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# NGAL is a novel target in hypertension by modulating the NCC-mediated renal Na balance

**Short title: NGAL modulates the renal Na balance**

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## **Abstract**

**Background:** The expression of neutrophil gelatinase-associated lipocalin (NGAL/lcn2) is directly modulated by mineralocorticoid receptor activation but its role in blood pressure control is unclear.

**Methods:** a potential relationship between NGAL plasma levels, systolic blood pressure and urinary Na excretion was assessed in the STANISLAS cohort. The specific role of NGAL/lcn2 in salt-sensitive hypertension was studied using lcn2-depleted mice (lcn2 KO) fed with low Na diet (0Na).

**Results:** we show that NGAL plasma levels positively correlate with systolic blood pressure (SBP), whereas they negatively correlate with urinary Na excretion in subjects of the STANISLAS cohort. Prolonged feeding of lcn2 KO mice with a 0Na diet induced lower SBP than that of the control group

(WT), suggesting a role for NGAL/lcn2 in Na-balance homeostasis. Short-term or prolonged 0Na increased Na-Cl cotransporter (NCC) phosphorylation in the cortex of WT mice, which was prevented in lcn2 KO mice. Recombinant mouse lcn2 injections in lcn2 KO mice induced NCC phosphorylation in the kidney cortex, associated with decreased urinary Na excretion. *Ex-vivo* experiments using kidney slices from lcn2 KO mice showed increased NCC phosphorylation by recombinant murine lcn2. In addition, recombinant murine lcn2 induced activation of CamK2 $\beta$  phosphorylation in lcn2 KO mice and in kidney slices, providing an underlying mechanism involved in lcn2-induced NCC phosphorylation. Indeed, the inhibition of CamK2 $\beta$  prevented NCC phosphorylation induced by recombinant lcn2 in kidney slices.

**Conclusion:** we highlight a novel role of NGAL/lcn2 as a modulator of the activity of the renal sodium transporter NCC affecting salt-sensitive blood pressure

### **List of nonstandard abbreviations and acronyms**

**ABPM:** Ambulatory Blood Pressure Monitoring

**ASDN:** Aldosterone-sensitive distal nephron

**CamK2 $\beta$ :** Calcium/calmodulin-dependent protein kinase II  $\beta$  subunit

**CCD:** Cortical collecting duct

**DCT:** Distal convoluted tubule

**ENaC:** Epithelial Na channel

**Lcn2:** Lipocalin 2

**NCC:** Na-Cl cotransporter

**NKCC2:** Na-K-Cl cotransporter 2

**NGAL:** Neutrophil Gelatinase-associated lipocalin

**SBP:** Systolic blood pressure

### **Introduction**

High blood pressure is a silent killer. An increasing proportion of the population suffers from hypertension resistant to standard therapies<sup>1</sup> that are based on our current understanding of the mechanisms that control blood pressure. High blood pressure continues to be the biggest single contributor of disease and mortality, responsible for approximately 10 million deaths per year. Hypertension-associated comorbidities, such as cardiovascular (CV) disease, stroke, chronic kidney disease (CKD), and cognitive impairment, as well as risk factors, such as diabetes, stress, and aging, are increasing<sup>1</sup>. The number of people affected by hypertension will increase in the coming decades due to increased exposure to the main environmental determinants of high blood pressure, such as an excess intake of calories and salt, resulting in an additional 560 million extra people who will be affected in the next 20 years<sup>1</sup>. It is well recognized that the kidney is either the culprit or accomplice in hypertension: renal Na homeostasis is central to optimal extracellular volume control and the long-

term setting of blood pressure<sup>1</sup>. Several pathophysiological mechanisms have been identified that relate to the control of renal Na balance, either directly (acting on tubular Na transport) or indirectly (as a consequence of inflammation, fibrosis, or vascular dysfunction). Blood pressure is an inheritable trait and an estimated 30% of variance in blood pressure relates to genetic factors. Our understanding has progressed from the study of rare Mendelian hypertensive phenotypes, highlighting the role of renal ion transporters and their regulators. The salt sensitivity of blood pressure is critically dependent on the renal handling of salt<sup>2</sup>. Major Na transporters expressed in renal distal nephrons, such as Na-K-Cl cotransporter 2 (NKCC2) and the Na-Cl cotransporter (NCC) epithelial sodium channel (ENaC), have been proven to be involved in genetic hypertensive/hypotensive disorders and their pharmacological blockade (with diuretics/natriuretics) is clinically useful in controlling blood pressure.

The renin-angiotensin-aldosterone system is recognized as a major actor in blood pressure control, and mineralocorticoids have been shown to be particularly involved in high blood pressure and its CV comorbidities<sup>1</sup>. Aldosterone plays an important role in renal Na balance, as well as in CV comorbidities, affecting inflammatory and pro-fibrotic processes<sup>3</sup>. We recently identified neutrophil gelatinase-associated lipocalin (NGAL) as a novel aldosterone target and demonstrated that its genetic inactivation prevents the effects of mineralocorticoids in the CV system, including inflammation, fibrosis, and increased blood pressure<sup>4,5</sup>. However, it is difficult to dissociate the separate primary effects of NGAL on fibrosis, inflammation, and blood pressure, as they are intimately interrelated.

NGAL, or its murine ortholog lipocalin2 (lcn2), is a 25Kd protein that can be produced by several cell types (including renal tubule cells and immune cells) and secreted into the surrounding medium, acting as a paracrine molecule. NGAL has been shown to be involved in a variety of processes, such as iron sequestration, thus affecting several cellular functions. It has pro-inflammatory and chemotactic properties, inducing the recruitment of immune cells to injured tissues. NGAL is best known as a marker of renal injury and has been proposed to be essential for the promotion of CKD progression by modulating proliferation<sup>6</sup>. It is also involved in macrophage polarization, promoting differentiation into “inflammatory” macrophages versus “repair type” macrophages<sup>6</sup>. It is secreted by the thick ascending limb of Henle’s loop, distal tubules and collecting ducts and excreted into the urine<sup>7</sup>. Interestingly, the NGAL receptor 24p3 is expressed in the distal nephron<sup>8</sup>, suggesting direct local action. We therefore hypothesized that NGAL may participate in the control of blood pressure not only via its pro-inflammatory and pro-fibrotic actions but also directly due to its involvement in renal Na balance.

