concentration-dependent suppression by spironolactone of macrophage-derived cytokines. Data are in agreement with a

TABLE 3. Correlations: ΔMAP or ΔSBP and Δplasma cytokine (spironolactone treated)

	ΔIFN-γ	ΔIL-17A	ΔTNF-α	ΔIL-6	ΔIL-1β	ΔIL-10	ΔK^+
ΔMAP	$P = 0.06$ $r = -0.3$ $n = 47$	$P = 0.3$ $r = 0.2$ $n = 36$	$P = 0.4$ $r = -0.1$ $n = 41$	$P = 0.5$ $r = 0.1$ $n = 44$	$P = 0.09$ $r = 0.5$ $n = 14$	$P = 0.5$ $r = -0.1$ $n = 34$	$P = 0.004^{**}$ $r = -0.4$ $n = 50$
ΔSBP	$P = 0.05^{*}$ $r = -0.3$ $n = 48$	$P = 0.3$ $r = 0.2$ $n = 36$	$P = 0.2$ $r = -0.2$ $n = 42$	$P = 0.6$ $r = 0.09$ $n = 44$	$P = 0.1$ $r = 0.4$ $n = 14$	$P = 0.6$ $r = -0.08$ $n = 40$	$P = 0.03^{*}$ $r = -0.3$ $n = 50$
ΔDBP	$P = 0.1$ $r = -0.2$ $n = 47$	$P = 0.2$ $r = 0.2$ $n = 36$	$P = 0.7$ $r = -0.06$ $P = 41$	$P = 0.5$ $r = 0.1$ $n = 44$	$P = 0.09$ $r = 0.5$ $n = 14$	$P = 0.6$ $r = -0.09$ $n = 39$	$P = 0.03^{*}$ $r = -0.3$ $n = 50$
ΔUACR	$P = 0.1$ $r = -0.2$ $n = 40$			$P = 0.9$ $r = -0.02$ $n = 38$			
ΔK^+	$P = 0.4$ $r = 0.1$ $n = 43$			$P = 0.7$ $r = -0.08$ $n = 41$			

Correlations of mean arterial blood pressure (MAP), SBP, DBP or urine albumin/creatinine ratios (UACR) differences and differences in plasma cytokine levels after 16 weeks treatment with spironolactone. IFN-γ change upon spironolactone intervention is negatively correlated to SBP and MAP changes. Potassium changes were negatively correlated with blood pressure changes (MAP, SBP and DBP). *Indicates $P < 0.05$. $P < 0.05$ was considered statistically significant.

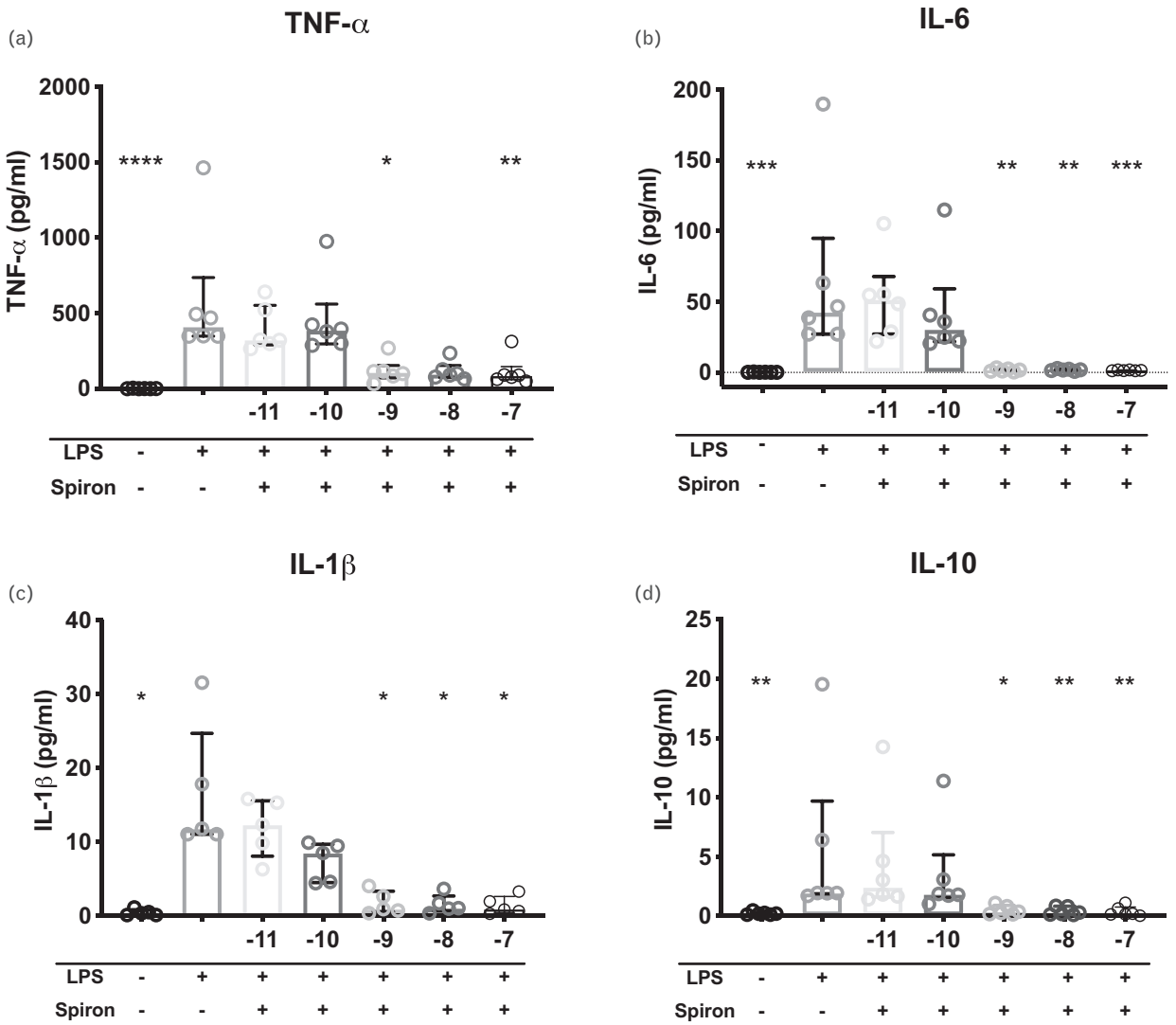

FIGURE 4 Effect of spironolactone on lipopolysaccharide-induced cytokine release from THP-1 (human monocyte cell line) differentiated macrophages. LPS stimulation led to a significant increase of TNF-α, IL-6, IL-1β and IL-10) (from 30 up to 650 times) above vehicle. Co-incubation with spironolactone caused a significant attenuation in the accumulation of TNF-α, IL-6, IL-1β and IL-10 ($n = 8$). Spironolactone was added at concentrations 10^{-11} to 10^{-7} mol/l (shown as -11 up to -7). Data are presented as median with interquartile range in pg/ml. Data were analysed by one-way ANOVA with multiple comparisons using Dunnett posthoc test (normally distributed data after log-transformation - IL-1β and IL-6) or the Friedman test with multiple comparisons followed by Dunn' posthoc test (non-normally distributed data - IL-10 and TNF-α). $P < 0.05$ was considered significant. Spironolactone interventions and vehicle-treated cells were compared to the LPS control. *Indicate $P < 0.05$, *** indicate $P > 0.001$ and **** indicate $P < 0.0001$.

nonredundant effect of spironolactone on blood pressure through reduced inflammation and elevated potassium. Patients suffering from diabetes have prevalent and often difficult-to-treat hypertension associated with low-grade inflammation [34,35]. IL-17A is a salt-sensitive pro-hypertensive T-cell cytokine stimulated by aldosterone [12,17] similar to macrophage-derived cytokines [14,36]. The absolute level of plasma IL-17A in the patients with T2DM and hypertension was comparable to plasma IL-17A concentration in disorders associated with IL-17A, for example psoriasis and rheumatoid arthritis [37–39]. However, the lack of any significant relation between aldosterone and IL-17A before intervention and any change in IL-17A following spironolactone indicates that aldosterone-MR may not have any direct in-vivo effect on IL-17A in humans. This is in contrast to in-vitro cell studies and in-vivo animal studies from mice and rats [17,40]. On the contrary, IL-17 may be more dependent on ANGII than aldosterone, as a direct ANGII type 1 (AT1) receptor-mediated relation has been shown in preclinical studies [18,19]. Patients with hypertension and carotid atherosclerosis treated with telmisartan and/or a cholesterol-lowering statin (rosuvastatin) showed reduced IL-17/Th-17 frequency, Th-17/Treg ratio, blood plasma IL-6, IFN-γ and TNF-α levels upon treatment alone or in combination [41]. ACEi and ARBs reduce IL-17A and suppress Th1-derived cytokines including IFN-γ and IL-6 in preclinical models [40,42]. In these in-vivo studies, the effect of ACEi/ARB could be related to inhibition of the ANGII-AT1 receptor pathway and/or to lower levels of circulating aldosterone and mineralocorticoid receptor

activation. Our study shows that aldosterone-mineralocorticoid receptor signalling could account for stimulatory effects on IL-6 and IFN-γ, while they indicate that IL-17A may depend more on ANGII-AT1 than MR. If IL-17A depends predominantly on ANGII/AT1 receptor interaction, the patients in our cohort would have suppressed IL-17A before receiving spironolactone. On the basis of these data, the hypothesis that the blood pressure lowering effect of spironolactone is related to IL-17A reduction was refuted.

Rather than IL-17A, the present data point at IFN-γ as sensitive to spironolactone in patients *in vivo*. There was a robust decrease in IFN-γ both within and between groups in response to spironolactone although the changes in IFN-γ and blood pressure did not relate directly. These data agree with a contribution of aldosterone-mineralocorticoid receptor to IFN-γ release *in vivo* in patients with T2DM and hypertension. The data fit cross-sectional observations and preclinical studies. Thus, IFN-γ is elevated in patients with hypertension and T2DM and in diabetic kidney disease [43,44]. IFN-γ activates macrophages to release TNF-α, IL-6 and IL-1β [45,46]. Our observation of lower IL-6 agrees with such a relation. IFN-γ suppresses anti-inflammatory cytokines like IL-10 and TGF-β that inhibit macrophage activation [47,48], which also fits our observations of lower IL-10 in placebo group. ANGII or DOCA/salt-induced hypertension in rats increased IFN-γ production [49,50]. Mineralocorticoid receptor deficiency in T-cells decreased IFN-γ in kidneys and aortas [51]. Knockout of IFN-γ in mice blunted the blood pressure response to ANGII [52]. In ANGII-infused mice, IFN-γ receptor knockout caused reduced cardiac hypertrophy, reduced cardiac macrophage and T-cell infiltration and less fibrosis [53]. Although preclinical and clinical studies imply that IFN-γ deficiency protects against hypertension [52] and ARB and statin treatment in hypertension patients lead to a decrease in plasma IFN-γ [41], no mechanistic link between IFN-γ and blood pressure has been shown in patients. The inverse relation between IFN-γ and blood pressure decrease could indicate complex relations and that IFN-γ may not directly affect, for example vascular resistace. We conclude that IFN-γ is a relevant therapeutic target for MR blockers.

The positive correlation between baseline blood pressure and baseline serum aldosterone was only seen for diastolic blood pressure and not for systolic or mean arterial blood pressure. Before intervention at baseline, all patients received at least three anti-hypertensive drugs and at least one ACEi/ARB. This treatment would be expected to decrease serum alsosterone levels. Thus, the observed positive relation between serum aldosterone and blood pressure in patients with T2DM and treatment-resistant hypertension may reflect the aldosterone escape phenomenon wherein aldosterone returns to pretreatment levels despite ACEi/ARB treatment [54].

The data implicate IL-6 as sensitive to spironolactone *in vivo* and *in vitro*. IL-6 is pro-inflammatory and produced by macrophages in response to IFN-γ and associated with blood pressure elevation in human studies [55,56]. ANGII induced IL-6 production through mineralocorticoid receptor in humans [57]. In patients with hypertension, serum IL-6 levels were increased and associated with SBP and treatment with an ARB (irbesartan) decreased IL-6 levels [58]. In a preclinical study with hypertensive rats, treatment with a neutralizing IL-6 antibody reduced blood pressure [59]. Chronic infusion of aldosterone in mice increased IL-6 production sensitive to treatment with eplerenone and anti-IL-6 neutralizing antibody [60]. These observations match the present data and indicate a non-redundant inhibitory effect of spironolactone on IL-6 *in vivo* that could be a consequence of IFN-γ suppression. Moreover, at baseline, IL-6 related directly to albuminuria and spironolactone reduced albuminuria, effects that could be related in the patients.

The in-vitro data on macrophages show a potent and direct cellular reduction of cytokines production by spironolactone. Of interest for the in-vivo interpretation, the in-vitro effect of spironolactone on macrophages may not only be mediated by mineralocorticoid receptor, as spironolactone targets other receptors as well [61,62]. A previous ex-vivo study with monocytes showed that the suppression of cytokines by spironolactone was unrelated to interaction with mineralocorticoid receptor and that aldosterone alone cannot induce cytokine production [16]. Such a possibility should be considered when interpreting the in-vivo additive effect of spironolactone on top of ACEi/ARB. In the present study, we observed no changes in plasma TNF-α and IL-1β in patients after spironolactone while both cytokines were suppressed by spironolactone *in vitro*. This discrepancy could be related to a more dominant contribution of the ANGII-AT1 pathway *in vivo,* masking any residual influence of spironolactone on the production of TNF-α, IL-1β and IL-6 [63].

Our data also revealed that IL-6 was more sensitive to higher doses of spironolactone both *in vivo* and *in vitro*; this was however not the case for IFN-γ *in vivo*. A signicant reduction in IL-6 was observed in patients receiving 50 mg/day of spironolactone and unchanged in patients receiving 12.5 or 25 mg/day spironolactone, and IFN-γ was significantly reduced in patients that received 25 mg/day only. The patients included in this study were all treatment resistant, and higher doses of spironolacton reveal more resistance, thus this could be reflected on cytokine levels as well.

In summary, patients with diabetes and treatment-resistant hypertension experience an additive blood pressure lowering effect of spironolactone associated with systemic suppression of macrophage-derived IL-6 and T-cell IFN-γ. It is concluded that spironolactone exerts anti-inflammatory effects most likely by suppressing selected T-cell and macrophage-derived pro-hypertensive cytokines *in vivo* in patients with diabetes and treatment-resistant hypertension. It can be speculated that such anti-inflammatory effects contribute to the additional antihypertensive effects of spironolactone.

Strengths and limitations

The present study used biobanked plasma samples stored at −80°C for up to 6 years, which could have affected stability of the analytes of interest. Samples did not experience repeated freeze-thaw cycles and were treated alike. In-house inter-assay variability were acceptable. Most values were in the

low measuring range of the assay and variation could thus be relatively more important. With these drawbacks, the study was in patients; it was interventional and placebo-controlled and was powered primarily to detect blood pressure differences. The number of observations were sufficient if cytokines would drive blood pressure changes.

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Conflicts of interest

The authors declare no conflicts of interest.

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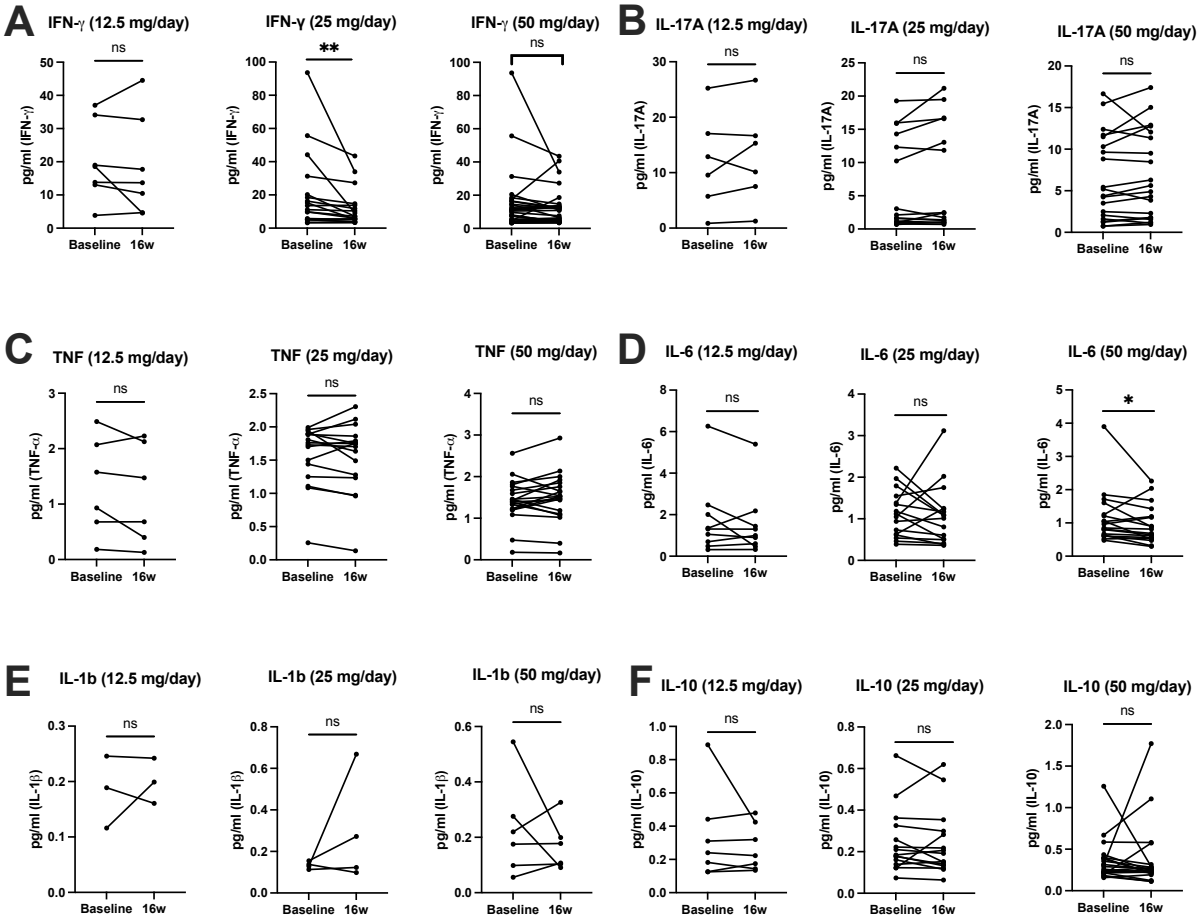
SUPPLEMENTARY FIGURES

Figure S1



STUDY II

Amiloride decreases plasma Tumor Necrosis Factor and Interleukin-6 but not Interleukin-17A in patients with treatment resistant hypertension and type 2 diabetes

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ABSTRACT

Deficiency of the pro-inflammatory cytokine interleukin-17A (IL-17A) is protective against hypertension in animals. Dendritic cells are salt-sensitive and depend on the epithelial sodium channel (ENaC) for activation and to promote Th-17 conversion. We hypothesized that anti-hypertensive treatment with the ENaC blocker, amiloride, and the consequent increase in plasma potassium, reduce plasma IL-17A and other T-helper and macrophage-derived cytokines in patients with diabetes and resistant hypertension. Cytokines (IL-17A, IFN-γ, TNF, IL-6, IL-1β, and IL-10) were determined by multiplex immunoassays in paired plasma samples, before/after amiloride treatment, from patients with type 2 diabetes mellitus (T2DM) and treatment-resistant hypertension included in an interventional, open-label, non-randomized, study (*n*=60, amiloride 5-10 mg/day, 8 weeks). Sensitivity of LPS-stimulated THP-1 macrophages to amiloride and benzamil was determined *in vitro*. In patients with T2DM, baseline mean arterial blood pressure (MAP) and diastolic blood pressure (DBP) correlated positively to baseline plasma IL-1β. Baseline systolic blood pressure (SBP) correlated with IL-17A. Amiloride reduced plasma TNF and IL-6. In THP-1 macrophages, amiloride decreased LPS-induced IL-6, IL-10, and IL-1β, but not TNF (*P*=0.07 at 1-100 nmol/L). The more selective ENaC blocker, benzamil, reduced all 4 cytokines (TNF, IL-6, IL-10, and IL-1β) at 1 nmol/L. Thus, ENaC inhibitors lower macrophage-derived cytokines (TNF and IL-6) *in vitro* and *in vivo* in patients with T2DM and treatment-resistant hypertension. In conclusion, amiloride exerts anti-inflammatory actions *in vivo* relevant for organ protection and hypertension.

Keywords Treatment-resistant hypertension, benzamil, potassium, macrophage, interleukin-17A, macrophages, dendritic cells

INTRODUCTION

The involvement of the adaptive immune system in the pathogenesis of hypertension has been shown in several animal studies. Mice devoid of T-lymphocytes are protected against experimental hypertension, and adoptive transfer of T-lymphocytes into these mice restored their hypertensive responsiveness to angiotensin II (ANGII) (1-4). T-lymphocytes play a pivotal role in the pathogenesis of hypertension. Interleukin-17A (IL-17A)-producing Th-17 lymphocytes have been in focus in both preclinical and clinical studies of hypertension. In animals, plasma IL-17A is increased in ANGII- and deoxycorticosterone acetate (DOCA)-salt induced hypertension, and deficiency or blockade of IL-17A or IL-17A receptor (IL-17RA) in mice reduced blood pressure (5-8). Hypertensive patients with T2DM have increased IL-17A in serum compared to normotensive patients with T2DM (5). Patients with non-controlled hypertension have higher plasma IL-17A than patients with controlled hypertension (9). Th-17 cell proliferation and IL-17A production can be stimulated by high salt concentration *in vitro,* and *in vivo,* high-salt diet enhanced the severity of experimental multiple sclerosis in mice (10-12). The causal relation between IL-17A and blood pressure is less well established in patients. The epithelial sodium channel (ENaC) is expressed in both T and B-lymphocytes and dendritic cells (13-16). Hyperactive ENaC in lymphocytes is related to refractory hypertension (16). ANGII and salt stimulate monocyte, macrophage, dendritic cell, and T-lymphocyte infiltration in the kidneys and vasculature and promote blood pressure elevation, sodium retention, and end-organ damage (5, 14, 17). Dendritic cells are the most potent initiators of immune response and T-cell activation. High sodium concentrations prime and activate dendritic cells to accumulate γ-ketoaldehydes also mentioned as isoketals, which adducts to self-proteins and promote autoimmune-like responses of T-cells (14, 18). These isoketal protein-adducts promote both DOCA-salt and ANGII-hypertension in mice, Th-17 conversion *ex vivo,* and increase the production of pro-inflammatory cytokines produced by dendritic cells (IL-6, IL-1β, and IL-23) and T-cells (IFN-γ and

IL-17A) (14, 18). Adoptive transfer of salt-stimulated dendritic cells into mice that received low dose ANGII, primed hypertension (14). The salt-sensing kinase serum glucocorticoid kinase 1 (SGK-1) mediates salt-induced expression of α and γ subunits of ENaC in dendritic cells, facilitates sodium entering, and promotes hypertension (19, 20). Blocking ENaC with amiloride reduces blood pressure in hypertensive patients (21, 22), and prevents isoketal accumulation and IL-1β production in salt stimulated dendritic cells (14, 18). These studies put together suggest a mechanistic link between salt-sensitive hypertension and dendritic cell ENaC-dependent activation with subsequent stimulation of Th17 cells and IL-17A production. There is very little available information from patients *in vivo* on the significance of ENaC activity for immune cell activation. In the present study it was therefore hypothesized that ENaC blockade with amiloride reduces plasma IL-17A along with other T-cell (IFN-γ), dendrite (IL-1β) and macrophage (IL-6, TNF, IL-1β)-derived cytokines in resistant hypertensive patients with T2DM, and that plasma cytokine levels would relate to blood pressure. Patients with diabetes would a priori be well suited to address the question since they have subclinical inflammation with elevated levels of pro-inflammatory cytokines including IL-1β, IL-6, and TNF (23-26). *In vitro* studies have shown that increased extracellular potassium levels inhibit T-cell activation and IL-17A production (27-29). It was further hypothesized that an amiloride-induced reduction in plasma cytokines would relate to plasma potassium elevation. Third, since cytokines could mediate end organ injury, it was hypothesized that the cytokine level and changes relate to u-albumin. The hypotheses were tested in stored plasma samples from patients with T2DM and hypertension who participated in a clinical trial where amiloride lowered blood pressure and albuminuria (21). This cohort included patients with T2DM and treatment resistant hypertension from an open-label intervention study with amiloride (5-10 mg/day for 8 weeks). Concentrations of IL-17A, IFN-γ, IL-6, TNF, IL-1β, and IL-10 were determined in paired plasma samples before and after

amiloride intervention. Positive *in vivo* findings prompted experiments *in vitro* with macrophages treated with LPS and ENaC blockers directly.

METHODS

Patient cohorts

Type 2 diabetes patients with treatment resistant hypertension

Type 2 diabetes patients with treatment resistant hypertension were included in a multicenter, double-blind, randomized, placebo-controlled spironolactone interventional study (30). Followed by a two-week wash-out period after the double-blind trial, 80 patients were invited to an observational open-label extension study where they were treated with 5-10 mg amiloride per day. Of these patients, complete datasets of urine and blood pressure analyses were available from 60 patients, and for plasma analyses, dataset were available from 70 patients (21). The study protocols were approved by the Ethical Committee of the Region of Southern Denmark (S-20090135, S-20180121) and the Danish Health and Medicines Agency, EudraCT 2009-017033-22. The study was performed in accordance with the Helsinki Declaration and Good Clinical Practice provided by the International Conference of Harmonization (ICH-GCP) rules and was registered at ClinicalTrials.gov as NCT01062763 and NCT02122731 (21). GCP monitoring was done by the Department of Clinical Pharmacology, University of Southern Denmark. Briefly, patients aged between 30-75 years and were treatment-resistant hypertensive defined as blood pressure >130/80 mmHg measured as day-time average by ambulatory blood pressure monitoring, despite 3-drug antihypertensive treatment of different classes, including angiotensin converting enzyme inhibitor (ACEi) and angiotensin II receptor blockers (ARBs). After 4 weeks of amiloride intervention with 5 mg/day, the dose was titrated up to 10 mg/day if blood pressure did not reach less than 130/80 mmHg. If potassium increased above 5.5 mmol/L amiloride intervention was discontinued. Patients were considered

compliant when taking more than 80% of study medication, which was decided by counting returned tablets. The primary outcome of the previous study was significant blood pressure reduction. Average daytime blood pressure was reduced by 6.0/3.7 mmHg (P<0.001/P<0.0001) (21). Urine albumin creatinine ratios (UACR) were significantly reduced (21). In the present study, plasma samples before and after amiloride intervention were analyzed for cytokine abundance by commercially available immunoassays.