## **Materials and methods**

A detailed Methods section is provided in the Data Supplement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

### **Human cohort**

A detailed description of the STANISLAS cohort has been previously published<sup>9</sup>. The STANISLAS cohort was established with the primary objective of investigating gene-gene and gene–environment interactions in the field of cardiovascular diseases. To assess the effect of genetics on the variability of intermediate phenotypes on the transition toward disease, the families were deemed healthy and free of declared acute and/or chronic illness at baseline (V1). The fourth visit (V4) enabled a follow-up of 18 to 23 years (mean of 18.5 years). The collected information was enriched by the measurement of new circulating proteins and detailed clinical phenotyping. The study protocols for all examinations were reviewed and approved by the local ethics committee of CPP-Est 3, France. All participants provided written informed consent to participate in the study. Among the 1705 subjects who participated in the last visit, NGAL measurements were performed for the first 500. In total, 133 subjects were excluded from the analyses because they were taking an anti-hypertensive treatment. The participant characteristics (n= 367) are presented in Supplemental Table S1.

### **Mice**

Experiments were performed using C57BL/6J wildtype mice (from the Janvier Labs company, France) and C57BL/6j *Lcn2* knockout<sup>10</sup> and wildtype littermate controls. Animals were housed in a climate-controlled facility CEF (Centre d'Explorations Fonctionnelles of the Cordeliers Research Center, Agreement no. B75-06-12) with a 12-h/12-h light/dark cycle. Mice were fed a standard laboratory diet (Safe, France (for more information regarding the detailed composition see <http://www.safe-diets.com/wp-content/uploads/2018/01/DS-SAFE-A04.pdf>) and water. The influence of sex was not investigated in this study and the analysis was performed on male mice only. All experiments were conducted in accordance with the institutional guidelines and recommendations for the care and use of laboratory animals put forward by Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes (the project was approved by the “Charles Darwin” ethics committee of the Sorbonne Université—Project Authorization number APAFIS#7420 2016102718076839 v3).

### **Statistics**

All analyses were performed using GraphPad Prism V6.01 (GraphPad Software, San Diego, CA) software. The two-tailed significance level was set to  $P < 0.05$ . Results are expressed as the mean  $\pm$  SEM. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test (two groups and two treatments) or one-way ANOVA followed by Tukey's multiple comparisons test (more than two conditions) or unpaired or paired t tests (two groups).

## RESULTS

We first investigated the possible correlation between plasma NGAL and blood pressure or urinary Na excretion for 367 normotensive (e.g., average daytime ABPM 124/79 mm Hg) participants of the STANISLAS cohort (Table S1). The results from crude and adjusted models for an association between NGAL measurements and blood pressure and urinary sodium, as assessed in a spot urine sample, are shown in Table 1. We observed a positive correlation between plasma NGAL levels and office and 24-h systolic blood pressure, whereas there was no correlation with diastolic blood pressure (Table1). There was a negative correlation between NGAL plasma levels and urinary Na ( $p=0.038$  to  $0.046$ ) (Table1). These correlations were observed with the two models tested (model 1: adjusted for sex and age; model 2: model 1 + diabetes, BMI, smoking, and total cholesterol) and suggest a role for NGAL in blood pressure control related to Na balance.

We used a mouse model with constitutive *lcn2* gene inactivation (*lcn2* is the murine ortholog of NGAL) and applied various challenges to test this relationship and the possible underlying mechanisms. We first analyzed whether *lcn2* is an aldosterone-modulated gene in the mouse kidney. Two days of a low-salt diet induced mRNA expression of *lcn2* in the renal cortex (Figure. 1A) and micro-dissected tubules of the aldosterone-sensitive distal nephrons (ASDNs) in the distal convoluted tubules (DCTs) and cortical collecting ducts (CCDs), but not in the cortical thick ascending limbs (Figure 1B). mRNA expression of NCC is unaffected in the DCT from mice fed with low-salt diet (Figure S1A) at difference from mRNA expression of the  $\alpha$ ENaC subunit that is also increased in both DCTs and CCDs following the low-salt diet (Figure S1B). At steady state, *lcn2* KO mice showed normal systolic blood pressure (WT vs *lcn2* KO:  $115.8 \pm 2.34$  mmHg vs  $119.1 \pm 2.56$  mmHg;  $n=9$ ) and normal plasma levels of Na, K, Cl, Ca, Mg,  $\text{cHCO}_3^-$ , and pH (Table 2). In addition, urinary excretion at steady state of Na, K, Cl, Ca, and Pi was similar between WT and *lcn2* KO mice, whereas the urinary concentration of Mg was lower in *lcn2* KO mice (Table 2). Gene expression of several ion transporters expressed in the proximal tubules was upregulated in the renal cortex, such as that of sodium-coupled neutral amino acid transporter 3 (SLC38A3), solute carrier family 22 member 13 (SLC22A13), and solute carrier family 7 member 13 (SLC7A13); whereas gene expression of ion transporters expressed in the ASDN, such as NCC, the  $\alpha/\beta/\gamma$  ENaC subunits, or NDCBE, was not altered (Figure 1C). Importantly, there was marked increased expression of the  $\beta$  and  $\gamma$  subunits of the epithelial Na channel in the distal colon, whereas the expression of the  $\alpha$  subunit was not modified (Figure 1C). The renal expression of DCT-specific apical  $\text{Mg}^{2+}$  channels TRPM6<sup>11</sup> and TRPM7 or its modulator (transcription factor Prospero Homeobox 1 (Prox-1))<sup>12</sup> is similar between WT and *lcn2* KO mice (Figure S1C). Overall, these data suggest that *lcn2* KO mice maintain the homeostasis of Na balance at the price of changes in the expression of ion transporters, and possibly ion transporter activity, in the kidney and distal colon.