Cytokine quantification

Using the mesoscale U-PLEX kit, 6 cytokines IFN-γ, IL-10, IL-6, IL-1β, IL-17A, and TNF were quantified in different analytes including plasma samples and cell culture medium. The multiplex assays were carried out as per manufactures protocol. In short, 10-spot 96-well plates were coated with 6 biotinylated antibodies conjugated to 6 plate linkers. All plasma samples or cell culture medium was added to wells undiluted or diluted 1:5, respectively in duplicates along with standard calibrators. All samples were incubated with shake O/N at 4°C. After detection antibody incubation, plates were developed and read using Mesoscale 2X read buffer and the MSD instrument reader MESO QuickPlex SQ 120. 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. Concentrations are given as pg/ml and data are presented as median with interquartile range. Paired samples from T2DM patients (n=70) were analyzed on the same plates so that any possible inter-assay variation would not interfere with the differences within each patient. Plasma cytokine concentrations below detection range or with CV>20% were excluded from analyses.

Cell culture

Human macrophages were differentiated from the human monocyte cell line (THP-1, ACCT). Cells were cultured in RPMI medium (Gibco™, ThermoFisher Scientific) supplemented with 10% heat-inactivated FBS (Gibco™, ThermoFisher Scientific), 100 U/ml penicillin (Gibco™, ThermoFisher Scientific), 100 ug/ml streptomycin (Gibco™, ThermoFisher Scientific), L-glutamine 292 ug/ml (Gibco™, ThermoFisher Scientific) and Sodium pyruvate 1 mM (Gibco™, ThermoFisher Scientific) and cultured at 37 °C and 5% CO_2. THP-1 monocytes were seeded in in 96-well plates (25.000 cells/well in 200 ul), and cultured for 3 days until the confluence of approximately 100.000 cells/ml. The culture medium was refreshed on day 3, 7, and 10. On day 3, monocytes were differentiated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 hours. Then, PMA was washed out by refreshing the medium. The cells were allowed to rest for 6 days with change of medium on day 7. On day 10 culture medium was refreshed again and the differentiated THP-1 macrophages were stimulated with 10 ng/ml LPS (Sigma Aldrich) with and without amiloride or benzamil (Sigma-Aldrich) at different concentrations (10^{-11}-10^{-7} mol/L) for 6 hours. Culture supernatants were then harvested and stored at -20 °C for cytokine quantification.

Statistics

All cytokine results are shown as paired lined figures. Statistical significance between baseline and after amiloride intervention in plasma cytokine concentrations were determined using paired Student's t-test when normal distributed or Wilcoxon's test for non-parametric tests. A difference was considered significant if the P-value was less than 0.05. The interventional effect of amiloride and benzamil on THP-1 differentiated macrophages were analyzed by One-way ANOVA or Friedmann's test with repeated measures followed by Tukey's or Dunn's multiple comparison tests respectively. For normally distributed data Pearson's correlations were carried out and for non-

normally distributed data spearman correlations were performed. Statistical analyses was performed in GraphPad Prism version 9.

RESULTS

Effect of amiloride on T-cell-derived cytokine production in patients with T2DM and resistant hypertension

In T2DM patients with treatment-resistant hypertension, plasma IFN-γ and IL-17A concentrations did not change in response to amiloride intervention (5-10 mg/day for 8 weeks) (figure 1A-B).

Effect of amiloride on macrophage-derived cytokine production in patients with T2DM and resistant hypertension

In patients with T2DM a minor, but statistically significant, decrease was observed in plasma TNF and IL-6 concentrations in response to amiloride (figure 2A-B). Plasma IL-1β was unchanged after amiloride intervention (figure 2C), and a tendency towards a decrease in plasma IL-10 was observed (*P*=0.08, figure 2D).

The relation between plasma cytokines, blood pressure, and urine albumin/creatinine ratios in T2DM patients

Amiloride lowered mean blood pressure in patients with T2DM by 6.3/3.0 mmHg (21). At baseline, plasma TNF and IL-6 concentrations did not correlate to blood pressure, and the amiloride-mediated reduction in these cytokines did not correlate significantly to blood pressure decrease (table 1). Plasma IL-1β correlated positively to MAP and DBP, but no relation was observed after amiloride intervention (table 1). Plasma IFN-γ related inversely to SBP, and plasma IL-17A related positively to SBP at baseline (table 1). UACR was significantly reduced by amiloride in this trial (21). Baseline

UACR correlated positively to baseline SBP (figure 3B), but not to MAP or DBP (figure 3A, C). Baseline UACR correlated positively to plasma IL-17A, TNF, IL-6, and IL-1β (table 1).

The relation between plasma potassium, blood pressure, and plasma IL-17A, TNF, and IL-6 after amiloride intervention

Plasma potassium in patients with T2DM and resistant hypertension was significantly increased by 0.5 (SD, 0.6) mmol/L after amiloride intervention (21). In these patients, amiloride mediated plasma potassium elevation did not relate to plasma TNF or IL-6 decrease, or blood pressure reduction either (table 1).

The effect of ENaC blockade on in vitro human macrophage cytokine production

THP-1 differentiated human macrophages (*n*=6) were stimulated with LPS (10 ng/ml) for 6 hours with addition of amiloride and benzamil at increasing concentrations in separate experiments (10^{-11}-10^{-7} mol/L) or with vehicle (PBS). LPS stimulation alone significantly increased the release of TNF, IL-6, IL-1β, and IL-10 compared to vehicle-treated cells. Co-incubation with amiloride and LPS for 6 hours had no effect on TNF secretion (*P*=0.07 at 1-100 nmol/L), but decreased IL-1β significantly at 1 nmol/L amiloride, and IL-6 and IL-10 were decreased at 10-100 nmol/L amiloride. Co-incubation with benzamil, a more specific and potent ENaC blocker, significantly reduced cytokine accumulation of TNF, IL-6, IL-1β, and IL-10 at 1 nmol/L (figure 4).

DISCUSSION

The present study showed that in patients with T2DM and treatment-resistant hypertension where amiloride lowered blood pressure and albuminuria (21), there was a reduction in macrophage-derived plasma TNF and IL-6, while IL-17A and IL-1β were unchanged. *In vitro,* human macrophages were sensitive to benzamil in the nanomolar concentration range, supporting a direct role for ENaC in immune cell activation.

Increased UACR is independently associated with higher risk of hypertension in the general population (31, 32). In the present study, UACR correlated positively to SBP in T2DM patients with hypertension at baseline. Furthermore, plasma TNF, IL-6, and blood pressure decreased after amiloride treatment in T2DM patients but there was no significant correlation between these parameters. This however, does not exclude a relation. In preclinical studies, targeting pro-inflammatory cytokines like TNF, IL-6, IL-1β, and IL-17A with neutralizing antibodies or gene deletion, lowered blood pressure in different experimental hypertension models (1, 5, 7, 33-36). Clinical trials testing impact of cytokines directly are limited but it has been shown that suppression of TNF significantly reduced blood pressure in patients with rheumatoid arthritis (37). IL-1β suppression reduces cardiovascular event rates but did not influence blood pressure in humans (38). Suppression of IL-17A in psoriasis and arthritis patients is an efficient anti-inflammatory approach, but whether blood pressure is affected in these patients has not been investigated directly so far (39-42). In the present study, we found that IL-17A was not affected by amiloride which indicates that IL-17A may not depend on ENaC activity *in vivo* in patients with T2DM and resistant hypertension. The patients in the present protocols all received ACEi/ARB as part of inclusion and diuretic treatment. This alters the sensitivity of IL-17A and IFN-γ production in hypertensive patients (43-45). Thus, ACEi/ARB suppression of IL-17A in the analyzed patients could shadow for minor, amiloride-mediated, anti-inflammatory effects on IL-17A. Th-17 cell differentiation and IL-17A

production can be induced by the cytokines IL-1β and TGF-β (46). Studies have shown that salt-stimulated dendritic cells increase IL-1β production, promote hypertension, and Th-17 cell conversion with IL-17 production from naïve T-cells (14, 18). IL-1β is elevated in hypertensive patients and is related to age-related hypertension (47). In cystic fibrosis patients, ENaC-mediated sodium influx in PBMCs leads to inflammasome activation and downstream IL-1β production (48). These studies suggest a mechanistic link between ENaC-mediated stimulation of inflammatory cytokines including IL-1β and IL-17. However, *in vivo* there were no changes in plasma IL-1β or IL-17A after amiloride intervention in T2DM patients with resistant hypertension.

Studies have shown expression of the α and γ subunits of ENaC in dendritic cells and circulating lymphocytes (13, 14), but to our knowledge, the full complement of subunits is not present. The α-subunit is the minimal requirement for conduction of current and channel activation. We previously found in patients with T1DM that administration of 20-40 mg amiloride yielded plasma concentrations of 79 ± 7 nmol/L 12 hours after administration (49). This suggests that the significant effects on cytokines are likely related to ENaC and not to other known targets of amiloride, including sodium hydrogen exchanger 3 (NHE3), α-adrenoceptors, and urokinase plasminogen activator (uPA) with half-maximal inhibition (IC50) in the micromolar concentration range, corresponding to 100-1000 times higher concentrations than obtained in patient plasma. The *in vitro* experiments suggested that macrophages stimulated by LPS via TLR depend on ENaC activity directly based on the low concentrations of amiloride and benzamil. Macrophages were stimulated and activated by high salt concentrations and accumulated isoketal protein-adducts similar to dendritic cells and this was mediated by ENaC activity (14). Alpha and γ subunits of ENaC were detected in salt stimulated splenic $CD11c^+$ antigen-presenting cells from mice. $CD11c^+$ is abundantly expressed on dendritic cells, monocytes, and macrophages and is a known marker of antigen-presenting cells. Studies elucidating the anti-inflammatory effects of ENaC blockade with amiloride are scarce, however, *ex*

vivo studies have shown that amiloride treatment of monocytes from cystic fibrosis patients alleviated augmented cytokine production (48), and LPS-stimulated alveolar macrophage-derived TNF was significantly lowered by amiloride treatment *ex vivo* (50). The present data confirm these studies and show that specific blockade of ENaC exerts anti-inflammatory effects by reducing TNF and IL-6.

Amiloride treatment mediated an increase in plasma potassium concentration (21). Studies show that elevated extracellular potassium concentrations mediated membrane depolarization of T-lymphocytes and suppress Ca^{2+} influx which is needed for T-cell activation and T-cell cytokine production, including IL-17A (27-29). Pro-inflammatory cytokine response correlates with increased expression and activity of potassium channels, and blockade of these suppress T-cell proliferation and inflammation (27, 28). Amiloride elevated plasma potassium significantly in the present study, but blood pressure reduction and plasma TNF and IL-6 changes did not relate to plasma potassium increases in these patients.

In summary, the present study shows that ENaC blockade with amiloride in hypertensive patients does not affect plasma IL-17A or IL-1β, but TNF and IL-6 are significantly reduced. This is supported by *in vitro* amiloride and benzamil intervention studies using LPS-stimulated macrophages. It is concluded that amiloride exerts direct anti-inflammatory effects by suppressing macrophage-derived TNF and IL-6 in hypertensive patients with diabetes. It can be speculated that this effect of amiloride could contribute to blood pressure-lowering and organ protection.

Strengths and limitations

The present study was not powered with cytokines as the primary outcome but included 70 patients, which sufficed to detect small differences. The study was not placebo-controlled and should therefore be considered explorative. The majority of patients were Caucasians, while persons of African descend display higher salt and ENaC sensitivity which may have underestimated the effect. All

patients had T2DM, and therefore some degree of low-grade inflammation compared to essential hypertensive patients without diabetes. Samples were stored for 4-5 years at -80 °C before analyses which may have affected cytokine stability and absolute levels. Used samples were not subjected to multiple freeze-thaw cycles. All patients received ACEi/ARBs during and before intervention, and since these anti-hypertensive drugs have previously shown to have anti-inflammatory effects there is a chance that the plasma cytokines analyzed in these patients may already have been suppressed at baseline. With these limitations, the data were interventional and from the human patient setting and thus provide valuable and important information that could supplement the multiple preclinical studies on this topic.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figures Legends

Figure 1: T-cell-derived plasma cytokines in hypertensive patients with T2DM. A-B) Plasma IL-17A and IFN-γ were unchanged in T2DM and patients after amiloride intervention. Data are presented as paired samples in pg/ml and compared by paired Wilcoxon's test. $P<0.05$ was considered significant.

Figure 2: Macrophage-derived plasma cytokines in hypertensive patients with T2DM. A+B) In patients with T2DM and resistant hypertension, plasma TNF and IL-6 were significantly decreased after amiloride intervention. **C+D)** Plasma IL-1β and IL-10 were unchanged. Data are presented as paired samples in pg/ml and compared by paired Wilcoxon's test. *$P<0.05$ was considered significant.

Figure 3: The relation between blood pressure and urine albumin creatinine ratios (UACR) at baseline in patients with T2DM and resistant hypertension. A+C) Mean arterial pressure (MAP) and diastolic blood pressure (DBP) did not correlate to UACR at baseline. **B)** Systolic blood pressure (SBP) correlated positively with UACR at baseline. Correlations were done with Spearman's correlation test.

Figure 4: ENaC blockade in THP-1 differentiated macrophages inhibited cytokine production. A-D) Amiloride co-incubation with LPS reduced IL-6, IL-1β, and IL-10 cytokine production compared to LPS/vehicle-stimulated macrophages (n=6). **E-H)** Macrophage cytokine production was more sensitive to and significantly lowered by benzamil co-incubation. Data are presented as median with interquartile range in pg/ml, and statistical comparisons were performed using the Friedmann's test. P<0.05 was considered statistically significant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Tables

Table 1: The relation between plasma cytokines, blood pressure, UACR, and plasma K^+ at baseline and after amiloride intervention in patients with T2DM and resistant hypertension

Patients with T2DM and treatment resistant hypertension (baseline)				
	MAP	**SBP**	**DBP**	**UACR**
p-IFN-γ	P = 0.059	P = 0.02*	P = 0.2	P = 0.9
	r = -0.3	r = -0.3	r = -0.2	r = -0.02
p-IL-17A	P = 0.1	P = 0.02*	P = 0.4	P = 0.006**
	r = 0.2	r = 0.3	r = 0.1	r = 0.4
p-TNF	P = 0.3	P = 0.2	P = 0.6	P = 0.0009***
	r = 0.1	r = 0.2	r = 0.1	r = 0.4
p-IL-6	P = 0.8	P = 0.8	P = 0.8	P = 0.04*
	r = -0.04	r = -0.04	r = -0.03	r = 0.3
p-IL-1β	P = 0.04*	P = 0.06	P = 0.05*	P = 0.02*
	r = 0.4	r = 0.4	r = 0.4	r = 0.4
p-IL-10	P = 0.3	P = 0.2	P = 0.5	P = 0.2
	r = 0.2	r = 0.2	r = 0.1	r = 0.2
Patients with T2DM and treatment resistant hypertension (Δ – values)				
Cytokines	**ΔMAP**	**ΔSBP**	**ΔDBP**	**ΔK^+**
Δp-IFN-γ	P = 0.8	P = 0.6	P = 0.6	
	r = -0.05	r = -0.1	r = 0.1	
Δp-IL-17A	P = 0.07	P = 0.04*	P = 0.2	
	r = 0.-0.3	r = -0.3	r = -0.2	
Δp-TNF	P = 0.8	P = 0.5	P = 0.5	P = 0.1
	r = 0.03	r = 0.1	r = -0.1	r = -0.2
Δp-IL-6	P = 0.4	P = 0.4	P = 0.9	P = 0.2
	r = -0.1	r = -0.1	r = -0.02	r = -0.2
Δp-IL-1β	P = 0.4	P = 0.5	P = 0.2	
	r = 0.2	r = 0.1	r = 0.3	
Δp-IL-10	P = 0.2	P = 0.1	P = 0.7	
	r = -0.2	r = -0.3	r = -0.1	
ΔK^+	P = 0.5	P = 0.5	P = 0.9	
	r = -0.1	r = -0.1	r = -0.02	

Figure 1

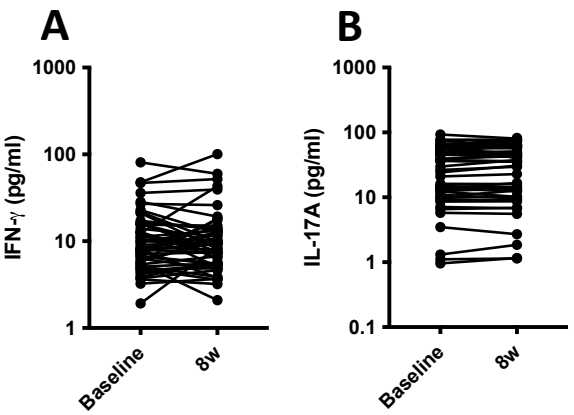


Figure 2

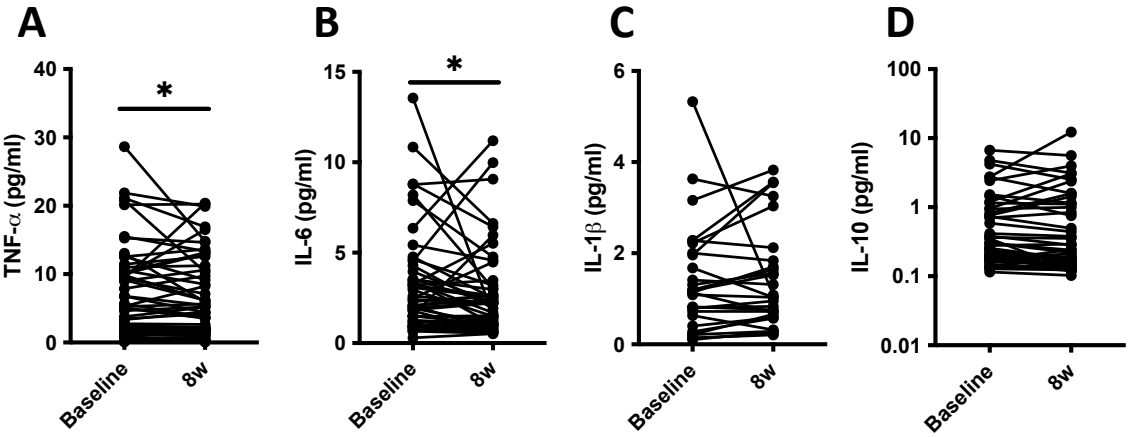


Figure 3

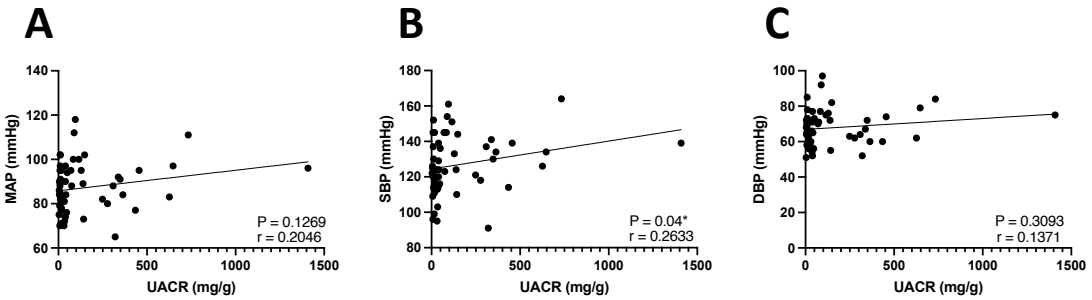
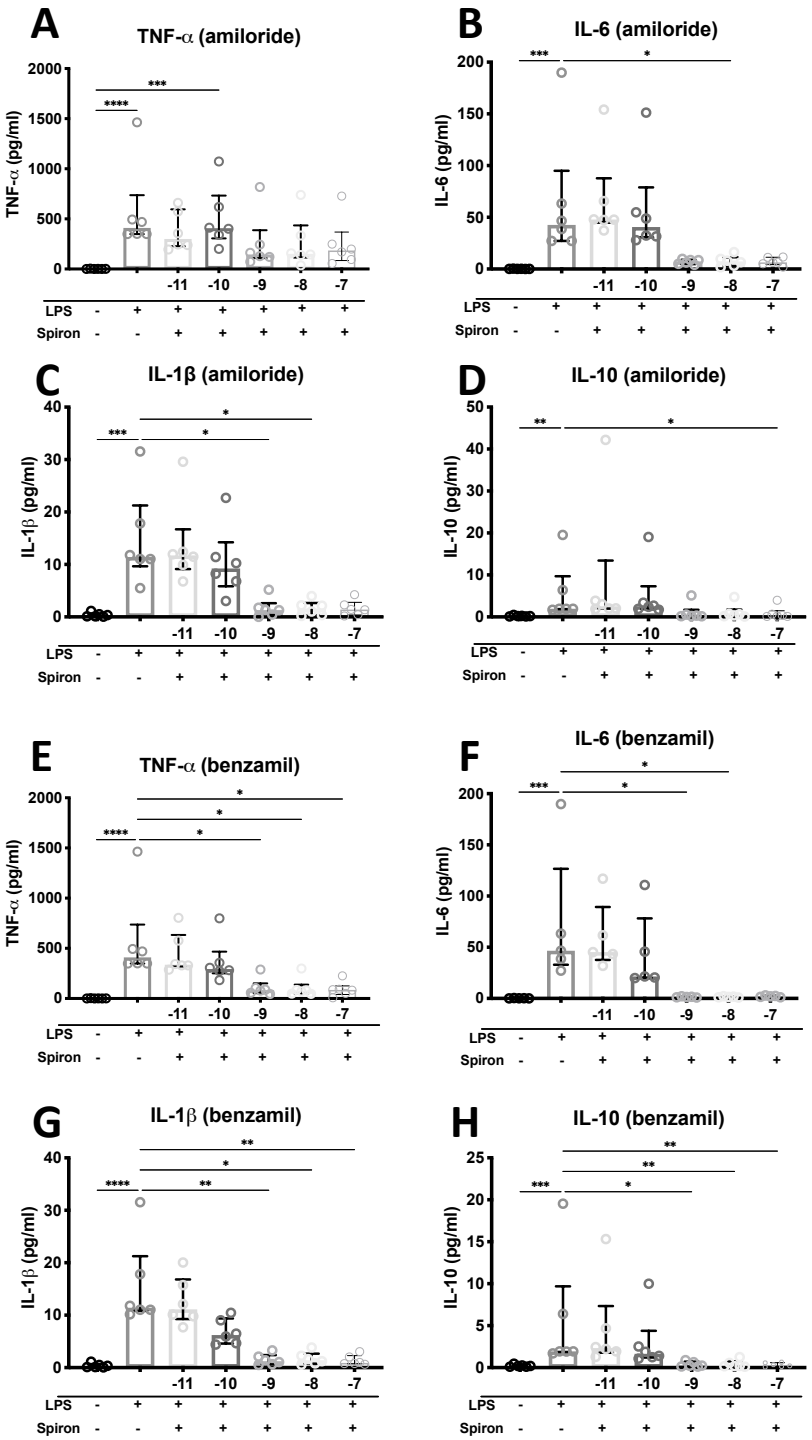


Figure 4


A
TNF-α (amiloride)
TNF-α (pg/ml)
B
IL-6 (amiloride)
IL-6 (pg/ml)
C
IL-1β (amiloride)
IL-1β (pg/ml)
D
IL-10 (amiloride)
IL-10 (pg/ml)
E
TNF-α (benzamil)
TNF-α (pg/ml)
F
IL-6 (benzamil)
IL-6 (pg/ml)
G
IL-1β (benzamil)
IL-1β (pg/ml)
H
IL-10 (benzamil)
IL-10 (pg/ml)
-11 -10 -9 -8 -7
LPS
Spiron

STUDY III

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

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Running title: Effect of spironolactone on IL-17A and kidney injury markers.

Key Words: Aldosterone, hypertension, cytokine, calcineurin, interleukins,

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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). It was hypothesized that 1-year intervention with the MR-antagonist spironolactone lowers IL-17A along with other cytokines and reduces kidney epithelial injury markers in kidney transplant recipients. Plasma and urine samples were obtained from kidney transplant recipients from a double-blind randomized clinical trial with spironolactone (*n*=39) or placebo (*n*=41). Plasma concentrations of cytokines IFN-γ, IL-17A, TNF, IL-6, IL-1β, and IL-10 were determined before and after 1-year treatment. Urine excretion of calbindin, clusterin, KIM-1, osteoactivin, TFF3 and VEGF were analyzed. Blood pressure and plasma aldosterone concentration at inclusion did not relate to plasma cytokines or to urine injury markers. Plasma cytokines did not change after spironolactone intervention. Plasma IL-17A and IL-1β increased in the placebo group. The injury markers calbindin and TFF3 decreased significantly in urine after spironolactone intervention with no changes in the placebo group. Blood pressure at baseline correlated positively with urine TFF3 levels. 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. Baseline treatment with angiotensin-converting-enzyme inhibitor and/or angiotensin II receptor blockers was not associated with changed levels of plasma IL-17A or the injury markers. In conclusion, spironolactone has no direct effect on plasma IL-17A levels in stable kidney transplant recipients but decreases kidney injury markers calbindin and TFF3.

New & Noteworthy

The mineralocorticoid receptor antagonist spironolactone had no direct anti-inflammatory effects on plasma IL-17A but reduced distal nephron epithelial injury markers independent of blood pressure and plasma K^+ changes in kidney transplant recipients.

INTRODUCTION

Kidney allograft injury with decline in 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 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 Th17 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 Th1 cells (12). However, only few clinical studies have shown 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. Taken together, IL-17A could be a, non-redundant, contributor to allograft dysfunction by direct effects and indirectly through hypertension. 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).

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 (20-22). Thus, elevated K^+ could suppress renal inflammation.

Mineralocorticoid receptors are expressed in T-lymphocytes, B-lymphocytes, dendritic cells, and macrophages, and aldosterone promotes the Th-17 lineage differentiation (23-26). 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 (27, 28). 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 (29). The present 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 concentration of IL-17A along with other related T-lymphocyte and macrophage-derived cytokines and that this suppression relates to renal epithelial protection (30, 31). The 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 (30).

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 (30-32). 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 (30). In short, prevalent kidney transplant patients 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 (30, 31). 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 (31). 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 (31). Baseline data, urine, and blood samples collected at study inclusion and after 1 year intervention for the first 80 patients who had completed the first year of the SPIREN trial were used. At the time of the present analysis, the blinding of the original trial was not compromised since all patients had 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 (31). 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, 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 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. 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 plasma samples from 24hr LPS-incubated (10 ng/ml) whole blood 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 control plasma sample 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, 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, IL-6, and 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. The different numbers of "n" for each analyte is a result of this exclusion criteria. 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, data was reassessed by including all datapoints obtained (below and within detection range) except the ones with CV>20% (table S2).

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. In short, pre-coated 96-well mesoscale plates were blocked with blocker A 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 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 (*Mesoscale Discovery, Denmark*). 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. Data are presented as 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 (31).

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 (31).

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 by a two-way ANOVA analysis followed by a Bonferroni's multiple-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 (31). 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% ($p<0.05$)) (31). There were no episodes of acute rejection during the study period. Plasma samples from 3 patients in the spironolactone group and 2 patients from the placebo group were not available since sample volume was too small, due to usage in previous analysis.