We investigated how *lcn2* KO mice adapt to an acute change in Na intake by subjecting the mice to a switch from a chow diet to a low-Na diet and examining urinary excretion of ions and aldosterone. As expected, urinary Na excretion decreased (Figure 2A) and aldosteronuria increased (Figure 2B & Table S2) following the switch to a low-Na diet in WT mice, with virtually no urinary Na excretion 48 h after the switch. By contrast, *lcn2* KO mice showed a loss of salt during the 4- to 8-h collection period after the switch, with higher urinary Na excretion than that of WT mice (Figure 2A). Urinary Na excretion between *lcn2* KO mice and their WT littermates was similar during the 8- to 24-h period and afterwards (Figure 2A). Aldosteronuria rapidly increased in both groups but the increase was more pronounced in the *lcn2* KO mice over the four days of low Na regimen (Figure 2B & Table S2), suggesting compensation to avoid the continuous loss of Na. Urinary excretion of K, Cl, and Ca were similar at all time points between *lcn2* KO and WT mice, whereas urinary Mg excretion was lower in *lcn2* KO mice at that basal state and 24 h after the switch to a low-Na diet (Figure S3).

We assessed how *lcn2* KO mice adapt to a prolonged low-Na diet challenge by maintaining *lcn2* KO and WT littermates on a low-Na diet for four weeks following switching. At the end of the four-week challenge, plasma ion concentrations in WT mice were similar to those measured during the chow-diet period, whereas *lcn2* KO mice showed hypokalemia and hypomagnesemia (Table 2). The 24-h urinary concentrations of various ions were similar between WT and *lcn2* KO mice after four weeks on a low-Na diet (Table 2), but the blood pressure was lower in the *lcn2* KO than WT mice (Figure 2C). Of note, a switch back to a normal Na diet for one week corrected the difference in blood pressure in the *lcn2* KO mice (with no effect in WT mice), suggesting salt-sensitivity of the blood pressure drop associated with *lcn2* deficiency (Figure 2C). To test this hypothesis, we used a model of salt-sensitive hypertension in C57Bl6 mice that combines NO synthase inhibition by L-NAME and a high-salt diet<sup>13</sup>: the switch from a NSD to a HSD diet or low dose L-NAME treatment did not affect systolic blood pressure, unless a combination of HSD and low L-NAME treatment is used (Figure S3). Similar results for HSD diet or low dose L-NAME treatment are observed when WT and *lcn2* KO mice are compared (Figure 2D). However, the addition of L-NAME together with HSD for one week increased the systolic blood pressure of WT mice but not that of *lcn2* KO mice (Figure 2D), resulting in a difference in systolic blood pressure at the end of the protocol that was blunted in *lcn2* KO mice (Figure 2E).

The combination of hypotension, hypokalemia, and hypomagnesemia observed in *lcn2* KO mice upon a prolonged low-Na diet suggests a Gitelman-like syndrome, as previously reported in NCC KO mice on a low-Na diet<sup>14</sup>. Moreover, the L-NAME and high-salt diet salt-sensitive hypertension model is NCC dependent<sup>13</sup>. We therefore analyzed phospho/total NCC protein ratios as a proxy of its function. The increase in the phosphoT53/total NCC ratio observed in WT mice after two days or four weeks of a low-Na diet was fully blunted in *lcn2* KO mice (Figure 3A-B). We next tested whether the injection

of recombinant murine lcn2 (rlcn2) into WT and Lcn2 KO mice would affect 24-h urinary Na excretion, as well as phospho T53/total NCC protein ratios. Injection of rlc2 had no effect on 24-h urinary Na excretion in WT mice but strongly decreased it in lcn2 KO mice, suggesting increased Na reabsorption (Figure 3C). The phosphoT53/total NCC protein ratio was higher in lcn2 KO mice than WT mice 24-h after injection of rlc2 (Figure 3D).

Overall, these data suggest that the aldosterone target NGAL/lcn2 modulates NCC activity, resulting in increased renal Na reabsorption and salt-sensitive hypertension. We sought to identify the underlying mechanism linking NGAL/lcn2 and NCC phosphorylation by investigating whether the calmodulin kinase pathway may be involved. Indeed, we were particularly interested in the calcium/calmodulin-dependent protein kinase II  $\beta$  subunit (CamK2 $\beta$ ), as analysis of public single-cell sequencing data showed expression of CamK2 $\beta$  in the distal nephron only, including the DCTs, CNTs, and principal and intercalated cells of the CD (Figure S4). Of note, CamK2 $\beta$  subunit autophosphorylation on the Thr-287 residue converts the kinase into a constitutively active form. Acute administration of rlc2 in vivo increased both the phosphoT287/total CamK2 $\beta$  and phosphoT53/total NCC protein ratio in lcn2 KO mice but not WT mice (Figure 4A-B). We tested whether CamK2 $\beta$  is directly involved in lcn2-modulated NCC phosphorylation using kidney slices from lcn2 KO mice and exposed them to rlc2 for 10 min in the presence or not of KN93, a CamK2 $\beta$  phosphorylation inhibitor. The increase in the phosphoT287/total CamK2 $\beta$  protein ratio was induced by rlc2 in kidney slices but prevented by the CamK2 $\beta$  inhibitor KN93 (Figure 4C). The CamK2 $\beta$  inhibitor also blunted the increased phosphoT53/total NCC protein ratio induced by rlc2 (Figure 4D), suggesting a link between CamK2 $\beta$  phosphorylation induced by lcn2 and subsequent NCC phosphorylation.

## Discussion

In this study, we showed the aldosterone (aldo)/mineralocorticoid receptor (MR) target NGAL to be a novel regulator of NCC and renal Na balance in mice. This may also be relevant in humans, as high plasma NGAL levels are associated with higher blood pressure in a cohort of untreated normotensive participants and reduced urinary Na excretion.

We previously identified NGAL (also known as 24p3 or lipocalin2) as a novel aldo/MR target in global transcriptomic analysis of the heart of MR overexpressing mice<sup>4,15</sup> and showed that the aldo-bound MR binds to the hormone response element present in the -794 to +36 NGAL promoter<sup>4</sup>. Over the last several years, we have reported that aldo/MR modulates NGAL expression in several cell targets, such as endothelium/vascular smooth muscle, adipocytes, retina, and inflammatory and immune cells, including dendritic cells<sup>6</sup>. Importantly, this has been confirmed by independent teams in, for example, neutrophils, the vasculature, and the kidneys<sup>16,17</sup>. Viau *et al.* and Paragas *et al.*



reported that *lcn2* is expressed in distal nephrons<sup>7,18</sup>. Here, we show that a low-Na diet increased aldosterone levels and induced *lcn2* expression in the whole kidney and distal nephrons, especially DCTs and CCDs. Previous reports have suggested similar findings: RNaseq analysis identified differential *lcn2* expression in distal tubule cells isolated from mice subjected to low-Na/high Na-diet challenge<sup>19,20</sup>.

NGAL plays an important role in cardiorenal diseases. NGAL was initially reported as a biomarker of renal failure. However NGAL is more than a simple biomarker and has direct pathogenic roles in various pathological situations. The use of *lcn2* KO mice, recombinant NGAL/*lcn2*, or neutralizing NGAL/*lcn2* antibody administration has highlighted the deleterious role of NGAL/*lcn2* in animal models of CV and renal diseases<sup>6</sup>. We and others have shown NGAL/*lcn2* to be profibrotic<sup>5,21</sup> and an immunomodulator in mineralocorticoid-induced hypertension<sup>21,22</sup>. The correlation between NGAL and/or the NGAL-MMP9 complex with CV risk (independently of renal dysfunction) underscores the potential harmful impact of NGAL in human disease<sup>23,24</sup>.

Salt sensitivity occurs in half of the hypertensive population and is an independent risk factor for CV disease and mortality<sup>25</sup>. The handling of salt by the kidneys is a key component of salt sensitivity and several ion transporters have been shown to be drivers of renal Na homeostasis. Among them, sodium chloride cotransporter (NCC) activation is recognized to be a key factor in the maintenance of salt-induced hypertension. NCC is the pharmacological target of the widely used thiazide as a diuretic. Rare genetic diseases leading to disturbances in blood pressure and/or Na balance have revealed NCC, or the signaling pathways associated with NCC regulation, to be one of the genes responsible for Gitelman syndrome. Interestingly, mice with *lcn2* gene deletion appear to show a normal Na balance at steady state, probably due to adaptation of various Na transporter/channel activities in the kidneys and distal colon. The changes in renal gene expression we report in the basal state for ion transporters expressed in the proximal tubule mimic those reported by Grimm *et al.* in the absence of NCC phosphorylation in SPAK (sterile 20/SPS1-related proline/alanine-rich kinase) KO mice<sup>26</sup>. Prolonged challenge with a low-Na diet led to disequilibrium and revealed hypotension associated with hypokalemia and hypomagnesemia, which are typical features of Gitelman syndrome. However, *lcn2* KO mice do not completely mimic Gitelman syndrome, with the absence of metabolic alkalosis. A mouse NCC KO model showed hypomagnesemia and hypocalciuria in the basal state and hypotension (without hypokalemia) after two weeks of a low-Na diet<sup>14</sup>. This suggests a possible defect in NCC activity in *lcn2* KO mice, which we confirmed by the analysis of a well-accepted proxy of NCC activity, the phosphoT53/total NCC ratio. The administration of recombinant *Lcn2 in vivo*, as well as *ex vivo* incubation of kidney cortex slices from *lcn2* KO mice, led to increased NCC phosphorylation, underscoring the direct role of *lcn2* in modulating NCC activity in mice.

We were interested in the calcium/calmodulin-dependent protein kinase II  $\beta$  (CamK2 $\beta$ ) subunit (an accessory subunit of the calmodulin complex), as renal expression of CamK2 $\beta$  appears to be restricted to the ASDN, suggesting a possible role in renal ion transport activity. Importantly, CamK2 $\beta$  activation by various processes (for example, oxidative stress or calmodulin binding) results in a conformational change that relieves intrasteric autoinhibition and allows autophosphorylation of Thr-287, which converts the kinase into a constitutively active form and confers Ca<sup>2+</sup>-independent activity<sup>27</sup>. A recent report showed that the pharmacological inhibition of CamK2 by the compound KN93 increases the active form of the NCC (phospho-NCC) at the luminal membrane and also increases NCC activity in murine DCT15 cells<sup>28</sup>. However, here, we report an opposite effect in native renal tissues, in which phosphorylation of NCC was activated by *lcn2*, both *in vivo* and *ex vivo*, in kidney slices. We have no explanation for such a discrepancy. Whether NCC is a direct target of CamK2 and whether CamK2/ CamK2 $\beta$  also modulates NCC activity independently of *lcn2* is yet to be explored in the native kidney.

The population genetics of blood pressure have shown that individual genetic loci have only small effects on blood pressure (1 mmHg systolic on average) and explain only 3% of heritability thus far. This is likely related to the fact that population studies do not discriminate between several types of hypertension. In preclinical models, *lcn2* gene inactivation has been shown to reduce the blood pressure increase associated with mineralocorticoid challenge<sup>5</sup>, CKD<sup>18</sup>, and obesity<sup>29</sup>. The association of common single-nucleotide polymorphisms in the gene encoding NGAL (*lcn2*) with elevated blood pressure (BP) was analyzed in Hong Kong Chinese subjects<sup>30</sup>. Carriers of the GGTCC haplotype had higher systolic BP than noncarriers, suggesting that genetic variants in *lcn2* may affect BP<sup>30</sup>. We report here that plasma NGAL levels are associated with blood pressure and urinary Na excretion in the STANISLAS cohort in normotensive (on average) participants. Whether *lcn2* polymorphism and/or circulating NGAL levels are associated specifically with salt-sensitive hypertension is yet to be explored.

### **Perspectives**

We show the aldosterone target NGAL/*lcn2* to be a novel modulator of Na transport in the distal nephron, enhancing NCC phosphorylation via CamK2b subunit activation, leading to salt-sensitive hypertension in the mouse. Our limited clinical study (367 normotensive participants -on ambulatory blood pressure monitoring - in a population-based cohort with an ad lib diet and a spot urine sample taken off) suggests that this could also occur for renal Na balance and blood pressure control in humans. Further studies are required to explore this possibility in larger cohorts and to analyze potential gene polymorphisms that modulate NGAL levels and their correlation with blood pressure and Na balance.