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

When comparing baseline cytokine levels 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 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 were based on the two data inclusion criteria CV<20% and within detection range. It was verified that the excluded datapoints did not affect the results by reassessing data including all datapoints below detection range with CV<20%. Number of datapoints excluded in each assay is shown (table S1 https://tinyurl.com/2ss6b2tz). Analyses of data including all datapoints did not change the results (table S2 https://tinyurl.com/2ss6b2tz).

Effect of spironolactone on plasma concentration of macrophage-derived cytokines

Baseline levels of the four macrophage-derived cytokines (IL-6, TNF, 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). Analyses of all data points included revealed increased levels of plasma IL-β upon placebo intervention with no effects observed in spironolactone-treated patients and no significant difference between groups was observed (table S2 https://tinyurl.com/2ss6b2tz).

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

It was previously shown that 24hr mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) remained stable after spironolactone intervention in the kidney transplant patients. (31). SBP was significantly increased in placebo treated patients

after intervention, but not different between groups. This blood pressure increase was due to an increase in blood pressure during day-time blood pressure recording (31). Placebo-treated patients increased blood pressure by 7 mmHg after 1 year treatment (31). When correlating 24hr SBP, DBP, or MAP to plasma cytokine levels at study entry, no significant correlations were observed for any of the analyzed cytokines (table 2). Correlation of plasma IL-17A increase and SBP increase in the placebo group revealed no significant relation (table S3 https://tinyurl.com/2ss6b2tz). Plasma aldosterone concentrations at baseline did not relate significantly to MAP, SBP, or DBP at baseline in the patients (table 2). There was no significant correlation between plasma aldosterone and plasma cytokine levels at study entry (table 2). Blood pressure and plasma aldosterone changes upon spironolactone intervention did not relate to cytokine changes (table S3 https://tinyurl.com/2ss6b2tz).

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 (31). Since treatment with ACEi and ARBs may impact levels of the measured cytokines, we performed a subgroup analyses of cytokine levels in patients treated with ACEi/ARB vs no ACEi/ARB (no drug) at baseline (figure S1 https://tinyurl.com/2ss6b2tz) and after intervention (delta-values) in both the spironolactone and placebo groups (figure S2 https://tinyurl.com/2ss6b2tz). At baseline, plasma cytokine levels were not different between patients receiving ACEi/ARB and no drug (figure S1A-E https://tinyurl.com/2ss6b2tz) except for plasma IL-10, which was significantly decreased in patients receiving ACEi/ARB compared to patients treated with no drug (figure S1F https://tinyurl.com/2ss6b2tz). 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 https://tinyurl.com/2ss6b2tz).

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 https://tinyurl.com/2ss6b2tz). 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 https://tinyurl.com/2ss6b2tz). 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 https://tinyurl.com/2ss6b2tz).

Spironolactone treatment in kidney transplant patients caused a decrease in urine TFF3 and Calbindin

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 1). 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 ($p<0.05$) whereas no changes were observed in the placebo group (figure 3). No significant differences after treatment were observed in clusterin, KIM-1, osteoactivin, and VEGF levels. No correlation was observed between plasma aldosterone or plasma IL-17A and urinary kidney injury markers at baseline (table 2), however blood pressure (MAP, SBP and DBP) correlated positively with urinary TFF3 at baseline (table 2), but no relation was observed between blood pressure changes and TFF3 changes upon spironolactone intervention (table S3 https://tinyurl.com/2ss6b2tz). DBP changes correlated positively with calbindin changes and plasma aldosterone changes correlated positively with KIM-1 changes (table S3 https://tinyurl.com/2ss6b2tz).

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 (31). 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 https://tinyurl.com/2ss6b2tz). 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 https://tinyurl.com/2ss6b2tz).

DISCUSSION

In the present study, plasma IL-17A, IFN-γ, TNF, IL-6, IL-1β and IL-10 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, 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, however a small but significant increase in 1-year placebo-treated patients was observed for IL-17A. When reassessing data including all datapoints below detection level with CV<20%, plasma IL-1β levels increased in placebo-treated patients, however since the difference observed is based on very low concentrations of IL-1β and almost 50% of the data are below detection limit, any interpretation of this result should be done with caution.

The concentration of plasma IL-17A in the kidney transplant patients of the present study 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 groups, the IL-17A increase could be causally related. However, there was no significant relation. Since the difference was small it cannot be excluded that the significant increase is a coincidental random finding. Cytokines circulate in 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 percentage 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 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). However specific mechanism in humans have not been investigated. If IL-17A is more dependent on ANGII-AT1 actions, it is possible that IL-17A was already suppressed at baseline and a MR-blockade mediated suppression would be less pronounced. The plasma IL-17A increase observed in placebo-treated kidney transplant patients may reflect a less pronounced MR-mediated effect on IL-17A production. The data were re-analyzed according to antihypertensive treatment with ACEi and/or ARBs. When groups were compared, ACEi/ARBs did not influence the cytokine concentration in kidney transplant recipients since no effect was seen in patients treated with and without ACEi/ARBs in the placebo group, and no cytokine-lowering effect was observed in spironolactone-treated patients with and without ACEi/ARB. Of interest, in paired analyses, plasma IL-17A and IL-10 increased in placebo and ACEi/ARB treated patients. These data indicate that ACEi/ARBs may not be potent inhibitors of IL-17A but if anything, then rather promote IL-17A. Thus, in stable kidney transplant patients, ACEi/ARB treatment does not exclude beneficial non-redundant effects of spironolactone.

One confounding factor could be the treatment with prednisolone. *In vivo* and *ex vivo* studies in humans have shown an indication of prednisolone to reduce IL-17A levels both on transcript and protein levels (40, 41). However, with more prednisolone treated patients in the placebo group this cannot account for increased IL-17A concentration. Rather, an underlying inflammatory response in the placebo-treated patients could explain the IL-17A increase. We previously reported that spironolactone increased plasma K^+ in this cohort (31). Increased extracellular [K^+] is associated with suppression of T cell activation and T-cell cytokine production including IL-17A *in vitro* (22, 42, 43). Moreover, it has been shown that increased expression and activity of K^+-channels correlate with pro-inflammatory cytokine response, and that blockade of subtypes of potassium channels inhibits T-cell proliferation and inflammation

(20, 21). However, in the present cohort, the increase in plasma potassium concentration in the spironolactone group did not relate significantly to a decrease in plasma IL-17A levels after 1 year. It cannot be excluded that the increase in K^+ in the spironolactone-treated patients could have contributed to stabilize plasma IL-17A, since K^+ did not change in the placebo group where plasma IL-17A increased.

All patients in the included cohort received immunosuppressive calcineurin inhibitors (CNIs). CNIs prevent graft rejection by predominantly inhibiting interleukin-2 (IL-2) production. Interleukin-2 is a critical component in maintaining the regulatory T-cell (Treg)/Th-17 axis, where IL-2 promotes polarization of Tregs and inhibits Th-17 cells. Deficiency of IL-2 in 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. 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). Studies have shown that 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). There was no relation

between cytokines and injury markers in response to spironolactone and the protective action could thus be direct. The distal nephron localization of calbindin is in line with a direct protective action of the MR antagonist since MR is expressed from the distal convoluted tubules over connecting tubule and including the collecting ducts. 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). Thus, MR has some overlap in expression with TFF3 and a direct effect is therefore possible.

In the present study spironolactone-induced changes in urinary calbindin and TFF3 in patients did not relate to spironolactone-induced changes in plasma IL-17A, K^+, aldosterone or blood pressure. Also, ACEi/ARB treatment of kidney transplant patients was not associated with any changes in urinary calbindin and TFF3 levels at baseline or after intervention. However, the treatment effect related predominantly to patients receiving ACEi/ARBs again suggesting that this pre-treatment does not exclude beneficial therapeutic effect of MR-blocker.

In conclusion spironolactone suppressed epithelial injury markers TFF3 and calbindin, associated with the distal nephron, and had no potent but a non-redundant anti-inflammatory effect on IL-17A production in kidney transplant patients.

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 and IL-1β 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 may have additional but minor anti-inflammatory and direct protective effects in the distal epithelium of the nephron.

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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.

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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)** One year spironolactone intervention had no effect on plasma IL-17A concentrations (n=23), **D)** but an increase in the placebo group (n=26) was observed ($p<0.05$*). Data are presented as pg/ml where 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 (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 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).

Figure 3: Effect of spironolactone treatment for 12 months on kidney injury markers in spot urine samples from kidney transplant patients. A + I) After 1-year spironolactone intervention, Calbindin and TFF3 were significantly decreased in urine samples of kidney transplant patients (P<0.05*). **B-H + J-L)** No significant changes were observed in any of the other kidney injury markers upon spironolactone or placebo intervention. The variable number of samples was caused by some samples being below measuring range of the assay: Calbindin (spironolactone: n=36, placebo: n=38), Clusterin (spironolactone: n=34, placebo: n=36), KIM-1 (spironolactone: n=37, placebo: n=37), Osteoactivin (spironolactone: n=37, placebo: n=38), TFF3 (spironolactone: n=27, placebo: n=21) and VEGF (spironolactone: n=37, placebo: n=39). Normally distributed data were analyzed with a paired t-test and non-normally distributed data were analyzed by the Wilcoxon test (KIM-1 spironolactone, osteoactivin placebo and TFF3 spironolactone). Data are presented as pg/ml where urinary kidney injury marker ratios at baseline and after 1-year intervention are connected by lines. P-values<0.05* were considered statistically significant.

Figure 4: Relation between change in plasma K^+ concentration after spironolactone and ΔIL-17A, ΔCalbindin and ΔTFF3 after spironolactone intervention. No significant correlation between K^+ and plasma IL-17A, urinary calbindin and TFF3 changes after 1-year spironolactone treatment was observed. Data were not normally distributed and therefore Spearman's correlations were carried out.

Tables Legends

Table 1: Plasma cytokine, plasma K^+, and urinary kidney injury marker levels

Cytokine and kidney injury markers are presented as pg/ml median with interquartile range. Plasma K^+ concentrations are presented as mmol/L mean± SD. 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. $P<0.05$ was considered significant.

Table 2: Results of correlation analyses

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

	Spironolactone			Placebo							
Assay	**Baseline (pg/ml)**	**1yr (pg/ml)**	**Within-group Comparison p-value**	**Baseline (pg/ml)**	**1yr (pg/ml)**	**Within-group Comparison p-value**	**Between-group comparison (baseline) p-value**	**Between-group comparison (1yr) p-value**	**Between-group comparison (Δ-values) p-value**	**Two-way ANOVA (Within group) Placebo p-value**	**Two-way ANOVA (Within group) Spironolactone 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	0.1	0.9
TNF	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	0.9	0.9
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	0.2	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	-	-
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	0.9	<0.05*
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	0.9	0.9
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	0.1
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***	0.9	<0.0001***

Table 2: Correlations between blood pressure and cytokines; 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	**P-values**	**r-values**	**Test**	**Paired samples (n)**
MAP vs IFN-γ	0.4	-0.1	Pearson	59
MAP vs IL-17A	0.4	-0.1	Pearson	49
MAP vs TNF	0.7	-0.05	Pearson	62
MAP vs IL-6	0.8	0.03	Spearman	61
MAP vs IL-1β	0.4	0.1	Pearson	41
MAP vs IL-10	0.9	0.01	Spearman	62
MAP vs p-aldo	0.2	0.1	Spearman	67
SBP vs IFN-γ	0.3	-0.1	Pearson	59
SBP vs IL-17A	0.2	-0.2	Pearson	49
SBP vs TNF	0.5	-0.1	Pearson	62
SBP vs IL-6	0.5	0.1	Spearman	59
SBP vs IL-1β	0.5	0.1	Pearson	41
SBP vs IL-10	0.6	-0.07	Spearman	62
SBP vs p-aldo	0.8	67	Pearson	67
DBP vs IFN-γ	0.5	-0.08	Pearson	61
DBP vs IL-17A	0.7	-0.06	Pearson	50
DBP vs IL-1β	0.4	0.1	Pearson	41
DBP vs TNF	0.9	-0.01	Pearson	64
DBP vs IL-10	0.7	0.06	Spearman	64
DBP vs IL-6	0.9	-0.01	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	0.8	0.04	Pearson	58
p-aldo vs TNF	0.5	-0.09	Pearson	74
p-aldo vs IL-6	0.9	0.02	Spearman	71
p-aldo vs IL-1β	0.5	-0.1	Pearson	45
p-aldo vs IL-10	0.2	0.1	Spearman	73
MAP vs u-calbindin/crea ratio	0.7	0.06	Pearson	64
MAP vs u-clusterin/crea ratio	0.2	0.2	Pearson	60
MAP vs u-KIM-1/crea ratio	1.0	0.003	Pearson	63
MAP vs u-Osteoactivin/crea ratio	1.0	0.002	Pearson	63
MAP vs u-TFF3/crea ratio	0.005**	0.4	Pearson	47
MAP vs u-VEGF/crea ratio	0.4	0.1	Pearson	63
SBP vs u-calbindin/crea ratio	0.7	0.07	Pearson	64
SBP vs u-clusterin/crea ratio	0.2	0.2	Pearson	60
SBP vs u-KIM-1/crea ratio	0.9	0.02	Pearson	63
SBP vs u-Osteoactivin/crea ratio	0.5	0.09	Pearson	63
SBP vs u-TFF3/crea ratio	0.01**	0.4	Pearson	47
SBP vs u-VEGF/crea ratio	0.2	0.2	Pearson	63
DBP vs u-calbindin/crea ratio	1.0	0.002	Pearson	66
DBP vs u-clusterin/crea ratio	0.3	0.1	Pearson	62
DBP vs u-KIM-1/crea ratio	0.9	0.01	Pearson	65
DBP vs u-Osteoactivin/crea ratio	0.7	-0.05	Pearson	65
DBP vs u-TFF3/crea ratio	0.008**	0.4	Pearson	49
DBP vs u-VEGF/crea ratio	0.8	0.03	Pearson	65
P-aldo vs u-calbindin/crea ratio	0.8	-0.03	Spearman	77
P-aldo vs u-clusterin/crea ratio	0.1	0.2	Spearman	72
P-aldo vs u-KIM-1/crea ratio	0.5	0.07	Spearman	76
P-aldo vs u-Osteoactivin/crea ratio	0.8	0.03	Spearman	76
P-aldo vs u-TFF3/crea ratio	0.5	0.1	Spearman	55

P-aldo vs u-VEGF/crea ratio	0.5	-0.08	Spearman	76
P-IL-17A vs u-calbindin/crea ratio	0.6	0.08	Pearson	47
P-IL-17A vs u-TFF3/crea ratio	0.2	0.2	Pearson	33

Figure 1

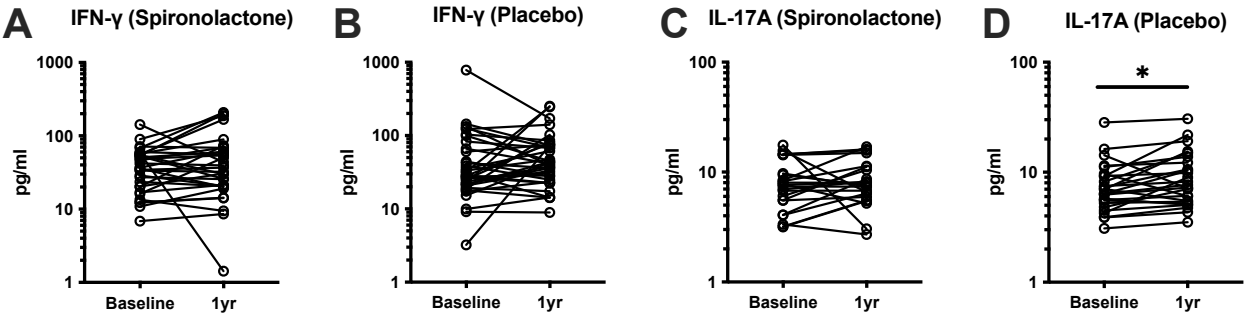


Figure 2

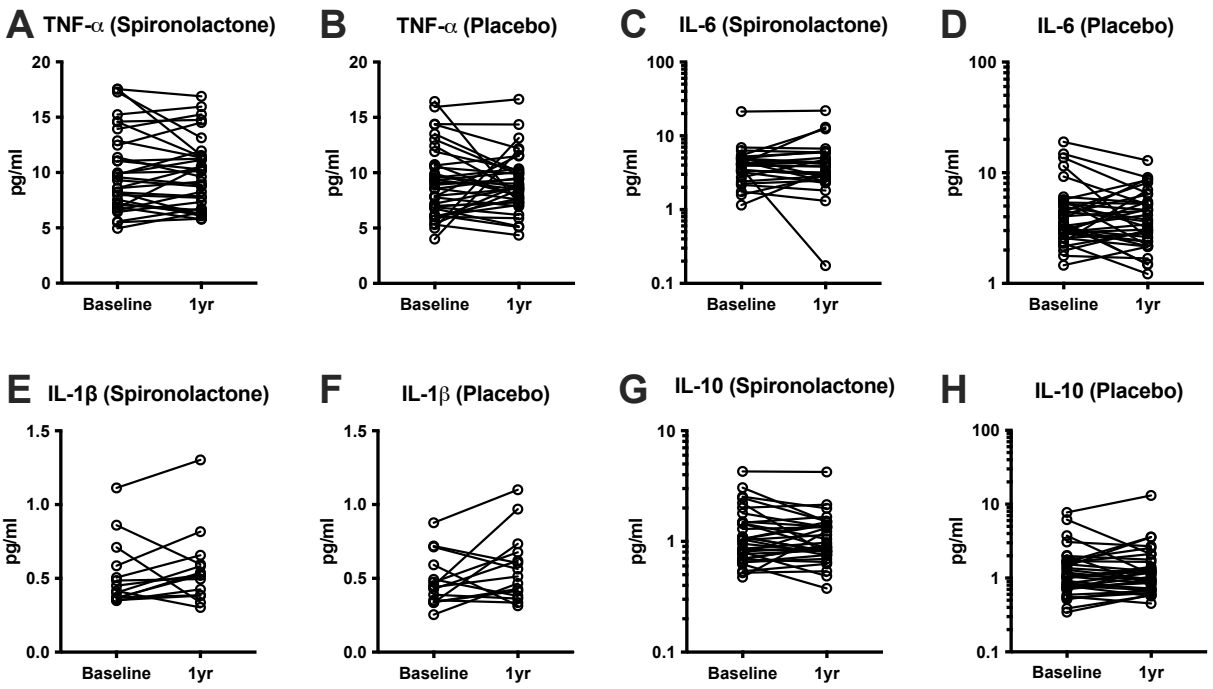
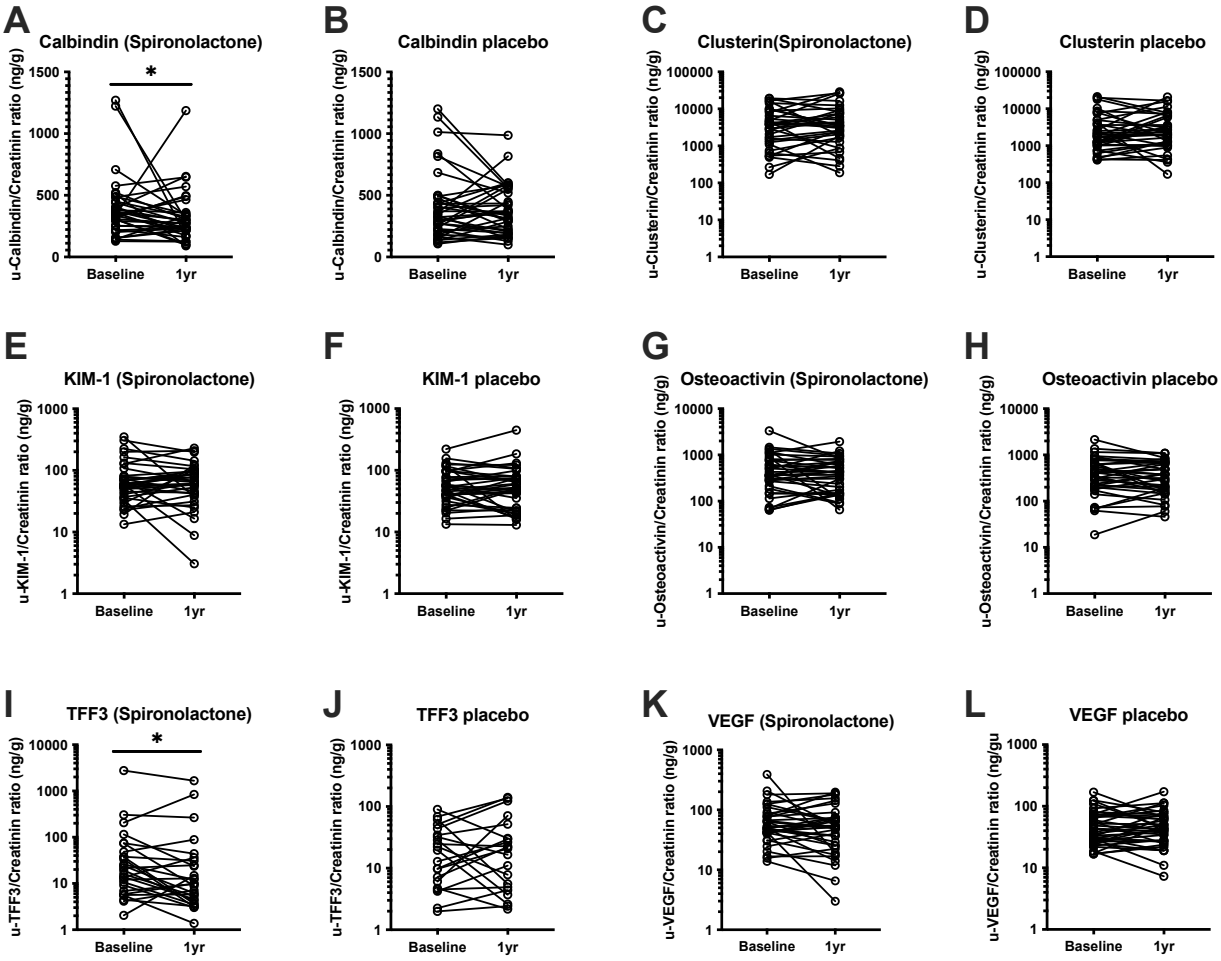


Figure 3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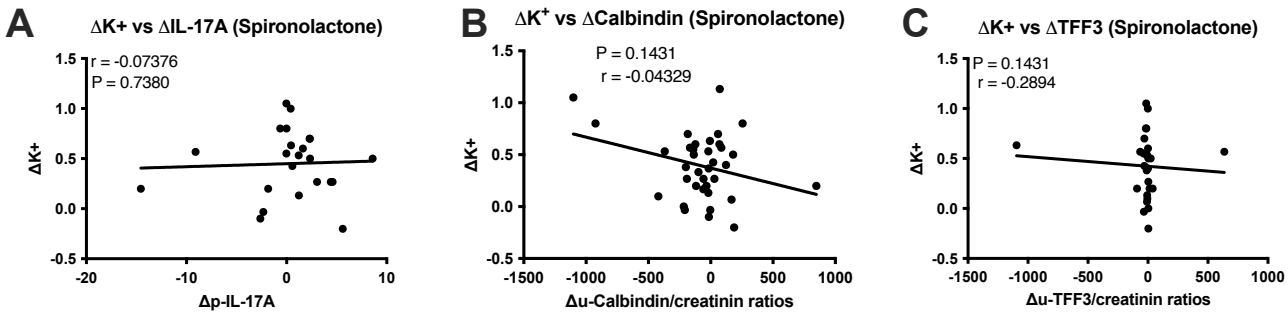
u-Calbindin/Creatinin ratio (ng/g)
1500
1000
500
0
*
Baseline
1yr
B
Calbindin placebo
u-Calbindin/Creatinin ratio (ng/g)
1500
1000
500
0
Baseline
1yr
C
Clusterin(Spironolactone)
u-Clusterin/Creatinin ratio (ng/g)
100000
10000
1000
100
10
1
Baseline
1yr
D
Clusterin placebo
u-Clusterin/Creatinin ratio (ng/g)
100000
10000
1000
100
10
1
Baseline
1yr
E
KIM-1 (Spironolactone)
u-KIM-1/Creatinin ratio (ng/g)
1000
100
10
1
Baseline
1yr
F
KIM-1 placebo
u-KIM-1/Creatinin ratio (ng/g)
1000
100
10
1
Baseline
1yr
G
Osteoactivin (Spironolactone)
u-Osteoactivin/Creatinin ratio (ng/g)
10000
1000
100
10
1
Baseline
1yr
H
Osteoactivin placebo
u-Osteoactivin/Creatinin ratio (ng/g)
10000
1000
100
10
1
Baseline
1yr
I
TFF3 (Spironolactone)
u-TFF3/Creatinin ratio (ng/g)
10000
1000
100
10
1
*
Baseline
1yr
J
TFF3 placebo
u-TFF3/Creatinin ratio (ng/g)
1000
100
10
1
Baseline
1yr
K
VEGF (Spironolactone)
u-VEGF/Creatinin ratio (ng/g)
1000
100
10
1
Baseline
1yr
L
VEGF placebo
u-VEGF/Creatinin ratio (ng/gu
1000
100
10
1
Baseline
1yr

Figure 4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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

Supplementary Tables

Table S1: Samples excluded

Assay	Spironolactone: *n (%)* Exclusion criteria: CV>20%, samples below detection range.	Placebo: *n (%)* Exclusion criteria: CV>20%, samples below detection range.	Spironolactone: *n (%)* Exclusion criteria: CV>20%	Placebo: *n (%)* Exclusion criteria: CV>20%
IFN-γ	8 (21)	5 (12)	8 (21)	5 (12)
IL-17A	16 (42)	15 (37)	7 (18)	4 (10)
TNF-α	7 (18)	4 (10)	6 (15)	2 (5)
IL-6	12 (31)	5 (12)	10 (26)	4 (10)
IL-1β	25 (64)	26 (63)	10 (26)	11 (27)
IL-10	5 (13)	6 (15)	4 (10)	5 (12)

Table S1: Data points excluded from the cytokine analyses have been presented as numbers (%) excluded from both the spironolactone and placebo group. Left side of table) Numbers of data points excluded based on exclusion criteria: CV>20% and below detection range. Right side of table) Numbers of data points excluded based on exclusion criteria: CV>20%. All datapoints below detection limit were included.

Table S2: Plasma cytokine levels in kidney transplant recipients before (baseline) and after treatment with placebo or spironolactone for 12 months (all datapoints included)

	Spironolactone			Placebo							
Assay	Baseline (pg/ml)	1yr (pg/ml)	Within-group Comparison p-value	Baseline (pg/ml)	1yr (pg/ml)	Within-group Comparison p-value	Between-group comparison (baseline) p-value	Between-group comparison (1yr) p-value	Between-group comparison (Δ-values) p-value	Two-way ANOVA (within group) placebo p-value	Two-way ANOVA (within group) spironolactone p-value
IFN-γ	36.9 [20.2-55.9] n = 31	36.9 [20.6-68.6] n = 31	0.2	28.5 [21.0-65.0] n = 36	42.2 [25.8-74.7] n = 36	0.6	0.8	0.2	-	-	-
IL-17A	6.4 [3.2-9.2] n = 32	7.1 [4.0-10.0] n = 32	0.4	5.6 [3.0-7.8] n = 37	6.1 [4.0-10.5] n = 37	0.003**	0.4	0.6	-	-	-
TNF-α	9.3 [7.0-12.7] n = 33	9.6 [6.7-11.5] n = 33	0.6	8.6 [6.4-10.7] n = 39	8.7 [7.4-10.4] n = 39	0.7	0.2	0.7	0.9	0.9	0.9
IL-6	4.1 [2.6-5.0] n = 29	3.8 [2.6-5.5] n = 29	1.0	3.4 [2.7-5.5] n = 37	3.6 [2.4-5.4] n = 37	0.5	1.0	1.0	-	-	-
IL-1β	0.4 [0.3-1.2] n = 29	0.3 [0.2-0.5] n = 29	0.2	0.3 [0.3-0.5] n = 30	0.4 [0.3-0.6] n = 30	0.03*	0.7	0.03*	0.09	0.2	0.09
IL-10	1.0 [0.7-1.5] n = 35	0.9 [0.8-1.4] n = 35	0.2	1.0 [0.7-1.6] n = 36	1.0 [0.7-1.4] n = 36	0.7	0.9	0.9	-	-	-

Table S2: Data analyses including all data points (below detection limit data are included). Plasma cytokine concentrations are presented as pg/ml median with interquartile range. Within-group comparison was performed with paired t-test or Wilcoxon test and between group comparison was performed using an unpaired t-test or Mann-Whitney test dependent on normal distribution of data for normally distributed data a two-way ANOVA was performed followed by a with a post-hoc Bonferroni multiple comparison test (TNF-a and IL-1b).

Table S3: Correlations between blood pressure changes and cytokine changes; aldosterone changes and cytokine changes; and aldosterone changes and epithelial injury marker changes in kidney transplant recipients after spironolactone intervention.

	Spironolactone			
Parameters	**P-values**	**r-values**	**Test**	**Paired samples (n)**
ΔMAP vs ΔIFN-γ	0.5	-0.1	Spearman	25
ΔMAP vs ΔIL-17A	0.4	-0.2	Spearman	17
ΔMAP vs ΔTNF-α	0.8	-0.1	Spearman	24
ΔMAP vs ΔIL-6	0.9	0.03	Spearman	21
ΔMAP vs ΔIL-1β	0.8	0.08	Pearson	11
ΔMAP vs ΔIL-10	1.0	-0.01	Spearman	27
ΔMAP vs Δp-aldo	1.0	0.003	Pearson	30
ΔSBP vs ΔIFN-γ	0.5	0.2	Spearman	25
ΔSBP vs ΔIL-17A	0.4	-0.2	Spearman	17
ΔSBP vs ΔTNF-α	0.8	-0.1	Spearman	24
ΔSBP vs ΔIL-6	0.9	0.03	Spearman	21
ΔSBP vs ΔIL-1β	0.8	0.08	Pearson	11
ΔSBP vs ΔIL-10	1.0	-0.01	Spearman	27
ΔSBP vs Δp-aldo	1.0	0.003	Pearson	30
ΔDBP vs ΔIFN-γ	0.6	0.1	Spearman	25
ΔDBP vs ΔIL-17A	0.3	-0.3	Spearman	17
ΔDBP vs ΔIL-1β	0.8	0.08	Pearson	11
ΔDBP vs ΔTNF-α	0.8	-0.05	Spearman	24
ΔDBP vs ΔIL-10	0.7	0.07	Spearman	27
ΔDBP vs ΔIL-6	0.7	0.1	Spearman	21
ΔDBP vs Δp-aldo	0.7	0.06	Pearson	30
Δp-aldo vs ΔIFN-γ	0.5	-0.1	Spearman	31
Δp-aldo vs ΔIL-17A	0.4	-0.2	Spearman	23
Δp-aldo vs ΔTNF-α	0.8	-0.05	Spearman	32
Δp-aldo vs ΔIL-6	0.3	-0.2	Spearman	27
Δp-aldo vs ΔIL-1β	0.7	-0.1	Pearson	14
Δp-aldo vs ΔIL-10	0.3	0.2	Spearman	34
ΔMAP vs Δu-calbindin/crea ratio	0.9	-0.03	Spearman	29
ΔMAP vs Δu-clusterin/crea ratio	0.2	0.3	Spearman	27
ΔMAP vs Δu-KIM-1/crea ratio	0.9	0.02	Spearman	30
ΔMAP vs Δu-Osteoactivin/crea ratio	0.7	-0.07	Spearman	30
ΔMAP vs Δu-TFF3/crea ratio	0.6	0.1	Spearman	22
ΔMAP vs Δu-VEGF/crea ratio	0.1	-0.3	Spearman	30
ΔSBP vs Δu-calbindin/crea ratio	0.07	0.4	Spearman	27
ΔSBP vs Δu-clusterin/crea ratio	0.9	0.02	Spearman	25
ΔSBP vs Δu-KIM-1/crea ratio	0.9	-0.02	Spearman	28
ΔSBP vs Δu-Osteoactivin/crea ratio	0.9	0.02	Spearman	28
ΔSBP vs Δu-TFF3/crea ratio	0.9	0.02	Spearman	20
ΔSBP vs Δu-VEGF/crea ratio	0.3	0.2	Spearman	28
ΔDBP vs Δu-calbindin/crea ratio	0.04*	0.4	Spearman	27
ΔDBP vs Δu-clusterin/crea ratio	0.9	0.03	Spearman	25
ΔDBP vs Δu-KIM-1/crea ratio	0.5	0.1	Spearman	28
ΔDBP vs Δu-Osteoactivin/crea ratio	0.2	0.3	Spearman	28
ΔDBP vs Δu-TFF3/crea ratio	0.8	0.7	Spearman	20
ΔDBP vs Δu-VEGF/crea ratio	0.2	0.3	Spearman	28
ΔP-aldo vs Δu-calbindin/crea ratio	0.3	-0.2	Spearman	36

ΔP-aldo vs Δu-clusterin/crea ratio	0.6	0.08	Spearman	34
ΔP-aldo vs Δu-KIM-1/crea ratio	0.02*	0.4	Spearman	37
ΔP-aldo vs Δu-Osteoactivin/crea ratio	0.2	0.2	Spearman	37
ΔP-aldo vs Δu-TFF3/crea ratio	0.6	0.1	Spearman	27
ΔP-aldo vs Δu-VEGF/crea ratio	0.09	0.3	Spearman	37
ΔP-IL-17A vs Δu-Calbindin/crea ratio	0.8	-0.06	Spearman	22
ΔP-IL-17A vs Δu-TFF3/crea ratio	1.0	0.02	Spearman	18
		Placebo		
ΔP-IL-17A vs ΔP-SBP	0.5	0.2	Spearman	19

Table S3: Univariate correlations have been performed on parameters as indicated.

Supplementary figure legends

Figure S1: Baseline plasma cytokine and urinary kidney injury marker levels in patients treated with and without ACEi/ARBs. A-E) Baseline plasma IFN-γ, IL-17A, TNF-α, IL6 and IL-1β levels were not different between patients treated with and without (no drug) ACEi/ARBs. **F)** Baseline plasma IL-10 was significantly decreased in patients treated with ACEi/ARBs than patients that were not. **G-L)** Baseline urinary calbindin/creatinine, clusterin/creatinine, KIM-1/creatinine, osteoactivin/creatinine, TFF3/creatinine, and VEFG/creatinine ratios were not different between patients treated with and without ACEi/ARBs. Non-parametric Mann-Whitney test was used for comparison between unpaired samples. Data are presented as pg/ml median with interquartile range. $P<0.05$** was considered statistically significant.

Figure S2: Plasma cytokine changes (delta-values) in spironolactone and placebo treated patients with and without ACEi/ARBs. A-J) Plasma cytokine changes were not different between patients treated with and without ACEi/ARBs in both the spironolactone and placebo groups. Non-parametric Mann-Whitney test was used for comparison between unpaired samples. Data are presented as delta-values. $P<0.05$ was considered statistically significant.

Figure S3: Urinary kidney injury marker changes (delta-values) in spironolactone and placebo treated patients with and without ACEi/ARBs. A-J) Urinary kidney injury marker changes were not different between patients treated with and without ACEi/ARBs in both the spironolactone and placebo groups. Non-parametric Mann-Whitney test was used for comparison between unpaired samples. Data are presented as delta-values. $P<0.05$ was considered statistically significant.

Figure S4: Plasma cytokine and urinary kidney injury marker paired analyses in patients treated with and without ACEi/ARBs in both spironolactone and placebo groups. A-B, E-F) Plasma IL-17A and IL-10 were unchanged after 1yr intervention in spironolactone and placebo treated patients without ACEi/ARBs (no drug). **C-D, G-H)** Plasma IL-17A and IL-10 was significantly increased in patients treated with ACEi/ARBs in the placebo group and not in the spironolactone group. **I-J, M-N)** Urinary calbindin and TFF3 were unchanged in patients treated with no ACEi/ARBs in both spironolactone and placebo treated patients. **K-L, O-P)** Urinary Calbindin and TFF3 were both significantly decreased in patients treated with ACEi/ARBs in the spironolactone group and not in the placebo. For paired comparisons a non-parametric Wilcoxon test or parametric t-test was performed on normally and non-normally distributed data respectively. Data are presented as pg/ml where cytokine concentrations and urinary kidney injury/creatinine ratios at baseline and after 1-year intervention are connected by lines. $P<0.05$* and $P>0.01$** was considered statistically significant.

Supplementary figures

Figure S1

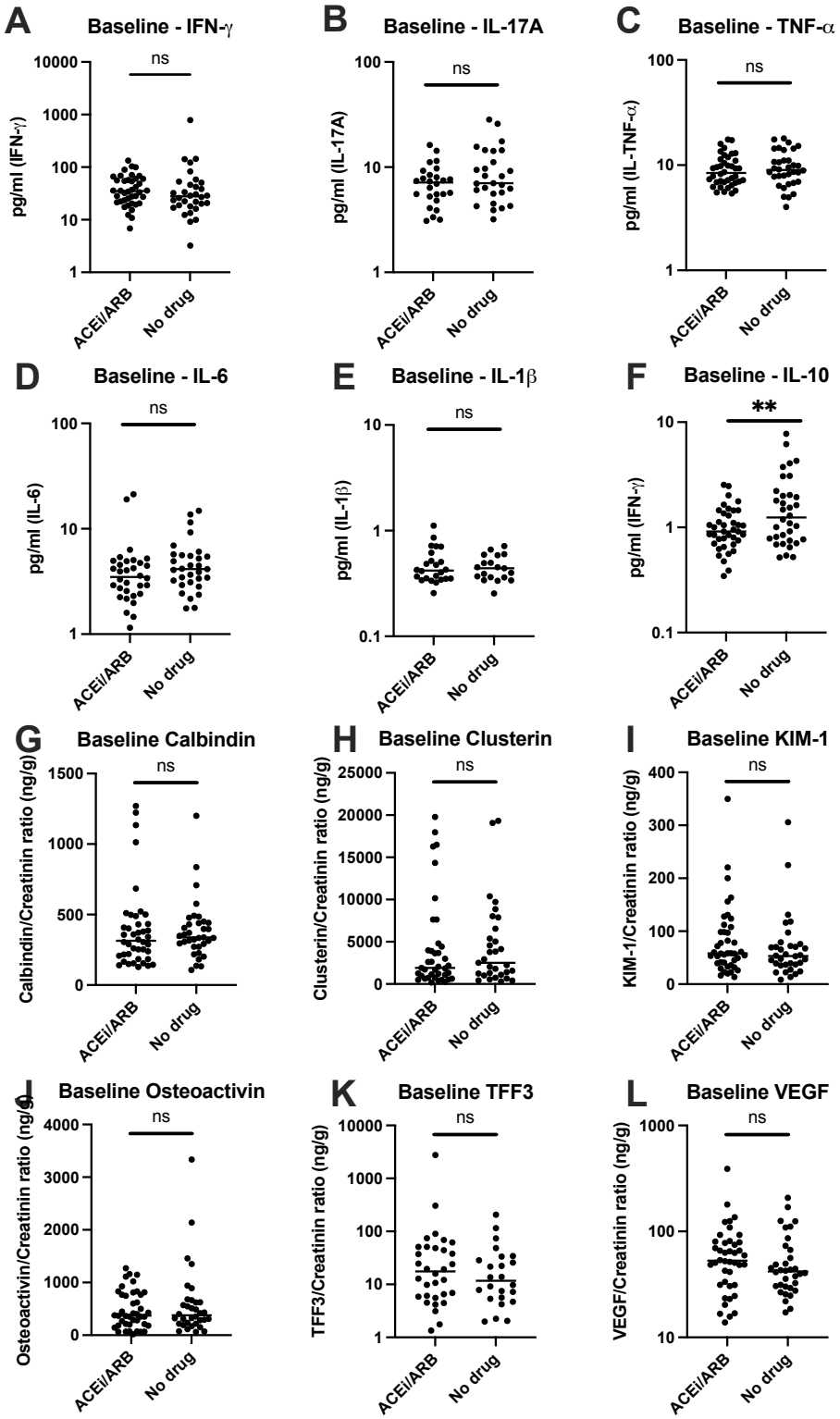
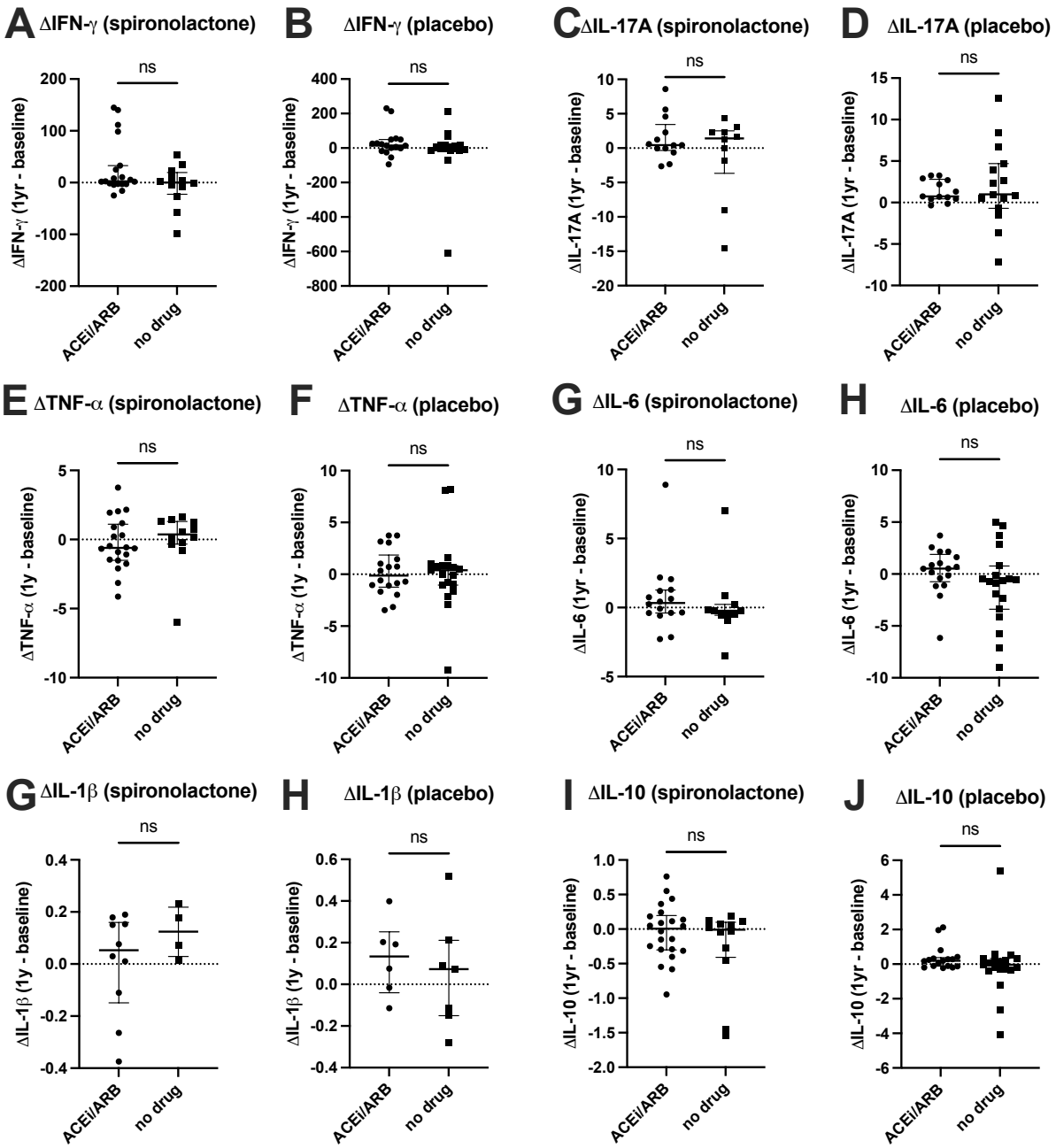


Figure S2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B ΔIFN-γ (placebo)
C ΔIL-17A (spironolactone)
D ΔIL-17A (placebo)
E ΔTNF-α (spironolactone)
F ΔTNF-α (placebo)
G ΔIL-6 (spironolactone)
H ΔIL-6 (placebo)
G ΔIL-1β (spironolactone)
H ΔIL-1β (placebo)
I ΔIL-10 (spironolactone)
J ΔIL-10 (placebo)
ns
ACEi/ARB
no drug
ΔIFN-γ (1yr - baseline)
ΔIL-17A (1yr - baseline)
ΔTNF-α (1y - baseline)
ΔIL-6 (1yr - baseline)
ΔIL-1β (1y - baseline)
ΔIL-10 (1yr - baseline)

Figure S3

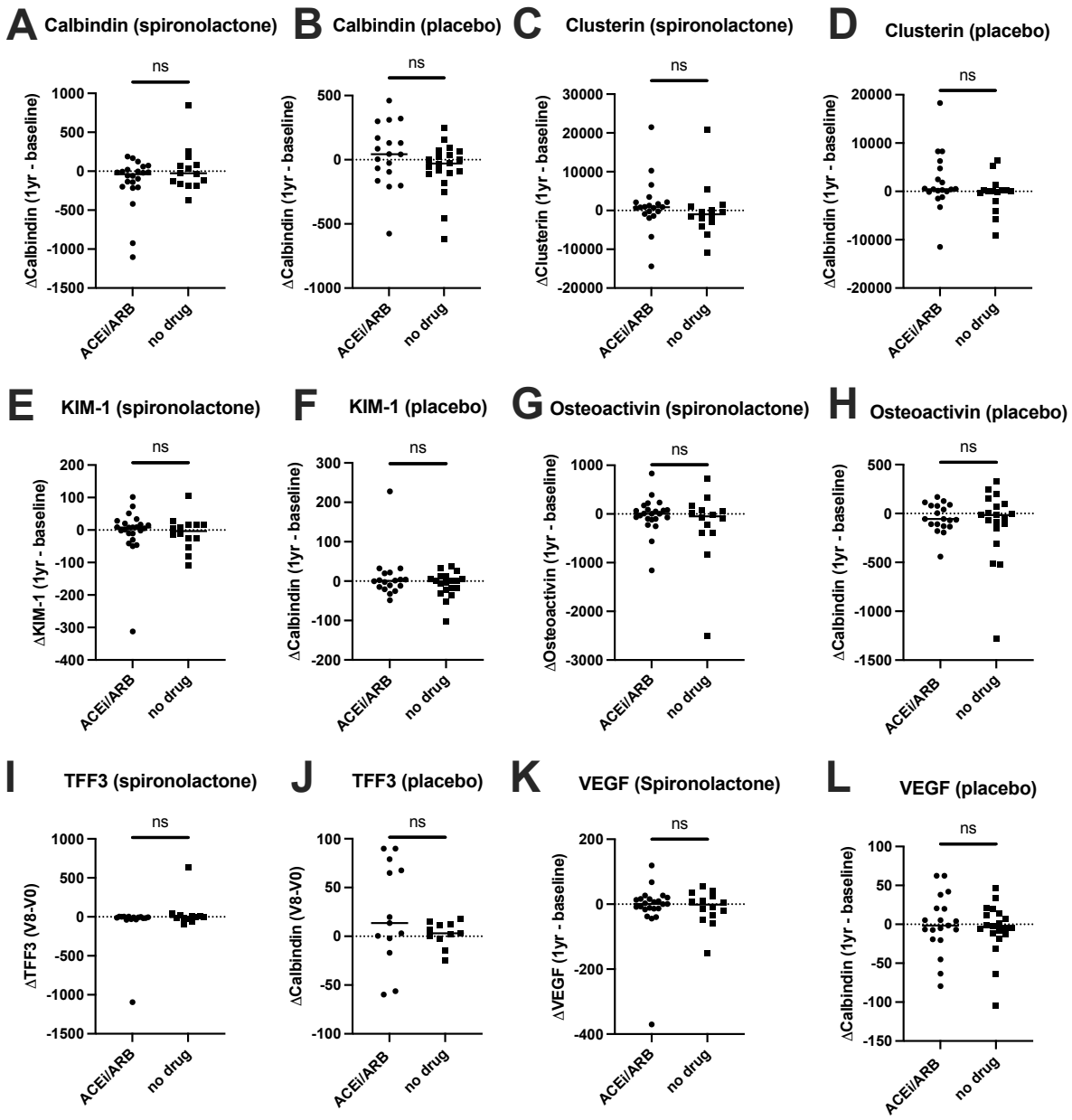
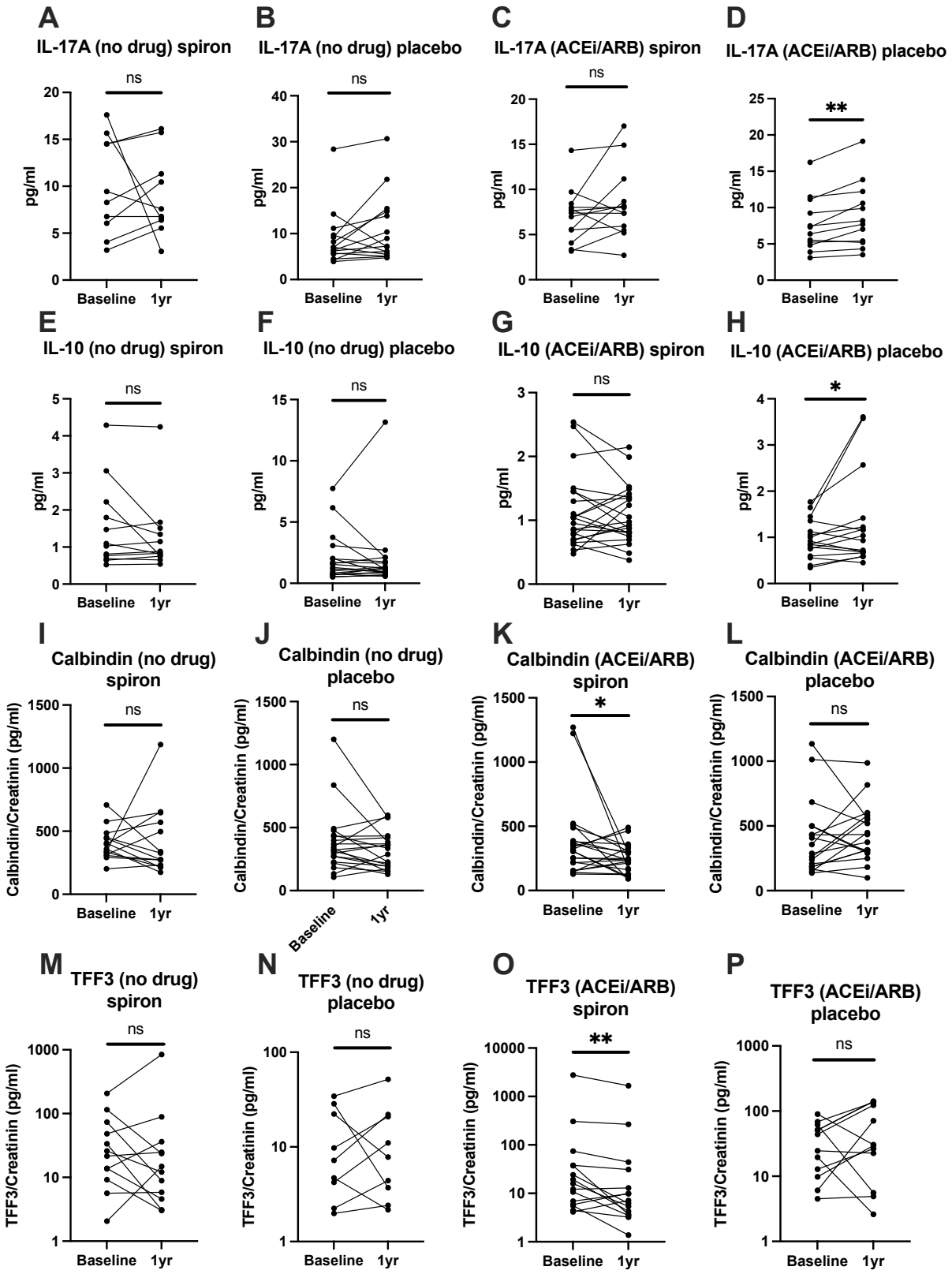

A Calbindin (spironolactone)
B Calbindin (placebo)
C Clusterin (spironolactone)
D Clusterin (placebo)
E KIM-1 (spironolactone)
F KIM-1 (placebo)
G Osteoactivin (spironolactone)
H Osteoactivin (placebo)
I TFF3 (spironolactone)
J TFF3 (placebo)
K VEGF (Spironolactone)
L VEGF (placebo)
ns
ΔCalbindin (1yr - baseline)
ΔClusterin (1yr - baseline)
ΔKIM-1 (1yr - baseline)
ΔOsteoactivin (1yr - baseline)
ΔTFF3 (V8-V0)
ΔCalbindin (V8-V0)
ΔVEGF (1yr - baseline)
ACEi/ARB
no drug

Figure S4


A
IL-17A (no drug) spiron
B
IL-17A (no drug) placebo
C
IL-17A (ACEi/ARB) spiron
D
IL-17A (ACEi/ARB) placebo
E
IL-10 (no drug) spiron
F
IL-10 (no drug) placebo
G
IL-10 (ACEi/ARB) spiron
H
IL-10 (ACEi/ARB) placebo
I
Calbindin (no drug) spiron
J
Calbindin (no drug) placebo
K
Calbindin (ACEi/ARB) spiron
L
Calbindin (ACEi/ARB) placebo
M
TFF3 (no drug) spiron
N
TFF3 (no drug) placebo
O
TFF3 (ACEi/ARB) spiron
P
TFF3 (ACEi/ARB) placebo
pg/ml
Calbindin/Creatinin (pg/ml)
TFF3/Creatinin (pg/ml)
ns
*
**
Baseline
1yr

STUDY IV

Interleukin 17A lowers blood pressure at baseline and after Angiotensin II-hypertension in conscious unrestrained male mice

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Short title: Hypotensive action of interleukin-17A

Figures: 4

Total word count: 4720 words

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Abstract

Interleukin 17A (IL-17A) is a candidate mediator of inflammation-driven hypertension, but its direct effect on blood pressure is obscure. The present study was designed to test the hypothesis that systemic IL-17A concentration-dependently increases blood pressure and amplifies ANGII-induced hypertension in mice. Blood pressure was measured by indwelling chronic femoral catheters before and during IL-17A infusion w/wo angiotensin II (ANGII, 60ng/kg/min) in male FVB/n mice. Baseline blood pressure was recorded, and 3 experimental series were conducted: **1)** IL-17A infusion with increasing concentrations over 6 days (2 series with IL-17 from 2 vendors, *n*=11); **2)** ANGII infusion with IL-17A or vehicle (9 days, *n*=11) and **3)** acute bolus infusions with 4 different concentrations (*n*=5). Plasma IL-17A and IL-6 concentrations were determined by ELISA. Increased IL-6 levels would indicate biologic activity of infused IL-17A. IL-17A infusion decreased mean arterial- and systolic blood pressure (MAP, SBP) significantly, but heart rate was unchanged. In these mice, plasma IL-17A and IL-6 concentrations increased up to 3500 and 2.4-fold, respectively, above baseline. ANGII infusion increased MAP (~25 mmHg) while co-infusion of IL-17A attenuated hypertension by 4.0 mmHg, and plasma IL-17A increased 350-fold above baseline. Acute IL-17A bolus infusion did not change blood pressure or heart rate. IL-17A receptor and IL-6 mRNA was detected in aorta, heart, and kidneys of mice after IL-17A infusion. High concentrations of IL-17A reduce baseline blood pressure and increases IL-6 formation in male FVB/n mice. It is concluded that IL-17A is less likely to drive hypertension as the sole cytokine mediator *in vivo*.