## Summary

We show that plasma NGAL concentrations positively correlate with systolic blood pressure and negatively correlate with urinary Na excretion in normotensive subjects of the STANISLAS cohort. In mice, we identified a role of NGAL/lcn2 in renal Na handling and systolic blood pressure control via NCC modulation.

## Author contributions

F.J design the study. B.B, S.M, K.U, M. S and L.L carried out the experiments. B.B, S.M, C.X and L.L analyzed the data. B.B made the figures. B.B and F.J drafted the paper. All authors revised the paper and approved the final version of the manuscript.

## Disclosure

The authors have no conflict of interest to declare.

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### **Novelty and relevance**

#### **What's new?**

Neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin2) is a novel modulator of NCC activity in the aldosterone-sensitive distal nephron and contributes to renal Na balance and blood pressure control.

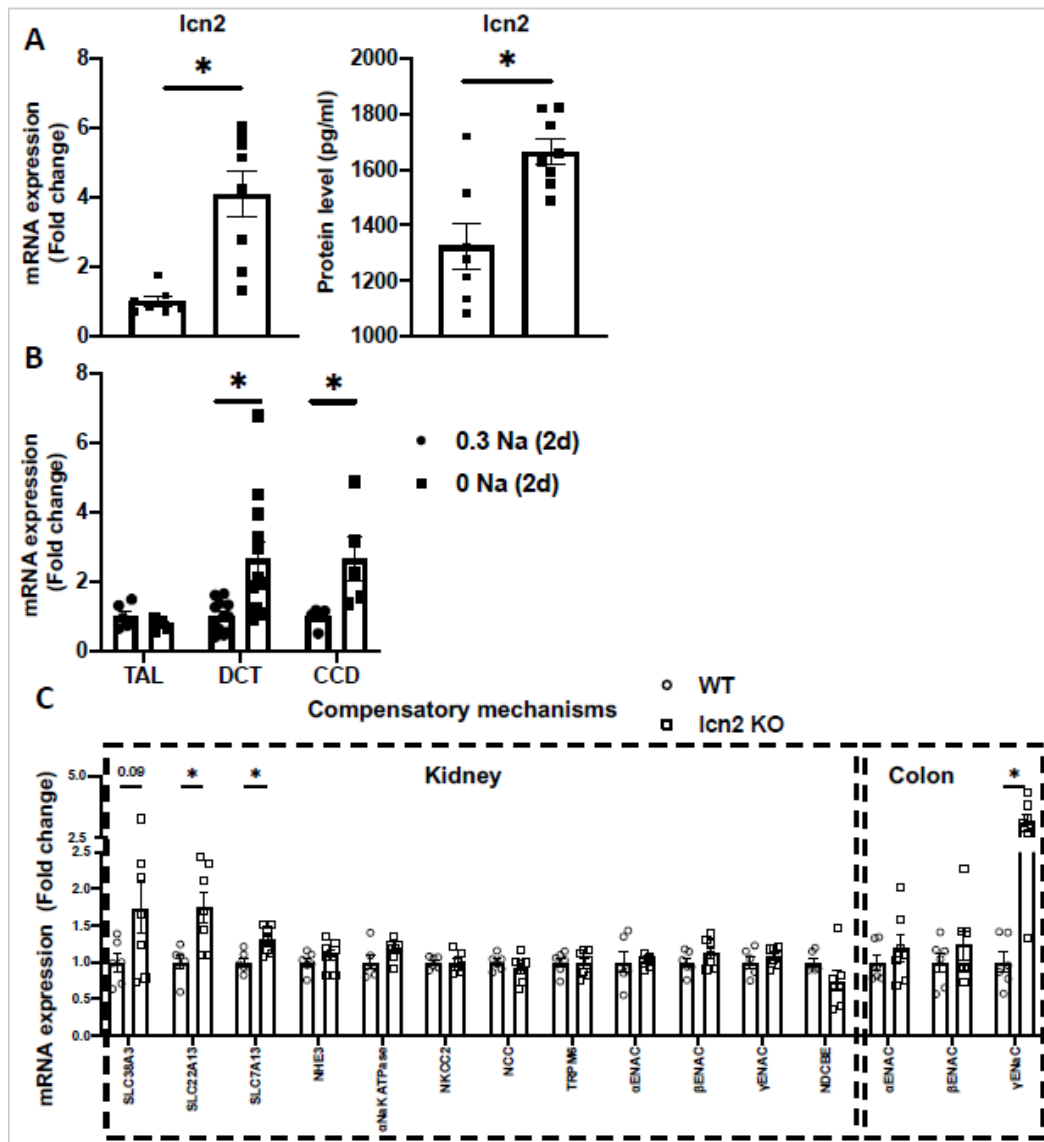
#### **What is relevant?**

The precise role of NGAL/lcn2 in blood pressure control is unknown. Our study broadens our knowledge on the molecular mechanisms of salt-sensitive hypertension

#### **Clinical/Pathological implication**

NGAL may be a risk factor for salt-sensitive hypertension and a target for blood pressure regulation.