INTRODUCTION

The immune system contributes to the pathogenesis of hypertension. Splenocyte transfer from hypertensive rats recapitulate hypertension in normotensive recipient rats, and athymic mice are protected from deoxycorticosterone acetate (DOCA)-induced hypertension (1, 2). Recombinase-activating gene $(RAG\text{-}1)^{-/-}$ mice lacking T and B lymphocytes, are unable to sustain blood pressure elevation after ANGII or DOCA-salt treatment, and adoptive transfer of T, but not B lymphocytes restored their hypertensive responsiveness (3). Salt-sensitive hypertensive Dahl rats with inflammation responded with a reduction in blood pressure to immunosuppressive drugs including T-cell inhibitors like mycophenolate mofetil and calcineurin inhibitor tacrolimus (4, 5). Severe combined immunodeficient (SCID) mice devoid of lymphocytes exhibit attenuated ANGII-induced hypertension (6). Thus, T-lymphocytes may promote hypertension. Th-17 cell-derived interleukin 17A (IL-17A) has gained significant attention as an important salt-sensitive hypertensive cytokine (7-9). Patients with hypertension have increased plasma IL-17A concentrations (8, 10, 11). Knockout of IL-17A or IL-17A receptor (IL-17RA) or treatment with IL-17 neutralizing antibodies in ANGII-induced hypertensive mice reduced their blood pressure (8, 9, 12, 13). However, the preclinical data on the role of IL-17A in hypertension are not consistent. In experimental DOCA-salt and ANGII-induced hypertension models, anti-IL-17 treatment reduces blood pressure in DOCA-salt hypertensive animals (13, 14). In other studies, there was no effect of anti-IL-17A treatment on blood pressure in ANGII-hypertensive mice although protective effects in the vasculature was observed (15, 16). IL-17A knockout mice treated with DOCA and ANGII developed hypertension not different from wild-type mice (17). In a high-fat diet fed LDL receptor knockout mice with reduced kidney function and atherosclerosis, IL-17A neutralizing antibody treatment was athero-protective but did not change blood pressure (18). Some of the discrepant data may arise from different experimental models with different degrees of systemic

inflammation. It has been shown that local overexpression of IL-17A in skin keratinocytes in mice led to systemic endothelial dysfunction and arterial hypertension with elevated levels of multiple downstream cytokines, e.g., IL-6 and TNF-a (19). By contrast, constitutive overexpression of IL-17A in $CD4^+$ and $CD8^+$ T cells in mice resulted in 15 times higher circulating level of IL-17A with no change in 14 other cytokines, which was not associated with a change in systolic blood pressure nor cardiac hypertrophy (20). While this observation suggests that a selective systemic increase in IL-17A is not sufficient to alter blood pressure, acute intraperitoneal injection of IL-17A yields systolic hypertension in mice (21) and in pregnant rats (22, 23) mitigated by IL-17A soluble receptor (24). Continuous infusion of IL-17A using osmotic minipumps in mice elevated blood pressure (25, 26).

All together the data indicate that administration routes and co-morbidities are important confounders for the effect of IL-17A. The present study was designed to test the hypothesis that IL-17A infusion precipitates concentration-dependent acute elevation of arterial blood pressure in a setting with no confounding systemic or local inflammation.

METHODS

Animal and ethical approvals

In all animal experiments, male wild-type (WT) FVB/n mice aged 10-12 weeks (Envigo, Indianapolis, IN) were used. Male mice are generally more prone to hypertension which is why female mice were not omitted (27-29). Animal experimental protocols were approved by the Danish National Animal Experiments Inspectorate under the Danish Ministry of Justice (approval number: 2020-15-0201-00470). Animal care followed the guidelines of the National Institutes of Health. Mice were housed at the Biomedical Laboratory at the University of Southern Denmark. Mice were kept on a 12:12 hours light/dark cycle and had free access to

water and normal rodent chow. The total use of mice with all mice lost and mice that completed the study are shown in a consort-like diagram (figure S1).

Blood pressure measurements in conscious mice

Procedures were done as described in detail before (30-32) and details on procedures and experimental series are given in the supplement methods. In brief, 3 series of experiments were conducted as follows. Series 1) Step-up protocol: Mice received continuous i.v. infusion of IL-17A with increasing concentrations (3.2 up to 320 ng/kg/min) over 6 days (*n*=11); Series 2) Mice received continuous i.v. infusion of ANGII (60 ng/kg/min) with IL-17A (32 ng/kg/min) or vehicle (saline) for 9 days (*n*=11); Series 3) Mice received acute bolus infusions (455 up to 3640 mg/kg, 910 mg/kg, (*n*=5).

Plasma concentration of IL-17A and IL-6

IL-17A and IL-6 were measured in EDTA plasma samples (non-stressed) taken at baseline and after IL-17A infusion from all mice, and IL-6 was measured in IL-17A stimulated 3T3 fibroblast cells, using the commercially available one-spot mesoscale IL-17A and IL-6 UPLEX immunoassay (*Mesoscale Discovery, Denmark*). Procedures are described in supplementary methods.

Statistical analyses

Normal distribution of blood pressure measurements between mice was assessed during different periods of the experiment at baseline and infusion during dose 1-3, on one average blood pressure value per mouse at each period, using the D'Agostino or the Shapiro-Wilks test. In the step-up protocol, blood pressure and heart rate differences between baseline and after dose 1, 2, and 3 infusions were analyzed by one-way ANOVA with repeated measures followed

by Dunnett's multiple comparison post-hoc test or the Friedman's test followed by Dunn's multiple comparison post-hoc test. Blood pressure differences induced by IL-17A from the two vendors were analyzed by two-way ANOVA with mixed effect analysis followed by Tukey's multiple comparison post-hoc test. Blood pressure changes induced by bolus infusions were analyzed using the one-way ANOVA with repeated measures followed by Tukey's multiple comparison post-hoc test. In ANGII co-infusion experiments, blood pressure and heart rate differences between mice that received ANGII-vehicle and ANGII-IL-17A was assessed by two-way ANOVA with mixed analyses followed by Tukey's multiple comparison test. For plasma IL-6 and IL-17A levels a paired student's t-test was used to compare baseline plasma cytokine levels with plasma cytokine levels after IL-17A infusion. P-values less than 0.05 were considered statistically significant. All statistical analyses were conducted using GraphPad Prism 9 software. All data are presented as mean ± SEM or as median (interquartile range).

RESULTS

Chronic concentration-graded IL-17A infusion in male mice lowered blood pressure.

In mice (*n*=5) infused with increasing concentrations (3, 16, and 32 ng/kg/min) of IL-17A (R&D systems) over 6 days (protocol 1a, supplemental material) mean arterial blood pressure (MAP) decreased significantly during infusion with 32 ng/kg/min (*P*=0.05*), and heart rate was unchanged (figure S2A-B). When mice were infused with higher concentrations of IL-17A (3.2, 32, and 320 ng/kg/min) from two different vendors (BioLegend and R&D systems) that used two different expression systems (CHO-cells and E. Coli) over 8 days (protocol 1b, supplemental material) no blood pressure elevation, but a lowering in blood pressure was observed and heart rate was unchanged (figure S2C-D).

Recombinant murine IL-17A expressed in E. Coli (R&D systems) may theoretically contain endotoxins (e.g., LPS) which potently lowers blood pressure (33). According to the vendor,

endotoxin level was less than 0.1 endotoxin unit per 1 ug, much lower than recommended levels for preclinical studies (34). To test whether endotoxin from E. Coli masked a pro-hypertensive effect of IL-17A, recombinant murine IL-17A expressed by mammalian CHO-cells (BioLegend) was tested. Since there was no difference in blood pressure recordings in mice that received IL-17A produced by eukaryotic cells or by E. Coli (2 vendors) or between low and high increasing IL-17A concentrations (figure S2E) blood pressure recording data was pooled from all mice in the step-up concentration experiments (n=11). When mice were given 3 consecutive increasing doses of IL-17A corresponding to concentrations 3-16-32 ng/kg/min or 16-32-320 ng/kg/min from two different vendors, significant blood pressure reductions were observed. MAP was significantly reduced during IL-17A infusion at all 3 doses (figure 1A-B). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) was significantly reduced at 320 ng/kg/min IL-17A, and heart rate was unchanged when compared to baseline measurements at all 3 IL-17A infusion doses (figure 1C-D).

Thus, contrary to our hypothesis chronic i.v. infusion of IL-17A decreased blood pressure in healthy wild-type FVB/n male mice.

Chronic infusion with IL-17A increased plasma IL-17A concentrations and stimulated IL-6 production in all mice.

Plasma IL-17A levels in increased significantly in mice that received IL-17A (figure 1E). In the step-up experiment, IL-17A increased from 0.7 (0.5-1.1) pg/ml at baseline to 2523 (84-8252) pg/ml after IL-17A infusion. Mice receiving the higher concentrations of IL-17A (3-32-320 ng/kg/min) showed increased levels of plasma IL-17A compared to mice receiving the lower concentrations of IL-17A (3-16-32 ng/kg/min, figure 1E).

The biological activity of the recombinant IL-17A protein was examined *in vitro* by stimulating cultured NIH 3T3 fibroblast cells with 10 ng/ml IL-17A for 24 hours (figure S4). Interleukin-

6 (IL-6) is both an upstream and downstream factor of Th-17 mediated IL-17A release (35). Culture supernatants showed a significant increase of IL-6 compared to vehicle (PBS) treated cells (figure S4). The biological activity of IL-17A was examined *in vivo* by measuring plasma IL-6 in the step-up protocol. Plasma IL-6 concentration increased significantly in mice infused continuously with IL-17A in the step-up experiment (figure 1E). Messenger RNA expression of IL-17RA and IL-6 was further assessed in tissues from mice that received continuous IL-17A for 8 days. With total RNA from the kidney, left cardiac ventricle and aorta of mice, PCR amplification of cDNA yielded positive signals and only in the presence of RT and cDNA (figure S4). Thus, IL-17A was not subject to instant degradation, IL-17RA was present in the kidneys, heart and aorta, and the increase in plasma IL-6 indicates *in vivo* biological activity of infused IL-17A.

Acute bolus i.v. infusion of IL-17A in mice had no blood pressure elevating effect

Two series of bolus infusion experiments were conducted. There were no changes in blood pressure or heart rate (n=5) in the first series with consecutive acute administration of 455 µg/kg and 1820 µg/kg (figure S3). To ensure catheter patency, all experiments concluded with an ANGII bolus infusion that increased blood pressure and decreased heart rate, and by acetylcholine (Ach) infusion, where blood pressure decreased, and heart rate increased (figure S3). In the second series (n=5), mice were given 4 boli infusions of IL-17A at higher concentrations (455 µg/kg, 910 µg/kg, 1820 µg/kg, and 3640 µg/kg). Again, no blood pressure changes were observed (figure 2A, C) and heart rate was also unchanged (figure 2B-C). A significant increase in plasma IL-17A was measured in all mice that received acute bolus infusion of IL-17A up to $2.5x10^6$ fold from baseline level before infusion (figure 2D).

Thus, no acute blood pressure effects were observed after IL-17A infusion in healthy male mice.

IL-17A did not accentuate ANGII mediated blood pressure elevation

Mice received continuous i.v. infusion of IL-17A (32 ng/kg/min) or saline for 2 consecutive days. At baseline, before infusion, MAP, SBP, and DBP between groups were comparable.

IL-17A infusion in mice reduced DBP and MAP by 4-6 mmHg, and mice infused with saline showed a blood pressure reduction by 10 mmHg in SBP and MAP. Therefore, no difference between groups was observed in blood pressure during IL-17A/vehicle infusion (figure 3A-C). ANGII co-infusion (60ng/kg/min) increased MAP significantly from 107 (99-111) to 132 (126-134) mmHg and 104 (97-110) to 128 (122-129) mmHg in the IL-17A and saline groups respectively (P=0.0001). ANGII co-infusion also increased SBP and DBP by ~23 mmHg above baseline (P=0.0001) in both groups. During ANGII co-infusion MAP, SBP, and DBP were attenuated in mice that received IL-17A and ANGII compared to ANGII with vehicle (figure 3A-C). Furthermore, heart rate decreased significantly upon co-ANGII infusion from 647 (639-659) to 584 (546-600) BPM and 669 (646-679) to 590 (573-626) BPM in mice that received saline and IL-17A respectively. In the IL-17A-ANGII infused mice with attenuated blood pressure, heart rate was significantly increased compared to vehicle mice (figure 3D). Thus, IL-17A administration did not accentuate ANGII-induced increase in blood pressure.

ANGII does not increase IL-17A plasma levels

In mice co-infused with ANGII and IL-17A, plasma IL-17A levels increased significantly compared to baseline levels and this was not seen in saline-infused mice (figure 4A-B). Plasma IL-17A after prolonged ANGII infusion (9 days) at 60ng/kg/min (n=5) in WT FVB/n mice from a separate protocol was not different from baseline levels (figure S4).

ANGII infusion increased plasma IL-6 levels in 3 out of 4 mice in each group (figure 4C-D). The addition of IL-17A to ANGII caused increased production of IL-6 in these 3 mice

compared to ANGII/vehicle mice. However, this increase was not statistically significant (n=4 in each group, figure 4D).

DISCUSSION

The present study shows that graded chronic and acute bolus infusion of IL-17A to conscious mice increased plasma IL-17A levels up to 3500 times above baseline but did not have hypertensive action alone or in combination with ANGII. The recombinant IL-17A was biologically active and stimulated release of IL-6 to plasma in mice and to media from fibroblast cell cultures. Chronic infusion of IL-17A lowered blood pressure and attenuated ANGII-induced hypertension, while this was not observed by acute boli infusions. It is concluded that IL-17A has no direct cardiac or vascular pro-hypertensive effects *in vivo* in FVB/n mice. After chronic infusion, the blood pressure drop was not accompanied by increased heart rate, which suggests a direct cardio-depressive or sympatholytic action of IL-17A. This implies that if IL-17A increases blood pressure, this is likely by a synergistic or concrete action with other cytokines in states of chronic inflammation.

The blood pressure lowering effects of IL-17A after chronic exposure are in contrast to several previous preclinical studies which concluded that IL-17A promotes blood pressure elevation (19, 21, 25, 26). In most studies, IL-17A or IL-17RA were manipulated either by targeted gene deletion or by neutralizing antibodies on top of conventional chronic hypertension models (8, 9, 12, 13). Most studies did not report on circulating levels of IL-17A and used tail-cuff measurements for blood pressure. By contrast, a recent study achieved a stable and constant 15 times higher circulating level of IL-17A with no change in 14 other cytokines, which was not associated with a change in systolic blood pressure and no cardiac hypertrophy as measured in freely moving mice by carotid artery telemetry in mice (20). Since IL-17A is typically administered by acute injections or osmotic minipumps in published studies (21-23, 25, 26), it

was imperative in the present study to validate that peptide infusion resulted in elevated plasma concentrations *in vivo* and that IL-17A was not prematurely degraded or adhered to the infusion line. Immunoassays confirmed graded increases of plasma IL-17A after infusions. IL-6 in combination with TGF-b, stimulates IL-17 release from Th-17 cells, and IL-17 stimulates IL-6 release from fibroblasts, and hereby function as both a downstream and upstream factor of IL-17A production (35-37). In the present study, infused IL-17A led to increased IL-6 synthesis and release *in vivo,* showing intact biological reactivity towards the cytokine. The *in vitro* increase in IL-6 release from NIH-3T3 mouse fibroblast cells after IL-17A confirmed the potency stated by vendors.

The present experimental setup allows online continuous blood pressure recordings in freely moving unstressed animals while taking undisturbed non-stressed small-volume plasma samples directly from the arterial line. The present technique and radiotelemetry recording allow detection of small differences and diurnal variation and are gold-standards for blood pressure measurements in experimental hypertension models (38, 39). Positive (ANGII) and negative (Ach) *in vivo* control boli confirmed the ability of the recording system to detect small and transient responses. Other studies in mice showing elevated blood pressure after IL-17A infusion used tail-cuff (19, 21, 25, 26). These differences in recording techniques could be the reason for the discrepancies. The blood pressure effects of IL-17A may also be affected by mouse strain. Previous studies have predominantly used C57BL/6J strain to infuse IL-17A (19, 21, 25, 26). Although we detected IL-17A receptor and IL-6 expression in heart, kidney, and aortic tissue in FVB/n mice, we cannot exclude that the C57BL/6J strain, could be more sensitive to IL-17A or that the IL-17A receptor expression level or localization changes during inflammation versus at baseline. The present data showed that the FVB/n strain mice reacted to ANGII with blood pressure elevation, also shown by other groups (40), and similar to other mouse strains like Swiss-Webster (41) and C57BL/6J mice (42). FVB/n mice are more

susceptible to kidney injury after ANGII infusion and unilateral nephrectomy (43) while several studies show that C57BL/6J mice are less susceptible to kidney injury (44) and salt-sensitive hypertension (45). Thus, with detection of the IL-17A receptor in the kidneys, heart and aorta, detection of inflammatory response by increased levels of plasma IL-6 after IL-17A infusion, and elevation of blood pressure after ANGII infusion, the FVB/n strain appears well suited to study blood pressure changes imposed by IL-17A.

In summary, IL-17A infusion increases *in vivo* plasma concentration of IL-17A and IL-6, but does not mediate direct cardiac or vascular hypertensive effects in mice that received continuous or acute high dose IL-17A. ANGII-hypertension with co-infusion of IL-17A in mice reaching 350 times higher plasma IL-17A levels than baseline, did not accentuate blood pressure, but rather attenuated blood pressure and showed that ANGII-stimulated inflammatory factors alone were not enough to co-stimulate IL-17A mediated hypertensive actions.

Perspectives

Several studies have suggested that IL-17A act in concert with other pro-inflammatory cytokines like IFN-g, TNF-a, and IL-6 or regulatory T-cells to induce inflammatory pathogenesis in experimental hypertension and in other inflammatory disorders like systemic sclerosis, psoriasis, and arthritis (46-48). In this study, we show that IL-17A alone does not have hypertensive effects and also that ANGII-mediated hypertension did not elicit inflammatory stimulation of IL-17A. Our results indicate that IL-17A by itself, is not a hypertensive agent, and other unknown factors may be needed for IL-17A induced hypertension. With the limitation of an animal study, protocols with anti-IL-17A antibodies in clinical use should delineate, in hypertensive patients, the role of IL-17A in human patient settings. Further mechanistic studies of IL-17A pathogenesis in hypertension are needed.

Conflict of interest

The authors declare no conflicts of interest. PBL Hansen is an employee and shareholder at AstraZeneca.

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Figure Legends

Figure 1: Blood pressure measurements of mice receiving continuous i.v. IL-17A for 8 days. Blood pressure measurements (*n*=11) are presented as circadian rhythm average day/night blood pressure measurements (left side) and average blood pressure measurements at baseline, during doses 1 (3.2), 2 (16 or 32), and 3 (32 or 320) ng/kg/min. (right side). **A)** Mean arterial pressure (MAP) was significantly decreased at all 3 doses of IL-17A infusion. **B-C)** Systolic and diastolic blood pressures (SBP, DBP) were significantly decreased during high dose IL-17A infusion. **D)** Heart rate (HR) was unchanged. **E)** Plasma IL-17A and IL-6 were markedly elevated above baseline levels. Grey and black circles represent mice that received lower and higher concentrations of IL-17A respectively. For statistical comparison between baseline and dose 1, 2, and 3 blood pressure and heart rate, one-way ANOVA with repeated measures followed by Tukey's multiple comparison test was used (MAP, heart rate) or Friedmann's test followed by Dunn's multiple comparison test was performed (SBP, DBP). Differences in plasma cytokine concentrations were analyzed with paired t-tests. $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Data are presented as mean ± SEM or median with interquartile range.

Figure 2: Blood pressure measurements of mice receiving i.v. IL-17A bolus infusions (*n*=5). A-B) Mean arterial pressure (MAP) and heart rate (HR) pressure recordings of bolus infusion with 4 different concentrations (455, 910, 1820, and 3640 mg/kg) with 10. min interval. No blood pressure changes were observed during IL-17A infusion. **C)** Bolus infusion 1-4 did not affect MAP when compared to baseline or glu/hep infusion MAP. During ANGII and ACh infusion, MAP increased with 15 mmHg and decreased with 10 mmHg respectively when compared to baseline levels and heart rate (HR) was unchanged. **D)** Plasma IL-17A levels were markedly elevated after IL-17A infusion. For statistical comparison between baseline and

after IL-17A infusion MAP or HR measurements, One-way ANOVO with repeated measures followed by Tukey's multiple comparison test was performed. For baseline and after IL-17A infusion comparison of plasma IL-17A levels, a paired t-test was performed. $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Data are presented as mean ± SEM.

Figure 3: Blood pressure measurements of mice receiving i.v. ANGII with IL-17A (n=5) or saline (*n*=5). Blood pressure measurements are presented as average 12-hour (day/night) blood pressure measurements (left side). Blood pressure measurements at baseline, during IL-17A/saline infusion and ANGII infusion are presented as average measurements over 3, 2, and 7 days respectively of 5 mice (right side). **A)** Mean arterial pressure (MAP), **B)** systolic blood pressure (SBP), and **C)** diastolic blood pressure (DBP) were all increased upon ANGII infusion in both groups. **D)** Heart rate was decreased upon ANGII infusion in both groups. Blood pressure changes between groups during IL-17A/saline infusion alone was not different. MAP, SBP, and DBP were all significantly attenuated during ANGII co-infusion with IL-17A when compared to vehicle mice. Data are presented as median with interquartile range. For statistical comparison, a two-way ANOVA with mixed analysis was performed followed by Tukey's multiple comparison test. $P<0.05$ was considered statistically significant. (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$)

Figure 4: Plasma IL-17A and IL-6 quantification. A) Plasma IL-17A was unchanged in mice that was given ANGII infusion with saline, **B)** but significantly increase in those given IL-17A. **C)** Plasma IL-6 increased in 3 of the mice that were given ANGII with saline or IL-17A, where IL-17A addition increased IL-6 by approximately 3-fold, however not statistically

significant (n=4 in each group). For statistical comparison paired t-test or Mann Whitney tests were carried out. $P<0.05$ was considered statistically significant (*$P<0.05$).

SUPPLEMENTARY

METHODS

Catheterization of mice

After 1 week of acclimatization, mice were anesthetized and prepared for surgical implantation of chronic indwelling catheters in the femoral artery and vein for measurements of arterial blood pressure and venous infusions as described by us (30, 49). Catheters consisted of a micro-renathane tip (0.340-0.380 mm outer diameter) connected to polyethylene tubing and were exteriorized through a subcutaneous tunnel to the back of the neck. Catheters were always filled with heparin (100 U/ml isotonic glucose) at 10 ml/hr. The exterior part was attached to a swivel, enabling the mice to move freely without disturbance. For analgesia, after surgery mice were given one bolus s.c. injection of 0.1 mg/kg Temgesic followed by 1 day of continuous infusion of 3.75 mg/kg/day Temgesic. After surgery, mice were given a 4-day recovery time before continuous blood pressure and heart rate measurements every 5 min. began. Baseline measurements were conducted for 3 days, followed by measurements as per experimental protocol (figure S1).

Experimental protocol 1a: Chronic i.v. IL-17A infusion in mice – step-up concentration (vendor 1: CHO expressed)

Recombinant mouse IL-17A (R&D Systems, CHO-expressed) was infused chronically i.v. in mice in a dose-step-up protocol (n=5). After 4 days of recovery upon surgery and 3 days of baseline blood pressure measurements, mice received 3.2 ng/kg/min IL-17A for 2 days, followed by 16 ng/kg/min IL-17A for 2 days and finally 32 ng/kg/min IL-17A for 4 days. The lowest dose was determined based on a previous i.p. IL-17A injection study and was adjusted to initial animal body weight and given continuously (21). Mean arterial blood pressure (MAP), systolic and diastolic blood pressure (SBP and DBP) and heart rate was measured during the

whole protocol and mean of every 5 min. recordings were collected. Whole blood was collected into EDTA vials from conscious undisturbed mice (max 200 mL) from the arterial catheter at baseline before IL-17A infusion and after 8 days of IL-17A infusion. At the end of protocol, mice were euthanized, and organs were harvested.

Experimental protocol 1b: Chronic i.v. IL-17A infusion in mice – step-up concentration (vendor 2: E. Coli expressed)

The same protocol was repeated with higher concentrations of recombinant IL-17A from another vendor (BioLegend, murine IL-17A expressed in E. Coli) along with CHO-expressed IL-17A (R&D systems) at 3.2 ng/kg/min, 32 ng/kg/min, and 320 ng/kg/min (*n*=4 and *n*=2 respectively).

Experimental protocol 2: Continuous IL-17A or vehicle infusion followed by ANGII-infusion

After baseline measurements, mice were given 32 ng/kg/min IL-17A (BioLegend, *n*=5) or vehicle (saline, *n*=5) by continuous i.v. infusion for 2 days, followed by addition of 60 ng/kg/min ANGII for 7 days. Blood pressure recordings were obtained for the whole protocol every 5 min. Baseline and after-intervention EDTA whole blood along with organs were collected as mentioned above. One plasma sample from the group of mice that received saline is missing since it was not possible to withdraw blood at the end of the experiment.