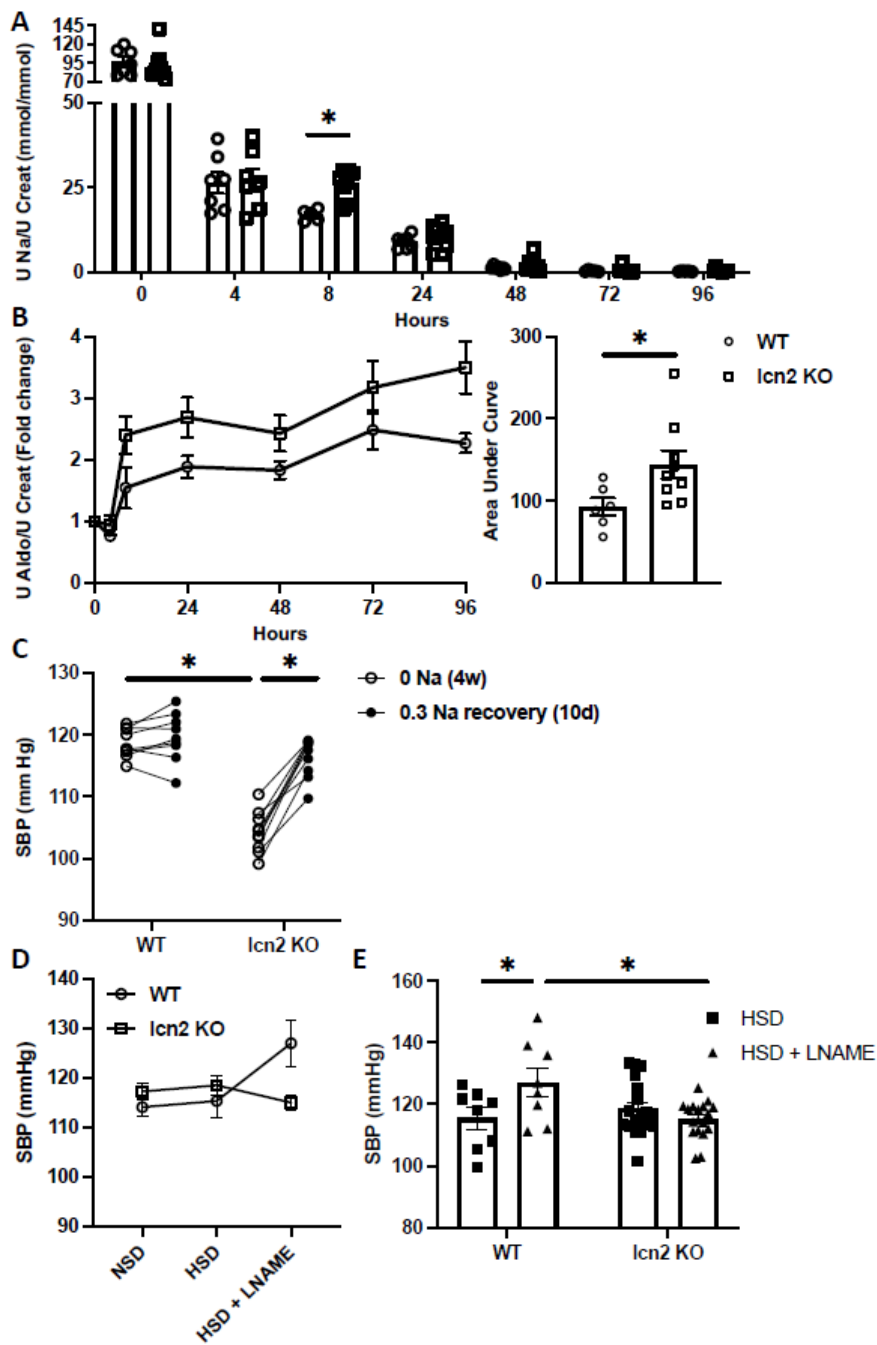
### **Figure 1. A low-salt diet induces specific lcn2 expression in the distal nephron**



(A) mRNA expression (left panel) and protein expression (right panel) of *lcn2* in the renal cortex of WT mice fed a normal-salt (0.3 Na) or low-salt (0 Na) diet for 2 days. Unpaired t tests were used for the statistical analysis,  $n=7-8$ .  $*P<0.05$ . (B) mRNA expression of *lcn2* in microdissected tubules from the renal cortex: thick ascending limb (TAL), distal convoluted tubule (DCT), and collecting duct (CCD) from WT mice fed a 0.3 Na or 0 Na diet. Unpaired t tests were used for the statistical analysis,  $n=4-12$ .  $*P<0.05$ . (C) Gene expression of several ion transporters expressed in the renal proximal tubule and distal colon of WT and *lcn2* KO mice (*lcn2* KO). Unpaired t tests were used for the statistical analysis,  $n=6-7$ .  $*P<0.05$ .