Experimental protocol 3: IL-17A acute bolus infusions

Acute administration of IL-17A (BioLegend) at different doses was carried out. IL-17A was given as an i.v. bolus infusion in 2 series of experiments. In the first series, mice were first given a 50 ml glucose-heparin bolus followed by IL-17A boli infusions with the concentrations

455 mg/kg and 1820 mg/kg with 10 min. interval between each bolus infusion (*n*=5). In the second series of bolus experiments, mice were given one glucose-heparin bolus followed by four bolus infusions of 50-100 ml of IL-17A with the concentrations 455 mg/kg, 910 mg/kg, 1820 mg/kg, and 3640 mg/kg (*n*=5). All bolus infusions were given with 10 min. interval. EDTA whole blood was collected at baseline and after IL-17A infusion. After IL-17A infusion, mice were given i.v. boli of ANGII (60 ng/kg) and Ach (0.005 mg/kg) by slow infusion over 10 min. Blood pressure was measured every 2 seconds.

Cell culture

NIH 3T3 fibroblast cells were cultured in T25 flasks using DMEM media (Sigma-Aldrich, Denmark) supplemented with L-glutamine (Sigma-Aldrich, Denmark), 100 U/ml penicillin (ThermoFisher, Denmark), 100 mg/ml streptomycin (ThermoFisher, Denmark), Glutamax (ThermoFisher, Denmark), 10% newborn calf serum (ThermoFisher, Denmark). When 80% confluent, cells were treated with 10 ng/ml IL-17A for 24 hours. Culture supernatants were harvested and analyzed on an IL-6 immunoassay to check for IL-6 production.

Immunoassays: IL-17A and IL-6

In short, IL-17A and IL-6 antibodies were used to coat mesoscale one-spot 96-well plates for 1 hour at room temperature (RT) with shaking at 750 rpm on an ELISA plate shaker. After coating, wells were washed 3 times with PBS with 0.05% tween-20 (PBS/Tw). Then, plasma, culture supernatants and standard calibrators were added to the wells with MSD diluent and incubated for 1 hour at RT with shaking at 750 rpm on an ELISA plate shaker. Dilution of plasma from each series was optimized for obtaining values within detection range. After analyte incubation, wells were washed 3 times with PBS/Tw followed by IL-17A or IL-6 detection antibodies incubation for 1 hour at RT with shaking. Finally, wells were washed 3

times with PBS/Tw and developed using mesoscale 2X read buffer and read using the MESO QuickPlex SQ 120 reader. Concentrations of IL-17A and IL-6 were calculated by the mesoscale Workbench software and given as pg/ml based on a 4-parameter analysis of fitted calibrator curve obtained by serial dilutions of the given calibrators. Data are presented median with interquartile range. Recombinant IL-17A was used as a positive control for the IL-17A immunoassay and determining inter-assay variation (4,2%).

RT-PCR detection of IL-6 and IL-17A-Receptor mRNAs

Total RNA was isolated from the kidney, heart, and aorta of mice that received IL-17A for 8 days (step-up protocol 1) by homogenizing in TriZol (Sigma-Aldrich, Denmark) using the TissueLyzer II (QIAGEN, Germany). Lysates were subjected to chloroform extraction, and RNA was precipitated with isopropanol. RNA concentrations were measured using the Nano Photometer (IMPLEN, Germany). RNA was stored at -80 °C until further use. Purified RNA was converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad, USA) as per manufactures protocol using a Thermal cycler (VWR ® Doppio, USA). For PCR, 50 pmol of cDNA was mixed with 10 µM of forward primer (Sigma-Aldrich, Denmark, table S1), 10 µM of reverse primer (Sigma-Aldrich, Denmark, table S1), and 10 µL of Taq DNA polymerase w/Standard buffers (Molecular Biology, ThermoFisher Scientific, Denmark). The mixture was amplified by 35 cycles (15s at 95°C; 15s at 56°C; and 15s at 75°C) in a thermal cycler (Doppio). The PCR products were separated by electrophoresis on a 2% agarose gel with GelRed™ Nucleic Acid stain (Biotium, USA) in Tris acetate-EDTA buffer (TAE, Sigma Aldrich, Denmark) along with a molecular weight marker (GeneRuler 50 bp DNA ladder, ThermoFisher Scientific, Denmark). The gels were photographed using GelDoc 2000 (Bio-Rad).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Diagram with number of mice used in the 3 animal experiments.

Figure S2: Blood pressure measurements of mice receiving continuous i.v. IL-17A for 8 days from two different vendors. A) Mice were continuously infused with IL-17A at 3 consecutive doses: 3.2-16-32 ng/kg/min (R&D systems, *n*=5). Mean arterial pressure (MAP) was significantly decreased during IL-17A infusion with 32 ng/kg/min B) but heart rate was unchanged. **C)** In a second series of experiments (protocol 1b), MAP was significantly decreased in mice that received continuous IL-17A infusion at concentrations 3.2 and 320 ng/kg/min using recombinant protein from two different vendors (BioLegend and R&D systems, *n*=7) **D)** and heart rate was unchanged (*P*=0.08 during infusion with 320 ng/kg/min). **E)** Blood pressure changes were not different between vendors or between the different concentrations. For statistical comparison between MAP and heart rate at baseline and during infusion with different doses of IL-17, one-way ANOVA with repeated measures followed by Dunnett's post-hoc multiple comparison test was used. For assessing difference between blood pressure using two different vendors unpaired Mann Whitney tests were performed, since data were not normally distributed. P<0.05 was considered statistically significant (**P<0.01, ***P<0.001). Data are presented as mean ± SEM or median with interquartile range (E).

Figure S3: Blood pressure measurements of mice receiving i.v. IL-17A bolus infusions (*n*=5). A-B) Mean arterial pressure (MAP) and heart rate (HR) recordings of bolus infusion with 2 different concentrations (455 and 1820 mg/kg) with 10. min interval. No blood pressure changes were observed during IL-17A infusion. MAP was significantly increased and decreased upon ANGII and Ach infusion respectively compared to baseline blood pressure, and HR had opposite effects. For statistical comparison One-way ANOVO with repeated

measures followed by Tukey's multiple comparison test was performed. $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Data are presented as median with interquartile range.

Figure S4: Expression of IL-17RA and IL-6 in kidney, heart and aortic tissue of mice infused with IL-17A. A) Chronic ANGII (60 ng/kg/min) infusion in FVB/n WT mice from a separate protocol, had no effect on plasma IL-17A after infusion. **B)** NIH 3T3 fibroblasts stimulated with 10 ng/ml IL-17A for 24 hours increase IL-6 production. IL-17RA and IL-6 mRNA was expressed in **C)** kidney, **D)** heart, **E)** and aortic tissues from mice that received continuous i.v IL-17A infusion for 8 days.

Table S1

Target	Forward primer	Reverse primer	bp
IL-17RA	gtcacattcactctaagcaag	cttgggaactgtggtatttg	144
IL-6	tccttcagagagatacagaaac	ttctgtgactccagcttatc	124
TBP	cagccttccaccttatgctc	ttgctgctgctgtctttgtt	169

Figure 1

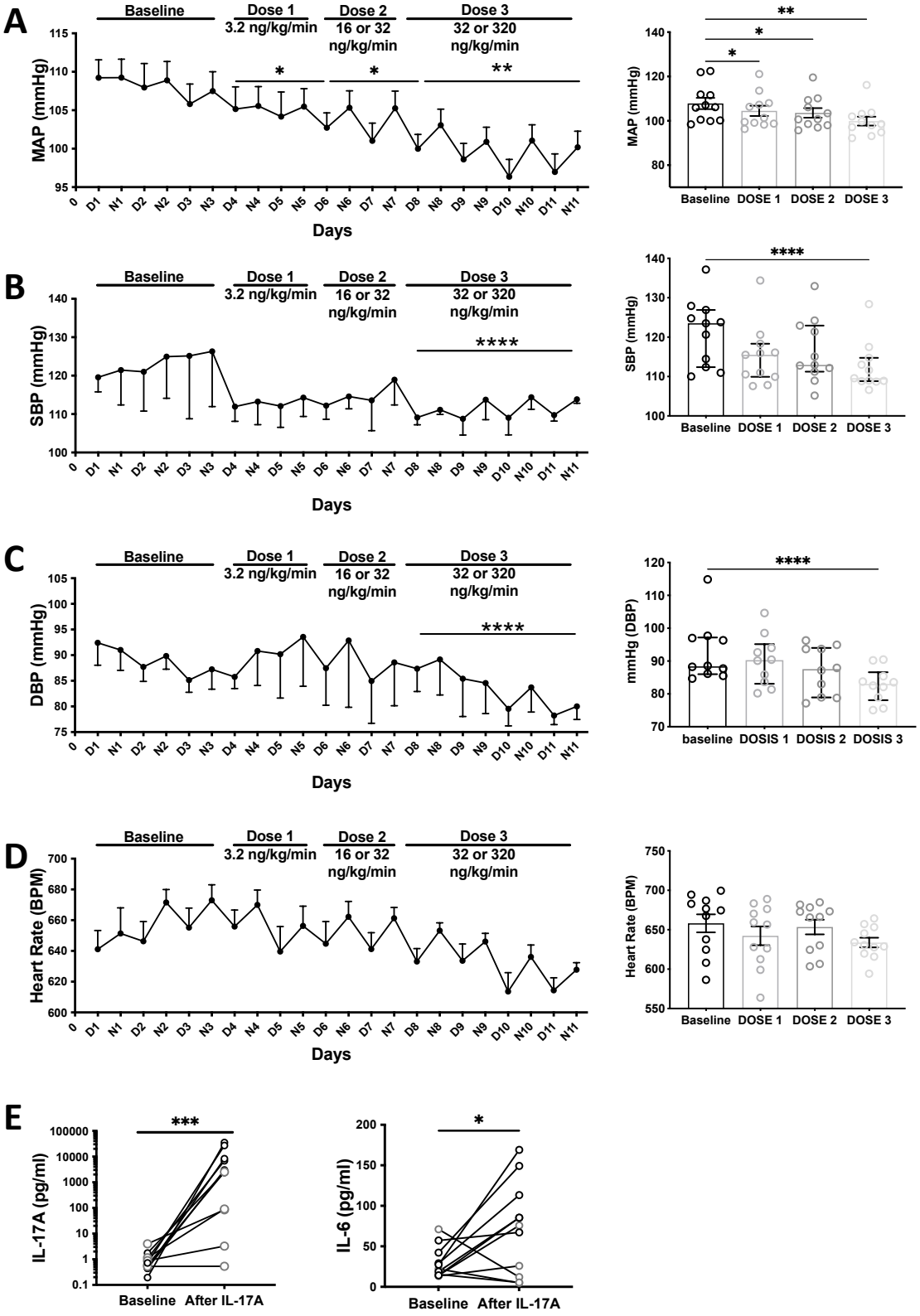

A
Baseline
Dose 1
3.2 ng/kg/min
Dose 2
16 or 32
ng/kg/min
Dose 3
32 or 320
ng/kg/min
MAP (mmHg)
Days
Baseline DOSE 1 DOSE 2 DOSE 3
B
SBP (mmHg)
Baseline DOSE 1 DOSE 2 DOSE 3
C
DBP (mmHg)
mmHg (DBP)
baseline DOSIS 1 DOSIS 2 DOSIS 3
D
Heart Rate (BPM)
E
IL-17A (pg/ml)
Baseline After IL-17A
IL-6 (pg/ml)
Baseline After IL-17A

Figure 2

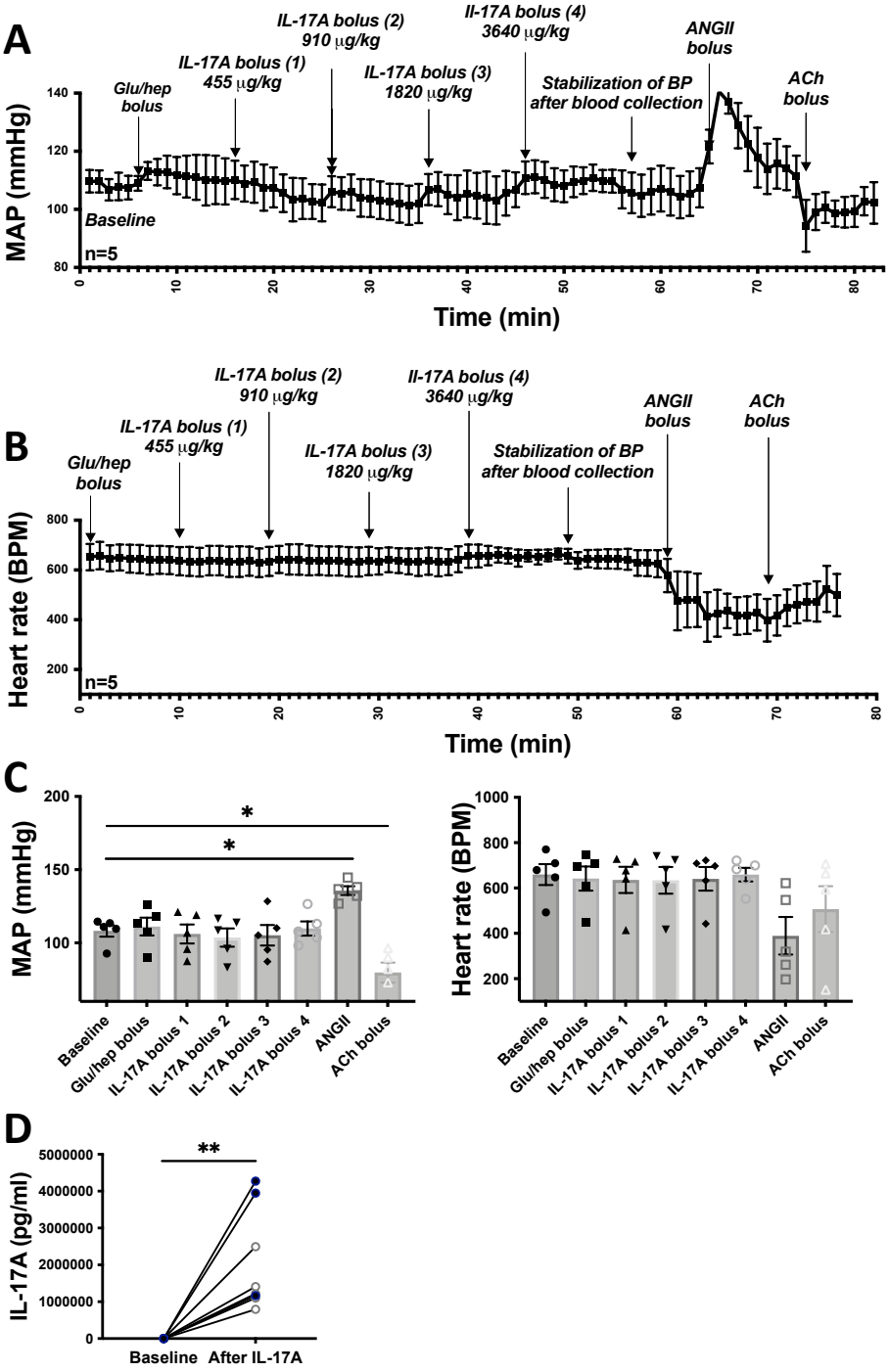

A
MAP (mmHg)
Time (min)
Glu/hep bolus
IL-17A bolus (1) 455 μg/kg
IL-17A bolus (2) 910 μg/kg
IL-17A bolus (3) 1820 μg/kg
Il-17A bolus (4) 3640 μg/kg
Stabilization of BP after blood collection
ANGII bolus
ACh bolus
Baseline
n=5
B
Heart rate (BPM)
Time (min)
C
MAP (mmHg)
Heart rate (BPM)
Baseline
Glu/hep bolus
IL-17A bolus 1
IL-17A bolus 2
IL-17A bolus 3
IL-17A bolus 4
ANGII
ACh bolus
D
IL-17A (pg/ml)
Baseline
After IL-17A

Figure 3

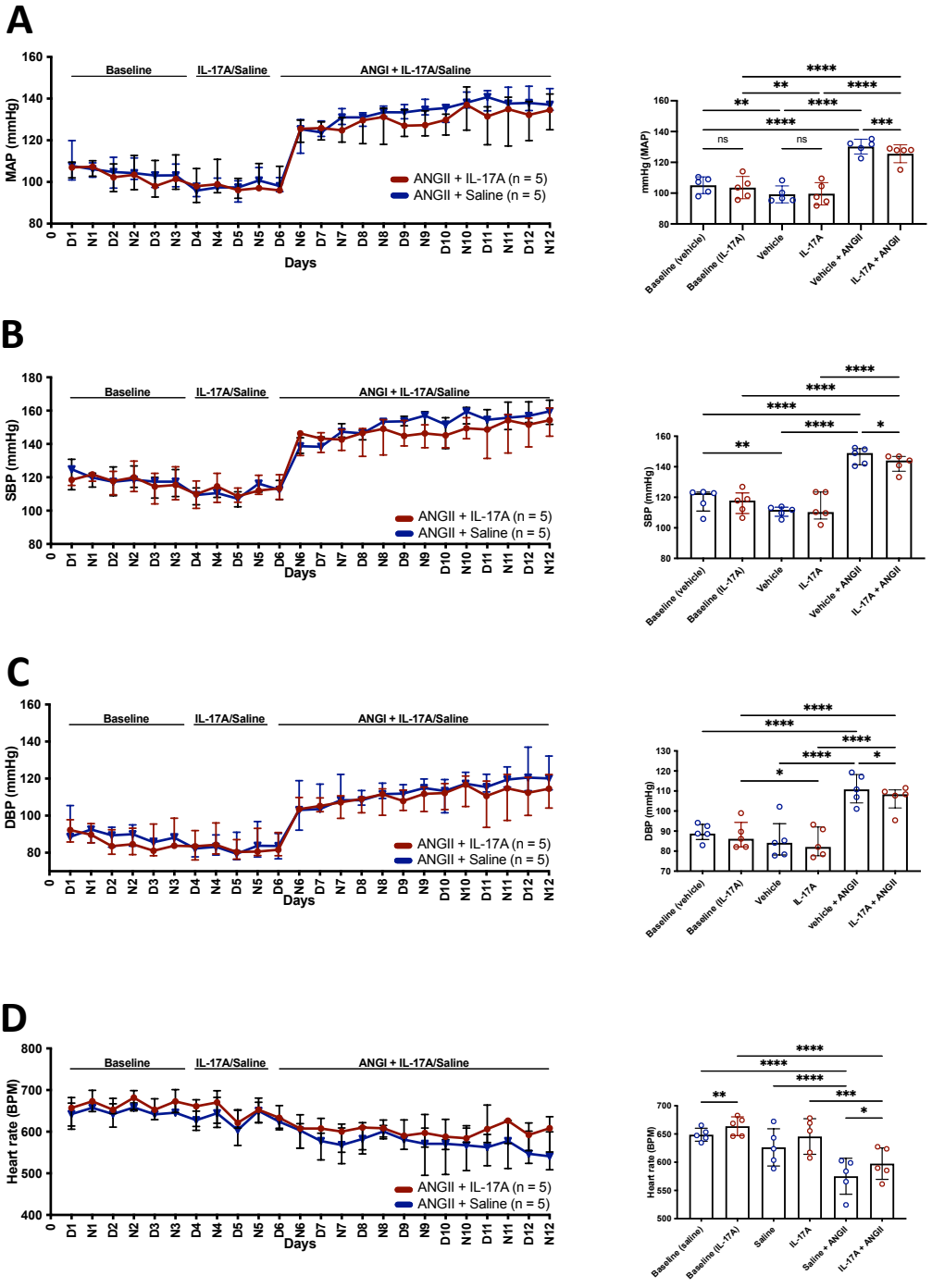
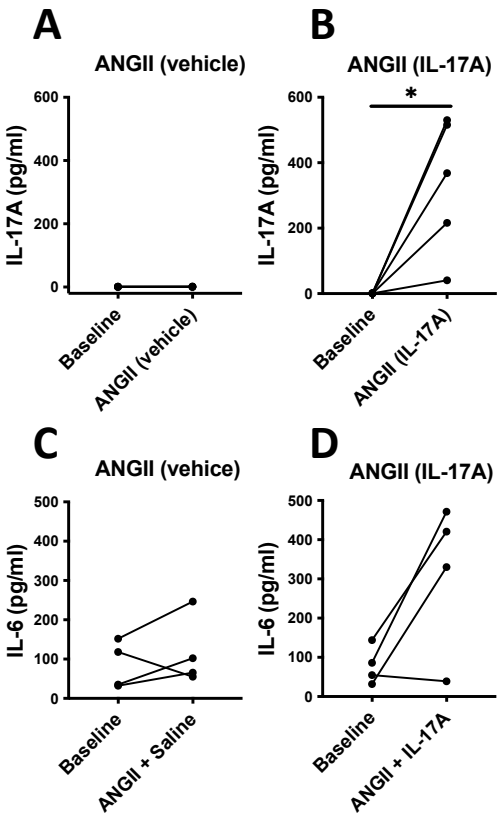

A
Baseline
IL-17A/Saline
ANGI + IL-17A/Saline
MAP (mmHg)
Days
ANGII + IL-17A (n = 5)
ANGII + Saline (n = 5)
mmHg (MAP)
Baseline (vehicle)
Baseline (IL-17A)
Vehicle
IL-17A
Vehicle + ANGII
IL-17A + ANGII
ns
B
SBP (mmHg)
C
DBP (mmHg)
D
Heart rate (BPM)
Saline
Saline + ANGII

Figure 4


A
ANGII (vehicle)
IL-17A (pg/ml)
600
400
200
0
Baseline
ANGII (vehicle)
B
ANGII (IL-17A)
*
IL-17A (pg/ml)
600
400
200
0
Baseline
ANGII (IL-17A)
C
ANGII (vehice)
IL-6 (pg/ml)
500
400
300
200
100
0
Baseline
ANGII + Saline
D
ANGII (IL-17A)
IL-6 (pg/ml)
500
400
300
200
100
0
Baseline
ANGII + IL-17A

Supplementary figures

Figure S1

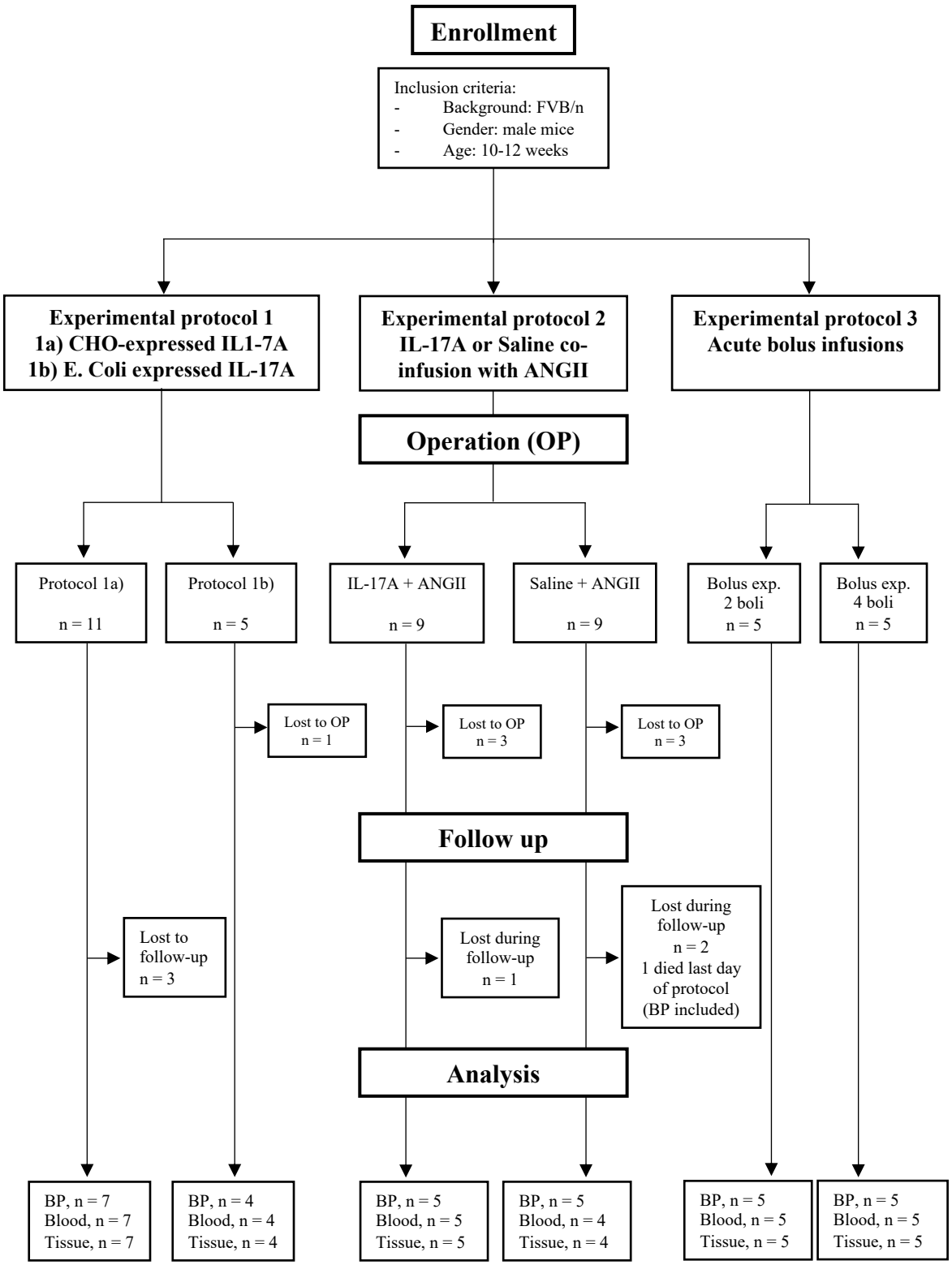


Figure S1: Diagram with number of mice used in the 3 animal experiments.

Figure S2

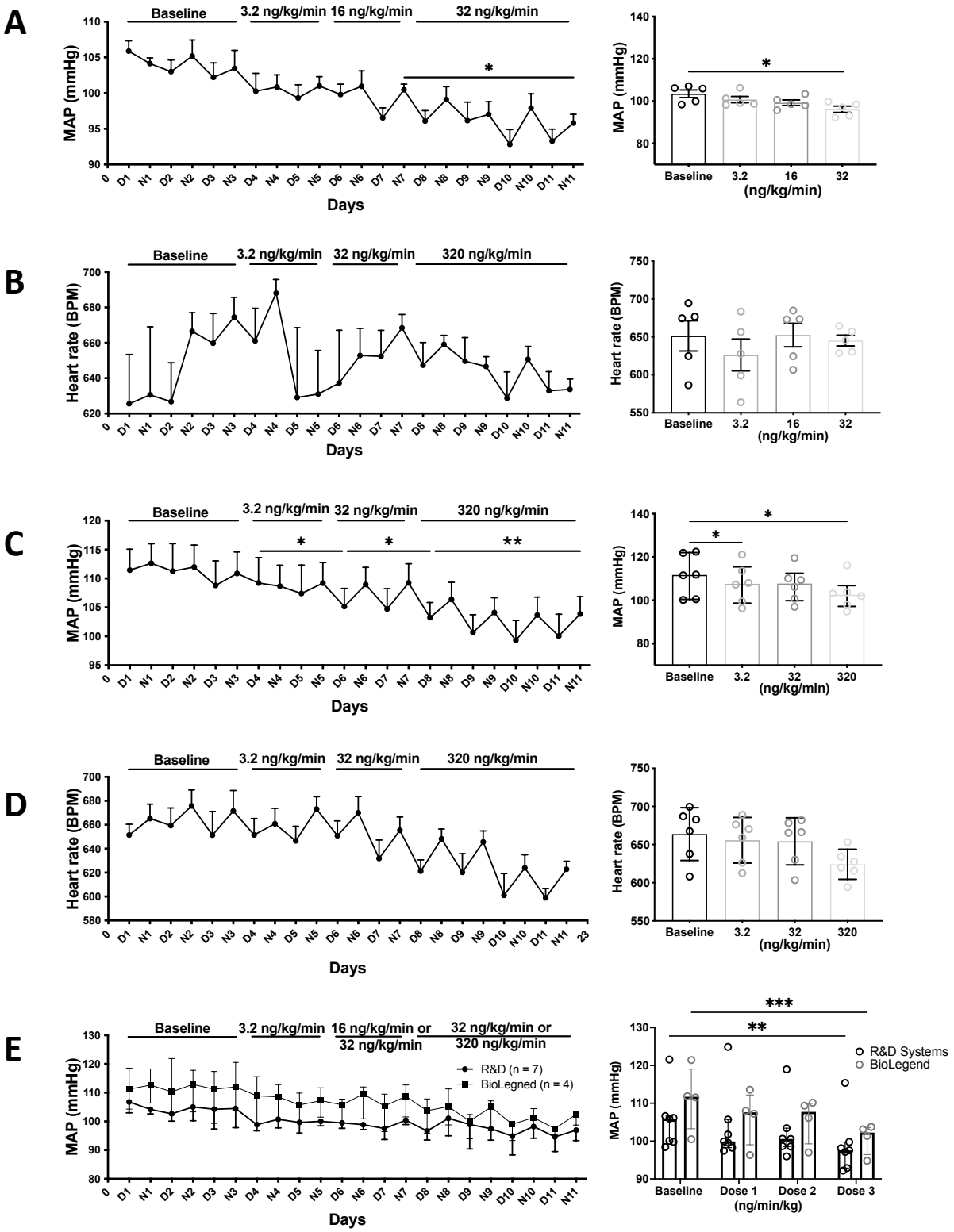

A
Baseline
3.2 ng/kg/min
16 ng/kg/min
32 ng/kg/min
MAP (mmHg)
Days
(ng/kg/min)
B
Heart rate (BPM)
C
320 ng/kg/min
D
E
32 ng/kg/min or
320 ng/kg/min
R&D (n = 7)
BioLegned (n = 4)
R&D Systems
BioLegend
Dose 1
Dose 2
Dose 3
(ng/min/kg)

Figure S3

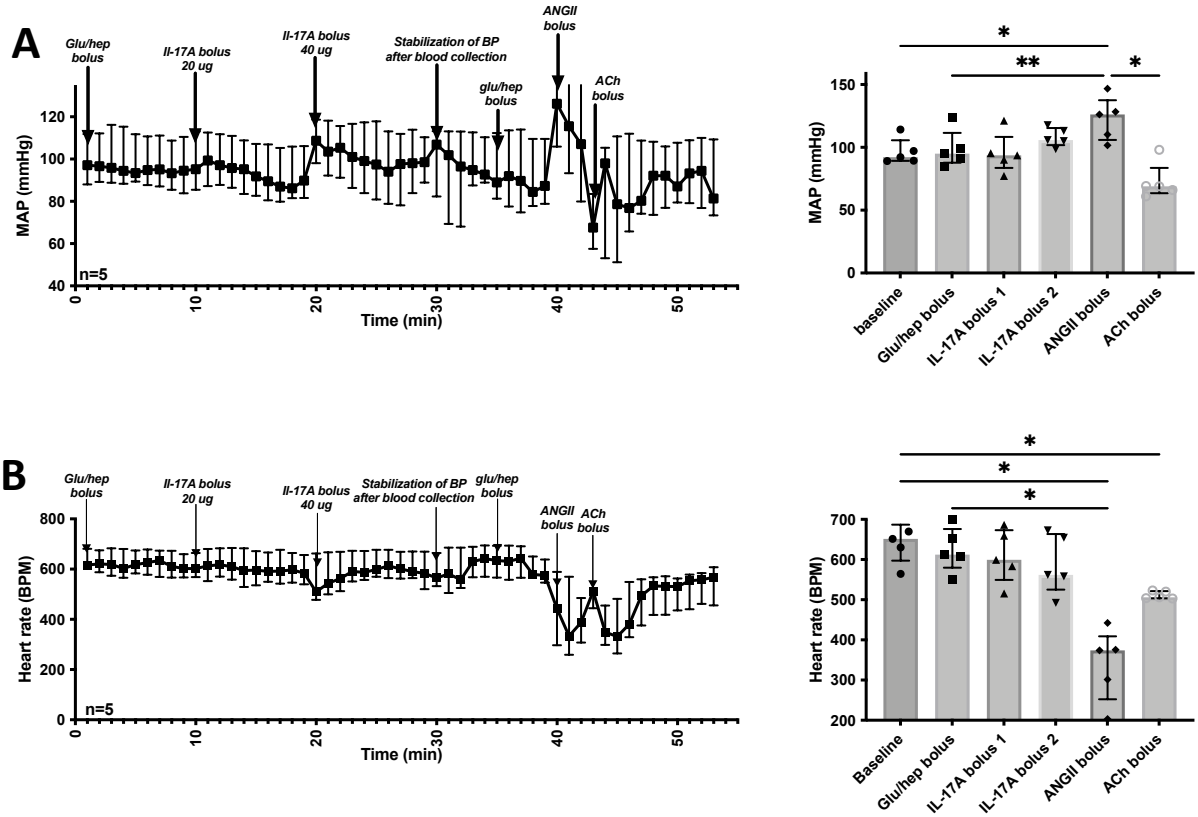
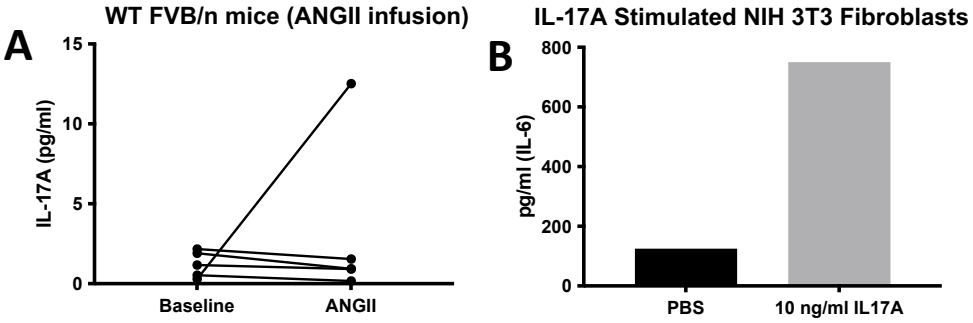
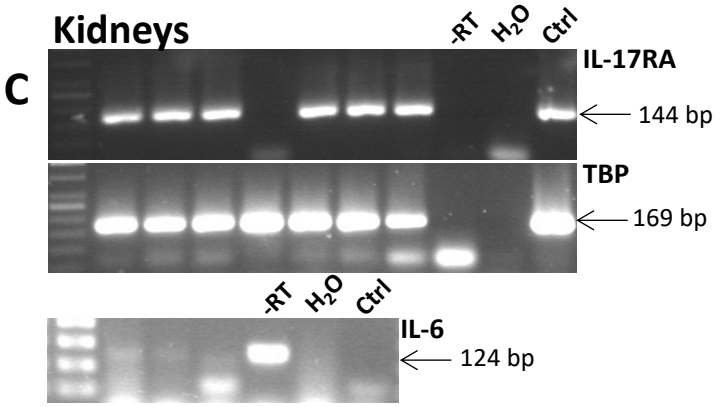
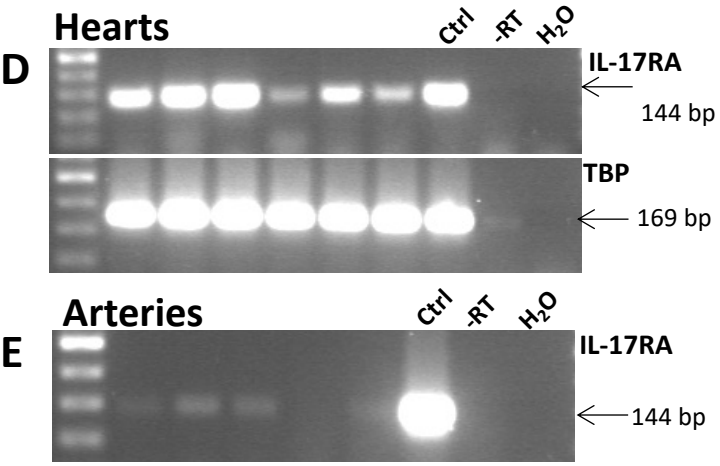

A
Glu/hep bolus
Il-17A bolus 20 ug
Il-17A bolus 40 ug
Stabilization of BP after blood collection
glu/hep bolus
ANGII bolus
ACh bolus
MAP (mmHg)
n=5
Time (min)
baseline
Glu/hep bolus
IL-17A bolus 1
IL-17A bolus 2
ANGII bolus
ACh bolus
B
Heart rate (BPM)
Baseline

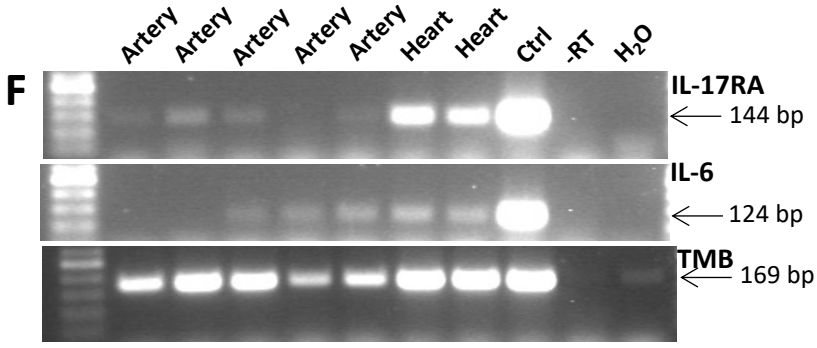
Figure S4







Arteries and hearts of mice chronically infused with IL-17A



DISCUSSION

The following chapter provides a general summary of the results of all 4 studies of this PhD, a discussion of the obtained results, and the advantage and limitations of the used methods and techniques.

SUMMARY OF RESULTS

CLINICAL DATA (MR BLOCKADE WITH SPIRONOLACTONE)

Plasma cytokine analyses in samples from the two double-blind, randomized, controlled trials with T2DM patients with resistant hypertension and kidney transplant patients, revealed no effect of MR antagonism with spironolactone on the IL-17A production. In these patients no relation was observed between baseline and after-intervention blood pressure and cytokine levels, except for T2DM patients, where IFN-γ related inversely to SBP after spironolactone intervention. In T2DM patients a minor but significant reduction in IFN-γ and IL-6 was observed, and this reduction was not seen in placebo-treated patients. Also, a positive relation was observed between plasma aldosterone and DBP, and between UACR and plasma IL-6 at baseline in these patients. Spironolactone-mediated increased plasma potassium related inversely to blood pressure reduction in T2DM patients but did not relate to cytokine changes. *In vitro* studies with LPS-stimulated THP-1 macrophages confirmed a spironolactone-mediated reduction in IL-6, TNF, IL-1β, and IL-10. In kidney transplant patients, urinary kidney injury markers calbindin and TFF3 were significantly reduced after intervention, and baseline levels of these markers related to blood pressure, however with no relation to IL-17A or other cytokines or to plasma aldosterone at baseline. In conclusion MR antagonism with spironolactone in T2DM patient with resistant hypertension and kidney transplant patients, may have additional anti-inflammatory effect by suppressing T-cell and macrophage-derived cytokine production, and direct protective effects in the distal epithelium of the nephron.

CLINICAL DATA (ENaC BLOCKADE WITH AMILORIDE)

In the interventional study with amiloride in T2DM patients with resistant hypertension, ENaC blockade with amiloride suppressed plasma TNF and IL-6 significantly. In these patients, plasma IL-1β and IL-17A correlated to blood pressure at baseline, and UACR correlated positively to plasma IL-17A, TNF, IL-6, IL-1β, and SBP at baseline. There was no correlation observed between blood pressure-decrease, plasma cytokine decreases of TNF and IL-6, or plasma potassium increase after amiloride intervention. In *in vitro* LPS-stimulated THP-1 macrophages, amiloride suppressed IL-6, IL-1β, and IL-10, whereas benzamil, a more potent ENaC blocker, suppressed TNF, IL-6, IL-1β, and IL-10 at 1nM concentrations. In conclusion, ENaC blockade with amiloride had no effect on IL-17A but exerted an anti-inflammatory effect

by suppressing macrophage-derived cytokine production (TNF and IL-6), independent of blood pressure and plasma potassium concentrations in T2DM patients.

PRE-CLINICAL DATA (EFFECT OF IL-17A INFUSION ON BLOOD PRESSURE AND ANGII-HYPERTENSION)

In the experiments with FVB/n mice chronic and acute i.v. infusion of IL-17A revealed no blood pressure elevating effect, but instead during chronic infusion, a blood pressure lowering effect was observed, with no change in heart rate. Chronic co-infusion of ANGII with IL-17A did not accentuate blood pressure, but instead attenuated blood pressure compared to mice co-infused with vehicle, and heart rate was accordingly increased in mice that received IL-17A compared to vehicle. Plasma concentrations of IL-17A were increased by up to 3500 times above baseline levels. Downstream cytokine production of IL-6 was increased in mice that received IL-17A. In conclusion, IL-17A by itself, is not a hypertensive agent, and other unknown factors may be needed to explain IL-17A induced hypertension.

GENERAL DISCUSSION OF FINDINGS

THE RELATION BETWEEN IL-17A AND MR- OR ENAC-MEDIATED HYPERTENSION

In the present study, hypertensive patients with T2DM, and kidney transplant patients included in the respective controlled trials intervened with either the MR antagonist, spironolactone, or the ENaC blocker, amiloride, showed no changes in plasma IL-17A concentrations compared to baseline levels. In T2DM patients however, baseline plasma IL-17A related to SBP. These observations are in contrast to the hypotheses of this study, but the studies were consistent and did not support any role for MR or ENaC to support IL-17A across the tested patient categories.

ALDOSTERONE-MR AND IL-17A

A great amount of the studies indicating a hypertensive role of IL-17A are based on animal experiments using different models of hypertension with either targeted gene deletion or neutralizing antibodies against IL-17A (79, 90). Only little clinical data is available. Plasma IL-17A elevation in hypertensive patients compared to respective controls has been documented (72, 90-92, 155). The effect of MR-antagonism on plasma IL-17A in hypertensive patients has not been shown before, but *in vitro* and pre-clinical studies have suggested a MR-mediated Th-17 proliferating role. *In vitro* stimulation of dendritic cells with aldosterone

mediated Th-17 conversion, and DOCA administration enhanced disease severity of experimental multiple sclerosis in mice, which further showed with increased IL-17A expressing cells in the CNS. These events were suppressed by MR-antagonism with spironolactone (139). DOCA-salt hypertensive rats are shown to have elevated plasma IL-17A, and MR-antagonism with spironolactone reduced blood pressured and plasma IL-17A (94). In these rats, IL-17A neutralizing antibody treatment lowered blood pressure as well (94). These studies raise the question if a possible beneficial anti-hypertensive effect of spironolactone could be exerted by inhibition of the IL-17A axis. It must however, also be mentioned that anti-hypertensive treatment with diuretics in the DOCA-salt hypertensive mice lowered blood pressure with no change in IL-17A levels (94), questioning the direct effect of IL-17A on blood pressure.

Our clinical data reveal no MR-dependent IL-17A production in patients with T2DM and treatment resistant hypertension (155) (study I) or in kidney transplant patients (study III), where spironolactone intervention reduced blood pressure significantly in the T2DM patients only (143, 146). Others have shown that IL-17A is important in ANGII-mediated hypertension (79, 90), and both preclinical and clinical studies have shown an anti-inflammatory effect of ACEi and ARBs by reduced levels of IL-17A and other pro-inflammatory cytokines (156-158). If IL-17A predominantly depend on ANGII/AT1 receptor interaction, the hypertensive T2DM patients (study I, II) and kidney transplant patients (study III) of the present study would have suppressed IL-17A levels before receiving spironolactone, since most patients received at least 3 anti-hypertensive drugs and at least 1 ACEi/ARB prior and during experiment. Since absolute plasma IL-17A concentrations were comparable across studies, our data did not suggest that IL-17A at baseline in patients that received ACEI/ARBs was markedly different from those who did not receive ACEi/ARBs. In the kidney transplant patients, where plasma IL-17A was elevated after 1 year placebo treatment, a sub-analysis was performed to elucidate if patients that received ACEi/ARBs had lower levels of IL-17A compared to patients that were not treated with ACEi/ARBs in both placebo and spironolactone treated groups. The analyses revealed no differences in plasma IL-17A. Based on these data, it is concluded that the blood pressure lowering effect of spironolactone is not related to IL-17A. But based on previously published work, it could be speculated that IL-17A exerts pro-inflammatory effects in concert with other cytokines to contribute indirectly to inflammation-mediated hypertension. The direct role of IL-17A on blood pressure is questionable and needs further investigation.

ENaC AND IL-17A

ENaC is expressed on adaptive immune cells including lymphocytes and dendritic cells (129, 159). Salt-stimulated dendritic cells produce isoketal protein-adducts dependent on ENaC activity, promote hypertension in mice, and prime Th17 proliferation *in vitro* (129). The involvement of immune-cell-expressed ENaC in hypertension has been documented in both patients and mice (129, 159), but mechanistic studies of IL-17A contribution are scarce. In clinical settings, IL-17A neutralizing antibodies have shown promising results in psoriasis and arthritis disease severity but blood pressure outcomes have not been investigated (85, 87-89). In study II, hypertensive T2DM patients treated with the ENaC blocker amiloride showed no changes in plasma IL-17A. SBP and UACR related positively to plasma IL-17A at baseline. Plasma TNF, IL-6, and IL-1β related positively to UACR at baseline, indicating a strong cytokine association to baseline albuminuria in T2DM hypertensive patients. Preclinical studies have shown that reduction of pro-inflammatory cytokines mediates reduced kidney injury using different experimental hypertensive and kidney injury models (79, 95, 103, 160). However, cytokine decrease of TNF and IL-6 and UACR decrease did not correlate after amiloride intervention. Furthermore, as mentioned previously, the T2DM patients all received ACEi/ARBs and diuretics, which exert anti-inflammatory effects as well. Therefore, these patients could have been IL-17A suppressed prior to amiloride intervention and shadow any minor amiloride-mediated anti-inflammatory effects. Taken together, although SBP related to baseline plasma IL-17A in T2DM patients, while ENaC blockade with amiloride lowered blood pressure, it did not change plasma IL-17A, which suggest that IL-17A may not directly be related to the ENaC-regulated part of blood pressure in diabetes patients *in vivo but* may act in concert with other pro-inflammatory cytokines to promote hypertension.

IS THE IL-6 AND IFN-GAMMA ASSOCIATION TO HYPERTENSION, ANGII-AT1 OR MR-ALDOSTERONE DEPENDENT?

The clinical data on patients with T2DM and treatment resistant hypertension revealed spironolactone-mediated suppression of plasma IFN-γ and IL-6. Although blood pressure was lowered by spironolactone, there was no correlation between blood pressure and cytokine levels at baseline or after intervention, except for IFN-γ, which correlated inversely to SBP after intervention. These clinical data were supported by *in vitro* macrophage studies where cell excretion of IL-6 along with TNF, IL-1β, and IL-10 was significantly lowered by spironolactone, suggesting an additive anti-inflammatory effect of spironolactone.

IS IL-6 RELATED TO BLOOD PRESSURE THROUGH MR OR ANGII-AT1 MECHANISMS?

The anti-inflammatory effect of spironolactone has only scarcely been investigated in clinical settings. In a randomized double-blind crossover study, it was shown that aldosterone i.v. infusion over 14 hours mediated increased plasma IL-6 concentration in normotensive sodium restricted individuals (*n*=11) with no effect on blood pressure, serum potassium, or high sensitivity C-reactive protein (161). In another crossover study, angiotensin II infusion in 14 salt-replete normotensive subjects mediated elevated serum IL-6 concentrations, which was prevented by spironolactone (161). In line with these clinical studies, ANGII hypertension in mice was shown to be IL-6 dependent, and MR blockade in these mice lowered serum IL-6 (162-164). However, since hypertension was induced by ANGII, and aldosterone infusion did not impact blood pressure, whether aldosterone-MR mediated hypertension is IL-6 dependent, is not clearly elucidated from these studies. One study investigating this, showed that ANGII-hypertension-mediated elevation of plasma IL-6 in mice was suppressed with spironolactone after 1 week infusion, but after 2 weeks IL-6 suppression was eliminated (164). In DOCA-salt hypertensive mice, plasma IL-6 increased after 1 week but after 2 weeks IL-6 was at baseline control levels (164). Furthermore, IL-6 knock out did not protect mice against ANGII or DOCA-salt hypertension (164). However, others have shown blood pressure reducing effects with neutralizing IL-6 antibodies in hypertensive animals (165), and ARB treatment in hypertensive patients lowered IL-6 plasma concentrations (166). These experiments suggest that MR actions may contribute to elevation of plasma IL-6 at the early stages of hypertension mediated by ANGII-AT1 and MR, but the connection between IL-6 and blood pressure is more likely linked to ANGII rather than aldosterone or MR. In line with this, hypertensive T2DM patients (study I) and kidney transplant patients (study III) of the present analyses, did not show any correlation between blood pressure and plasma IL-6 at baseline or between serum/plasma aldosterone and plasma IL-6 at baseline. However, when assessing a subpopulation of the T2DM patient cohort for follow-up amiloride intervention (study II), these patients revealed a relation between baseline plasma IL-6 and SBP. This relation has been shown by others as well (166). The anti-inflammatory effect by spironolactone-mediated IL-6 lowering has been supported by both *ex vivo* and *in vivo* studies. An *ex vivo* study show that spironolactone mediate suppression of IL-6 production by SARS-CoV2 Spike (S) protein-stimulated human aortic endothelial cells (167), and an *in vitro* study show that aldosterone directly induce IL-6 production via MR in THP-1 macrophages (168). In mice, aldosterone infusion mediated

macrophage infiltration of the myocardium, and this was abrogated by MR blockade with eplerenone or anti-IL-6 treatment, but with no data on blood pressure (168). Altogether these studies suggest that MR-antagonism, besides its anti-hypertensive and reno-protective effects, exert an anti-inflammatory effect by IL-6 suppression, and that this anti-inflammatory effect does not directly impact blood pressure. Instead, IL-6-inflammation-mediated hypertension is suggested to be more related to ANGII-AT1 hypertensive actions, but this link needs further investigation to identify mechanisms.

IS IFN-GAMMA PRODUCTION, MR OR ANGII-AT1 DEPENDENT IN HYPERTENSION?

Preclinical studies have shown that IFN-γ is elevated in ANGII and DOCA/salt induced hypertension in rats (169, 170), and that MR deficiency in T-cells lowered production of IFN-γ in kidneys and aortas after ANGII infusion (171). Clinical data on spironolactone mediated anti-inflammatory effect are not available, but anti-inflammatory effects by other anti-hypertensive medicaments including ARBs have been reported (158). Furthermore, targeted gene deletion of IFN-γ blunted ANGII-mediated hypertension in mice (95). These findings show that IFN-γ is elevated during hypertension, but the direct effect on blood pressure via ANGII-AT1 or MR has not been clearly established. In study I, although spironolactone intervention lowered plasma IFN-γ, we saw an inverse correlation between IFN-γ and blood pressure decrease, which can be speculated about to indicate that the spironolactone-mediated decrease in plasma IFN-γ may not directly be associated to the spironolactone-mediated blood pressure decrease and may instead be an expression of the overall effects of MR blockade.

The *in vitro* experiment of study I showed spironolactone to have a suppressive effect on TNF, IL-1β, and IL-10 as well. These cytokines were unaltered in the T2DM patients and kidney transplant patients after spironolactone intervention. In line with our findings, others have found spironolactone to have no effect on serum TNF levels in 6 diabetic nephropathy patients (172). Bendtzen and colleagues have shown that spironolactone inhibits proinflammatory cytokine (i.e., TNF, IL-6, IFN-γ) production in LPS stimulated human mononuclear cells, but that this anti-inflammatory effect of spironolactone may be unrelated to MR antagonism, since aldosterone alone could not induce cytokine production and MR blockade with the spironolactone metabolite canrenone had no effect on cytokine production (173, 174). Plasma IL-10 was increased in T2DM placebo treated patients and unaffected or stabilized in spironolactone treated T2DM patients, this could suggest higher levels of inflammation in

placebo treated patients and therefore counteractingly increased levels of the anti-inflammatory IL-10 in these patients.

Thus, the additive anti-inflammatory effect of spironolactone was addressed by detection of reduced plasma IL-6 and IFN-γ, and increased IL-10 in placebo treated patients, and in line with other human, animal, and *in vitro* cell studies. The direct relation of IL-6 and IFN-γ to hypertension may be more dependent on ANGII-AT than aldosterone-MR actions, moreover, the exact mechanism of spironolactone mediated anti-inflammatory effects is still unclear since these effects have shown not to depend on MR by others.

IS ENAC ASSOCIATED WITH TNF AND IL-6 CYTOKINE PRODUCTION IN HYPERTENSION?

Since amiloride suppressed TNF and IL-6 in T2DM patients, we sought to investigate the direct suppressive effect on these macrophage-derived cytokines *in vitro*. TNF and IL-6 are known as early response cytokines primarily produced by macrophages (175). These two cytokines are elevated during hypertension in both preclinical (164, 176-179) and clinical studies (180-182). TNF deficiency in animals by either targeted gene deletion or neutralizing antibodies have shown to protect against ANGII induced hypertension (176, 178). However, anti-TNF treatment in DOCA-salt induced hypertensive rats, had no effect on blood pressure, but reduced kidney injury (177). In humans, TNF antibody treatment in rheumatoid arthritis patients has shown beneficial effects on disease severity and blood pressure was reduced (183). Furthermore, TNF has also shown to promote cardiac hypertrophy in ANGII animal models, and deficiency of TNF has shown to exert organ protection (184). Furthermore, TNF plays a major inflammatory role in autoimmune disorders like psoriasis and arthritis (185). Effects of anti-IL-6 treatment on blood pressure has only been documented in mice (164, 165). These studies however, showed opposing results as mentioned previously.