**Figure 2. *lcn2* gene inactivation increases urinary Na excretion and reduces blood pressure upon a low-salt diet**

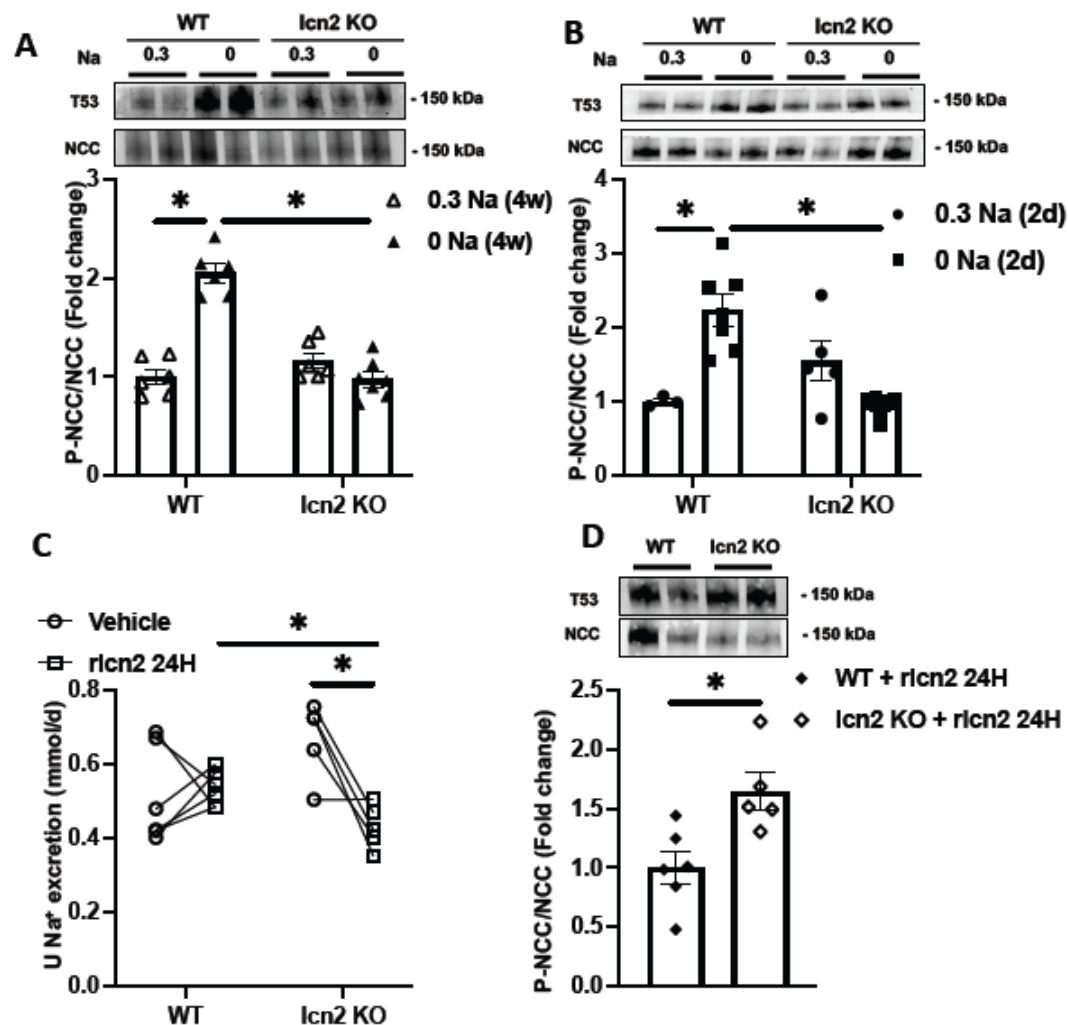




(A) Urinary Na excretion measurements in WT and lcn2 KO (lcn2 KO) mice fed a low-salt diet (0 Na) for four days. (B) Urinary aldosterone excretion measurements in WT and lcn2 KO mice fed a low-salt (0 Na) diet for four days (left panel) and quantification of the area under the curve (right panel). Unpaired t tests were used for the statistical analysis,  $n=6-9$ .  $*P<0.05$ . (C) Systolic blood pressure (SBP) measurements in WT and lcn2 KO mice fed a 0 Na diet for four weeks following, with a period of 10 days under a normal-salt diet (0.3 Na). Student t tests were used for the statistical analysis,  $n=8$ .  $*P<0.05$ . (D) SBP measurements of WT and lcn2 KO mice under a normal-salt diet (NSD), high-salt diet (HSD), or HSD +

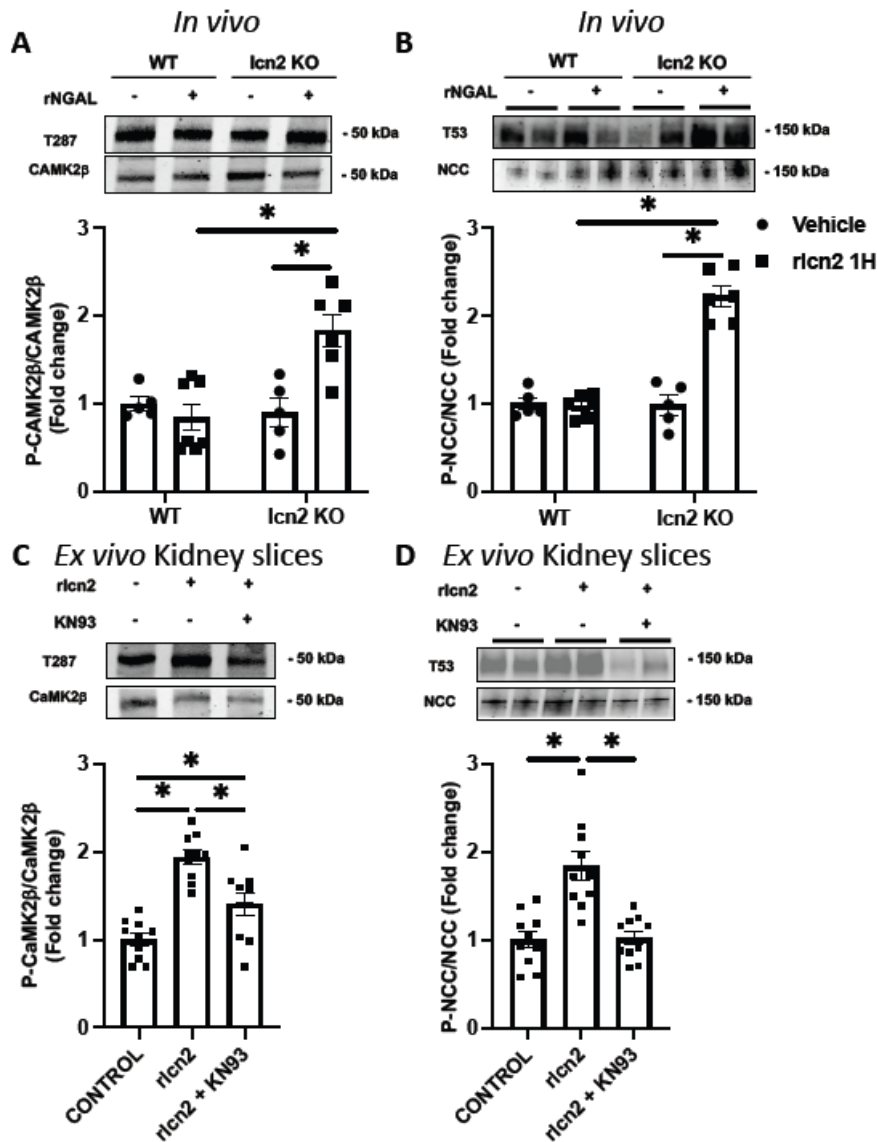
LNAME challenge and (E) quantification. Two-way ANOVA was used for the statistical analysis,  $n=3-7$ .  $*P<0.05$ .