Taken together, studies suggest that TNF and IL-6 may be related to elevated blood pressure, independent of aldosterone-MR but may depend more on ANGII-AT mechanisms. In study II, hypertensive T2DM patients treated with amiloride, showed reduced TNF and IL-6. Macrophage-derived cytokine-lowering by ENaC blockade was confirmed by *in vitro* studies using both amiloride and benzamil, where benzamil was a more potent suppressor of cytokine production compared to amiloride, which also has other targets including sodium hydrogen exchanger 3 (NHE3), a-adrenoceptors, and urokinase plasminogen activator (uPA). Although ENaC expression has not been shown on macrophages directly, α and γ subunits of ENaC have

been shown to be expressed on salt stimulated splenic $CD11c^+$ antigen presenting cells from mice. This is a marker that is abundantly expressed on antigen presenting cells including dendritic cells, monocytes, and macrophages (128, 131). Furthermore, high salt stimulation of macrophages has shown accumulation of isoketal protein adducts as in dendritic cells where this was mediated through ENaC (129). To our knowledge anti-inflammatory effects of amiloride in hypertensive patients have not been investigated before. However, amiloride intervention on monocytes from patients with cystic fibrosis, alleviated augmented cytokine production (186), and *in vitro* studies with LPS stimulated alveolar macrophages treated with amiloride showed to reduce TNF significantly (187). Thus, our results indicate that macrophages may express ENaC, and we conclude that amiloride exerts anti-inflammatory effects by suppressing macrophage-derived TNF and IL-6 in hypertensive patients with diabetes. It can be speculated that this effect of amiloride could contribute to blood pressure lowering and organ protection.

SPIRONOLACTONE AND ITS RENO-PROTECTIVE EFFECTS

In kidney transplant patients, spironolactone intervention reduced urine calbindin and TFF3 without any changes seen in placebo treated patients. Baseline urinary TFF3 correlated positively to blood pressure. The panel of markers included in this study represent injury at different segments of the nephron including glomerulus (VEGF), proximal tubule (KIM-1), thick ascending limb of Henle's loop (TFF3), distal tubule, and the collecting ducts (Calbindin) (188, 189). Calbindin is primarily produced by distal tubular and collecting ducts cells, and function as an extracellular calcium-binding protein (190, 191). Calbindin has been implicated in distal tubular cell injury with increased production in patients and *in vitro* (192, 193). Both preclinical and clinical studies have shown increased urinary calbindin levels in acute kidney injury (189, 194, 195). TFF3, produced by tubular epithelial cells in the thick ascending limb of Henle's loop and the early portion of the distal tubule of the nephron (196-198), is increased in patients with CKD, and relates directly to CKD severity (198-200). Although plasma aldosterone at baseline did not relate to calbindin or TFF3, the localization of these markers at the distal site of the nephron overlaps with MR localization from the distal convoluted tubules over the connecting tubule including the collecting ducts and could facilitate direct reno-protective actions of MR antagonism. Although previously published studies suggest cytokine suppression to be related to injury protection (78, 95, 103), in this study there was no relation between cytokines and injury markers in response to spironolactone, and therefore the protective actions by spironolactone could thus be directly MR-mediated. A good indicator for

protection in these patients could be comparisons of urinary albumin/creatinine ratios before and after intervention. However, since this is one of the main parameters of the study, the interventional code was not revealed until end of study, and the original work has also not been published yet.

DOES INCREASED PLASMA POTASSIUM LEVELS HAVE ANTI-INFLAMMATORY EFFECTS?

In all three interventional studies amiloride and spironolactone intervention induced elevated plasma potassium (132, 143, 146, 151). Previous studies have shown that increased extracellular potassium concentrations mediated membrane depolarization of T-lymphocytes and suppress Ca^{2+} influx which is needed for T-cell activation and T-cell cytokine production including IL-17A (201-203). Also, pro-inflammatory cytokine response correlates with increased expression and activity of potassium-channels, and blockade of these suppress T-cell proliferation and inflammation (201, 202). Furthermore, potassium channel blockers attenuate renal fibrosis and organ damage in animals (204). In the present study, spironolactone mediated blood pressure lowering in T2DM patients was inversely related to spironolactone-mediated plasma potassium increases. This relation was not seen in kidney transplant patients, possibly because blood pressure was unaltered. However, in amiloride treated patients, although blood pressure was reduced and plasma potassium increased, no correlation was observed between these two parameters. We did also not see any relation between plasma cytokine reduction and potassium increases in any of the analyzed patients of the present study, suggesting that the anti-inflammatory effects by spironolactone and amiloride were independent of potassium increase.

THE DIRECT ROLE OF IL-17A IN HYPERTENSION IN PRECLINICAL STUDIES

When assessing the direct effects of IL-17A on blood pressure in mice, using both chronic and acute infusion models, IL-17A had no hypertensive role despite a 3500 times increase in plasma IL-17A levels above baseline. Chronic infusion with step-up doses of IL-17A mediated blood pressure lowering without changes in heart rate, and bolus i.v. infusions of IL-17A revealed no acute effects on blood pressure. The elevated plasma IL-17A in mice increased plasma IL-6, and IL-17A stimulated fibroblast cells secreted increased levels of IL-6 *in vitro*. Hereby the biological activity of recombinant murine IL-17A was confirmed. Chronic co-infusion of ANGII and IL-17A in mice attenuated blood pressure compared to mice co-infused with ANGII and saline. This study indicates that IL-17A has no direct cardiac or vascular pro-

hypertensive effects *in vivo* in FVB/n mice. Rather, IL-17A in very high doses, lowered blood pressure and heart rate. These parallel changes in blood pressure and heart rate suggests a sympatholytic effect or cardio-suppressive effect of IL-17A that could account for the effect on blood pressure. The finding that IL-17A does not mediate direct hypertensive effects is in line (97), and in contrast (84, 96, 99, 100) to other studies. In these studies, IL-17A was given to mice by different administration routes including daily i.p. injections, s.c. infusion using osmotic minipumps, and dermal overexpression resulting in elevated blood pressure in mice (84, 96, 99, 100), whereas systemic overexpression in T-cells in mice had no effect on blood pressure (97). Previous studies implicating a pro-hypertensive role of IL-17A, is based on indirect experiments using deficiency of IL-17A or IL-17RA by either knockout or neutralizing antibodies in animals on top of different experimental hypertensive models, showing protection against hypertension (78, 79, 90, 95). In the present study, we were able to measure continuous blood pressure recordings in freely moving unstressed mice during chronic and acute i.v. IL-17A infusion, using chronic indwelling catheters in the femoral artery and vein. This technique resembles the radio telemetry technique, but with possibility for continuous i.v. infusion, and considered the gold standard for blood pressure measurements in experimental hypertension models and allow detection of small differences and diurnal variations (205, 206). Previously published data showing blood pressure elevation after IL-17A administration mostly used the tail cuff method for blood pressure measurements, and plasma IL-17A levels were not reported (84, 96, 99, 100). This is a non-invasive but less expensive alternative to the indwelling catheter method, which requires handling, restraint, and warming of mice, that are all factors that could affect blood pressure, and major limitations of this method. These different approaches could be the reason for the discrepancies between studies.

Moreover, in our study we used FVB/n mice, whereas others have primarily used C57BL/6J mice when investigating the role of IL-17A administration and showing blood pressure elevation (84, 96, 99, 100). Infusion with ANGII induced blood pressure elevation in FVB/n mice up to 25 mmHg as shown previously by others (207, 208), and similarly to other mouse strains including Swiss-Webster (209) and C57BL/6J mice (210). Although IL-17RA expression in FVB/n mice was shown in both kidney, heart, and aortic tissue, it cannot be ruled out that C57BL/6J mice could be more susceptible to inflammatory stimulation by IL-17A. The difference between mice strains towards inflammatory response by cytokines has not been investigated much, but one study recommends using FVB/n mice when studying inflammatory

diseases in murine models (211). Thus, the FVB/n strain should be well suitable to study blood pressure changes imposed by IL-17A.

Altogether, study IV implicates strong evidence that IL-17A does not exert direct hypertensive actions in mice, despite markedly high levels of biologically active plasma IL-17A and expression of its receptor in the kidneys, heart, and aorta. IL-17A co-infused with ANGII did not accentuate ANGII-mediated hypertension, but rather attenuated blood pressure. Thus, ANGII-stimulated inflammatory factors were not enough to promote IL-17A mediated hypertension in this study. Although we do not see any effects directly by IL-17A on blood pressure, IL-17A has shown to be elevated in hypertensive patients. Therefore, if IL-17A exerts any pro-hypertensive effects this is most likely by synergistic or concrete actions in combination with other cytokines in different states of chronic inflammation

METHODOLOGICAL CONSIDERATIONS

BLOOD PRESSURE MEASUREMENTS IN HUMANS

For precise diagnosis of hypertension, ambulatory blood pressure monitoring is considered the gold-standard for measuring if patients have constantly high blood pressure (212). With ambulatory blood pressure monitoring, blood pressure is recorded every 15-30 min. for 24hr and hereby daytime and night-time differences can be monitored. In study I-III, ambulatory blood pressure recordings were available from T2DM patients with resistant hypertension and kidney transplant patients. In T2DM patients, night-time measurements were used for analyses, and in kidney transplant patients average 24hr blood pressure measurements were available for analyses in the present study. Method of blood pressure recording was determined by the principal investigator of each study that were previously published (132, 143, 146).

MULTIPLEX IMMUNOASSAYS

For plasma cytokine and urinary kidney injury marker evaluation, we used mesoscale single and multiplex immunoassays with high sensitivity, measuring components down to 1 pg/ml. These assays enabled quantitative measurements of 6 different cytokines and 6 different injury markers. Human and mice material is very valuable and difficult to reproduce and must be used carefully. The mesoscale and multiplex assays enabled us to use only 50 ul in total from each sample to do plasma cytokine or urine kidney injury marker analyses in duplicates. This was especially an advantage when working with plasma samples from mice where only small sample volumes were available. By using the multiplex assay, we also avoided repeated freeze-thaw cycles as would have been the case if using single ELISAs for measuring each protein. We included a positive plasma and urine control in most of the analyses. Sometimes with limited space on the plates, controls were left out, but instead standards were compared between plates. A limitation of the LPS-stimulated plasma controls was that some of the analytes were very high in concentrations (e.g., IL-6 and TNF) whereas others were below detection limit (IL-17A and IL-1β). In order to get as many analytes as possible within detection range, control plasma samples were diluted 1:4. Based on recommendation and prior experience from multiplex experts from mesoscale, the maximal CV was set to 20% and it was decided prior to experiments, that all data included for analyses were within detection range with CV<20%.

BLOOD PRESSURE MEASUREMENTS IN MICE

In all animal experiments of this study, blood pressure measurements and i.v. infusions were carried out by using chronic indwelling catheters. This is an invasive procedure, where catheters are inserted in the femoral artery and vein, that enables blood pressure measurements directly measured from inside of the femoral artery. Blood pressure is easily affected by stress, environmental factors, and anesthesia (205, 213). Although invasive, time consuming, and expensive, for measurements of small differences and diurnal variations, invasive models of continuous blood pressure measurements using chronic indwelling catheters or radio telemetry in conscious live mice are the golden standard techniques (205, 206). Alternative techniques for blood pressure measurements in animals include the radiotelemetry and tail-cuff methods. The tail-cuff method is non-invasive and inexpensive, but requires handling, restraint, and warming of mice. Although mice are habituated to these conditions prior to experiments, these are all factors that can affect blood pressure during measurements. Tail-cuff measurements rely on flow or pulse following occlusion of the tail artery and gives a measure of peripheral systolic blood pressure. Although studies cite negligible differences between tail-cuff and telemetry methods, data are inconsistent (214). In radiotelemetry on the other hand, one catheter is implanted in the carotid artery or abdominal aorta and connected to a transmitter that records blood pressure measurements and transmit these to an external computer (215). Using chronic indwelling catheters in both the femoral artery and vein, enabled chronic i.v. infusion of substances (e.g., ANGII, IL-17A, and saline). This made it possible to investigate the direct effects of IL-17A on blood pressure, and to induce ANGII-mediated hypertension in mice. Besides blood pressure measurements, this model enables collection of “unstressed” arterial blood in conscious mice from the arterial line.

CONCLUSION

In conclusion, analyses from study I and III in, hypertensive T2DM- and kidney transplant patients, revealed no effect of spironolactone treatment on plasma IL-17A concentrations; no relation between blood pressure and plasma IL-17A; and no relation between plasma potassium increase and cytokine decrease. Spironolactone treatment in T2DM patients revealed anti-inflammatory effects by reducing plasma IFN-γ and IL-6, and possible direct renoprotection by lowering epithelial kidney injury markers TFF3 and Calbindin in kidney transplant patients.

Study II revealed a positive relation between plasma IL-17A and SBP at baseline, but despite blood pressure reduction by amiloride, no effect on plasma IL-17A was observed. Thus, IL17A did not relate to ENaC-mediated hypertension. Instead, amiloride exerted anti-inflammatory effects by reducing macrophage-derived cytokines TNF and IL-6 in hypertensive patients. This anti-inflammatory effect of reduced TNF and IL-6 was confirmed by *in vitro* studies in human LPS-stimulated macrophages as well, where a more potent ENaC inhibitor, benzamil lowered TNF, IL-6, IL-1β, and IL-10.

Finally, study IV demonstrates that IL-17A had no blood pressure elevating effects when administered acutely or chronically, despite a marked increase of plasma IL-17A and its downstream product IL-6. The animal studies revealed a blood pressure lowering effect of IL-17A. Although we did not see any direct effects by IL-17A on blood pressure in animals, or any relation between IL-17A and blood pressure in humans, IL-17A has previously been shown to be elevated in hypertensive patients and animals, therefore, if IL-17A exerts any pro-hypertensive effects, this would most likely be through synergistic actions in combination with other pro-inflammatory cytokines.

This study has shown new anti-inflammatory effects of known conventional diuretics that has not been documented before in humans, by lowering pro-inflammatory cytokines like IFN-γ, TNF, and IL-6. The anti-inflammatory cytokine-reducing effects of spironolactone and amiloride did not correlate to blood pressure reduction in the hypertensive patients analyzed in this study, suggesting a possible direct anti-inflammatory effect independent of blood pressure. This direct anti-inflammatory effect was also supported by the *in vitro* macrophage studies.

FUTURE PERSPECTIVES

Results of the present study did not indicate IL-17A to be related to hypertension or involved in mediating MR and ENaC-dependent parts of hypertension in patients. Direct test of IL-17 had no pro-hypertensive action in animals. Yet, preclinical studies have shown that IL-17A deficiency protects against hypertension, and plasma IL-17A has shown to be elevated in hypertension in humans and animals. However, the anti-hypertensive effects of IL-17A neutralizing antibodies in patients remains to be investigated directly. At Odense University Hospital, 100-200 psoriasis and arthritis patients are currently being treated with IL-17A neutralizing antibody (secukinumab) and TNF neutralizing antibody (etanacept), showing with promising clinical outcomes. From these patients, office blood pressure recordings are available, and blood pressure outcome analyses from these patients, was planned to be done, however this was not possible during this PhD study. Only one ongoing study (ClinTrials.Gov: NCT02144857) with psoriasis patients is investigating the direct effect of secukinumab, etanacept, and cyclosporin on cardiovascular parameters including blood pressure. Studies have also suggested that IL-17A together with other pro-inflammatory cytokines including TNF, IL-6, IL-1β and IFN-γ promote inflammation-mediated hypertension and contribute to disease severity of other autoimmune disorders like psoriasis, arthritis, and multiple sclerosis (86, 216, 217). Functional and mechanistic studies of IL-17A production and actions in concert with other pro-inflammatory cytokines *in vitro* and *in vivo* during hypertension would provide better understanding of the mechanisms leading to inflammation-mediated hypertension. For this purpose, other immune cells including dendritic cells, macrophages and regulatory T-cells must be further investigated in hypertension in humans and in animals as well. Dendritic cells have especially shown to be involved in salt-sensitive hypertension, and involves cytokine production of IFN-γ, IL-1β and IL-17A, and express ENaC (70, 129).

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DANSK RESUME

Hypertension er en stor risikofaktor for udvikling af kardiovaskulære- og kroniske nyresygdomme. På trods af behandlingsmuligheder med livstilsændringer og medikamenter har 12-15% af den hypertensive population stadig ukontrolleret blodtryksstigning. Heraf, vides det at type 2 diabetikere især udvikler svært behandlende resistent hypertension. Dyreforsøgsstudier har vist at det adaptive immunsystem (T-celler, makrofager og dendrit celler) bidrager signifikant til udvikling af hypertension. Hovedfokus i dette PhD studium er på de pro-inflammatoriske IL-17A producerende T-hjælper (Th)-17 cellers rolle i hypertension. Mineralocortikoidhormonet, aldosteron, som virker ved binding til dens receptor mineralocorticoid receptor (MR), er en velkendt mediator for udvikling af hypertension. Data tyder på at MR er udtrykt i T-lymfocytter, makrofager og dendritter. *In vitro* og *in vivo* studier har vist at Th-17 celle-differentiering er MR-afhængig, og hæmning af MR med spironolakton i hypertensive dyr nedsætter blodtryk og plasma IL-17A. Dette tyder på en mekanistisk sammenhæng mellem aldosteron-MR og Th17-medieret inflammation i patogenesen af hypertension. Ligeledes udtrykker cirkulerende lymfocytter, membran-salt kanalen ENaC. Dens funktion i Th17 lymfocytter er ikke belyst, dog har forsøg påvist en korrelation til hypertension. Der forekommer næsten ingen humane data. Baseret på tidligere studiers fund, har vi i dette studie undersøgt IL-17A-relation i immun-medieret hypertension ved to indgangsvinkler. **1)** Ved retrospektive eksplorative studier undersøgte vi effekten af kendte anti-hypertensive medikamenter (spironolakton og amilorid) på plasma IL-17A udtryk samt andre relaterede upstreams of downstreams cytokiner i hypertensive patienter. **2)** Ved direkte intervention i mus med IL-17A, adminsitreret i.v., kontinuert, og analyseret effekten direkte på blodtryk og hjertefrekvens.

De overordnede hypoteser af studiet er følgende: **1)** IL-17A er koncentrations-afhængigt, direkte pro-hypertensivt via vaskulære- og renale mekanismer. **2)** IL-17A bidrager non-redundant til immun-medieret hypertension via aldosterone-MR mekanismer; **3)** Dendrit og makrofager, og/eller Th17 lymfocyt-ENaC bidrager til IL-17A produktion in hypertensive patienter. Hypoteserne blev testet direkte ved IL-17A infusion i mus, og indirekte ved analyse af MR-antagonister og ENaC blokkere *in vitro* i LPS-stimulerede makrofag forsøg, samt *in vivo* i patient materiale fra interventionsstudier med hypertensive patienter fik spironolakton og amilorid. I disse prøver blev der målt cytokinet IL-17A, og andre cytokiner der bliver produceret downstream for IL-17A produktionen (IL-6), medierer IL-17A produktionen (IL-

6, IL-1b), produceret af makrofag aktivering (TNF, IL-6, and IL-10) eller Th-1 celle aktivering (IFN-g). Resultaterne fra disse analyser har udmundet I 4 manuskripter, som bliver præsenteret i det følgende.

Studie I: Mineralocorticoid receptor blokker spironolakton reducerer plasma interferon-γ og interleukin-6 i patienter med type 2 diabetes og behandlings-resistent hypertension

Hypotese: MR antagonisme med spironolakton, hæmmer Th-17 produceret IL-17A samt andre relaterede makrofag- og Th-1 celle-producerede cytokiner. Denne medierede reduktion af cytokiner relaterer til blodtryksreduktion, albuminuria, samt plasma kalium stigning.
Plasmaprøver blev analyseret for cytokin koncentrationer. Disse prøver var fra patienter med type 2 diabetes mellitus (T2DM) og behandlings-resistent hypertension, inkluderet i et dobbelt-blindet, placebo-kontrolleret interventionsstudie med spironolakton (25-50 mg/dag, i 16 uger). I det oprindelige primære studie blev det vist at spironolakton intervention i T2DM patienter medierede blodtryks- og albumin/kreatinin ratio (UACR) reduktion, samt plasma kalium stigning. I dette studium er det vist, at plasma IL-17A er upåvirket efter spironolakton intervention, men plasma IFN-γ og IL-6 er reducerede. Disse ændringer ses ikke for placebo-behandlede patienter. Ved inklusion ses det at serum aldosteron og nat-diastolisk blodtryk korrelerer positivt, men der er ingen korrelation mellem serum aldosteron og plasma cytokin niveauer eller mellem plasma cytokin og blodtryk, undtagen for IFN-γ, som korrelerer invers til blodtryksreduktion efter spironolakton intervention. Plasma kalium stigning korrelerer negativt med blodtryksreduktion, men ikke til plasma cytokin reduktion efter spironolakton intervention. Herudover er baseline plasma IL-6 positivt korreleret med baseline UACR. Ydermere, hæmmer spironolakton, direkte, cytokin udskillelsen fra LPS-stimulerede makrofager. Det konkluderes at blodtryksreduktion med spironolakton i T2DM patienter med behandlings-resistent hypertension ikke blev medieret af en Th-17-produceret IL-17A reduktion. I stedet har spironolakton en anti-inflammatorisk effekt *in vivo* ved nedsættelse af Th-1 og makrofag-produceret cytokiner (IFN-γ and IL-6).

Studie II: Amilorid nedsætter TNF og IL-6 men ikke IL-17A i patienter med hypertension og type 2 diabetes.

Hypoteser: ENaC blokering med amilorid reducerer plasma IL-17A i patienter med T2DM og resistent hypertension. Dette fald i plasma IL-17A er relateret til blodtryksfald samt plasma

kalium stigning medieret af amilorid, samt en relation mellem IL-17A og baseline UACR vil finde sted.

I et followup open-label studie med amilorid i 8 uger (5-10 mg/dag), blev 80 T2DM patienter med behandlings-resistent hypertension fra det forgående studie inviteret til deltagelse. I det primære studie blev det vist at amilorid nedsætter blodtryk, UACR og øger plasma kalium. I dette studie har vi vist at amilorid ikke har noget effekt på plasma IL-17A hos T2DM patienter, men i stedet ses TNF og IL-6 reduceret efter amilorid intervention. Ved baseline korrelerer plasma IL-1β positivt til nat-MAP og DBP. Baseline plasma IL-17A korrelerer positive til SBP, men plasma TNF og IL-6 korrelerer ikke blodtryk før eller efter amilorid intervention. Baseline UACR korrelerer positivt med baseline SBP og plasma IL-17A, TNF, IL-6, and IL-1β niveauer. Plasma kalium stigning korrelerede ikke til blodtryks fald eller plasma TNF of IL-6 fald efter amilorid intervention. Amilorid inkubation i LPS-stimuleret makrofager reducerer cytokin produktionen af IL-6, IL-1β, and IL-10, men TNF var upåvirket (P=0.07). Co-inkubation med benzamil, en mere potent ENaC hæmmer, reducerede alle 4 makrofag cytokiner i nmol/L kontraktionsrange. Det konkluderes ud fra disse data at amilorid-medieret blodtryksreduktion i hypertensive patienter ikke blev medieret af IL-17A reduktion. I stedet ses en direkte anti-inflammatrisk effekt af amilorid ved reduktion af TNF and IL-6 *in vitro* og *in vivo*. ENaC bidrager til makrofag-stimuleret cytokin produktion og kan være et relevant non-renal target til hæmning af inflammation og potentielt blodtryk i patienter med type 2 diabetes og hypertension.

Studie III: Mineralocorticoid receptor blokering med spironolakton har ingen direkte effekt på plasma IL-17A, men reducerer urin nyreskade markører i nyretransplanterede patienter.

Hypoteser: MR antagonisme med spironolakton reducerer IL-17A produktion i plasma, og dette relaterer til blodtryksændringer og renal epithelial beskyttelse i nyretransplanterede patienter.

Plasma og spot urin prøver fra nyretransplanterede patienter fra et multicenter, dobbelt-blindet, placebo-kontrolleret interventionsstudie med spironolakton (25-50 mg/dag) eller placebo i 3 år. Plasma og urinprøver fra dette studie ved inklusion og efter 1 års behandling blev analyseret for hhv. cytokiner og nyreskademarkører. Plasma IL-17A var uændret efter spironolakton intervention, men forøget i placebo behandlede patienter. Alle andre analyserede cytokiner var

uændret. Plasma aldosteron korrelerede ikke til MAP eller plasma IL-17A ved baseline. Plasma IL-17A korrelerede ikke til MAP ved baseline. Nyreskademarkørerne Calbindin og TFF3 var reduceret efter 1 års spironolakton intervention, uden ændringer i placebo behandlede patienter. TFF3 relaterede til blodtryk, men kun ved baseline. Der var ingen korrelationer mellem plasma aldosteron og urinskademarkører. ACEi og ARB behandling i disse patienter havde ingen effekt på cytokin- eller nyreskademarkør niveauer. Det konkluderes at MR blokade kunne stabilisere plasma IL-17A niveauer, og hæmmer epitheliale skadesmarkører, som er associeret med den distale del af nefronet.

Studie IV: Interleukin 17A nedsætter blodtryk ved baseline og efter ANGII-hypertension i vågne fritgående han FVB mus

Hypoteser: 1) Kronisk infusion med IL-17A i vågne mus øger blodtryk; 2) ANGII-induceret blodtryksstigning accentueres af IL-17A ved renale mekanismer; og 3) Akut bolus infusion med IL-17A forårsager akut blodtryksstigning ved vaskulære mekanismer i mus.

Kronisk i.v. infusion af IL-17A over 8 dage i en dosis-step-up model, medierede ingen hypertensive effekter i mus. I stedet medierede IL-17A-infusion hypotensive effekter. IL-17A infusion medierede heller ingen accentuerende effekt af ANGII-medieret hypertension i mus, men i stedet var blodtrykket reduceret i mus der fik IL-17A+ANGII i forhold til saltvands-kontroller. Akut bolusinfusion med IL-17A medierede ingen effekter på blodtryk. Plasma IL-17A niveauer blev målt til over 3500 gange så meget som baseline niveauer, og downstream produktet IL-6 var også målt forhøjet hos mus der fik Il-17A, og *in vitro* i fibroblast celler stimuleret med IL-17A. Det konkluderes, at IL-17A alene ikke har nogen direkte hypertensiv effekt, men i stedet reducerer blodtrykket.

Alt i alt viser de 3 kliniske studier med hypertensive patienter at MR-blokkering med spironolakton og ENaC blokkering med amilorid, ikke har nogen påvirkning på IL-17A produktionen i plasma. I stedet ses en anti-inflammatorisk effekt ved reduktion af cytokinerne IFN-γ, IL-6, and TNF. Kronisk og akut infusion med IL-17A i mus viser ingen direkte hypertensiv effekt af IL-17A, i stedet ses en hypotensiv effekt ved non-fysiologisk systemisk høje koncentrationer af IL-17A. Det konkluderes at IL-17A i sig selv ikke har en direkte hypertensiv effekt, men muligvis sammen med andre cytokiner og andre ukendte faktorer kan bidrage til inflammation-medieret hypertension.