**Figure 3. Icn2 enhances Na reabsorption through NCC activation**



(A) Western blot showing the ratio of phosphorylated (T53)/total NCC expression in WT and Icn2 KO (Icn2 KO) mice fed a normal-salt (0.3 Na) or low-salt (0 Na) diet for four weeks or (B) two days. Two-way ANOVA was used for the statistical analysis,  $n=3-7$ .  $*P<0.05$ . (C) Urinary Na excretion measurements for WT and Icn2 KO mice injected with vehicle or recombinant Icn2 (rIcn2) after 24 hours. Paired t test was used for statistical analysis between vehicle vs rIcn2 treatment in Icn2 KO group,  $n=6$ . Unpaired t tests were used for statistical analysis between WT and Icn2 KO group treated with rIcn2,  $n=5-6$ .  $*P<0.05$  (D) Western blot showing phosphorylated (T53) NCC expression in WT and Icn2 KO mice injected with recombinant NGAL after 24 hours. Unpaired t tests were used for the statistical analysis,  $n=5-6$ .  $*P<0.05$ .

**Figure 4. Short-term lcn2 administration increases phosphorylated NCC through CamK2 $\beta$  activation**



(A) Western blot showing the ratio of phosphorylated (T287)/total CamK2 $\beta$  and (B) phosphorylated (T53)/total NCC expression in the kidney cortex of WT and lcn2 KO (lcn2 KO) mice injected or not with recombinant lcn2 (rln2). Two-way ANOVA was used for the statistical analysis, n=5-7. \* $P$ <0.05. (C) Western blot showing the ratio of phosphorylated (T287)/total CamK2 $\beta$  and (D) phosphorylated (T53)/total NCC expression in kidney slices treated with recombinant lcn2 (rln2) with or without the CamK2 inhibitor (KN93). One-way ANOVA was used for the statistical analysis, n=10. \* $P$ <0.05

Parameters		NGAL mabC5		
		Beta	(95% CI)	P value
Urinary sodium	mod 1	0.70	(-5.55 _ 6.95)	0.83
	mod 2	0.92	(-5.27 _ 7.11)	0.77
Alb/Creat	mod 1	0.04	(-0.13 _ 0.22)	0.63
	mod 2	0.04	(-0.13 _ 0.22)	0.64
Na/K	mod 1	0.04	(-0.09 _ 0.16)	0.6
	mod 2	0.04	(-0.09 _ 0.17)	0.57
Na/Creat	mod 1	-0.09	(-1.74 _ -0.05)	0.038*
	mod 2	-0.09	(-1.71 _ -0.01)	0.046*
SBP office	mod 1	2.61	(0.76 _ 4.47)	0.006*
	mod 2	2.70	(0.88 _ 4.51)	0.004*
SBP 24 h	mod 1	1.41	(0.13 _ 2.69)	0.032*
	mod 2	1.46	(0.20 _ 2.72)	0.023*
SBP Day Time	mod 1	1.11	(-0.24 _ 2.46)	0.11
	mod 2	1.16	(-0.18 _ 2.49)	0.09
SBP Night Time	mod 1	1.58	(0.22 _ 2.94)	0.023*
	mod 2	1.65	(0.31 _ 2.99)	0.015*
DBP office	mod 1	0.81	(-0.39 _ 2.00)	0.19
	mod 2	0.82	(-0.34 _ 1.98)	0.17
DBP 24h	mod 1	0.37	(-0.56 _ 1.30)	0.43
	mod 2	0.37	(-0.56 _ 1.31)	0.43
DBP Day Time	mod 1	0.16	(-0.83 _ 1.15)	0.75
	mod 2	0.16	(-0.83 _ 1.16)	0.75
DBP Night Time	mod 1	0.44	(-0.58 _ 1.46)	0.39
	mod 2	0.45	(-0.58 _ 1.47)	0.39

**Table 1: Linear regressions for urinary measurements and blood pressure according to NGAL measurements**

Linear regression models were performed with adjusted sex and age (mod 1) and adjusted sex, age, diabetes, BMI, smoker, total cholesterol (mod 2). The statistical significance level was fixed at 0.05.

Samples	Parameters	0.3 Na		0 Na (4 weeks)	
		WT	KO NGAL	WT	KO NGAL
Blood	Na (mM)	147.6 ± 0.26	147.8 ± 0.05	148.7 ± 0.73	148.1 ± 0.4
	K (mM)	4.1 ± 0.08	3.9 ± 0.06	3.81 ± 0.06	3.6 ± 0.06 *
	Cl (mM)	107.1 ± 0.3	109.1 ± 0.48	109.4 ± 1.0	107.9 ± 0.48
	Ca (mM)	1.27 ± 0.01	1.26 ± 0.01	1.23 ± 0.01	1.23 ± 0.01
	Mg (mM)	0.88 ± 0.016	0.777 ± 0.021	0.834 ± 0.019	0.706 ± 0.031 **
	pH	7.346 ± 0.006	7.356 ± 0.009	7.343 ± 0.014	7.383 ± 0.007
	cHCO <sub>3</sub> <sup>-</sup> (mM)	30.3 ± 0.1	31.4 ± 0.31	29.2 ± 0.94	29.8 ± 0.29
Urine	Urine volume (ml)	1.43 ± 0.13	1.22 ± 0.11	1.37 ± 0.73	1.27 ± 0.73
	Urine Creatinine (mM)	2.67 ± 0.21	3.02 ± 0.18	3.89 ± 1.06	3.27 ± 0.84
	U Na/ U Créat (mM/mM)	96.63 ± 6.47	91.68 ± 6.74	4.02 ± 1.99	2.35 ± 1.60
	U K/ U Créat (mM/mM)	69.00 ± 4.99	71.11 ± 3.97	62.49 ± 4.69	67.88 ± 4.63
	U Cl/ U Créat (mM/mM)	77.62 ± 5.12	74.51 ± 5,18	7.64 ± 1.86	7.53 ± 1.77
	U Ca/ U Créat (mM/mM)	0.25 ± 0,02	0.21 ± 0.01 <i>p=0.055</i>	0.43 ± 0.42	0.47 ± 0.32
	U Mg/ U Créat (mM/mM)	4.31 ± 0.23	3.33 ± 0.25 *	5.81 ± 1.25	6.33 ± 1.46

**Table 2: Baseline electrolyte measurements in fluids from WT and KO NGAL mice and after 0 Na feeding during 4 weeks.**

Unpaired t test was used for statical analysis. n=7-10 \**p*<0.05 vs WT, \*\**p*<0.01 vs WT